Inhibitors of bacterial and mammalian hyaluronidase

Synthesis and structure-activity relationships

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
der Naturwissenschaftlichen Fakultät IV – Chemie und Pharmazie –
der Universität Regensburg

vorgelegt von

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2003

Die vorliegende Arbeit entstand in der Zeit von November 1999 bis Oktober 2003 unter der Leitung von Herrn Prof. Dr. A. Buschauer am Institut für Pharmazie Der Naturwissenschaftlichen Fakultät VI – Chemie und Pharmazie – der Universität Regensburg.

Das Promotionsgesuch wurde eingereicht im Oktober 2003.

Tag der mündlichen Prüfung: 21. November 2003

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für Griso und Otata

Ein guter, edler Mensch, der mit uns gelebt, kann uns nicht genommen werden; er lässt eine Spur zurück gleich jenen erloschenen Sternen, deren Bild nach Jahrhunderten die Erdbewohner sehen.

Thomas Carlyle

An dieser Stelle möchte ich mich bedanken bei:

Herrn Prof. Dr. A. Buschauer für die interessante Themenstellung, seine wissenschaftliche Anregungen, Diskussionen, Förderung und die kritische Durchsicht und Hilfestellung beim Verfassen dieser Arbeit.

Herrn PD Dr. G. Bernhardt für seine Unterstützung bei der Lösung experimenteller pharmakologischer Problemen.

Bei Herrn M. J. Jedrzejas (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) und Herrn D. J. Rigden (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil) möchte ich mich für die Durchführung der Kokristallisations-Experimente und die Aufnahme der Röntgenstrukturen bedanken.

Herrn A. Botzki danke ich ganz besonders für die LUDI Berechnungen, das Molekular Modelling und die zahlreichen wertvollen Diskussionen und Anregungen sowie das unermüdliche Korrekturlesen. Und vor allem für die langjährige persönliche Freundschaft, die stete Hilfsbereitschaft, Heiterkeit und die vielen unvergessliche Abende und Erlebnisse.

Frau L. Schneider, Frau A. Roithmeier, Frau E. Schreiber, Frau S. Bollwein und Frau K. Röhrl danke ich für die Unterstützung bei der Durchführung der pharmakologischen Testung.

Herrn Prof. Dr. E. von Angerer, Herrn Dr. G. Walter, Herrn Dr. F. Leurquin, Herrn Dr. C. Hutzler, Herrn Dr. D. Paper und Herrn Dr. C. Käsbauer danke ich für die zur Verfügung gestellten Substanzen.

Frau S. Heinrich und Herrn P. Richthammer für die Hilfsbereitschaft und Unterstützung in vielen organisatorischen und technischen Dingen.

Allen Mitarbeitern der analytischen Abteilungen der Fakultät für die Aufnahme der NMR- und Massenspektren, sowie der Durchführung der Elementar-analysen.

Allen Mitgliedern des Lehrstuhls danke ich für die Kollegialität und das gute Arbeitsklima.

Besonderen Dank gilt auch meinen Freunden: Tom, Thomas & Andrea, Fabien, Uta & Albert & Jona, Heidi & Achim, Christine H. & Martin R., Georgiana, Dirk & Simone, Michael K. sowie meiner gesamten Familie.

Besonderen Dank gilt auch meinen Laborkollegen Herrn A. Brennauer, Herrn St. Braun und Herrn A. Botzki für die Unterstützung bei fachlichen Problemen, das gute und heitere Laborklima sowie den vielen Aufheiterungen im Laboralltag.

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Chapter 1 Introduction

Chapter 1

Introduction

1. Hyaluronic acid

1.1 Structure and physicochemical properties

In 1934, hyaluronic acid (HA), a linear polymer of a disaccharide composed of *N*-acetylglucosamine and glucuronic acid linked together through alternating β-1,4 and β-1,3 glycosidic bonds, was isolated for the first time from the vitreous humor of bovine eyes by *Karl Meyer* (Meyer et al. 1934).

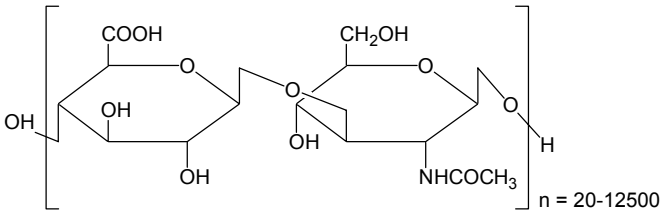

Fig. 1: Structure of hyaluronic acid

Hyaluronic acid (hyaluronan) is a member of the glycosaminoglycanes, a family of mostly linear polymers of high molecular weight composed of aminosugars (*N*-acetylglucosamine or *N*-acetylgalactosamine) and uronic acids (glucuronic or iduronic acid). The most important members of the glycosaminoglycanes are, apart from HA, chondroitin-, keratan- and dermatane sulphate, heparin and heparan sulphate. One important difference between HA and all other glycosaminoglycanes is the lack of sulphation - hyaluronan is strictly composed of non-sulphated sugar units shown in Fig. 1. The number of repeating disaccharide units can reach 10000 or more resulting in a molecular weight higher than $4 \cdot 10^6$ Da. The average length of a disaccharide unit is ~1 nm, thus a hyaluronan molecule of 10000 units could extend to 10 µm if stretched.

For decades, it was considered that hyaluronan chains in solution were random coils. The first indications that hyaluronan has preferred shapes were given by *J. E. Scott* (Scott 1989). Nuclear magnetic resonance confirmed the presence of an ordered structure in solution in which each disaccharide unit is twisted by 180 degrees compared with those ahead and behind it in the chain (cf. Fig. 2). The original orientation of the hyaluronan chain is achieved by a second twist, so that its structure is a two-fold helix. In solution, the HA-backbone is stabilised by internal hydrogen bonds and interaction with the solvent (Scott et al. 1991).

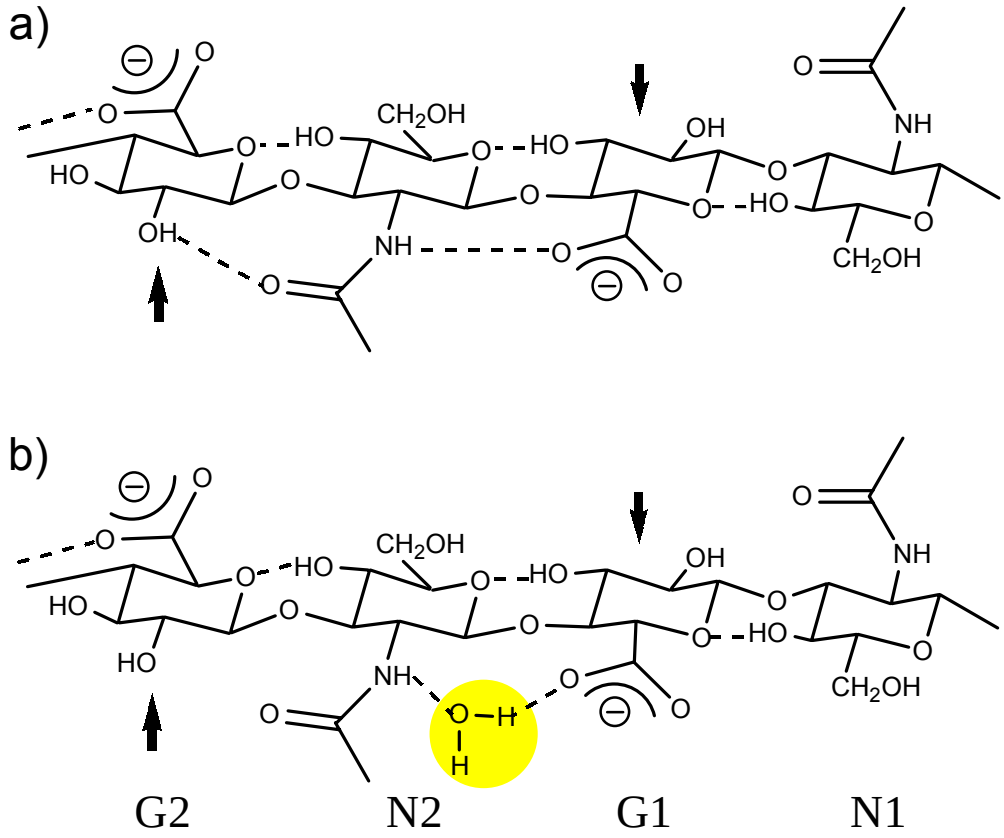

Fig. 2: Stabilisation of hyaluronic acid by internal hydrogen bonds: a tetrasaccharide unit of HA-chain a) in non-aqueous solution (DMSO); b) in aqueous solution; G = glucuronic acid, N = *N*-acetylglucoseamine. In aqueous solution, the two disaccharide units are distorted about 180° creating a twisting ribbon structure [according to *Scott* (Scott 1989)].

Due to the two-fold helix, the striking feature of this secondary structure is an extensive hydrophobic patch of about 8 CH-groups of 3 carbohydrate units. Thus, hyaluronan is amphiphilic, i.e. it has the properties of a highly hydrophilic material simultaneously with hydrophobic characteristics. By binding water, the

volume of HA increases by about 1000-fold compared to the non-hydrated state (Laurent et al. 1996). During the hydrated state the diffusion of e.g. proteins and electrolytes is substantially facilitated. In principle, all molecules can pass through a hyaluronan network, but with different velocity depending on their hydrodynamic volume.

1.2 Occurrence and physiological importance

Hyaluronan is a major constituent of the extracellular matrix, for example in the vitreous humor of the human eye (0.1-0.4 mg/g wet weight), in the synovial joint fluid (3-4 mg/ml), in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml), or in the pathological matrix that occludes the artery in coronary restenosis (http://www.glycoforum.gr.jp/).Hyaluronan is present in all vertebrates and also in the capsule of some *Streptococci* strains.
As hyaluronan serves as an essential structural element in the matrix, it plays an important role for tissue architecture. Furthermore, hyaluronan is important for cell proliferation, cell migration and cell growth as well as the metastasis of tumour cells. Morphogenesis, embryonic development, wound healing and inflammation are associated with an increase of hyaluronan production (Laurent et al. 1992; Laurent et al. 1996).

Hyaluronic acid interacts with a variety of receptors and binding proteins on the surface of cells (Laurent et al. 1992). The most common hyaluronan receptor and the most studied to date is CD44 (lymphocyte homing receptor). Furthermore, several hyaluronan binding proteins have been identified including the RHAMM (receptor for hyaluronan which mediates motility), ICAM-1 (intercellular adhesion molecule-1) and the LEC receptor (Liver Endothelial Cell clearance receptor) (http://www.glycoforum.gr.jp/; Laurent et al. 1996).

2. Hyaluronidases

2.1 History and occurrence

Hyaluronidase was first identified in an extract of mammalian testes and other tissues as a “spreading factor” that facilitated diffusion of antiviral vaccines, dyes, toxins injected subcutaneously (Duran-Reynals 1928). After the first isolation of hyaluronan by *Meyer et al.* (Meyer et al. 1934) and the identification of a HA degrading enzyme in bacteria (Meyer et al. 1937) it could soon be shown that the aforementioned spreading factor was an enzyme degrading hyaluronan (Chain et al. 1939). Similar hyaluronidase-like enzymes were detected and/or isolated from a large number of tissues and organisms e.g. liver, kidney, spleen, testes, uterus, placenta etc., from the venom of snakes, lizards, fish, bees, wasps, scorpions, spiders as well as from some bacteria, fungi and invertebrate animals. The isolated hyaluronidases differ in their molecular weight, substrate specificity and pH optima (Kreil 1995; Frost et al. 1996; Csoka et al. 1997). Although ubiquitously found, hyaluronidases are not well characterised and are a group of neglected enzymes due to difficult purification and lack of scientific interest over a larg period of time.

2.2 Classification of hyaluronidases

In 1971, K. *Meyer* (Meyer 1971) classified the hyaluronan degrading enzymes into three main families according to their catalytic mechanism (cf. Fig. 3).

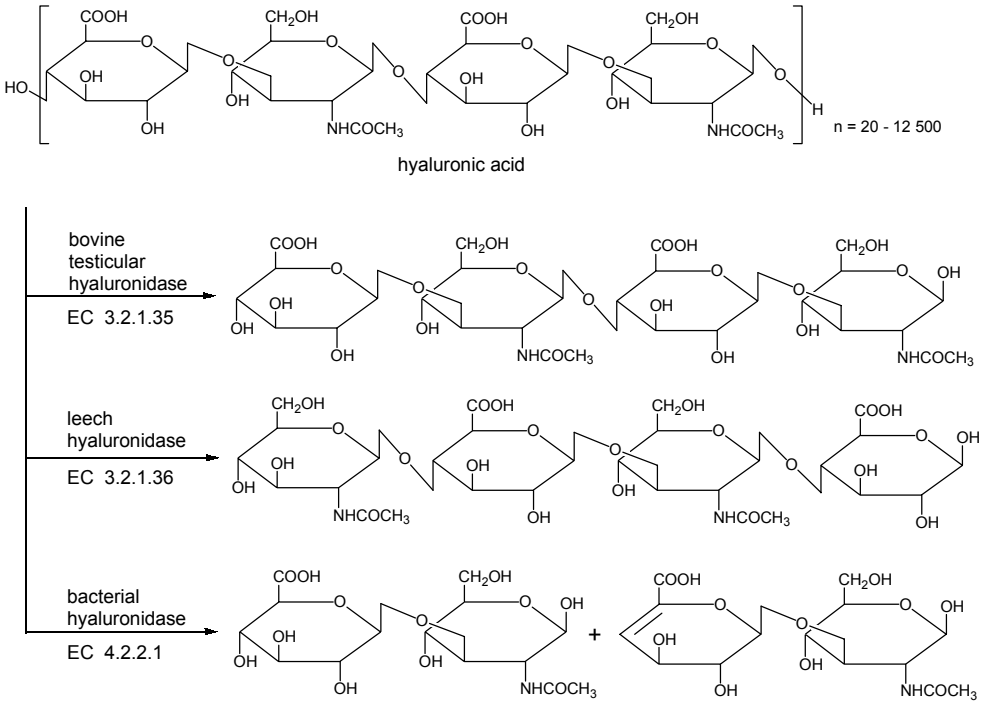

Fig. 3: Classification of hyaluronidases according to *Meyer* (Meyer 1971)

The first group of hyaluronidases are the hyaluronate 4-glycanohydrolases (EC 3.2.1.35) that degrade hyaluronan by cleavage of the 1,4-β-glycosidic bond to the tetrasaccharide as the main product. Furthermore, these enzymes degrade chondroitin, chondroitin 4- and 6-sulphate as well as in some cases dermatane sulphate. As a special characteristic, these enzyme also catalyse transglycosilation reactions (Cramer et al. 1994; Takagaki et al. 1994). The best known enzymes are the testicular, the bee venom and the lysosomal hyaluronidase.

The second type is represented by hyaluronidases from leeches and from hookworms. These enzymes are hyaluronate 3-glycanohydrolases (EC 3.2.1.36) that degrade HA by cleavage of the 1,3-β-glycosidic bond. The main product of this reaction is a tetrasaccharide, too.

The third group, the bacterial hyaluronidases (EC 4.2.2.1) are called hyaluronate lyases. These enzymes degrade hyaluronan by a β-elimination reaction to yield the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyluronic acid)-*D*-glucose as main product (Kreil 1995; Pritchard et al. 2000). The best known and characterised hyaluronate lyases are *S. pneumo-*

niae and *S. agalactiae* hyaluronate lyases (Jedrzejas 2000; Pritchard et al. 2000; Jedrzejas 2002).
As an alternative to the classification according to *Meyer* (Meyer 1971), hyaluronidases are divided in two main families – the procaryotic and eucaryotic hyaluronidases – according to amino acid sequence homology (Csoka et al. 1997; Csoka et al. 2001). In the last years, many mammalian hyaluronidase genes were decoded. The human genome contains six hyaluronidase-like genes, clustered on chromosome 3p21.3 (HYAL1, HYAL2 and HYAL3) and chromosome 7q31.3 (HYAL4, PH-20/SPAM1 and HYALP1) (Csoka et al. 2001). The testicular hyaluronidase, PH-20/SPAM1, was first identified by monoclonal antibodies present on the acrosomal membrane of sperm. Its homology to bee venom hyaluronidase led to its identification as a hyaluronidase that is essential for the penetration through the hyaluronan-rich cumulus mass that surrounds the ovum and consequently necessary for fertilisation (http://www.glycoforum.gr.jp/; Primakoff et al. 1985; Cherr et al. 1996).

In this study the bovine testicular hyaluronidase (BTH), the bee venom hyaluronidase (BVH) and the *S. agalactiae* hyaluronate lyase strain 4755 ($hylB_{4755}$) were used for pharmacological investigations. Therefore, these enzymes are briefly characterised in the following.

2.3 Bovine testicular hyaluronidase (BTH)

The bovine testicular hyaluronidase (EC 3.2.1.35) is an endo-glycanohydrolase that cleaves the 1,4-β glycosidic bond of hyaluronan. In addition to hyaluronic acid, BTH degrades chondroitin and chondroitin 4- and 6-sulphate, which are structurally related to HA. Depending on the BTH preparation, the used substrate, the hyaluronidase assay and the incubation conditions different pH optima were detected: pH value of 3.7 (Muckenschnabel et al. 1998), pH value of 5.2 (Gorham et al. 1975) and pH value of 7.5 (Meyer 1971).

In addition to the hydrolase activity, the bovine testicular hyaluronidase exhibits transglycosylase activity. *Cramer et al.* (Cramer et al. 1994) reported that saturated oligosaccharides with *N*-acetylglucosamine at the reducing end are pro-

duct of hyaluronan degradation. Hydrolase and transglycosylase activity takes place if oligosaccharides with 6 to 12 monomer units are available.

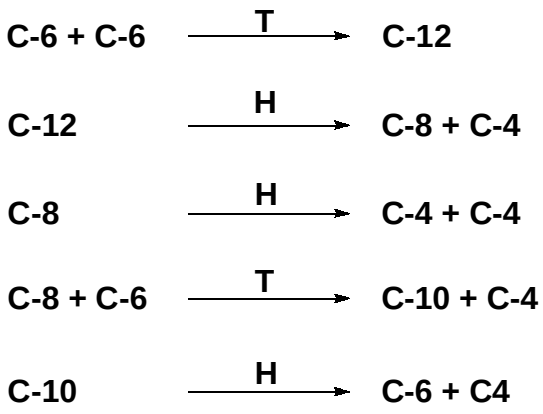

Fig. 4: Hydrolase (H) and transglycosylase (T) activity catalysed by BTH: C-4 to C-12 are HA oligosaccharides with *N*-acetylglucosamine at the reducing end (Cramer et al. 1994).

By contrast, *Takagaki et al.* (Takagaki et al. 1994) reported that saturated disaccharides are the smallest product of the degradation but tetrasaccharides are the main product.

Transglycosylation reactions are dependent on the pH value and the salt content of the incubation buffer. The optimal pH value for hydrolase activity is about 4-5, whereas pH 7 is optimal for transglycosylation reaction. In the presence of NaCl transglycosylase activity is partially inhibited and nearly completely inhibited at concentration higher than 0.5 M (Saitoh et al. 1995).

2.4 Bee venom hyaluronidase (BVH)

BVH is a member of the hyaluronate 4-glycanohydrolase (EC 3.2.1.35) like the bovine testicular hyaluronidase with similar properties and degradation mechanism. The crystal structure of the bee venom hyaluronidase was recently elucidated by *Markovic-Housley et al.* (Markovic-Housley et al. 2000; Markovic-Housley et al. 2002). In general, the glycosidases act via a double or a single nucleophilic displacement mechanism which results in either retention or inversion of the configuration of the anomeric carbon atom, respectively (Withers et

al. 1995; Markovic-Housley et al. 2002). The crystal structure of BVH suggests a catalytic mechanism, in which the amino acid residue Glu113 acts as the proton donor and the *N*-acetyl group of the substrate as the nucleophile (Markovic-Housley et al. 2002).
The bee venom hyaluronidase is a major allergen of bee venom and knowledge of the structural determinants responsible for the allergenic potency is expected to have importance for clinical implications.

2.5 *S. agalactiae* hyaluronate lyase ($hylB_{4755}$)

Streptococcus pneumoniae and *S. agalactiae* hyaluronate lyase degrade hyaluronic acid at the β-1,4-glycosidic linkage between D-glucuronic acid and *N*-acetyl-D-glucosamine. The product of this elimination reaction is the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (Kreil 1995; Pritchard et al. 2000). Furthermore, $hylB_{4755}$ also cleaves chondroitin and chondroitin sulphate but only with specific sulphation pattern (Pritchard et al. 2000). The three-dimensional structures of *S. pneumoniae* and *S. agalactiae* were recently elucidated by X-ray analyses (Jedrzejas et al. 2000; Li et al. 2000; Li et al. 2001; Jedrzejas et al. 2002). Based on the crystal structure and mechanistical studies, the mechanism of the elimination reaction was revealed (Jedrzejas et al. 2000; Kelly et al. 2001; Li et al. 2001; Jedrzejas 2002). The active centre of $hylB_{3502}$ is composed of two main parts, a catalytic group responsible for the substrate degradation and an aromatic patch responsible for the selection of cleavage sites on the substrate chains (Li et al. 2000). The proposed mechanism of catalysis is described in detail in chapter 5.
Investigations in our work group detected the highest hydrolytic activity of *S. agalactiae* hyaluronate lyase at a pH value of 5.0, independent of the used assay (Oettl et al. 2003), whereas *Ozegowski et al.* (Ozegowski et al. 1994) reported maximal activity at pH 6.3.
S. agalactiae hyaluronate lyase is a virulence factor which facilitates the spreading of the microorganisms and their toxins by degradation of hyaluronan. Human infection by this pathogen is one of the major causes of meningitis and septicaemia and many other serious diseases leading the death in neonates

(Dillon et al. 1987; Hynes et al. 2000). To study the role of hyaluronan and hyaluronidases in bacterial infection, the design and development of hyaluronate lyase inhibitors become more and more important.

3. Medical applications

In the late 1950s, the probably first medical application of hyaluronan to humans was a vitreous humor supplement/replacement during eye surgery.
Due to hyaluronan's high water-binding capacity and high viscoelasticity, HA is suitable for various medical and pharmaceutical applications. For example, given that HA retains moisture it is used in some cosmetics to keep skin young and fresh-looking.
A rapid increase of hyaluronan levels can occur in many clinical situation, for example during urticaria, the edema associated with wound healing and inflammation and the organ enlargement that occurs after transplantation. Furthermore, circulating levels of hyaluronan rapidly increase in situations such as shock, septicaemia and in burn patients (Frost et al. 1996; Natowicz et al. 1996; Csoka et al. 1997).
One of the most successful medical applications of HA is the use of sodium hyaluronate and a covalently cross-linked form of hyaluronan for the treatment of osteoarthritis (Balazs et al. 1989). It has been reported that sodium hyaluronate suppresses cartilage degeneration, protects the surface of articular cartilage (Fukuda et al. 1996), normalises the properties of synovial fluids (Asari et al. 1998) and reduces the perception of pain (Gotoh et al. 1993; Iwata 1993).
By cleaving hyaluronan in tissues, hyaluronidases increase the membrane permeability, reduce the viscosity and render the tissues more readily permeable to injected fluids (spreading effect). Thus, these enzymes could be used therapeutically to increase the speed of absorption, to promote resorption of excess fluids, to increase the effectiveness of local anaesthesia and to diminish tissue destruction by subcutaneous and intramuscular injection of fluids (Frost et al. 1996; Farr et al. 1997). Hyaluronidases are widely used in many fields like orthopaedia, surgery, ophthalmology, internal medicine, oncology, dermatology and gynaecology etc. (http://www.glycoforum.gr.jp/; Few 1987; Bertelli et al.

1994; Farr et al. 1997). Sperm hyaluronidase plays an important role for successful fertilisation in most mammals, including human (Primakoff et al. 1985; Lin et al. 1994).
Hyaluronidase has been investigated as an additive to chemotherapeutic drugs for augmentation of the anticancer effect (Spruss et al. 1995; Muckenschnabel et al. 1996; Baumgartner 1998; Muckenschnabel et al. 1998). There is evidence that hyaluronidase may have intrinsic anticancer effects and can suppress tumour development. Furthermore, it was reported, that treatment with hyaluronidase blocks lymph node invasion by tumour cells in an animal model of T cell lymphoma (Zahalka et al. 1995).
To prove and to affirm the role and the importance of hyaluronan and hyaluronidase in all aforementioned processes, selective and potent inhibitors are required.

4. Inhibitors of hyaluronidases

The first studies of hyaluronidase inhibitors were published half a century ago (Haas 1946; Dorfman et al. 1948; Meyer et al. 1951). In 1951, *Meyer et al.* (Meyer et al. 1951) reported on hyaluronidase inhibition by iron, cooper and zinc salts, heparin, polyphenols and flavonoids. It has also been reported that heparin and heparan sulphate are inhibitor of hyaluronidase, but the inhibition was achieved only at concentrations by far higher than physiological levels (Houck 1957; Wolf et al. 1984; Farr et al. 1997; Mio et al. 2002). The inhibitory activity of heparin and heparan sulphate was explained by the structural similarity of these oligosaccharides to hyaluronic acid. *Asada et al.* examined the effect of various types of alginic acid consisting of L-glucuronic acids and D-mannuronic acids on the bovine testicular hyaluronidase (Asada et al. 1997). The inhibition of the hyaluronidase by sodium alginate was dependent on the molecular weight – the higher the molecular weight, the stronger the inhibition. Based on these results, *Toida et al.* investigated *O*-sulphated glucosaminoglycanes whereby the fully sulphated compounds showed the highest inhibitory effect on the activity of hyaluronidases.

It was published that some flavones and flavone analogues were potent hyaluronidase inhibitors (Kakegawa et al. 1988; Kuppusamy et al. 1990; Kuppusamy et al. 1991; Kakegawa et al. 1992). The representative and widely accepted hyaluronidase inhibitors of this class of compounds are apigenin and kaempferol. Taken together the published results show that, in principle, flavones and related compounds are suitable to inhibit hyaluronidases but are unselective and only weakly active, i.e. at in millimolar concentration (cf. chapter 6).

Further compounds with inhibitory activity at millimolar concentration were detected, e.g. vitamin C, aescin; disodium cromoglycate, tranilast, traxanox, hederagenin, guanidine hydrochloride, L-arginine, norlignane, urolithin B etc. (Kakegawa et al. 1992; Tung et al. 1994; Facino et al. 1995; Jeong et al. 1999; Jeong et al. 2000; Akhtar et al. 2003). Indeed, the published data (% inhibition and IC_{50} values) of all aforementioned compounds are not comparable with each other because of differences in the applied test systems (e.g. incubation condition, enzymes and substrate concentrations).

It is apparent that the development of hyaluronidase inhibitors has barely begun. Due to the importance of hyaluronan and hyaluronidase in many clinical applications selective and potent inhibitors are worthwhile.

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Chapter 2

Scope of this thesis

To study the physiological and pathophysiological role of hyaluronan and hyaluronidase potent and selective hyaluronidase inhibitors are required as pharmacological tools. Additionally, such compounds are of potential therapeutic value for the treatment of a variety of diseases, e.g. cancer, arthroses or bacterial infections. As potent and selective hyaluronidase inhibitors are not known so far (cf. chapter 1), the goal of this thesis was to synthesise and identify lead-like compounds, to investigate their inhibitory effects on the *S. agalactiae* hyaluronate lyase, the bovine testicular hyaluronidase and the bee venom hyaluronidase and to study the structure-activity relationships.

For the design and development of enzyme inhibitors several approaches are known. The classical way of drug discovery and evaluation involves sequential testing of chemicals or extracts from biological materials in isolated organs followed by test in whole animals. Many new drugs were discovered by this classical approach during the 20th century. Following such approaches, it was published that isolated flavons and flavone related compounds as well as some natural products have inhibitory activities on hyaluronidase. As a first goal of this thesis new and diverse flavone derivatives should be synthesised and pharmacologically investigated as well as several isolated flavonoids and related compounds.

Due to the structural similarity to hyaluronic acid heparin and heparan sulphate were also considered as hyaluronidase inhibitors. Therefore, a set of sulphated and non-sulphated oligosaccharides were investigated for their inhibitory activities.

The X-ray structure of *S. pneumoniae* and *S. agalactiae* hyaluronate lyase were recently elucidated and the mechanism of the elimination reaction was re-

vealed. Based on mechanistical study of the hyaluronan degradation by bacterial hyaluronidase, the design and synthesis of a substrate analogue as a potential inhibitor was envisaged.

With the elucidation of the three-dimensional structure of receptors and enzymes, virtual screening and computer-based molecular design become of increasing importance. As a part of a project to design hyaluronidase inhibitors, a structure-based strategy to discover inhibitors of bacterial hyaluronan lyases (*S. agalactiae*, $hylB_{4755}$) has been carried out using the *de novo* design software LUDI. The main focus of this thesis was the synthesis and pharmacological evaluation of the compounds proposed by LUDI calculations[1]. Based on the results of this approach and to get more information about structural requirements for hyaluronate lyase inhibitory activity, differently substituted diphenylacrylic acids and diphenylpropionic acids as well as a set of indole derivatives were investigated as lead-like compounds.

To further elaborate structure-activity relationships of indole-type hyaluronidase inhibitors a series of 2-phenylindole derivatives was synthesised and pharmacologically investigated. For more detailed information about the interaction of 2-phenylindole derivatives with the amino acid residues inside the active site of *S. pneumoniae* hyaluronate lyase, a crystal structure of the enzyme-inhibitor complex should be elucidated[2].

[1] The construction of the $hylB_{4755}$ model and the LUDI calculations have been carried out by *Alexander Botzki* (Botzki 2004) as part of his PhD project.
[2] The co-crystallisation experiments have been carried out by *Mark J. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and *Daniel J. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

Chapter 3

Methods and assays for the determination of hyaluronidase activity

1. Classifications

A variety of methods has been developed over the years to measure the hyaluronidase activity, but many of these assays appear to be rarely used today (Stern et al. 1992; Muckenschnabel et al. 1998; Muckenschnabel et al. 1998; Mio et al. 2002). As there are many different assays for hyaluronidase activity the published methods are classified into groups based on the type of assay performed according to the classification of *Hynes et al.* (Hynes et al. 1994).

1.1 Chemical assays

The quantification of ***reducing sugars***, which are formed during the degradation of hyaluronic acid by hyaluronidases is one method to detect and to count hyaluronidase activity (Meyer et al. 1941; Hynes et al. 1994). For example, *Linker et al.* (Linker 1966) developed a colorimetric method to assay bacterial hyaluronidase based on the reduction of ferricyanide to ferrocyanide by reducing sugar.

The detection of the *N*-acetylglucosamine residues at the reducing end of hyaluronan degradation products as indicator of hyaluronidase activity is one of the most commonly used method to assay hyaluronidase (Meyer et al. 1941; Humphrey 1946; Humphrey 1946; Meyer 1947; Reissig et al. 1955; Ingham et al. 1979; Rouleau 1980; Hamai et al. 1989; Muckenschnabel et al. 1998; Muck-

enschnabel et al. 1998). A number of approaches have been developed for this method. One of the first assay was described by *Reissig et al.* (Reissig et al. 1955) in 1955. The optimised colorimetric method is based on the reaction of *p*-dimethylaminobenzaldehyde with *N*-acetylglucosamine to a coloured product which can be detected at 585 nm. Methods based on the assay of *Reissig et al.* (Reissig et al. 1955) have been used to study hyaluronidase from different sources for example from *Propionibacterium acnes* (Ingham et al. 1979), *S. dysgalactiae* (Hamai et al. 1989) and bovine testis (Muckenschnabel et al. 1998; Muckenschnabel et al. 1998).
A modified assay described by *Muckenschnabel et al.* (Muckenschnabel et al. 1998; Muckenschnabel et al. 1998) is one of the methods we used for the investigation of the hyaluronidase inhibitors. The advantage and the disadvantage of this method and the exact assay procedure are described later in this chapter (cf. Section 2. Colorimetric assay – Morgan-Elson assay).

Hyaluronate lyases (EC 4.2.2.1) differ from hyaluronidase from other sources by their mode of action. They cleave the hyaluronic acid by an elimination reaction resulting in an ***unsaturated hexuronic acid*** at the nonreducing ends (Kreil 1995). The detection and quantification of this unsaturated hexuronic acid can also be used to assay hyaluronidase activity (Greiling et al. 1965; Greiling et al. 1965; Linker 1966; Ohya et al. 1970). The formation of the elimination product can be detected and quantified spectrophotometrically by measuring the absorbance at a wavelength of 230-235 nm. Method and the detailed experimental procedure are described later in this chapter (cf. Section 4. UV difference spectroscopy).

1.2 Fluorogenic assays

Hyaluronic acid labeled with the fluorogenic reagent 2-aminopyridine has been used as substrate in a rapid, simple and sensitive fluorescence assay described by *Nakamura et al.* (Nakamura et al. 1990) for the detection of testicular hyaluronidase. After incubation with hyaluronidase, dilution with ethanol and centrifugation, the fluorescence of the supernatant was determined by fluoro-

spectrophotometry. It was found that the increase of the pyridylamino products is linearly correlated with the enzyme concentration under these conditions. The fluorogenic substrate has also been used for the determination of crude hyaluronidase e.g. hyaluronidase extract from liver (Nakamura et al. 1990; Hynes et al. 1994).

1.3 Indirect enzymoimmunological assays

Hyaluronectin, a hyaluronan-binding proteoglycan can be used as a probe in an indirect enzymoimmunological hyaluronidase assay (Delpech et al. 1987). Microtiter plates were coated with hyaluronic acid and incubated with hyaluronidase. After rinsing the residual, hyaluronic acid was incubated with hyaluronectin immune complexes conjugated with alkaline phosphatase, again rinsed and incubated with diluted conjugated antibodies. Hyaluronidase activity is indicated by a decrease in the absorbance measured at 405 nm. This method was developed for the detection of small amounts of hyaluronidase from the bovine testis, hepatoma cell lines, bee venom, leech, human sera and *streptomyces* species (Delpech et al. 1987; Hynes et al. 1994).
An ELISA-like assay for hyaluronidases and hyaluronidase inhibitors, similar to the assay of *Delpech et al.* (Delpech et al. 1987) was reported by *Stern et al.* (Stern et al. 1992). This assay is based on a high affinity biotinylated HA-binding peptide and the avidine-biotin reaction and seems to be a sensitive, rapid and simple assay.

1.4 Physicochemical assays

A ***turbidimetric assay,*** based on the observation that acidified hyaluronic acid forms a stable colloidal suspension in the presence of diluted serum was published by different authors (Hynes et al. 1994). After the degradation of the substrate with hyaluronidase the incubation mixture is remaining clear. Hyaluronidase activity is indicated by a decrease in the turbidity, measured with a spectrophotometer at around 600 nm. A sensitive, simple, reproducible and

economical semi quantitative microassay based on the turbidimetric assay was reported by *Ibrahim et al.* (Ibrahim et al. 1973).
The turbidimetric assay based on the precipitation of nondegradated hyaluronic acid with different reagents like cetyltrimethylammonium bromide is discussed later in this chapter (cf. 3. Turbidimetric assay).

A number of approaches have been developed for detection of hyaluronidase activity using the ***viscosity reduction*** of dissolved hyaluronic acid as indicator of enzyme activity (Meyer et al. 1941; Meyer 1947).
Meyer et al. (Meyer et al. 1941; Meyer 1947) described a standardised procedure in which many variations of this type of assay (substrate and buffer concentration, pH, temperature etc.) are combined. The viscosimetric assay is an assay which is reliable for investigations of different hyaluronidases (Tirunarayanan et al. 1968; Hynes et al. 1994).

The ***Mucin Clot Prevention*** (MCP) assay is based on the coprecipitation of native hyaluronic acid with protein to form mucin clot. The quality and character of the mucin clot is reduced when the substrate hyaluronic acid is degradated by hyaluronidase. The most widely used MCP assay was originally described by *Robertson et al*. (Robertson et al. 1940) and modified by *McClean et al*. (McClean 1943). Other modified MCP tests are described by *Unsworth* (Unsworth 1989), *Halperin et al.* (Halperin et al. 1987) and *Murphy* (Murphy 1972).

1.5 Plate (solid media) assays

Many assays have been developed for the detection of hyaluronidase using solid media (Hynes et al. 1994). One of the simple plate assay is described by *Smith et al.* (Smith et al. 1968). In this assay, petri dishes were coated with a mixture of agar and hyaluronic acid and incubated with bacteria. Hyaluronidase activity is detected as a zone of clearing around the bacteria in a cloudy background, resulting from acetic acid precipitation of a complex consisting of albu-

min and nondegradated hyaluronic acid. The same assay can be used to test isolated hyaluronidases instead of the whole bacteria (Hynes et al. 1989).

1.6 Radiochemical assays

A radiochemical method for detection of hyaluronidase activity was described by *Coulson* and *Girkin* in 1975 (Coulson et al. 1975). The principle of this radiochemical assay is that cetylpyridinium chloride precipitates the radioactive labelled hyaluronic acid but not the smaller polysaccharides obtained by digestion with hyaluronidase. In this procedure, hyaluronic acid is partially deacylated and then reacylated in the presence of [^{3}H]acetic anhydride. The radioactivity of the undigested substrate, precipitated with cetylpyridinium chloride is compared with the radioactivity of the blanks to get the hyaluronidase activity (Coulson et al. 1975; Hynes et al. 1994).

Hotez et al. (Hotez et al. 1992) described an alternative radiochemical assay for the detection of hookworm hyaluronidase activity. After incubation of the ^{3}H-labeled hyaluronic acid with hyaluronidase the degraded substrate is applied to a polyacrylamide gel, fixed and prepared for autoradiography. Hyaluronidase activity is observed as a decrease in the size of the labelled hyaluronic acid (Hotez et al. 1992; Hynes et al. 1994).

1.7 Spectroscopic assays

Benchetrit et al. (Benchetrit et al. 1977) developed a sensitive method for the detection of hyaluronidase based on a shift in maximal absorbance following interaction of anionic mucoploysaccharides with a carbocynine dye. The hyaluronidase activity is indicated by a decrease of the absorbance of the hyaluronan-dye complex. This method is useful for the detection and quantification of purified hyaluronidase, but it is not reliable for the detection of activity in crude preparations where other substances may interfere (Hynes et al. 1994).

A modification of this procedure was described for the assay of chondroitin sulphate depolymerase and hyaluronidase activity in *viridans Streptococci* by

Homer et al. (Homer et al. 1993; Homer et al. 1993). The principle of this assay is the reaction of undegradated hyaluronan with 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide to give a complex with a characteristic absorbance maximum at 650 nm. The increase in absorbance is directly proportional to the concentration of substrate interacting with the dye. Similar spectroscopic assays are described by *Coulson* (Coulson et al. 1975), *Pryce-Jones* (Pryce-Jones et al. 1979), *Pritchard* (Pritchard et al. 1993) and *Turner* (Turner et al. 1985).

1.8 Zymographic analysis

Zymography is a method with allows the visualisation of enzyme activity following electrophoretic fractions which has been used for the quantitative analysis of a number of hyaluronidases (Abramson et al. 1967; Liefländer et al. 1968; Herd et al. 1974; Fiszer-Szafarz et al. 1989; Hotez et al. 1992; Steiner et al. 1992; Hynes et al. 1994). Zymographic analysis can be carried out on a variety of solid supports such as agar (Abramson et al. 1967), cellulose acetate membranes (Herd et al. 1974) or acrylamide (Liefländer et al. 1968; Fiszer-Szafarz et al. 1989; Steiner et al. 1992; Yamagata 1996; Mio et al. 2002).

The electrophoresis on a cellulose acetate membrane is described by *Herd et al*. (Herd et al. 1974). After electrophoresis, the membrane is overlaid with a second membrane saturated with hyaluronic acid and incubated at 37 °C. After treating of the overlay membrane with alcian blue, the hyaluronidase activity is seen as white bands in a blue background.

Abramson and *Friedma*n (Abramson et al. 1967) used the electrophoresis on agar to detect hyaluronidase activity in concentrated preparations from *Staphylococcus aureus*, *Streptococcus pyogenes* and bovine testis.

Fiszer-Szafarz incorporated hyaluronic acid into polyacrylamide gels before electrophoresis (Fiszer-Szafarz et al. 1989; Hynes et al. 1994). The hyaluronidase activity is indicated by pink bands (polyacrylamide staining) in a blue background (undegradated hyaluronan staining).

2. Colorimetric assay – Morgan-Elson assay

For the investigation of the potential enzyme inhibitors described in this thesis, the hyaluronidase activity was determined by a modified colorimetric assay, which is based on the method of *Gacesa et al.* (Gacesa et al. 1981) and *Reissig et al. (Reissig et al. 1955)* and reported in detail by *Muckenschnabel et al*. (Muckenschnabel et al. 1998). The colorimetric assay (Reissig assay, Morgan-Elson assay) is based on the reaction of the *N*-acetyl-D-glucosamine (GlucNAc) at the reducing ends of hyaluronan and its fragments with *p*-dimethylamino-benzaldehyde resulting in a red coloured product.

As shown in Fig. 1, the postulated main product of the degradation of hyaluronic acid by the bovine testicular hyaluronidase is a tetrasaccharide with *N*-acetyl-D-glucosamine at the reducing end. The chromogens I and II are formed under alkaline conditions (100 °C, pH 9) of the Morgan-Elson reaction. The chromogen III, which is formed by elimination of water under acidic conditions (conc. HCl / glacial acetic acid) react in the final step with p-dimethylaminobenzaldehyde (Ehrlich´s reagent) to give the instable red - coloured product, which can be photometrically measured at 586 nm. The described structure of the red product was postulated by *Muckenschnabel et al*. in 1998 (Muckenschnabel et al. 1998) on the basis of HPLC-MS investigations.

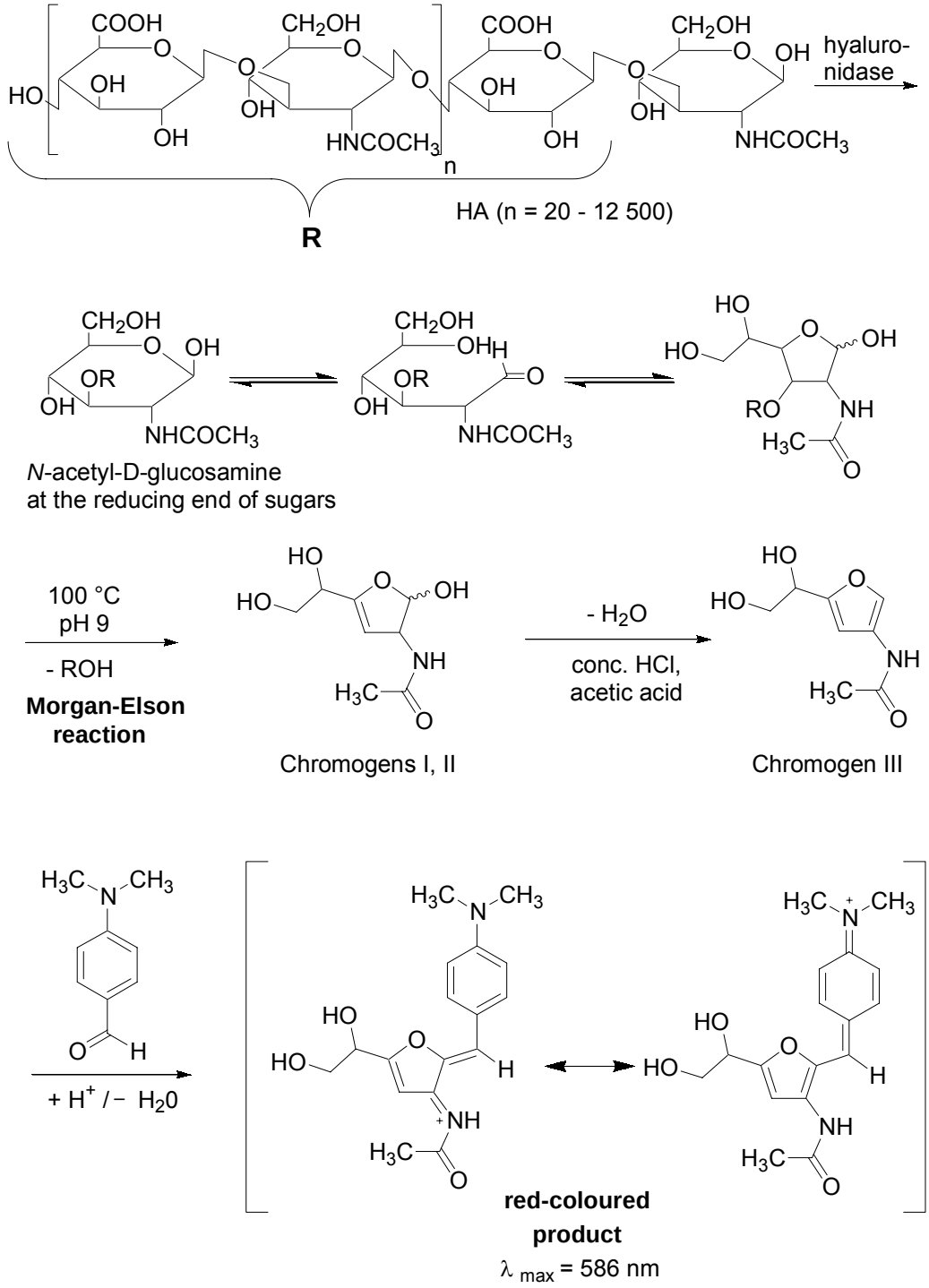

Fig. 1: Mechanism of the Morgan-Elson reaction - reaction of the *N*-acetyl-D-glucosamine with *p*-dimethylaminobenzaldehyde resulting in the red coloured product postulated by *Muckenschnabel et al*. (Muckenschnabel et al. 1998).

Effect of organic solvents on the enzyme activity

Our examined compounds were not soluble in the citrate-phosphate buffer, but soluble in organic solvents such as dimethylformamide, dimethylsulphoxide, methanol and ethanol. To find the appropriate solvent and the tolerated concentration bovine testicular hyaluronidase and hyaluronate lyase were investigated in the presence of different concentrations of various organic solvents. For this purpose the activities of the two enzymes were determined as a function of the solvent concentration in the incubation mixture. The effects of the solvents on the bovine testicular hyaluronidase are shown in Fig. 2. Whereas for all four solvents concentrations were accepted up to 4% higher concentrations resulted in weak or strong inhibition of the enzyme depending on the type of solvent.

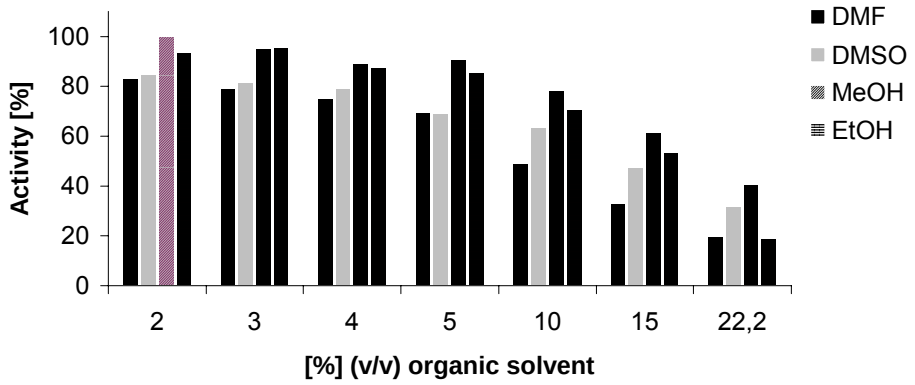

Fig. 2: Effect of DMF, DMSO, MeOH and EtOH on the activity of the bovine testicular hyaluronidase at optimum pH (3.6)

The effects of the four organic solvents on the hyaluronate lyase are shown in Fig. 3. Whereas the inhibitory effect of dimethylsulphoxide, methanol and ethanol at concentrations up to 4 % were negligible, concentrations higher than 5 % affected the enzyme activity negatively. The inhibitory effect of DMF was already significant at a concentration of 3 %.

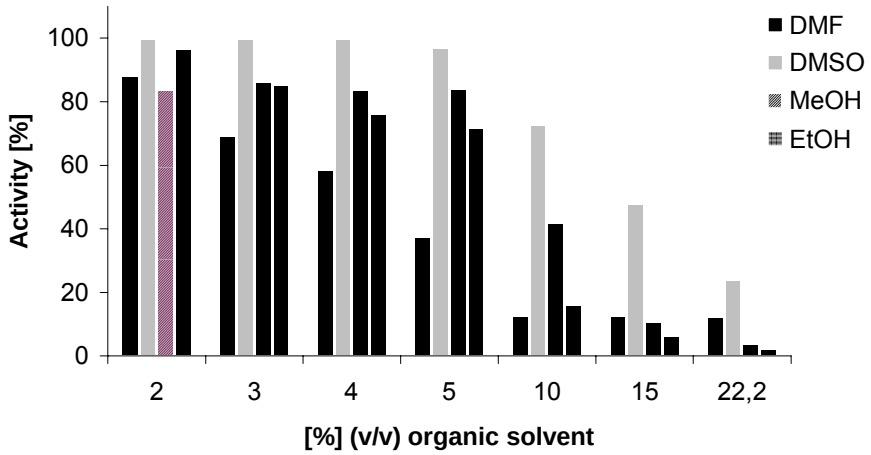

Fig. 3: Influence of the solvents DMF, DMSO, MeOH and EtOH on the activity of the bacterial hyaluronidase at optimum pH (5.0)

Because of the good solubility of the test compounds in DMSO and the low inhibitory effect of DMSO (< 4%) on the two enzymes, dimethylsulphoxide was used as solvent for pharmacological investigations.

Measurement of hyaluronidase activity

The procedure of the Morgan-Elson assay described in the literature was scaled down to minimise the required amounts of test compounds and enzymes. The enzyme concentrations and the incubation periods were optimised both for investigations at pH optimum and physiological pH. The incubation mixture, the enzyme concentrations, the incubation periods etc. of the modified assay are described in the following.

The test compounds (0.1 µM - 20 mM) dissolved in DMSO (7 µl), were incubated at 37 °C in an incubation mixture containing 60 µl of citrate-phosphate buffer (solution A: 0.1 M Na_2HPO_4 / 0.1 M NaCl, solution B: 0.1 M citric acid / 0.1 M NaCl; solution A and B were mixed in appropriate portions to adjust the required pH), 40 µl BSA solution (0.2 mg BSA per ml of water), 20 µl substrate solution (2 mg or 5 mg hyaluronic acid from rooster comb or *Streptococcus zooepidemicus* per ml of water), 33 µl H_2O and 20 µl enzyme solution (equiactive concentration: 8 IU BTH (pH 3.6), 40 IU BTH (pH 7.4), 0.4 IU $hyalB_{4755}$ (pH

5.0 and 7.4)). The pH of the incubation mixture was adjusted to the pH optimum of the enzymes (for BTH pH value of 3.6 and for $hylB_{4755}$ pH value of 5.0) and to physiological pH (7.4). The incubation time varied with the pH of the incubation mixtures: an incubation period of 1.5 h at optimum pH and 3 h at physiological pH for both enzymes.

The enzyme reaction was stopped by addition of 45 µl of alkaline borate solution and subsequent heating for 4.5 min in a boiling water bath. The alkaline borate solution was prepared immediately before use from the borate solution (17.3 g H_3BO_4 and 7.8 g KOH in 100 ml water) and the potassium carbonate solution (8.0 g K_2CO_3 in 10 ml water). After cooling on ice for 1 min 600 µl of *N,N*-dimethylaminobenzaldehyde (20.0 g *N,N*-dimethylaminobenzaldehyde dissolved in 25 ml concentrated hydrochloric acid and 75 ml glacial acetic acid; the solution was diluted with 4 volumes of glacial acetic acid immediately before use) was added and the mixture was incubated at 37 °C for 20 min. The resulting solution was transferred to 96 well plates and the absorbance of the coloured product was photometrically measured at 590 nm.

Enzyme activity was calculated from the formation of the red coloured product measured at 590 nm. The effect of the inhibitors on the enzyme activity was calculated according to the equation:

$$A\ \% = (B - C) / (D - E)$$

A: calculated enzyme activity

B: absorbance of the incubation mixture containing inhibitor

C: absorbance of the incubation mixture containing inhibitor in absence of the enzyme (enzyme solution replaced with buffer)

D: absorbance of the incubation mixture in absence of the inhibitor (inhibitor solution replaced with DMSO)

E: absorbance of the incubation mixture in absence of both enzyme and inhibitor (enzyme solution replaced with buffer, inhibitor solution replaced with DMSO)

The $IC_{50} \pm$ SEM values were calculated using the standard curves analysis of SigmaPlot™ (version 8.0) and are the means of three independent experiments performed in duplicate.

The Morgan-Elson assay is a useful method for the determination of hyaluronidase activity in the presence of inhibitors. With respect to the required incubation times, the assay is practicable and reproducible for the quantitation of enzyme activities. However, not all compounds can be examined with this assay. For instance, this assay is not suitable for the investigation of indole derivatives since the indole ring reacts with the Ehrlich´s reagent (*p*-dimethylaminobenzaldehyde) to a coloured product which is also detectable at 590 nm and falsifies the quantification of the red coloured product of the Morgan-Elson reaction (cf. chapter 9)

3. Turbidimetric assay

The turbidimetric measurement of hyaluronidase activity was carried out according to the method described by *Di Ferrante* (Di Ferrante 1956). The turbidimetric assay is based on the formation of insoluble complexes of cetyltrimethylammonium bromide (CTAB) and the remaining high molecular weight substrate (mw > 8 kDa) after incubation with enzyme (cf. Fig.4).

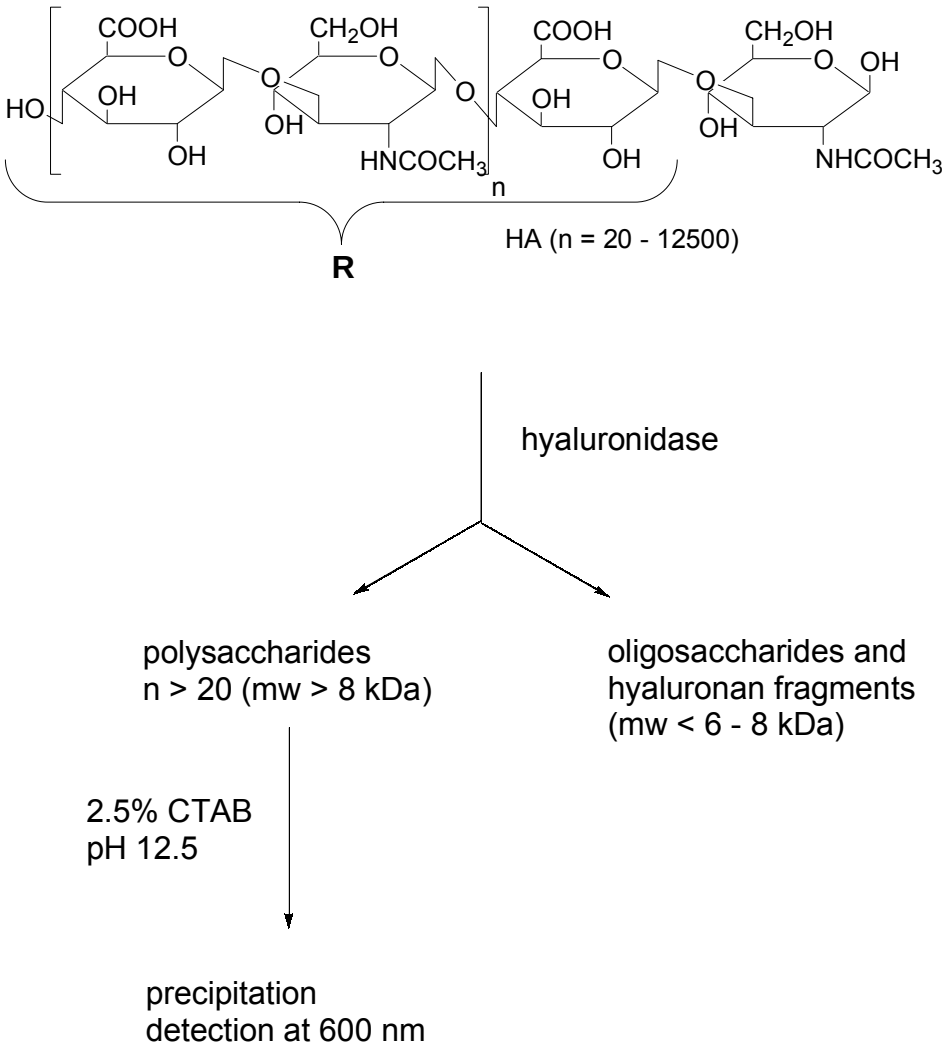

Fig. 4: Principle of the turbidimetric assay; precipitation of the polysaccharides with cetyltrimethylammonium bromide (Di Ferrante 1956).

The test compounds (0.1 µM - 20 mM) dissolved in DMSO (10 µl), were incubated at 37 °C in an incubation mixture containing 120 µl of citrate-phosphate buffer (solution A: 0.1 M Na_2HPO_4 / 0.1 M NaCl, solution B: 0.1 M citric acid / 0.1 M NaCl; solution A and B were mixed in appropriate portions to adjust the required pH), 30 µl BSA solution (0.2 mg BSA per ml of water), 30 µl substrate solution (2 mg hyaluronic acid from *Streptococcus zooepidemicus* per ml of water), 50 µl H_2O and 30 µl enzyme solution (equiactive concentration: 2 IU BTH (pH 3.6), 6 IU BTH (pH 7.4), 0.6 IU $hyalB_{4755}$ (pH 5.0) and 1 IU $hyalB_{4755}$ (pH 7.4)). The pH of the incubation mixture was adjusted to the pH optimum of the enzymes (BTH pH value of 3.6, $hylB_{4755}$ pH value of 5.0) and the physiological pH (7.4). The incubation time varied with the pH of the incubation mix-

tures: an incubation period of 1.5 h at optimum pH and 3 h at physiological pH for both enzymes. After addition of 720 µl of a 2.5 % cetyltrimethylammonium bromide solution (2.5 g CTAB dissolved in 100 ml 0.5 M sodium hydroxide solution, pH 12.5) the mixture was incubated at 25 °C for 20 min. Afterwards, the solution was transferred to UV cuvettes and the optical density was photometrically measured at 600 nm.
Enzyme activity was calculated from the formation of the precipitation measured at 600 nm. The effect of the inhibitors on the enzyme activity was calculated according to the equation

$$A\ \% = (B - C) / (B - D)$$

A: calculated enzyme activity
B: absorbance of the incubation mixture in absence of both enzyme and inhibitor (enzyme solution replaced with buffer, inhibitor solution replaced with DMSO)
C: absorbance of the incubation mixture in presence of inhibitor
D: absorbance of the incubation mixture in absence of the inhibitor (inhibitor solution replaced with DMSO)

For all examined compounds it can be excluded that the compounds precipitate with cetyltrimethylammonium bromide at the investigated concentrations. Furthermore, the inhibitors do not absorb light at the wave-length of 600 nm used in the turbidimetric assay.

The $IC_{50} \pm$ SEM values were calculated using the standard curves analysis of SigmaPlot™ (version 8.0) and are the means of three independent experiments performed in duplicate.

With respect to the required incubation times, the turbidimetric assay is a practicable and reproducible method for the quantification of enzyme activity. Furthermore, it is possible to investigate compounds which cannot be examined with the Morgan-Elson assay or with the UV differential spectroscopy assay (cf. section 4 of this chapter). Nevertheless, a disadvantage of this assay is the poor solubility of the examined compounds in the incubation mixture, a disadvantage which also applies for other methods.

4. UV difference spectroscopy

The hyaluronate lyases (EC 4.2.2.1) differ from hyaluronidase from other sources by their mode of action. They cleave the substrate hyaluronic acid by an elimination reaction resulting in an unsaturated hexuronic acid at the nonreducing ends (Kreil 1995; Pritchard et al. 2000) (cf. Fig. 5).

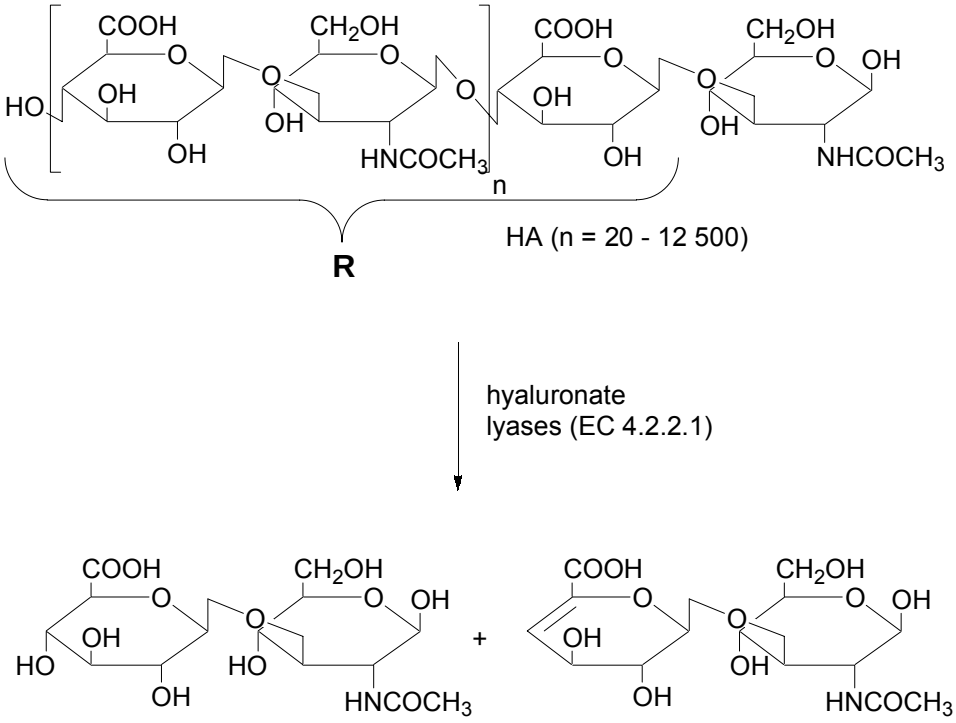

Fig. 5: Cleavage of hyaluronic acid by bacterial hyaluronidases (EC 4.2.2.1); 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyluronic acid)-*D*-glucosamine as main product

The activity of the hyaluronate lyases from *S. agalactiae* can also be determined by quantifying the unsaturated degradation product 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyluronic acid)-*D*-glucosamine and the unsaturated uronic acid residues of hyaluronan fragments photometrically at 232 nm according to *Greiling* (Greiling 1957).

To quantify the product formation, a set of matched tandem cuvettes, containing two compartments of equal pathlength (l = 4.375 mm) was used. The left compartment of the sample cuvette was filled with the incubation mixture A,

whereas the right compartment of the cell was filled with the solution B. The left compartment of the reference cuvette was filled with the incubation mixture C, the right compartment with the incubation mixture D (Table 1).

Table 1: Incubation mixtures used to quantify the formation of the unsaturated hexuronic acid.

Incubation mixture A	Incubation mixture B
50 µl inhibitor dissolved in DMSO	50 µl inhibitor dissolved in DMSO
300 µl citrate - phosphate buffer with NaCl (pH = 5.0)	300 µl citrate - phosphate buffer with NaCl (pH = 5.0)
200 µl BSA solution (0.2 mg / ml of water)	200 µl BSA solution (0.2 mg / ml of water)
250 µl HA solution (2 mg / ml of water)	250 µl H_2O
100 µl hyaluronate lyase dissolved in BSA solution (0.2 mg / ml of water)	100 µl hyaluronate lyase dissolved in BSA solution (0.2 mg / ml of water)

Incubation mixture C	Incubation mixture D
50 µl inhibitor dissolved in DMSO	50 µl inhibitor dissolved in DMSO
300 µl citrate - phosphate buffer with NaCl (pH = 5.0)	300 µl citrate - phosphate buffer with NaCl (pH = 5.0)
200 µl BSA solution (0.2 mg / ml of water)	200 µl BSA solution (0.2 mg / ml of water)
250 µl H_2O	250 µl HA solution (2 mg / ml of water)
100 µl BSA solution (0.2 mg / ml of water)	100 µl BSA solution (0.2 mg / ml of water)

The cuvettes were placed in the sample and the reference beam of the spectrophotometer and the increase in absorbance was monitored at 232 nm as function of time at 37 °C.

From the increase of absorbance (ΔA) at 232 nm per time (Δt) the enzymatic activity (A%) was calculated according to the following equation

$$A\% = (\Delta A / \Delta t) / (\Delta A_{max} / \Delta t)$$

where ΔA_{max} is equivalent to the absorbance of the incubation mixture containing DMSO instead of the inhibitor.

The $IC_{50} \pm$ SEM values were calculated using the standard curves analysis of SigmaPlot™ (version 8.0) and are the means of three independent experiments performed in duplicate.

The advantage of the UV difference spectroscopy assay is the short incubation time (5 to 10 minutes), the sensitivity and the good reproducibility of the assay. However, as an unsaturated hexuronic acid is determined, this assay can only be used for the investigation of inhibitors of hyaluronate lyases. Compounds, which show absorbance at 232 nm, the wave-length used for the detection of the double-bond formation cannot be tested with this assay. A further disadvantage of this assay is the poor solubility of the examined compounds in the incubation mixture, a disadvantage which also applies for other methods.

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Chapter 4

Investigations of sulphated oligosaccharides as inhibitors of hyaluronidases from bovine testis, bee venom and from *S. agalactiae*

1. Introduction

It was reported that apigenin (**1**) and kaempferol (**2**) are potent hyaluronidase inhibitors (Kuppusamy et al. 1990; Kuppusamy et al. 1991) (cf. Fig. 1). These two inhibitors are widely accepted as inhibitors of hyaluronidases and often used as positive controls for inhibition.

To verify the published results, we examined the effect of apigenin and kaempferol on the activity of the bovine testicular hyaluronidase at optimum pH (3.6). The inhibitory activities were determined in an optimised colorimetric assay based on the method of *Reissig et al.* (Reissig et al. 1955; Muckenschnabel et al. 1998).

As shown in Fig. 1 the inhibitory effects of apigenin (**1**) and kaempferol (**2**) on the bovine testicular hyaluronidase were only 56% and 36% at the highest concentration of 1 mM and 10 mM, respectively. Furthermore, the inhibition by compound **1** was not concentration dependent.

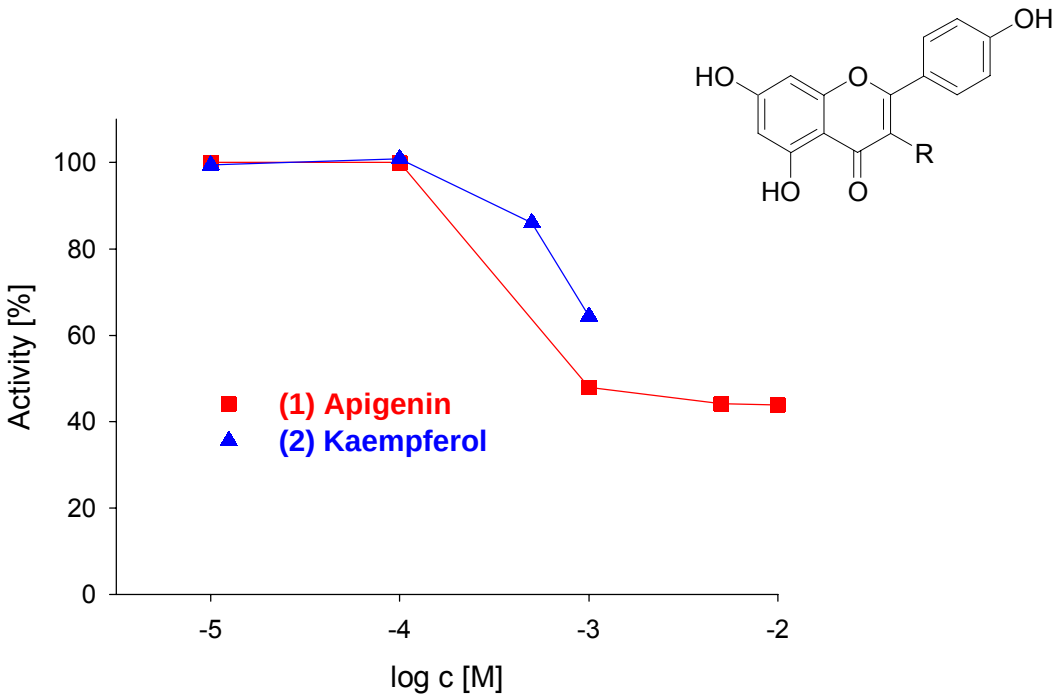


Fig. 1: Inhibitory effect of the classical inhibitors apigenin (**1**, R = H) and kaempferol (**2**, R = OH) on the activity of the bovine testicular hyaluronidase at optimum pH (3.6).

Asada et al. (Asada et al. 1997) examined the effect of various types of alginic acid consisting of L-glucuronic acids and D-mannuronic acids on the bovine testicular hyaluronidase. These investigations were stimulated by the structural resemblance of alginic acid to hyaluronic acid. The inhibition of the hyaluronidase by sodium alginate was dependent on the molecular weight – the higher the molecular weight, the stronger the inhibition.

It has also been reported that heparin, a sulphated glucosaminoglycane, is an inhibitor of hyaluronidase (Houck 1957; Wolf et al. 1984; Farr et al. 1997; Mio et al. 2002). The inhibition of hyaluronidase activity by heparin is achieved only at concentrations by far higher than physiological levels. The inhibition by heparin is non-competitive and the compound does not bind to the catalytic site of the enzyme. *Mio et al.* (Mio et al. 2002) reported that heparan sulphate, a glucosaminoglycane too, inhibited the hyaluronidase activity. The inhibitory activity of heparin and heparan sulphate was explained by the high structural similarity of these oligosaccharides to hyaluronic acid. Based on these results, *Toida et al.* (Toida et al. 1999) investigated *O*-sulphated glucosaminoglycanes whereby the

fully sulphated compounds showed the highest inhibitory effect on the activity of hyaluronidases.
In summary, oligosaccharides with sulphate and/or carboxylate as functional groups seem to be promising compounds as hyaluronidase inhibitors.

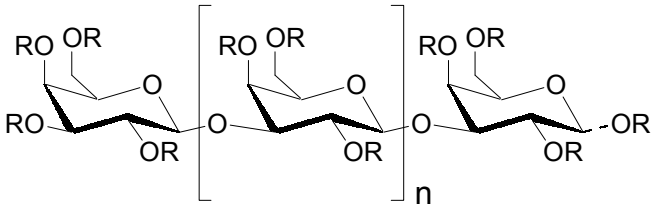
2. Pharmacological investigations

As part of our project on hyaluronidase inhibitors we studied a set of structurally different oligosaccharides against bovine testicular hyaluronidase (BTH), *Streptococcus agalactiae* hyaluronate lyase ($hylB_{4755}$) and bee venom hyaluronidase (BVH). The inhibitory effect of some sulphated and non-sulphated oligosaccharides (cf. Table 1) on the activity of hyaluronidases were examined.

2.1 The influence of sulphated and non-sulphated β-(1,4)-galacto-oligosaccharides on the activity of hyaluronidases

The first investigated compounds were a series of sulphated and non-sulphated β-(1,4)-galacto-oligosaccharides (**3**-**8**, cf. Table 1) with 3, 4, 5 or 8 sugar monomers and with a degree of sulphation (DS) ranging from 0.35 to 0.67 (DS = 1 equivalent to a sulphation of all hydroxyl groups). The inhibitory effects on the activity of the bovine testicular hyaluronidase (BTH), of the bee venom hyaluronidase (BVH) and of the hyaluronate lyase from *S. agalactiae* ($hylB_{4755}$) at optimum pH are summarised in Table.1.

Table 1: IC_{50} values of a series of sulphated and non-sulphated β-(1,4)-galacto-oligosaccharides investigated on BTH, BVH and $hylB_{4755}$.



No	β-(1,4)-galacto-oligosaccharides n / R	DS	BTH IC_{50} [µM][a] at pH = 3.6	BVH IC_{50} [µM][a] at pH = 3.6	$hylB_{4755}$ IC_{50} [µM][a] at pH = 5.0
3	n = 1 R = H or SO_3Na	0.67	**40**	**25**	**630**
4	n = 2 R = H	0	>> 1000	n.d.	>> 1000
5	n = 2 R = H or SO_3Na	0.56	**35**	**40**	**320**
6	n = 3 H	0	>> 1000	n.d.	>> 1000
7	n = 3 R = H or SO_3Na	0.59	**20**	**20**	**200**
8	n = 6 R = H or SO_3Na	0.35	**600**	n.d.	**4**

[a] the SEM values of the calculated IC_{50} values are in the range of 5-10%

As exemplarily shown for the sulphated β-(1,4)-tetragalactoside (**5**) in Fig. 2 the inhibitory effect on the bovine testicular hyaluronidase and on the bacterial enzyme at optimum pH (BTH pH 3.6, $hylB_{4755}$ pH 5.0) are in the micromolar range and concentration dependent. Whereas the obtained IC_{50} values of compound **5** were similar for the bovine testicular and the bee venom hyaluronidase (BTH 35 µM and BVH 40 µM), the IC_{50} value for the hyaluronate lyase was only

320 µM. The inhibitory effect on the bacterial hyaluronidase was 9-fold lower than on the bovine testicular hyaluronidase and the bee venom enzyme.

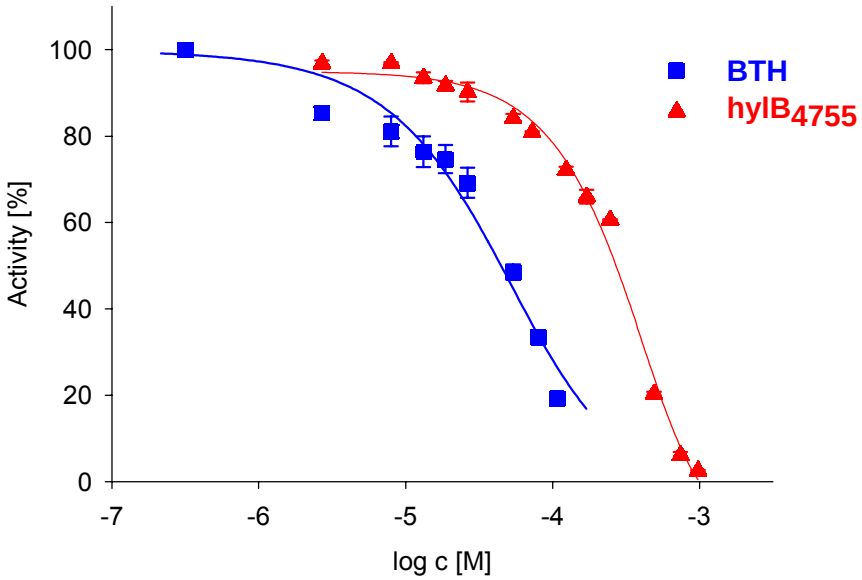


Fig. 2: Effect of the sulphated β-(1,4)-tetragalactoside **5** (structure cf. Table 1, DS = 0.56) on the activities of the bovine testicular hyaluronidase (IC_{50} = 35 µM, pH 3.6) and the bacterial hyaluronidase (IC_{50} = 320 µM, pH 5.0)

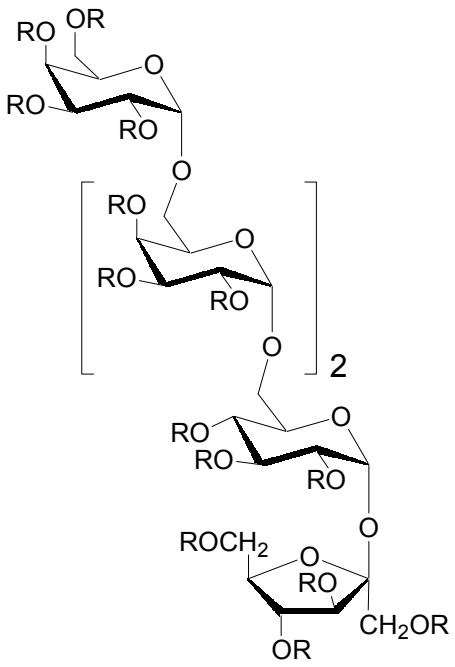
The highest activities on the bovine testicular and the bee venom hyaluronidase were found for the sulphated tri-, tetra- and pentasaccharides. The determined IC_{50} values were 40 µM / 25 µM (**3**, n = 1, DS = 0.67); 35 µM / 40 µM (**5**, n = 2, DS = 0.56); 20 µM / 20 µM (7, n = 3, DS = 0.59) (cf. Table. 1). The effect of the sulphated β-(1,4)-octagalactoside (**8**, n = 6, DS = 0.35) on the bovine testicular hyaluronidase was 15-fold lower than the effect of the sulphated compounds **3, 5** and **7**.

The IC_{50} values of the sulphated tri-, tetra- and pentasaccharides determined on the bacterial hyaluronidase ranged from 200 µM to 630 µM. These sulphated oligosaccharides had a 10- to 16-fold lower inhibitory effect on $hylB_{4755}$ in comparison to the bovine testicular and the bee venom hyaluronidase. Surprisingly the lowest IC_{50} value of 4 µM was measured for the sulphated β-(1,4)-octasaccharide (**8**, DS = 0.35). The extension of the chain length from 3 to 8 monomer units resulted in a 160-fold increase of the inhibitory activity on the hyaluronate lyase.

In conclusion, the optimal number of sugar residues seems to be 3, 4 and 5 for the inhibition of the bovine testicular and the bee venom hyaluronidase. The oligosaccharides with the chain length of 8 monomers showed the highest inhibitory activity on the bacterial enzyme. Independent of the chain length of the oligosaccharides, the sulphation was essential: all examined non-sulphated β-(1,4)-galacto-oligosaccharides were inactive at all three hyaluronidases.

2.2 Influence of the degree of sulphation on the potency

The pentasaccharide verbascose **9** (structure cf. Fig. 3) and two sulphated derivatives **10** and **11** which differed in the degree of sulphation (DS: 0.43 and 0.53) were selected to investigate the contribution of sulphation on the enzyme activity of the three hyaluronidases.



No	R	DS
9	H	0
10	H or SO_3Na	0.43
11	H or SO_3Na	0.53

Fig. 3: Structure of the verbascose derivatives **9**-**11**

The inhibitory effects of verbascose **9** and the sulphated derivatives **10** and **11** on the activity of the bovine testicular hyaluronidase are shown as concentration-response curves in Fig. 4. The IC_{50} value determined for the sulphated de-

rivative **10** (DS = 0.43) and **11** (DS = 0.53) were 30 µM and 3 µM, respectively. As expected, the non-sulphated pentasaccharide was ineffective on the bovine testicular hyaluronidase and on the hyaluronidases from bee venom as well as from *S. agalactiae* (data not shown). Obviously, a 20% increase in the degree of sulphation resulted in about 10-fold enhancement of the inhibitory activity.

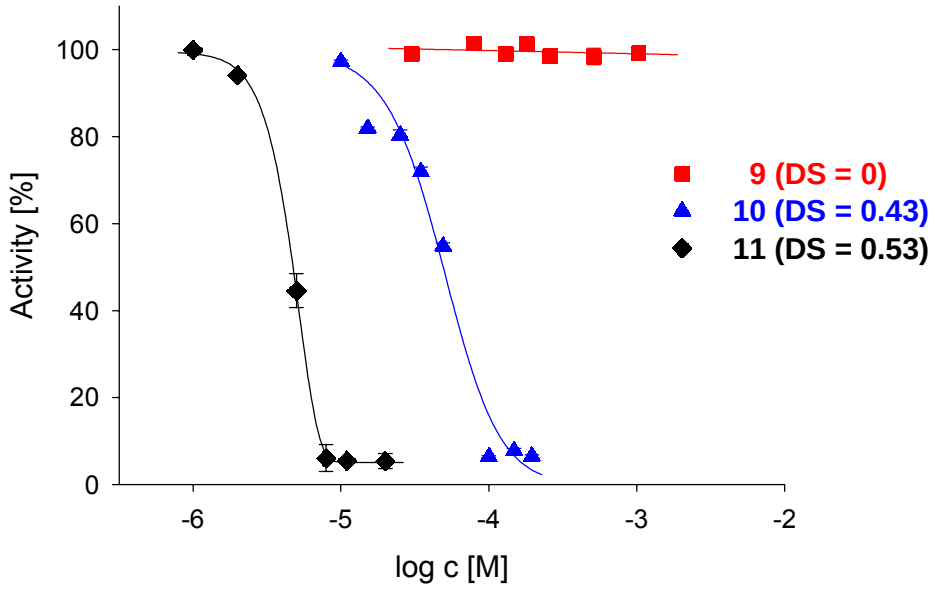
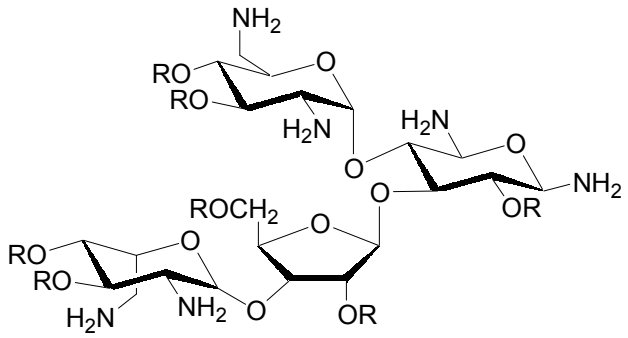


Fig. 4: Effect of the three verbascose derivatives **9**-**11** on the activity of BTH depending on the degree of sulphation (DS of 1 is equivalent to the sulphation of all hydroxyl groups)

The effects of verbascose and its sulphated derivatives on the hyaluronate lyase and on the bee venom hyaluronidase were similar to those on the bovine testicular enzyme. The IC_{50} values determined on $hylB_{4755}$ were 30 µM (DS = 0.43) and 1 µM (DS = 0.53) and for BVH 40 µM and 3 µM, respectively. An increase by 20% in the degree of sulphation resulted in about 30-fold and 13-fold enhancement of the inhibitory activity on the bacterial enzyme and the bee venom hyaluronidase, respectively. In summary, the potency of the pentasaccharides is dependent on the degree of sulphation: the higher the degree of sulphation, the higher the inhibitory activity.

2.3 Inhibitory effects of neomycin and planteose derivatives on the activity of the three hyaluronidases BTH, BVH and hylB$_{4755}$

As oligosaccharides with three, four or five monomers and a high degree of sulphation which appears to be the most promising derivatives, we examined the antibiotic neomycin (**12**) and its sulphated derivative **13** as well as planteose (**14**) and its sulphated derivative **15** – in order to confirm the aforementioned structure-activity relationships. The structures of the neomycin derivatives **12** and **13** and the planteose derivatives **14** and **15** are shown in Fig. 5 and Fig. 7, respectively.



No	R	DS
12	H	0
13	H or SO_3Na	0.62

Fig. 5: Structure of the neomycin derivatives **12-13**

The non-sulphated tetrasaccharide **12** was inactive on the bovine testicular and the bacterial hyaluronidase, whereas the sulphated compound showed a dose-dependent inhibition of both enzymes. The inhibition curves of the sulphated compound are depicted in Fig. 6. The IC_{50} value of the sulphated neomycin derivative **13** (DS = 0.62) determined on BTH was 60 µM, the IC_{50} value on hylB$_{4755}$ was 500 µM. The sulphated tetrasaccharide **13** was an 8-fold more potent inhibitor of the bovine testicular hyaluronidase than of the hyaluronate lyase.

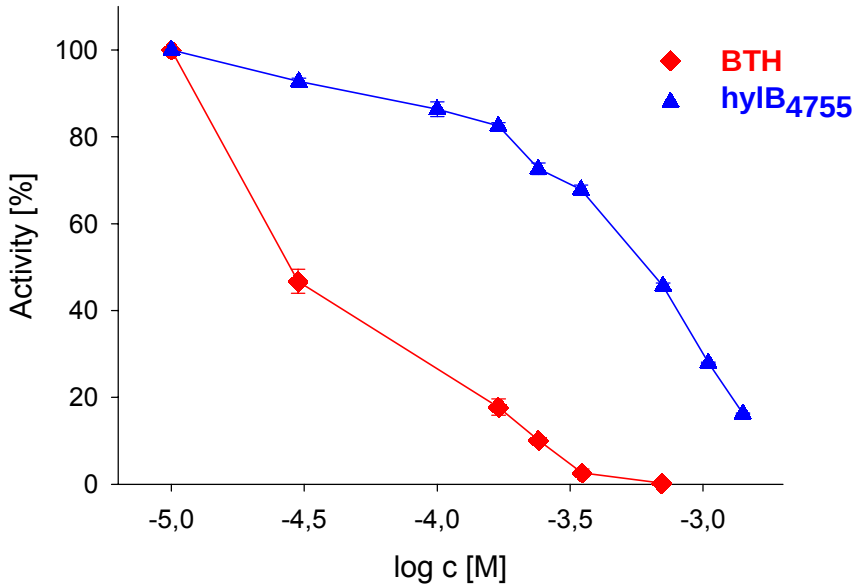
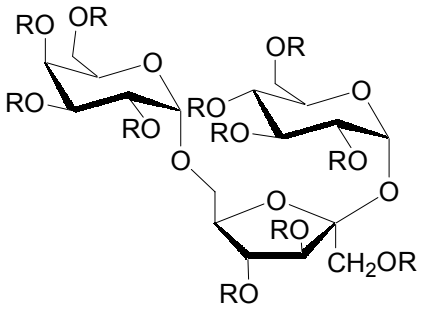


Fig. 6: Effect of the sulphated neomycin (**13**, DS = 0.62) on the activities of the bovine testicular hyaluronidase and the hyaluronate lyase at optimum pH (BTH pH 3.6; $hylB_{4755}$ pH 5.0)

As expected the non-sulphated trisaccharide **14** was inactive on the bovine testicular, the bee venom hyaluronidase and on the hyaluronate lyase (data not shown), whereas the sulphated derivative **15** showed concentration dependent inhibitions of all three hyaluronidases.



No	R	DS
14	H	0
15	H, SO_3Na	~ 1

Fig. 7: Structure of the planteose derivatives **14-15**

The inhibition curves of the sulphated planteose derivative **15** are depicted in Fig. 8. The determined IC_{50} value of compound **15** on the bovine testicular hyaluronidase of 4 µM was only 2-times lower than for the bee venom hyalu-

ronidase (8 µM; not shown in Fig. 8) and 4-times lower than for the bacterial enzyme (15 µM). In comparison to the sulphated neomycin analogue **13**, the sulphated trisaccharide **15** showed a 15-fold and a 30-fold higher inhibitory potency on the bovine testicular hyaluronidase and on the bacterial enzyme, respectively.

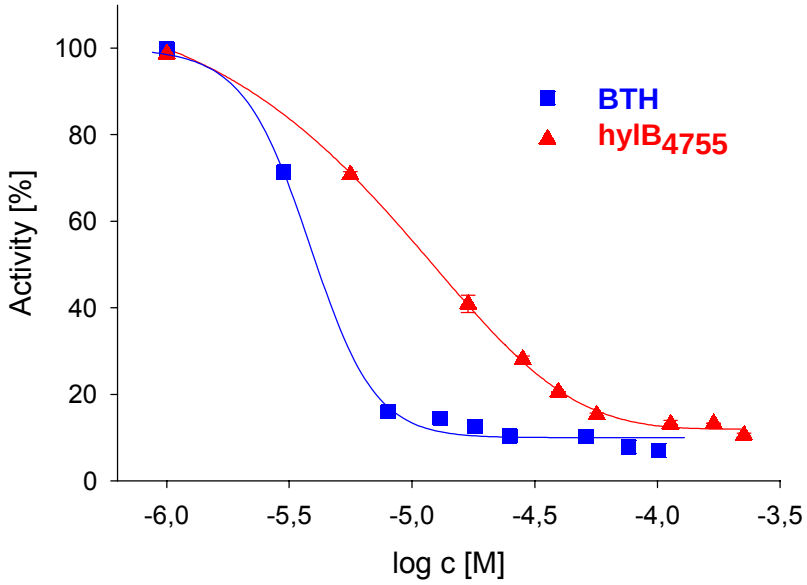


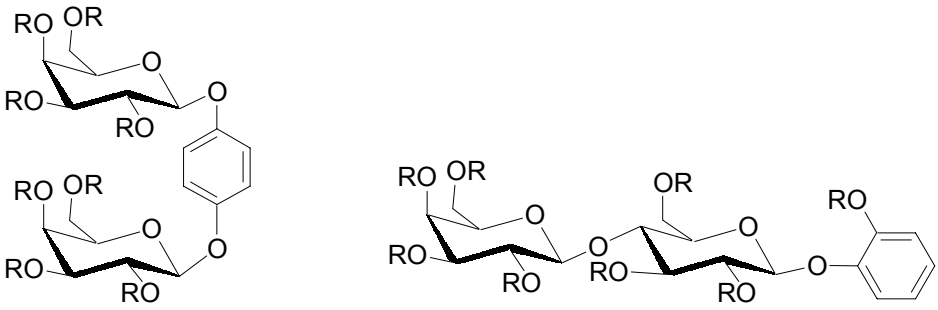
Fig. 8: Activities of BTH and $hylB_{4577}$ in the presence of the sulphated planteose derivative **15** with a degree of sulphation of approximately 1

In summary, the results obtained with neomycin and planteose derivatives support the idea that small oligosaccharides with a high degree of sulphation are the most promising compounds for further investigation.

2.4 Inhibition of hyaluronidases by the sulphates of hydrochinone digalactoside and 2-hydroxyphenyl monolactobioside

To investigate the influence of a planar ring system as spacer between monosaccharides or at the end of an oligosaccharide we tested the sulphated hydrochinone digalactoside (**16**) and the sulphated 2-hydroxyphenyl monolactobioside (**17**) (cf. Fig. 9). Compound **16** is composed by an aromatic ring be-

tween two galactose monomers, whereas compound **17** is composed by an aromatic ring at the end of a lactose unit.



16 **17**

Fig. 9: Structure of the sulphated hydrochinone digalactoside **16** and the sulphated 2-hydroxyphenyl monolactobioside **17** (R = H and/or SO_3Na)

The degrees of sulphation of the examined derivatives were 0.81 and 0.67 for compound **16** and **17**, respectively. The inhibitory activities determined on the bovine testicular, the bee venom and the bacterial hyaluronidase are summarised in Table 2.

Table 2: Effect of the sulphated hydrochinone digalactoside **16** and the sulphated compound **17** on the activities of hyaluronidases

	Compound		**BTH** IC_{50} **[µM]**[a]	**BVH** IC_{50} **[µM]**[a]	**hylB**$_{4755}$ IC_{50} **[µM]**[a]
No	R	DS	at pH = 3.6	at pH = 3.6	at pH = 5.0
16	R = H or SO_3Na	0.81	**15**	**6**	**80**
17	R = H or SO_3Na	0.67	**25**	n.d.	**350**

[a] the SEM values of the calculated IC_{50} values are in the range of 5-10%

The lowest inhibitory effects on the hyaluronidases were obtained for the sulphated compound **17**. The IC_{50} values of 2-hydroxyphenyl monolactobioside **17** were 25 µM determined on BTH and 350 µM on $hylB_{4755}$. The inhibitory activities of the sulphated hydroxychinone digalactoside **16** were also in the micromolar range with IC_{50} values of 6 µM (BVH), 15 µM (BTH) and 80 µM ($hylB_{4755}$). Whereas the inhibition of the bovine testicular enzyme by the two sulphated compounds were similar, the sugar derivative with the higher degree of sulphation (cf. compound **16** with DS = 0.81) showed a 4-fold increase of the inhibitory activity on the hyaluronate lyase.
The inhibitory activities of these two sulphated compounds, which possess a phenyl ring as structural element, are of the same potency as the investigated sulphated tri-, tetra- and pentasaccharides.

3. Summary

The results obtained in the present study suggest that sulphated sugar derivatives are useful as inhibitors of hyaluronidase, whereas the basic structures lacking sulphate groups are inactive. Furthermore, the degree of sulphation plays an important role on the inhibitory effect: the higher the degree of sulphation the higher the inhibitory activity. The major problem with the sulphated compounds is, that the sulphation results in mixture of reaction products, which are extremely difficult to separate. The degree of sulphation is a mean value and is not exactly known which hydroxy groups are sulphated and which are free. Compounds with sulphate groups in defined positions would be necessary for detailed structure-activity investigations.
As inhibitors of the bovine testicular and the bee venom hyaluronidase the sulphated oligosaccharides with three, four or five sugar units were the most active compounds. About the same activity was achieved with compounds having two sugar units and a phenyl ring. In summary, sulphated tri-, tetra-, pentasaccharides and/or sulphated disaccharides with a phenyl moiety are the most promising compounds for further investigations as inhibitors of BTH and BVH.
By contrast, in case of the hyaluronate lyase, the highest activity was found for the sulphated octasaccharide with a degree of sulphation of 0.35. It seems to

be, that an increase in the chain length of the oligosaccharides favours the inhibition of the hyaluronan lyases compared to the investigated hyaluronate hydrolases. The investigated sulphated saccharides were 100-500 times more active than the widely accepted hyaluronidase inhibitors apigenin (**1**) and kaempferol (**2**). In conclusion, oligosaccharides with sulphate groups in defined positions are necessary for further structure-activity studies.

4. Materials and Methods

4.1 Test compounds

The sulphated and non-sulphated oligosaccharides were kindly provided from *PD Dr. D. Paper* and *Dr. C. Käsbauer* (Institute of Pharmacy, University of Regensburg, Germany). The compounds were isolated, modified and analysed as described elsewhere (Käsbauer 1999; Käsbauer et al. 2001).

4.2 Enzymes and chemicals

Hyaluronic acid (HA) from rooster comb and bovine serum albumin (BSA) were purchased from Serva (Heidelberg, Germany). The investigated hyaluronidases were enzyme preparation from different sources.

Hylase® "Dessau", containing 1 500 IU (according to the declaration of the supplier) of lyophilised bovine testicular hyaluronidase (BTH) per vial was a gift from Pharma Dessau (Dessau, Germany).

Stabilised hyaluronate lyase, i.e., 200 000 IU (according to the declaration of the supplier) of lyophilised hyaluronidase (0.572 mg) from *Streptococcus agalactiae*, strain 4755 ($hylB_{4755}$), plus 2.2 mg BSA and 37 mg Tris-HCl per vial was kindly provided by id-Pharma (Jena, Germany).

Bee venom hyaluronidase (BVH) (Gmachl et al. 1993; Soldatova et al. 1998) was a gift from Dr. Zora Marcovic-Housley, University of Basel (Switzerland).

All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

4.3 Determination of enzyme inhibition

Hyaluronidase activity was quantified by the method of *Reissig et. al*. (Reissig et al. 1955) based on the Morgan-Elson reaction, a reaction between *N*-acetyl-D-glucosamine (NAG) at the reducing ends of sugars liberated from hyaluronic acid and *N,N*-dimethylaminobenzaldehyde. The assay was performed as described elsewhere in detail (Muckenschnabel et al. 1998; Muckenschnabel et al. 1998). The test compounds, dissolved in DMSO (0.1 µM - 5 mM), were incubated at 37 °C in an incubation mixture containing 60 µl of citrate-phosphate buffer (solution A: 0.1 M Na_2HPO_4 / 0.1 M NaCl, solution B: 0.1 M citric acid / 0.1 M NaCl; solution A and B were mixed in appropriate portions to adjust the required pH), 40 µl BSA (0.2 mg BSA per ml of water), 20 µl substrate solution (5 mg hyaluronic acid from rooster comb per ml of water) and 20 µl enzyme solution (equiactive concentration: 20 IU BTH, 1 IU $hylB_{4755}$ and 19 IU BVH). The pH of the incubation mixture was adjusted to the pH optimum of the enzymes (BTH and BVH pH = 3.6, $hylB_{4755}$ pH = 5.0).

The enzyme reaction was stopped by addition of alkaline borate solution and subsequent heating for 4.5 min in a boiling water bath. The alkaline borate solution was prepared immediately before use from the borate solution (17.3 g H_3BO_4 and 7.8 g KOH in 100 ml water) and the potassium carbonate solution (8.0 g K_2CO_3 in 10 ml water). After cooling on ice for 1 min 600 µl of *N,N*-dimethylaminobenzaldehyde (20.0 g *N,N*-dimethylaminobenzaldehyde dissolved in 25 ml concentrated hydrochloric acid and 75 ml glacial acetic acid; the solution was diluted with 4 volumes of glacial acetic acid immediately before use) was added and the mixture was incubated at 37 °C for 20 min. The solution was transferred to cuvettes or 96 well plates and the absorbance of the coloured product was measured with an Uvicon 930 UV spectrophotometer (Kontron, Eching, Germany) at 590 nm and with an automatised EL312E (BIO-TEK^R

Instruments INC., Highland Park, Winooski) microplate reader at 590 nm respectively.

Enzyme activity was calculated from the formation of the red coloured product measured at 586 nm. The effect of the inhibitors on the enzyme activity was calculated according to the equation:

$$A\ \% = (B - C) / (D - E)$$

A: calculated enzyme activity

B: absorbance of the incubation mixture containing inhibitor

C: absorbance of the incubation mixture containing inhibitor in absence of the enzyme (enzyme solution replaced with buffer)

D: absorbance of the incubation mixture in absence of the inhibitor (inhibitor solution replaced with DMSO)

E: absorbance of the incubation mixture in absence of both enzyme and inhibitor (enzyme solution replaced with buffer, inhibitor solution replaced with DMSO)

The $IC_{50} \pm$ SEM values were calculated using the standard curves analysis of SigmaPlot^{TM} (version 8.0) and are the means of three independent experiments performed in duplicate.

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Chapter 5

Design and synthesis of a substrate analogue as a potential inhibitor of hyaluronate lyases

1. Introduction

By cleaving hyaluronan, bacterial hyaluronate lyases, e. g. that of *S. agalactiae* (hylB$_{4755}$), facilitate the spreading of the microorganisms and their toxins in infectious diseases such as meningitis and septicaemia (Hynes et al. 2000). Potent inhibitors of hyaluronan lyases could be useful in studying the role of hyaluronan and hyaluronidases in bacterial infection. As part of our project on hyaluronate lyase inhibitors, the design of a substrate analogue as a potential inhibitor was envisaged.

The degradation of hyaluronic acid by bacterial hyaluronate lyases occurs at the β-1,4-glycosidic linkage between D-glucuronic acid and *N*-acetyl-D-glucosamine. The product of this elimination reaction is the unsaturated disaccharide yield 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyluronic acid)-*D*-glucose (Kreil 1995; Pritchard et al. 2000). Recently, the mechanism of the elimination reaction by *S. agalactiae* strain 3502 hyaluronate lyase (hylB$_{3502}$) was revealed (Jedrzejas et al. 2000; Li et al. 2001; Jedrzejas 2002). The active centre of hylB$_{3502}$ is composed of two main parts, a catalytic group responsible for the substrate degradation and an aromatic patch responsible for the selection of cleavage sites on the substrate chains (Li et al. 2000). The residues that form the aromatic patch of the enzyme are Trp371, Trp372 and Phe423, those of the catalytic group are His479, Tyr488 and Asn429.

The mechanism of the hyaluronan lyase reaction was postulated by *S. Li* and *M. J. Jedrzejas* (Li et al. 2001) as follows: In the first step, the positively charged cleft of the enzyme attracts and binds the negatively charged substrate

chain, whereby three disaccharide units can be accommodated into the cleft (cf. Fig. 1, only two of the three disaccharide units are drawn HA1 and HA2). In the second step, the aromatic patch of the active site interacts with the substrate chain and anchors it right position. In the third step, the glucuronic acid of HA1 is deprotonated at C-5 by His479 (cf. Fig. 1). At the same time, Tyr488 donates a proton to the glycosidic oxygen O-4 connecting D-glucuronic acid of HA1 and *N*-acetyl-D-glucosamine of HA2. The glycosidic bond is cleaved by 1,2-elimination forming the double bond of the unsaturated final product (fourth step). Finally (fifth step), the catalytic triad is regenerated: His479 loses its acquired proton and Tyr488 attracts a proton from the surrounding water molecules. Thus, the enzyme returns to the original state and a next cycle of catalysis can be started. All five steps of the mechanism are crucial for the degradation of hyaluronic acid. The protein acts as a proton exchanger, because the protein accepts one proton from the substrate and donates one proton from water molecules to the substrate to break the 1,4-β glycosidic bond in hyaluronan.

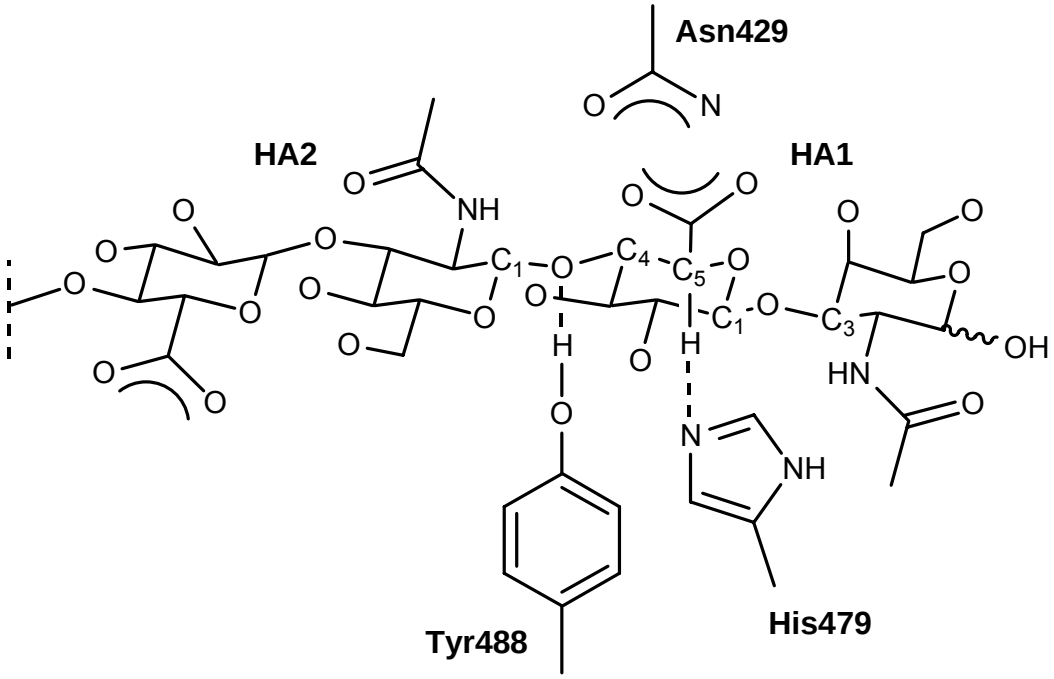


Fig. 1: Mechanism of the hyaluronan degradation by hyaluronate lyase according to *Li et al*. (Li et al. 2001). Schematical representation of the hyaluronic acid with HA1 and HA2 as disaccharide units and the position of the amino acids residues Tyr488, His497 and Asn429 relative to the substrate.

The *S. agalactiae* strain 4755 hyaluronate lyase (hylB$_{4755}$) shows extensive sequence identity of 98 % to the homologous enzyme of *S. agalactiae* strain 3502 (Baker et al. 2000; Ponnuraj et al. 2000). Even the whole structural architecture and the active site geometry are quite similar, thus the catalytic mechanism suggested for hylB$_{3502}$ is likely to be identical to that of hylB$_{4755}$.

Based on this mechanistical study of the hyaluronan degradation, we designed a substrate analogue as a potential inhibitor. One important step of this mechanism is the removal of the proton at C-5 of the glucuronic acid by the amino acid residues His479. By preventing the elimination of this proton, the cleavage of the substrate would be blocked. This could be achieved by replacing the hydrogen atom with a fluorine atom. As known, such substitutions (F vs. H) are commonly used for the development of enzyme inhibitors. To cleave the C-F bond an extra energy is required compared to the cleavage of the C-H bond. By introducing the fluorine atom at C-5 the binding properties of the whole molecule should be comparable to those of the substrate. As only the first three saccharide units of hyaluronan are directly involved in the cleavage, these three units were chosen as basic structure of our approach. The structure of the proposed inhibitor deduced from the hyaluronan structure in consideration of the cleavage mechanism, is shown in Fig. 2.

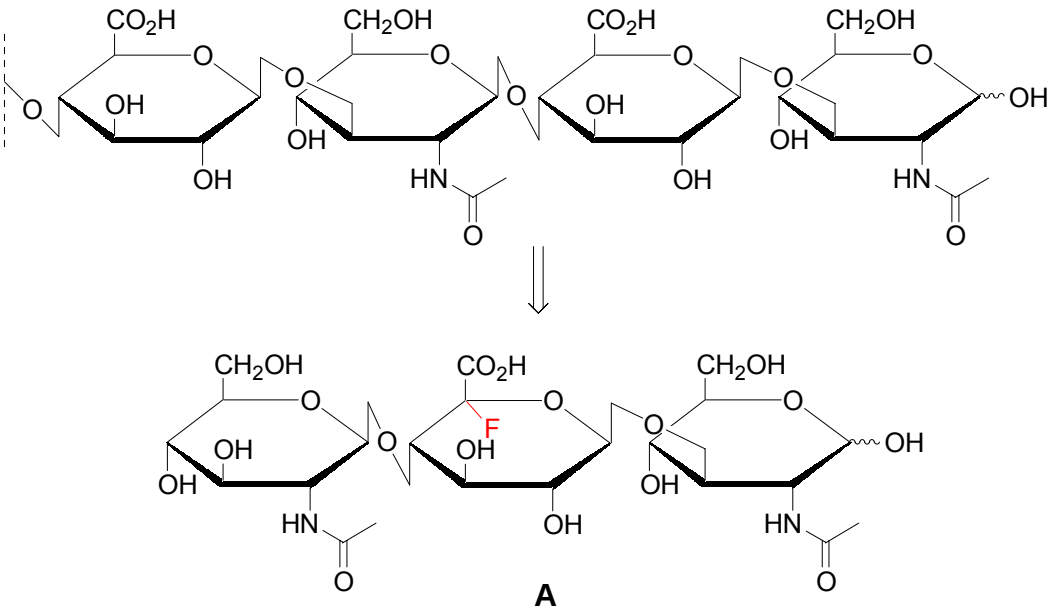


Fig. 2: Design of a fluorinated hyaluronan analogue as a potential substrate-like inhibitor of hyaluronate lyase

2. Synthetic strategies and chemistry

For the synthesis of the trisaccharide **A**, a general straightforward synthetic route had to be developed. Flanked by two *N*-acetylglucosamine units (**Ia** and **Ib**) D-glucuronic acid with a fluoro substituent in position 5 (**II**) is the central and most important building block (cf. Fig. 3). To synthesise the substrate analogue, it is necessary to protect the sugar monomers in specific positions, then to connect the three compounds to the trisaccharide and finally to cleave the protecting groups (PG) to get the desired product. To achieve the substantial structural similarity to the substrate, the linkage should be β-1,4 between *N*-acetyl-D-glucosamine and D-glucuronic acid and β-1,3 between D-glucuronic acid and *N*-acetyl-D-glucosamine (cf. Fig. 2 and Fig. 3).

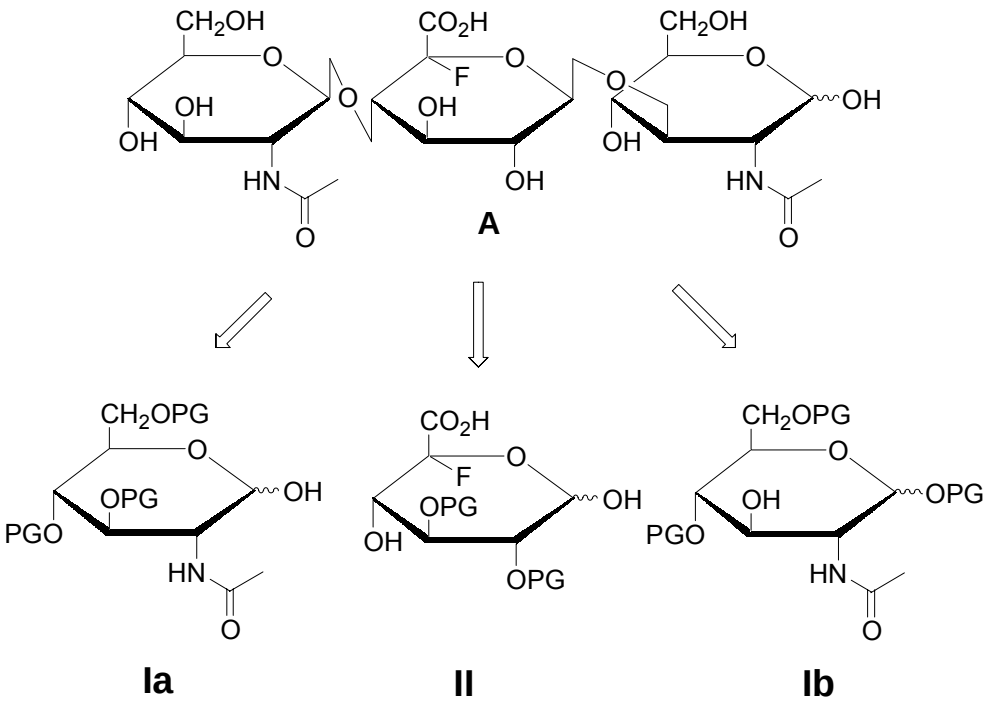
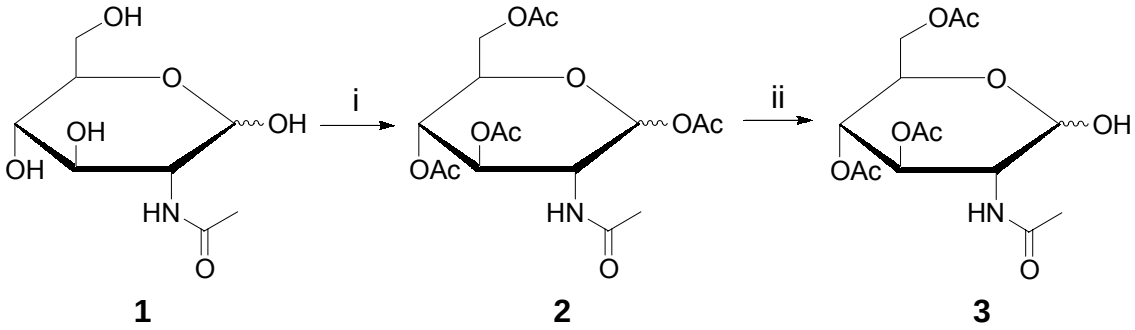


Fig. 3: First retrosynthesis considerations of the substrate analogue 2-acetylamino-2-deoxy-β-D-glucopyranosyl-(1→4)-5-fluoro-β-D-glucopyranuronosyl-(1→3)-2-acetylamino-2-deoxy-D-glucupyranose

2.1 Synthesis of the building blocks Ia and Ib

As the building block **Ia** has to be β-1,4 linked with the second monomer we protected *N*-acetyl-D-glucosamine (**1**) in the positions 3, 4 and 6 and kept position 1 unprotected for the linkage. The acetyl and the benzyl protecting groups were selected for the protection of *N*-acetyl-D-glucosamine (**1**) groups. The advantages of these two protecting groups are synthetic feasibility, mild cleavage conditions and stability during linkage reactions.

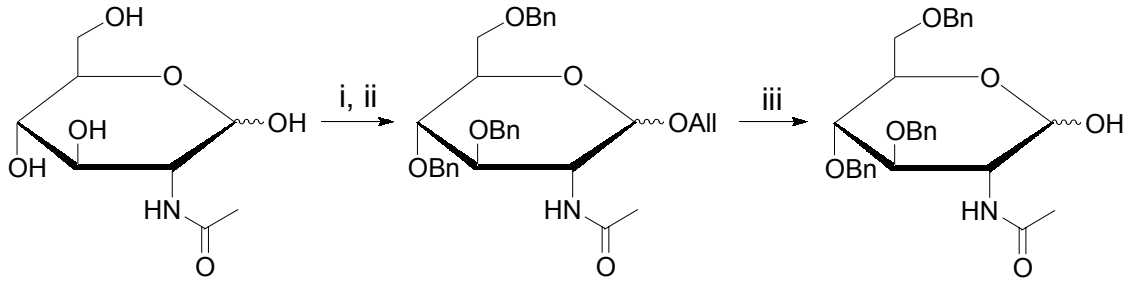
3,4,6-Tri-*O*-acetyl-2-acetylamino-2-deoxy-D-glucopyranose (**3**) was prepared in two steps (cf. Scheme 1). First, *N*-acetyl-D-glucosamine (**1**) was totally acetylated with acetic anhydride and sodium acetate at high temperature. In the second step, the acetyl group must be cleaved selectively in position 1 of compound **2**. This reaction was carried out with benzylamine in anhydrous tetrahydrofuran according to the method of *M. Sim et al.* (Sim et al. 1993).



1 **2** **3**

Scheme 1: Synthesis of 3,4,6-Tri-*O*-acetyl-2-acetylamino-2-deoxy-D-glucopyranose (**3**); reaction conditions: i) Ac_2O, NaOAc, ΔT; ii) $BnNH_2$, THF

To synthesise 2-acetylamino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranose (**5**) a different strategy was used starting with allyl protection of the hydroxy group in position 1, followed by benzylation of the remaining hydroxy groups and finally cleavage of the allyl ether (cf. Scheme 2).



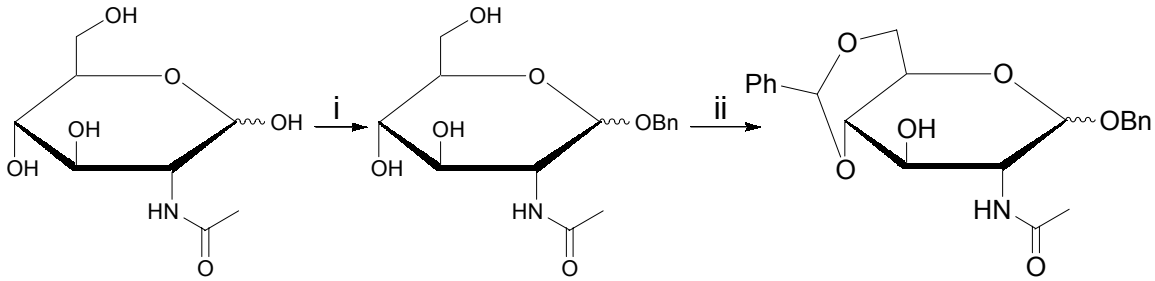
1 **4** **5**

Scheme 2: Synthesis of 2-acetylamino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranose (**5**); reaction conditions: i) AllOH, BF_3*Et_2O; ii) BnBr, KOH, THF; iii) $PdCl_2$, AcOH, NaOAc;

The allylation of the hydroxy group in position 1 with allyl alcohol and BF_3*Et_2O was carried out according to a method described by *A. Vasella et al.* (Vasella et al. 1991) with some modifications. The subsequent benzylation of the residual hydroxy groups was realised with benzyl bromide in anhydrous THF and potassium hydroxide as base according to the general procedure described by of *M. Shaban et al.* (Shaban et al. 1976). For the conversion of compound **4** to compound **5** by cleaving the allyl ether different standard methods were tried. The deprotection with ^{t}BuOK in DMSO at 60 °C and than I_2 in a bi-phase system (THF/H_2O) (Granier et al. 1998) did not lead to the desired product. As described by *Mereyala et al.* (Mereyala et al. 1993), the cleavage of the allyl ether should be achieved with $PdCl_2$ and CuCl, however, this reaction failed, too. Finally, 2-acetylamino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranose (**5**) was obtained by the deprotection of compound **4** with $PdCl_2$ under acidic conditions based on the method described by *S. Figueroa-Perez et al.* (Figueroa-Perez et al. 2000).

The building block **Ib** required for the preparation of the trisaccharide is again *N*-acetyl-D-glucosamine which should be β-1,3 linked with the fluorinated glucuronic acid **II**. Therefore, *N*-acetyl-D-glucosamine (**1**) should be protected in positions 1, 4 and 6, whereas the hydroxy group in position 3 should remain non-protected for the linkage.

The benzyl and the benzylidene groups were selected for the protection of *N*-acetyl-D-glucosamine to get benzyl 2-acetylamino-4,6-*O*-benzylidene-2-deoxy-D-glucopyranoside (**7**) (cf. Scheme 3). The advantages of these two protecting groups are the synthetic feasibility, the mild cleavage conditions and the stability during linkage reactions. The synthesis of compound **7** was carried out in two steps as shown in Scheme 3. In the first step the semi-acetal in position 1 of *N*-acetyl-D-glucosamine (**1**) was quantitatively converted to the acetal with benzyl alcohol in acetyl chloride according to the method of *Sutherlin et al.* (Sutherlin et al. 1997). Subsequently, the 4,6-benzylideneacetal **7** was obtained by treating compound **6** with $PhCH(OCH_3)_2$ and p-TsOH as catalyst in acetonitrile according to the method of *Aguilera et al.* (Aguilera et al. 1997) with some modifications.



1 **6** **7**

Scheme 3: Synthesis of benzyl 2-acetylamino-4,6-*O*-benzylidene-2-deoxy-D-glucopyranoside (**7**); reaction condition: i) BnOH, AcCl; ii) $PhCH(OCH_3)_2$, acetonitrile, p-TsOH

2.2 Synthesis of the fluorinated D-glucuronic acid II

As 5-fluoro-D-glucuronic acid has to be linked with two *N*-acetylglucosamine molecules in position 1 and 4, the fluorinated compound should be protected in the positions 2 and 3, whereas the hydroxy groups in the positions 1 and 4 should be ether free for linkage or protected with a group that can be selectively cleaved before linkage (cf. structure **II**).

Two different strategies were envisaged for the synthesis of this building block (cf. Fig. 4).

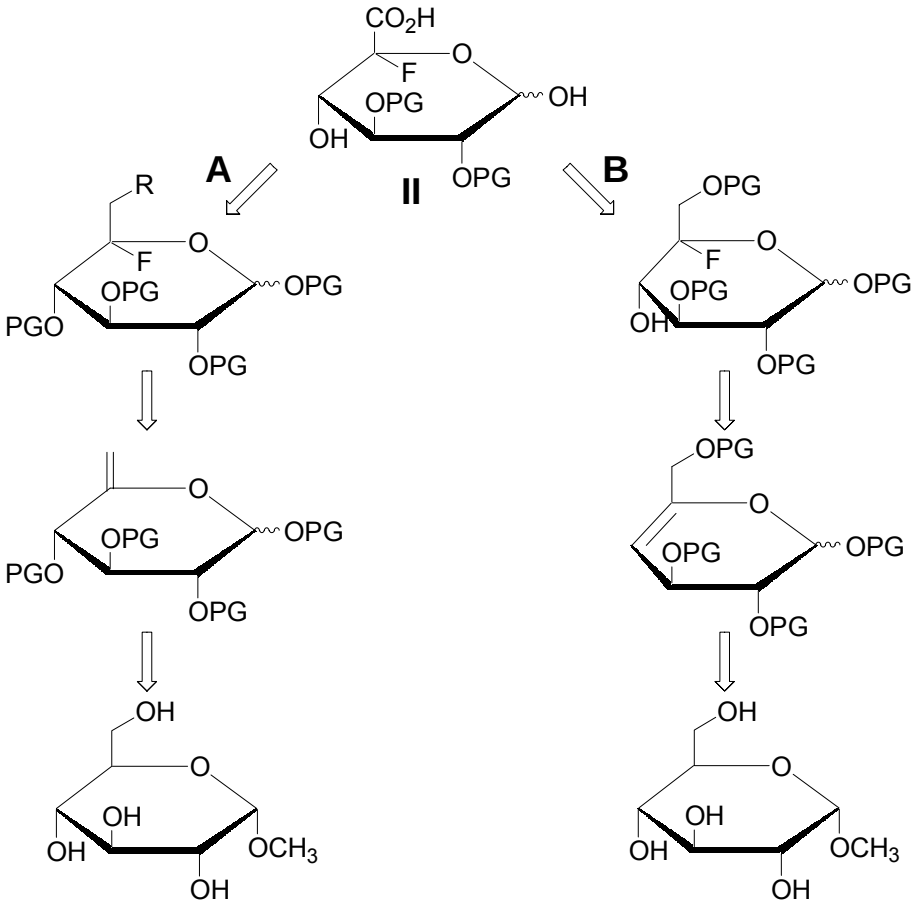


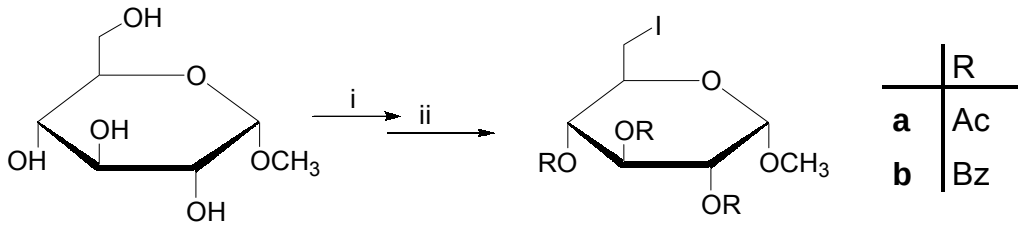
Fig. 4: Retrosynthetic routes for the synthesis of the fluorinated D-glucuronic acid

Starting from the commercially available methyl α-D-glucopyranoside according to route **A**, the double bond between the carbon atoms 5 and 6 will be introduced by an elimination reaction. By subsequent fluorination of this alkene, the fluorine atom would be introduced in position 5. Finally, the oxidation of the carbon atom in position 6 would lead to the desired fluorinated glucuronic acid, which can be linked with both other carbohydrate monomers **Ia** and **Ib**.

According to route **B**, the fluorine atom would be introduced by fluorination of an intermediate with C=C double bond in position 4. The pertinent alkene will be synthesised by an elimination reaction starting from the commercially available methyl α-D-glucopyranoside, too. The carboxylic acid in position 6 should be obtained by oxidation reaction.

Synthetic route A

The key reactions of route **A** are elimination, fluorination and oxidation. The elimination of HI on the protected 6-iodo-α-D-glucose derivative was the first method tried to introduce an alkene function. Therefore, the hydroxy group in position 6 of methyl α-D-glucopyranoside (**8**) was selectively substituted by iodine and the remaining hydroxy groups were subsequently acetylated or benzoylated. The iodination of compound **8** was successfully performed by treating the starting material with *N*-iodosuccinimide and triphenylphosphine in DMF (Garegg et al. 1978) or with iodine, triphenylphosphine and imidazole in acetonitrile (Garegg et al. 1982). The subsequent acetylation or benzoylation of the iodinated intermediate were carried out under standard conditions with acetic anhydride and sodium acetate or benzoyl chloride and pyridine, respectively to yield the products **9a** and **9b** (cf. Scheme 4).



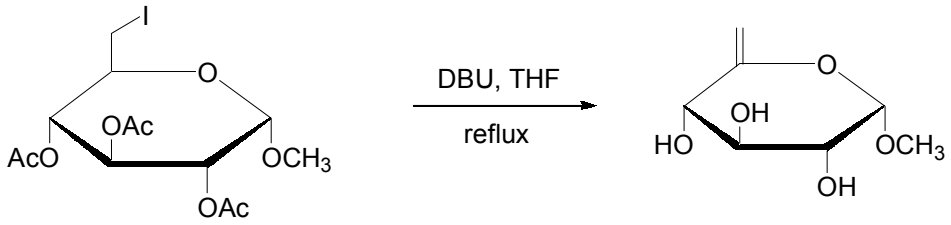
8 **9a, b**

Scheme 4: Synthesis of the protected methyl 6-deoxy-6-iodo-α-D-glucopyranoside **9a** and **9b**; reaction conditions: for **9a** i) NIS, Ph_3P, DMF; ii) Ac_2O, NaOAc; for **9b** i) I_2, Ph_3P, imidazole; ii) BzCl, pyridine

Due to difficult separation of the product from starting materials and low yield, an alternative route was envisaged: first protection of the hydroxy groups in the positions 2, 3 and 4 and then substitution of position 6 with iodine. Several methods were tried for this alternative route (Garegg et al. 1978; Garegg et al. 1982; Mirza et al. 1985), however, neither the acetylated glucose derivative nor the benzoylated compound could be iodinated. Therefore, the route shown in Scheme 4 was applied despite the aforementioned disadvantages.

In the next step the elimination of HI was accomplished according to the method described by *Mirza et al.* (Mirza et al. 1985) using 1,8-diazabicyclo-

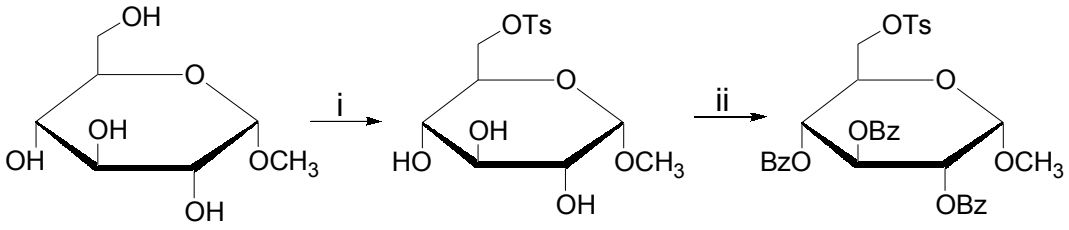
[5.4.0]undec-7ene (DBU) in THF. Compound **9a** could be converted to compound **10** by elimination and deacetylation, whereas the reaction did not work with the compound **9b** and could not be improved by variation of the reaction conditions (cf. Scheme 5).



9a **10**

Scheme 5: Synthesis of methyl 6-deoxy-α-D-*xylo*-hex-5-enopyranoside (**10**)

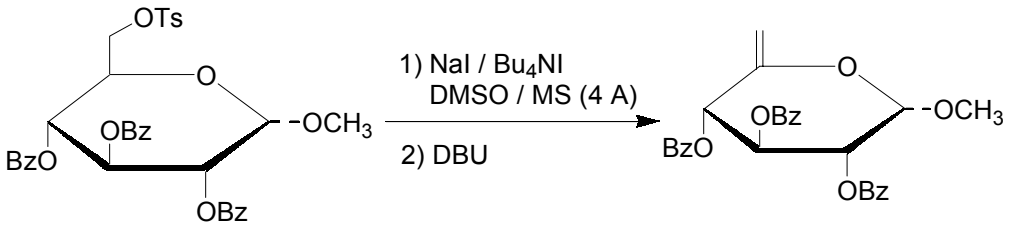
An alternative synthetic route for the synthesis of the alkene derivative started from the 6-tosylated glucose derivative **11**, which was prepared by treating methyl α-D-glucopyranose (**8**) with tosyl chloride in pyridine (Cramer et al. 1959). Then, methyl 2,3,4-tri-*O*-benzoyl-6-*O*-tosyl-α-D-glucopyranoside (**12**) was prepared by a benzoylation of compound **11** under standard conditions with benzoyl chloride and pyridine (cf. Scheme 6).



8 **11** **12**

Scheme 6: Protection of methyl α-D-glucopyranoside (**8**) with the tosyl protecting group in position 6 and the benzoyl group in the positions 2, 3 and 4; reaction conditions: i) TsCl, pyridine, 0 °C → RT; ii) BzCl, pyridine, RT

Finally, compound **12** was treated with sodium iodide, tetrabutylammonium iodide, molecular sieve and DBU in DMSO to give the unsaturated glucose derivative **13** (cf. Scheme 7) (Sato et al. 1988; Sato et al. 1991; Mereyala et al. 1993; Sato et al. 1993).



12 **13**

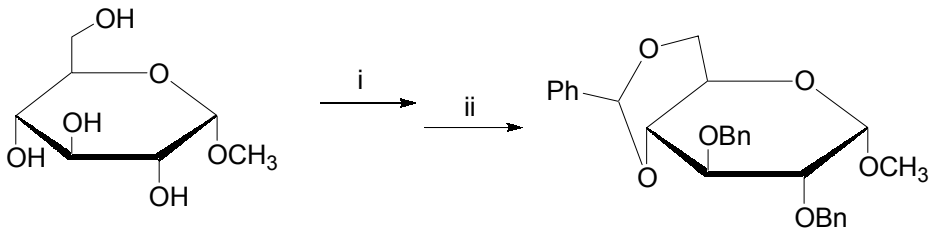
Scheme 7: Synthesis of methyl 2,3,4-tri-*O*-benzoyl-6-deoxy-α-D-*xylo*-hex-5-enopyranoside (**13**)

Following this synthetic route, the elimination product **13** could be obtained in a three step reaction instead of a four step reaction compared to the first strategy. Furthermore, the handling and the purification of the synthesised product were much more convenient.

The crucial step of the synthetic route **A** is the introduction of the fluorine atom in position 5 of the D-*xylo*-hex-5-eno-pyranoside **13**. In the literature, several methods are published for the introduction of a fluorine atom by an addition reaction. From the list of commercially available reagents 1-fluoropyridinium tetrafluoroborate, 1-fluoro-2,4,6-trimethylpyridinium triflate, *N*-fluorobenzenesulfonimide, selectfluor^{TM} and silver fluoride were the most common fluorinating agents (Maguire et al. 1993; Burkart et al. 1997; Albert et al. 1998; Vincent et al. 1999). With exception of silver fluoride, all other fluorination agents introduce the fluorine atom in position 6 instead of position 5, i.e. not adjacent to the oxygen atom. *Maguire et al.* (Maguire et al. 1993) reported the iodofluorination of an adenosine derivative with silver fluoride and iodine in acetonitrile at - 40 °C. All attempts to transfer this method to the preparation of the 5-fluorinated hexose derivative were unsuccessful. Variation of the reaction temperature from – 20 °C over – 10 °C to 0 °C or room temperature did not lead to the fluorinated compound.

Synthetic route B

In parallel to route **A**, the synthesis of the fluorinated D-glucuronic acid **II** via route **B** was explored. The hydroxy groups in position 4 and 6 of methyl α-D-glucopyranoside (**8**) were protected with benzaldehyde dimethyl acetal and camphorsulphonic acid in DMF according to a method of *Mallet et al.* (Mallet et al. 1993) with some modifications. The subsequent protection of the remaining hydroxy groups was carried out with benzyl bromide and sodium hydroxide to give methyl 2,3-di-*O*-benzyl-4,6-*O*-benzyliden-α-D-glucopyranoside (**14**) (cf. Scheme 8).



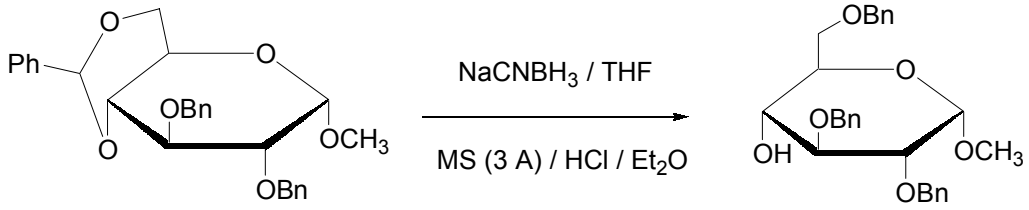
8 **14**

Scheme 8: Formation of the 4,6-benzylidene acetal and subsequent benzylation of positions 2 and 3; reaction conditions: i) $PhHC(OCH_3)_2$, DMF, camphorsulfonic acid; ii) BnBr, DMF, NaH

The reductive opening of benzylidene acetals using lithium aluminium hydride-aluminium chloride or sodium cyanoborohydrate has been reported (Bhattacharjee et al. 1969; Liptak et al. 1975; Garegg et al. 1981; Gelas 1981; Johansson et al. 1984). For 4,6-benzylidene acetals with bulky substituents such as benzyl groups at position 3, the regioselectivity of the dioxane ring opening is dependent on the reducing agents. By the reaction with $LiAlH_4/AlCl_3$ the benzyl group is directed to position 4 and position 6 remains free (Liptak et al. 1975), whereas with $NaCNBH_3$/HCl and $NaCNBH_3$/TFA the inverse result is achieved (Garegg et al. 1981; Garegg et al. 1982; Johansson et al. 1984).

To synthesise the sugar derivative **15** with the benzyl group in position 6, the $NaCNBH_3$ method was chosen. According to *Johansson et al.* (Johansson et al. 1984) the regioselective reductive ring-opening of the benzylidene acetals

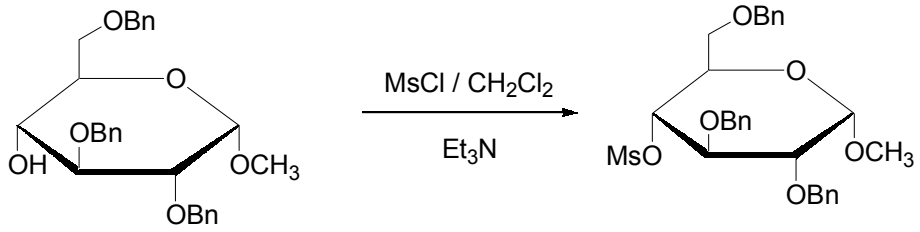
should be achieved with $NaCNBH_3$/TFA. Unfortunately, this reaction failed. However, a modified procedure (Garegg et al. 1981; Garegg et al. 1982) working at room temperature instead of 0 °C, changing the 3 Å molecular sieve from powdered to spherical shape and by extending the reaction time from 10 min to 16 h, gave compound **15** (cf. Scheme 9).



14 **15**

Scheme 9: Regioselective deprotection at position 4 by cleavage of the benzylidene acetal $NaCNBH_3$ under acidic conditions

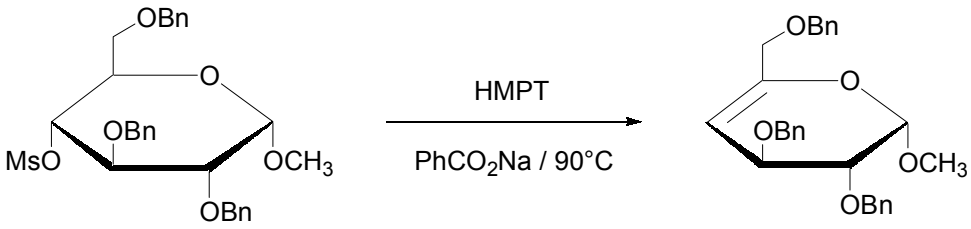
With respect to the introduction of a C=C double bond, the hydroxy group in position 4 was mesylated to obtain an efficient leaving group. Methyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (**15**) was treated with mesyl chloride and triethylamine in anhydrous dichloromethane according to a method described by *Bernet et al.* (Bernet et al. 1979) with some modifications (cf. Scheme 10).



15 **16**

Scheme 10: Synthesis the methane sulphonate **16**

For the subsequent elimination, methyl 2,3,6-tri-*O*-benzyl-4-mesyl-α-D-glucopyranoside (**16**) was heated with sodium benzoate in hexamethylphosphoric triamide (Gill et al. 1971) to give methyl 2,3,6-tri-O-benzyl-4-deoxy-β-L-*threo*-hex-4-enopyranoside (**17**).



16 **17**

Scheme 11: Synthesis of methyl 2,3,6-tri-O-benzyl-4-deoxy-β-L-*threo*-hex-4-enopyranoside (**17**)

The crucial step of the synthetic route **B** is similar to that of route **A**: the introduction of the fluorine atom in position 5. The fluorinated compound should be synthesised by fluorination of the double bond of compound **17**. The application of the fluorination methods described for route **A**, failed again. Obviously, the introduction of a fluorine atom at the carbon atom 5 adjacent to an oxygen atom is impossible by an addition reaction with fluorination agents.

3. Summary

Based on mechanistical studies of the hyaluronan degradation by hyaluronate lyases, we designed a substrate analogue consisting of three carbohydrate units including a fluorinated glucuronic acid as central building block as a potential inhibitor. The synthesis of the pertinent protected *N*-acetylglucosamine was successful, whereas the attempts to synthesise the fluorinated glucuronic acid failed. As the problems with the fluorination of hex-4-enopyranoside and hex-5-enopyranoside derivatives were not conquerable an alternative strategy should be envisaged, for instance, the synthesis of mechanism-based non-carbohydrate hyaluronidase inhibitors.

4. Experimental section

4.1 General conditions

- Starting materials and solvents were purchased from Acros Organics (Belgium), Lancaster Synthesis GmbH (Germany), Maybridge Chemical Company (United Kingdom), Sigma-Aldrich Chemie GmbH (Germany), Merck (Germany).

- Column chromatography was carried out using Merck Kieselgel 60 (0.063-0.200) and thin layer chromatography (TLC) was performed with Merck Kieselgel 60 F_{254} aluminium.

- Melting points (Mp) were determined on a BÜCHI 510 electrically heated copper block apparatus using an open capillary and are uncorrected.

- Elemental analysis were carried out by the department of microanalysis Regensburg. Compounds were dried in vacuo (0.1-1 Torr) at room temperature or with heating up to 80 °C for at least of 24 h prior to submission for elemental analysis.

- Infrared spectra (IR) were recorded on a BRUKER TENSOR 27 spectrophotometer. The wave number is given in cm^{-1}.

- Mass spectrometry analysis (MS) were performed on a Varian MAT 112 (PI-EIMS 70 eV) and on a Varian MAT 95 ($^{\oplus}$FAB-MS: methanol, glycerin, xenon) spectrometer. The peak-intensity is indicated relatively to the strongest signal in %.

- Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded using a Bruker AC-250 or ARX-300 or ARX-400 NMR spectrometer with per-deuterated dimethyl sulphoxide (DMSO-d_6) or deuterated chloro-

form ($CDCl_3$). The chemical shift δ is given in parts per million (ppm) with reference to the chemical shift of the residual protic solvent compared to tetramethylsilane (TMS, δ = 0 ppm). "s" indicates a singlet, "d" a doublet, “dd” a doublet of doublet, "t" a triplet, “q” a quartet, "m" a multiplet and "br" a broad peak. The multiplicity of carbon atoms (^{13}C-NMR) were determined by DEPT 135 and DEPT 90 (distortionless enhancement by polarisation transfer): “+” primary and tertiary carbon atom (positive DEPT 135 signal), “-“ secondary carbon atom (negative DEPT 135 signal), “C_{quart}” quaternary carbon atom.

4.2 Chemistry

1,3,4,6-Tetra-*O*-acetyl-2-acetylamino-D-glucopyranoside (2)

A solution of *N*-acetyl-D-glucosamine (5.0 g, 22.60 mmol), sodium acetate (2.22 g, 27.12 mmol) and acetic anhydride (22 ml) was stirred and heated at 110 °C for 30 min. After cooling to 60 °C, the reaction mixture was carefully poured into ice water (50 ml) and extracted with chloroform (3 x 30 ml). The combined organic layers were washed three times with a saturated solution of sodium hydrogen carbonate (30 ml) and three times with water (30 ml), dried over sodium sulphate, and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a white crystalline solid.

Yield: 8.55 g (21.96 mmol, 97 %, white crystalline solid)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 1.92 (s, 3H, C$\underline{H}_3$), 2.05 (s, 3H, C$\underline{H}_3$), 2.10 (s, 3H, C$\underline{H}_3$), 2.13 (s, 3H, C$\underline{H}_3$), 2.24 (s, 3H, C$\underline{H}_3$), 3.96-4.12 (m, 2H, C$\underline{H}_2$OH), 4.18-4.30 (m, 1H, H-5), (4.40-4.55 (m, 1H, H-4), 5.15-5.28 (m, 2H, H-2, H-3), 5.60 (d, 1H, ^{3}J = 7.4 Hz, NH), 6.20 (d, 1H, ^{3}J = 3.5 Hz, H-1)

$C_{16}H_{23}NO_{10}$ (389.35)

3,4,6-Tri-*O*-acetyl-2-acetylamino-2-deoxy-D-glucopyranose (3)

A solution of compound **2** (1.0 g, 2.57 mmol), benzylamine (0.41 g, 3.86 mmol) and anhydrous tetrahydrofuran (15 ml) was stirred at ambient temperature for 16 h. The reaction mixture was diluted with water (30 ml) and extracted three times with chloroform (50 ml). The combined organic layers were washed with diluted hydrochloric acid (1N, 1 x 50 ml), a saturated solution of sodium hydrogencarbonate (1 x 50 ml), a saturated solution of sodium chloride (1 x 50 ml) and water (1 x 50 ml) and dried over sodium sulphate. The solvent was removed in vacuo. The product was purified by column chromatography on silica gel eluting with ethyl acetate to give a colourless oil.
Yield: 0.23 g (0.66 mmol, 26 %, colourless oil)

^{1}H-NMR ($CDCl_3$):
δ [ppm] = 1.97 (s, 3H, C$\underline{H}_3$), 2.03 (s, 3H, C$\underline{H}_3$), 2.04 (s, 3H, C$\underline{H}_3$), 2.10 (s, 3H, C$\underline{H}_3$), 4.11-4.17 (m, 1H, H-5), 4.18-4.29 (m, 2H, C$\underline{H}_2$OH), 4.30-4.45 (m, 1H, H-2), 5.10-5.17 (m, 1H, H-3), 5.26-5.27 (m, 1H, H-1), 5.30-5.34 (m, 1H, H-4), 5.94 (d, 1H, ^{3}J = 7.4 Hz, N$\underline{H}$)

$C_{14}H_{21}NO_9$ (347.32)

1-*O*-Allyl-2-acetylamino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranoside (4)

To a solution of *N*-acetyl-2-amino-α-D-glucopyranoside (5.0 g, 22.60 mmol) in allyl alcohol (44.15 g, 0.76 mol), BF_3*Et_2O (5.13 g, 36.16 mmol) was added in one portion and stirred under a nitrogen atmosphere at 95 °C for 4.5 h. After evaporation of the solvent in vacuo, the residue was diluted with *N,N*-dimethylformamide (50 ml), treated with benzyl bromide (13.92 g, 81.36 mmol) and cooled with an ice bath to 0 °C. Sodium hydride (60 % in mineral oil, 2.98 g corresponding to 74.58 mmol NaH) was added in small portions, then the reaction mixture was stirred at room temperature for 15 h, treated with methanol (100 ml) and again stirred for 1 h. After the reaction mixture was concentrated and the residue was purified by chromatography on a silica gel column eluting

with a 1:2 (v/v) mixture of petrol ether 60-80 °C and ethyl acetate to give a colourless oil.

Yield: 1.44 g (2.71 mmol, 12 %, colourless oil)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 2.06 (s, 3H, C$\underline{H}_3$), 3.54-3.86 (m, 5H, H-2, H-4, H-5, C$\underline{H}_2$OH), 3.90-4.02 (m, 1H, H-3), 4.02-4.20 (m, 2H, C$\underline{H}_2$CH=CH$_2$), 4.23-4.34 (m, 1H, H-1), 4.50-4.98 (m, 6H, $C_6H_5C\underline{H}_2$), 5.15-5.22 (m, 1H, NH), 5.23-5.38 (m, 2H, CH$_2$CH=C$\underline{H}_2$), 5.76-5.91 (m, 1H, CH$_2$C$\underline{H}$=CH$_2$), 7.14-7.40 (m, 15H, C_6H_5)

$C_{32}H_{37}NO_6$ (531.65)

2-Acetylamino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranose (5)

To a solution of compound **4** (0.40 g, 0.83 mmol) and sodium acetate (0.53 g, 6.44 mmol) in acetic acid (95 %, 17 ml) palladium(II)-chloride (0.94 g, 5.28 mmol) was added. The reaction mixture was stirred at room temperature for 60 h, filtered through. Celite and diluted with dichloromethane (100 ml). The organic solution was washed with water (1 x 50 ml), saturated sodium hydrogencarbonate solution (1 x 50 ml) and again with water (1 x 50 ml), dried over sodium sulphate and concentrated in vacuo. The product was purified by chromatography on a silica gel column eluting with a 1:2 (v/v) mixture of petrol ether 60-80 °C and ethyl acetate to give a white solid.

Yield: 0.10 g (0.20 mmol, 25 %, white solid)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 2.06 (s, 3H, C$\underline{H}_3$), 3.48-3.82 (m, 5H, H-4, H-5, C$\underline{H}_2$OH), 4.00-4.31 (m, 2H, H-2, H-3), 4.48-4.90 (m, 4H, $C_6H_5C\underline{H}_2$), 4.72-4.90 (m, 2H, $C_6H_5C\underline{H}_2$), 5.08-5.12 (m, 1H, H-1), 5.45-5.50 (m, 1H, NH), 7.10-7.42 (m, 15H, C_6H_5)

$C_{29}H_{33}NO_6$ (491.58)

2-Acetylamino-1-*O*-benzyl-2-deoxy-D-glucopyranoside (6)

A solution of *N*-acetyl-D-glucosamine (8.76 g, 39.60 mmol), benzyl alcohol (100 ml) and acetyl chloride (2.43 g, 30.90 mmol) was stirred at 72 °C for 16 h. After cooling, the mixture was diluted with diethyl ether until a white solid precipitated. The solid was filtered and washed with diethyl ether and dried in vacuo.

Yield: 11.35 g (39.5 mmol, 100 %, white solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 1.85 (s, 3H, C$\underline{H}_3$), 3.44-3.76 (m, 3H, H-3, H-4, H-5), 4.44-4.48 (m, 1H, H-2), 4.53 (s, 2H, C$\underline{H}_2$OH), 4.67 (s, 1H, H-1), 4.72-4.75 (m, 2H, C_6H_5-C$\underline{H}_2$), 7.20-7.46 (m, 5H, C_6H_5), 7.88 (d, 1H, ^{3}J = 7.4 Hz, NH)

$C_{13}H_{21}NO_6$ (287.32)

Benzyl 2-acetylamino-4,6-*O*-benzylidene-2-deoxy-D-glucopyranoside (7)

To a solution of compound **6** (5.00 g, 17.40 mmol) in acetonitrile (150 ml) $PhCH(OCH_3)_2$ (6.89 g, 45.24 mmol) and p-toluenesulphonic acid (0.83 g, 4.36 mmol) were added. After the mixture was stirred at room temperature for 14 h, Et_3N (4 ml), hexane (100 ml) and methanol (100 ml) were added. The precipitated product was collected, washed with a warm 10:1:2 (v/v/v) mixture of hexane, dichloromethane and diethyl ether and dried in vacuo.

Yield: 4.37 g (10.94 mmol, 63 %, white solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 1.85 (s, 3H, NCOC$\underline{H}_3$), 3.46-3.58 (m, 1H, H-5), 3.83 (m, 1H, 1H, H-2), 3.66-3.80 (m, 3H, H-4, C$\underline{H}_2$), 4.15 (dd, 1H, ^{3}J = 3.6 Hz, ^{2}J = 5.5, H-3), 4.48 (d, 1H, ^{2}J = 12.5 Hz, $C_6H_5C\underline{H}_2$), 4.71 (d, 1H, ^{2}J = 12.5 Hz, $C_6H_5C\underline{H}_2$), 4.79 (d, 1H, ^{3}J = 3.2 Hz, H-1), 5.20 (d, 1H, ^{2}J = 5.5 Hz, OH), 5.62 (s, 1H, $C_6H_5C\underline{H}$), 7.28-7.48 (m, 10H, C_6H_5), 8.06 (d, ^{3}J = 7.9 Hz, NH)

$C_{22}H_{25}NO_6$ (399.44)

Methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-glucopyranoside (9a)

A solution of methyl-α-D-glucopyranoside (1.0 g, 5.15 mmol), *N*-iodosuccinimide (2.32 g, 10.30 mmol, 2eq) and triphenylphosphine (2.70 g, 10.30 mmol) in *N,N*-dimethylformamide (50 ml) was stirred at 50 °C for 2 h. After cooling to room temperature, the reaction mixture was diluted with water (30 ml) and extracted with chloroform (3 x 30 ml). The aqueous solution was concentrated under reduced pressure to give a brown viscose liquid (1.57 g). To the residue, sodium acetate (2.11 g, 25.75 mmol) and acetic acid anhydride (20 ml) were added and the mixture was stirred and heated for 40 min at 110 °C. After cooling to 60 °C, the reaction mixture was carefully poured into ice water (100 ml) and extracted with chloroform (3 x 30 ml). The combined organic layers were extracted three times with a saturated solution of sodium hydrogencarbonate (30 ml) and three times with water (30 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of petrol ether 60-80 °C and ethyl acetate to give a white solid.
Yield: 0.91 g (2.12 mmol, 41 %, white solid)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 2.01 (s, 3H, C$\underline{H}_3$), 2.06 (s, 3H, C$\underline{H}_3$), 2.08 (s, 3H, C$\underline{H}_3$), 3.13-3.18 (m, 1H, C$\underline{H}_2$), 3.28-3.32 (m, 1H, C$\underline{H}_2$), 3.48 (s, 3H, OC$\underline{H}_3$), 3.75-3.85 (m, 1H, H-5), 4.84-4.87 (m, 1H, H-4), 4.90-4.91 (m, 1H, H-2), 4.96-4.97 (m, 1H, H-3), 5.43-5.51 (m, 1H, H-1)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 3.6 (-, I$\underline{C}H_2$), 20.6 (+, $\underline{C}H_3$), 20.7 (+, $\underline{C}H_3$), 20.8 (+, $\underline{C}H_3$), 55.8 (+, O$\underline{C}H_3$), 68.6 (+, $\underline{C}$H), 69.7 (+, $\underline{C}$H), 70.9 (+, $\underline{C}$H), 72.5 (+, $\underline{C}$H), 96.7 (+, $\underline{C}$H), 169.7 (C_{quart}, C=O), 170.0 (C_{quart}, C=O), 170.1 (C_{quart}, C=O)

$C_{13}H_{19}IO_8$ (430.19)

Methyl 2,3,4-tri-*O*-benzoyl-6-deoxy-6-iodo-α-D-glucopyranoside (9b)

Iodine (27.18 g, 107.12 mmol) was added in small portions to a solution of methyl-α-D-glucopyranoside (10.00 g, 51.50 mmol), triphenylphosphine (30.26 g, 115.36 mmol), imidazole (15.88 g, 233.30 mmol), toluene (100 ml) and acetonitrile (200 ml) and the reaction mixture was stirred at 90 °C for 2 h. After evaporation of the solvent, water (300 ml) was added and shaken vigorously. The solution was extracted with toluene (3 x 100 ml) and the aqueous phase was concentrated under reduced pressure. The residue was dissolved in pyridine (70 ml), treated with benzoyl chloride (23.89 g, 0.17 mol) and stirred at ambient temperature for 16 h. The reaction mixture was diluted with dichloromethane (300 ml), washed with water (2 x 50 ml), a solution of potassium hydrogensulphate (3 x 50 ml, 1M) and again water (2 x 50 ml), dried over sodium sulphate and evaporated. The product was purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a white solid.

Yield: 3.81 g (6.18 mmol, 12 %, white solid)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.46 (s, 3H, OC$\underline{H}_3$), 3.82-3.91 (m, 1H, H-5), 3.94-3-95 (m, 2H, C$\underline{H}_2$), 3.99-4.07 (m, 1H, H-4), 5.15 (d, 1H, ^{3}J = 3.7 Hz, H-1), 5.30 (dd, 1H, ^{3}J = 3.7 Hz, ^{3}J = 10.1 Hz, H-2), 5.70 (dd, 1H, ^{3}J = 8.9 Hz, ^{3}J = 10.1 Hz, H-3), 7.34-8.15 (m, 15H, C_6H_5)

$C_{28}H_{25}IO_8$ (616.41)

Methyl 6-deoxy-α-D-*xylo*-hex-5-enopyranoside (10)

To a solution of compound **9a** (1.00 g, 2.32 mmol) in anhydrous tetrahydrofuran (50 ml) 1,8-diazabizyclo[5.4.0]undec-7-ene (DBU, 2.12 g, 13.92 mmol) was added. The reaction mixture was stirred and heated under reflux for 12 h. After removal of the solvent, the obtained oil was dissolved in methanol and shacked with a solution of NaOMe in MeOH (10 ml, 0.1M). The reaction mixture was

neutralised with acetic acid, concentrated under reduced pressure and purified by column chromatography on silica gel eluting with a 6:3:1 (v/v/v) mixture of diethyl ether, dichloromethane and ethanol to give a yellow oil.
Yield: 0.26 g (1.48 mmol, 64 %, yellow oil)

^{1}H-NMR ([D$_6$]acetone):
δ [ppm] = 3.38 (s, 3H, OC$\underline{H}_3$), 3.50-3.97 (m, 3H, H-2, H-3, H-4), 4.56 (d, 1H, ^{2}J = 2.2 Hz, =C$\underline{H}_2$), 4.74 (d, 1H, ^{3}J = 3.3 Hz, H-1), 4.77 (d, 1H, ^{2}J = 2.2 Hz, =C$\underline{H}_2$)

$C_7H_{12}O_5$ (176.17)

Methyl 6-*O*-tosyl-α-D-glucopyranoside (11)

To an ice-cold solution of methyl α-D-glucopyranoside (10.0 g, 51.50 mmol) in pyridine (90 ml), a solution of tosyl chloride (10.50 g, 55.07 mmol) in pyridine (25 ml) was added dropwise, and the mixture was stirred at ambient temperature for 48 h. After evaporation, the residue was diluted with chloroform (100 ml), washed with a solution of potassium hydrogensulphate (2 x 50 ml, 1M), a solution of sodium hydrogencarbonate (2 x 50 ml, 5 %) and water (1 x 50 ml), dried over sodium sulphate, and the solvent was removed under reduced pressure. The product was used without further purification.
Yield: 17.60 g (50.52 mmol, 98 %, white solid)

$C_{14}H_{20}SO_8$ (348.37)

Methyl 2,3,4-tri-*O*-benzoyl-6-*O*-tosyl-α-D-glucopyranoside (12)

To a solution of compound **11** (18.79 g, 53.94 mmol) in pyridine (50 ml), benzoyl chloride (25.02 g, 178.0 mmol) was added dropwise at 0 °C. Afterwards the reaction mixture was stirred at room temperature for 48 h, concentrated under reduced pressure and diluted with chloroform (200 ml). The solution was extracted with a solution of $KHSO_4$ (3 x 100 ml, 1M), a solution of $NaHCO_3$ (2 x

100 ml, 5 %) and water (2 x 100 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 2:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a white solid.
Yield: 15.35 g (23.23 mmol, 43 %, white solid)

^{1}H-NMR ($CDCl_3$):
δ [ppm] = 2.34 (s, 3H, $C_6H_4C\underline{H}_3$), 3.46 (s, 3H, $OC\underline{H}_3$), 4.02-4.09 (m, 1H, H-5), 4.14-4.21 (m, 2H, $C\underline{H}_2$), 4.52 (dd, 1H, ^3J = 3.6 Hz, ^3J = 10.0 Hz, H-2), 5.02 (d, 1H, ^3J = 3.6 Hz, H-1), 5.27 (t, 1H, ^3J = 9.6 Hz, H-3), 5.82 (t, 1H, ^3J = 9.6 Hz, H-4), 6.92-7.77 (m, 20H, C_6H_5)

MS (PI-DCIMS (NH_3)): m/z (%) = 678 ($[M+NH_4]^+$, 100), 524 ($[M-C_6H_4CH_3]^+$, 69)

$C_{35}H_{32}SO_{11}$ (660.70)

Methyl 2,3,4-tri-*O*-benzoyl-6-deoxy-α-D-*xylo*-hex-5-enopyranoside (13)

A mixture of compound **12** (5.0 g, 8.33 mmol), sodium iodide (6.24 g, 41.65 mmol), tetrabutylammonium iodide (1.54 g, 4.17 mmol) and molecular sieve 4 Å in DMSO (100 ml) was stirred under nitrogen at 100 °C. After 2 h, DBU (1.52 g, 100.0 mmol, 1.50 ml) was added and stirred for additional 2 h at 80 °C. The reaction mixture was poured into water and extracted with ethyl acetate (6 x 50 ml). The combined organic layers were dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 2:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a colourless oil.
Yield: 2.99 g (6.12 mmol, 73 %, colourless oil)

^{1}H-NMR ($CDCl_3$):
δ [ppm] = 3.62 (s, 3H, $OC\underline{H}_3$), 4.72 (s, 1H, $=C\underline{H}_2$), 4.90 (s, 1H, $=C\underline{H}_2$), 5.22-5.31 (m, 1H, H-1), 5.33-5.46 (m, 1H, H-2), 5.92-6.00 (m, 1H, H-3), 6.10-6.23 (m, 1H, H-4), 7.24-7.61 (m, 10H, C_6H_5), 7.79-8.09 (m, 5H, C_6H_5)

MS (PI-DCIMS (NH_3)): m/z (%) = 506 ($[M+NH_4]^+$, 100), 489 ($[MH]^+$, 35)

$C_{28}H_{24}O_8$ (488.50)

Methyl 2,3-di-*O*-benzyl-4,6-*O*-benzyliden-α-D-glucopyranoside (14)

A mixture of methyl-α-D-glucopyranoside (50.0 g, 257.45 mmol), benzaldehyde dimethyl acetal (48.96 g, 321.70 mmol), catalytic amounts (80 mg) of camphor-sulphonic acid (0.70 g, 3.01 mmol) and *N,N*-dimethylformamide (200 ml) was stirred at 100 °C in vacuo (water aspirator) for 2 h. The remaining solution was cooled with an ice bath to 0 °C and benzyl bromide (100.80 g, 0.59 mol) and *N,N*-dimethylformamide (300 ml) were added, followed by sodium hydride (60 % in mineral oil, 24 g corresponding to 0.6 mol NaH) in small portions. After stirring at ambient temperature for 15 h, methanol (100 ml) was added and stirring was continued for 1 h. The reaction mixture was concentrated in vacuo and diluted with dichloromethane (500 ml) and water (500 ml) and shaken vigorously. The aqueous phase was extracted with dichloromethane (3 x 50 ml) and the combined organic layers were washed with water (3 x 50 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of petrol ether 60-80 °C and ethyl acetate to give a white solid.
Yield: 80.84 g (174.78 mmol, 68 %, white solid)

^{1}H-NMR ($CDCl_3$):
δ [ppm] = 3.42 (s, 3H, OC$\underline{H}_3$), 3.50-3.87 (m, 4H, H-2, H-5, C$\underline{H}_2$), 4.02-4.10 (m, 1H, H-3), 4.20-4.32 (m, 1H, H-4), 4.56-4.60 (m, 1H, H-1), 4.65-4.98 (m, 4H, $C_6H_5C\underline{H}_2$), 5.05 (s, 1H, $C_6H_5C\underline{H}$), 7.20-7.51 (m, 15H, C_6H_5)

MS (PI-DCIMS (NH_3)): m/z (%) = 480 ($[M+NH_4]^+$, 100), 463 ($[MH]^+$, 39)

$C_{28}H_{30}O_6$ (462.54)

Methyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (15)

To a mixture of compound **14** (1.0 g, 2.16 mmol), $NaCNBH_3$ (1.7 g, 27.0 mmol), and molecular sieve 3 Å (spherical shape) in anhydrous THF (30 ml), a solution of hydrogenchloride in ether (2M) was added dropwise until the generation of gas ceased. Afterwards, the reaction mixture was stirred at room temperature for 16 h. After dilution with dichloromethane (150 ml) and water (150 ml), the organic phase was washed with 50 ml water and with a saturated solution of sodium hydrogencarbonate (2 x 50 ml), dried over sodium sulphate and evaporated under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 2:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a white solid.
Yield: 1.83 g (3.94 mmol, 36 %, white solid)

MS (PI-DCIMS (NH_3)): m/z (%) = 482 ($[M+NH_4]^+$, 100)

$C_{28}H_{32}O_6$ (464.56)

Methyl 2,3,6-tri-*O*-benzyl-4-mesyl-α-D-glycopyranoside (16)

To a solution of compound **15** (0.25 g, 0.54 mmol) in anhydrous dichloromethane (30 ml), triethylamine (330 µl, 2.38 mmol) and mesyl chloride (170 µl, 2.11 mmol) were added at 0 °C and stirred for 2 h. After dilution with a saturated solution of $NaHCO_3$ (100 ml), the reaction mixture was extracted with dichloromethane (3 x 50 ml). The combined organic layers were washed with a solution of $KHSO_4$ (1M, 50 ml), dried over $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 10:1 (v/v) mixture of chloroform and ethyl acetate to give a white solid.
Yield: 0.17 g (0.31 mmol, 56 %, white solid)

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.01 (s, 3H, SC$\underline{H}_3$), 3.39 (s, 3H, OC$\underline{H}_3$), 3.68-4.01 (m, 5H, H-2, H-4, H-5, C$\underline{H}_2$), 4.27-4.35 (m, 1H, H-3), 4.53-4.76 (m, 6H, $C_6H_5C\underline{H}_2$), 5.06-5.10 (m, 1H, H-1), 7.24-7.40 (m, 15H, C_6H_5)

MS (PI-DCIMS (NH_3)): m/z (%) = 560 ($[M+NH_4]^+$, 100)

$C_{29}H_{34}O_8S$ (542.64)

Methyl 2,3,6-tri-*O*-benzyl-4-deoxy-β-L-*threo*-hex-4-enopyranoside (17)

A solution of compound **16** (170 mg, 0.32 mmol), $PhCO_2Na$ (172 mg, 1.19 mmol) and HMPT (3 ml) was stirred at 90 °C for 95 min. After cooling, the reaction mixture was carefully poured into water (50 ml) and extracted with ether (3 x 50 ml). The combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 5:2 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a white solid.
Yield: 90 mg (0.2 mmol, 67 %, white solid)

MS (CI-MS (NH_3)): m/z (%) = 464 ($[M+NH_4]^+$, 3), 230 ($[M-2CH_2C_6H_5-OCH_3]^+$, 100)

$C_{28}H_{30}O_5$ (446.54)

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Chapter 6

Flavone and chalcone derivatives as inhibitors of hyaluronidases – synthesis and pharmacological investigations

1. Introduction

Flavonoids are a group of naturally occurring low molecular weight benzo-γ-pyranone derivatives. The immediate family members of flavonoids include flavones, flavanes, flavonols, isoflavones, anthocyanidines and catechins (cf. Fig. 1). The flavonoids all consist of a benzene ring (A) condensed with a heterocyclic six-membered ring (B) which carries a phenyl ring (C) in position 2 (flavonoids) or 3 (isoflavonoids) and a keto group in position 4.

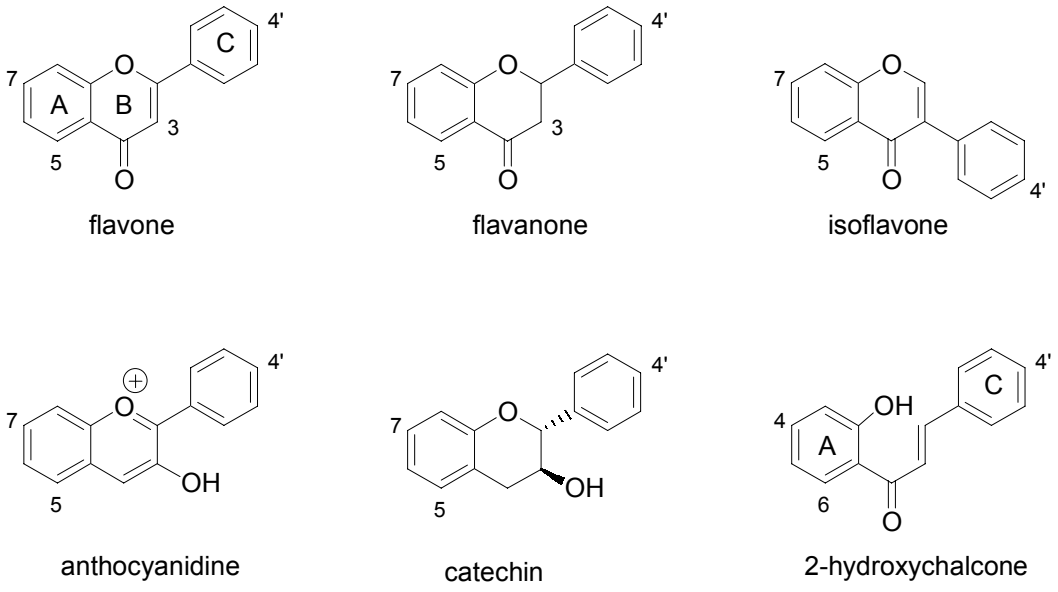
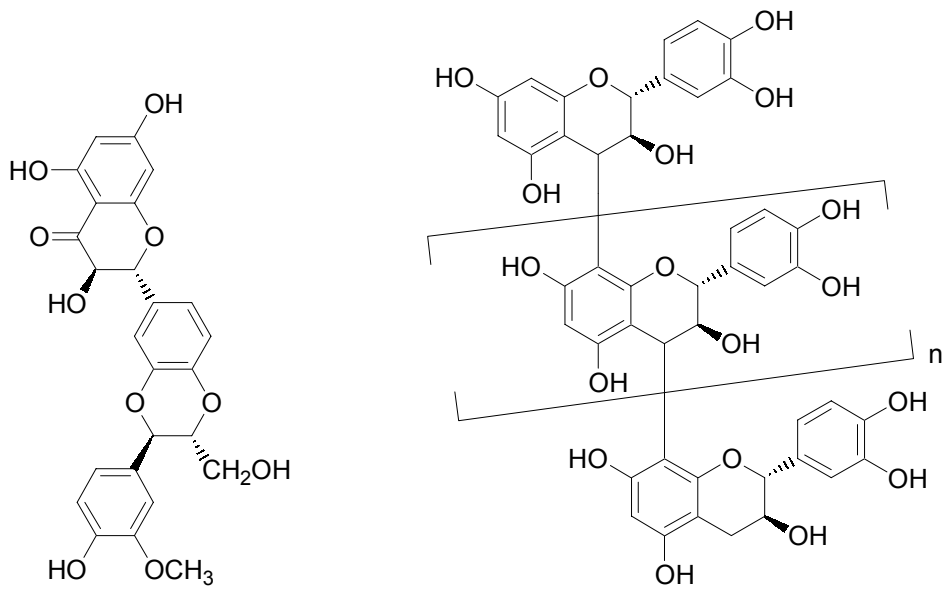


Fig. 1: Structure of chalcone, flavone and flavone related compounds

The difference between the flavane and the flavone structure is the lack of the double bond between C-2 and C-3. The structures of anthocyanidin and catechin are closely related to the flavone structure. The 2-hydroxychalcone structure correspond to a flavone with an opened ring B. The ortho-hydroxylated chalcone may be converted to flavone by intramolecular nucleophilic addition of the hydroxy group to the enone group.

Flavonoids are ubiquitous in vegetables, fruits, teas and other plants (Havsteen 1983; Hollman 2000). They exhibit a great variety of pharmacological and pharmaceutical functions which have been reviewed by *Havsteen* in 1983 (Havsteen 1983). It was reported that some flavone and flavone derivatives had anticancer, antiischemic, antiallergic, anti-inflammatory and several other activities (Harborne 1986; Deschner et al. 1991; Ferrandiz et al. 1991; van Acker et al. 2000). Furthermore, it was also reported that several structurally related flavonoids were potent hyaluronidase inhibitors. The inhibitory effect of the most important flavone compounds on hyaluronidases are summarised in the following.

In 1990, the group of *Kuppusamy* examined the effect of 31 flavonoids, sylibin (**1**) and condensed tannin (**2**) on the activity of the bovine testicular hyaluronidase (BTH) (Kuppusamy et al. 1990). From all investigated flavonoids only six compounds (apigenin, kaempferol, luteolin, morin, myricetin and quercetin) showed inhibitory activity on the enzyme between 29 % and 76 % at a concentration of 250 µM. Furthermore, the effect of sylibin (**1**) on BTH was 48 % at a concentration of 250 µM. Of the investigated compounds only condensed tannin (**2**) showed a complete inhibition of the bovine testicular enzyme at a concentration of 50 µM. *Kuppusamy et al.* (Kuppusamy et al. 1990) suggested a competitive inhibition for all active compounds. On the basis of these results, they concluded that the double bond between the positions 2 and 3 was crucial for the inhibitory activity. Also the keto group in position 4 and different substituents seem to be important for the inhibitory effect of the compounds. The introduction of hydroxy groups in the positions 5, 7 and 4' increase the potency, whereas the presence of a glycoside substituent completely reverses the inhibitory effect of the flavonoids on the bovine testicular hyaluronidase.



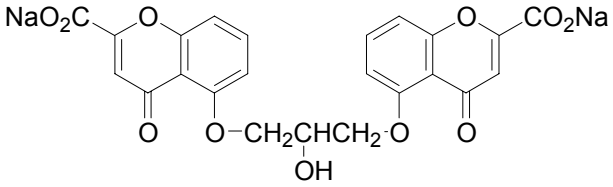
1 **2**

Fig. 2: Sylibin (**1**) and condensed tannin (**2**)

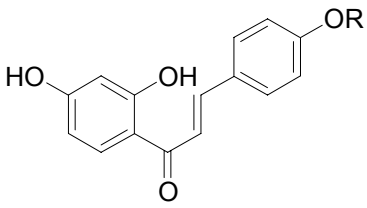
In 1991, the same working group investigated the effect of sylibin (**1**) and 12 flavone derivatives on the activities of five hyaluronidases from the venom of honey bee, scorpion, two rattle snakes and malayan cobra (Kuppusamy et al. 1991). The inhibition of the flavone compounds on the venom hyaluronidase was determined by a turbidimetric method. Additionally, the survival time of mice after the injection of the venom plus a flavonoid was measured. Naringenin, catechin and the four flavone-glycosides apiin, luteolin-7-glucoside, myricitrin and quercitrin were inactive on all five hyaluronidases. Apigenin, kaempferol, luteolin, myricetin, phloretin (chalcone) and quercetin showed variable inhibitions ranging from 30 % - 89 % at a concentration of 250 µM. Under these conditions, silybin (**1**) was the only compound that inhibited the activity of the bee and the scorpion venom hyaluronidases by 100 %. The authors concluded that flavones, flavonols and chalcones possess the general ability to inhibit venom hyaluronidases.

In 1992, the inhibitory effect of some natural products and synthetic anti-allergic drugs on the activation of bovine testicular hyaluronidase were investigated by *Kakegawa et al*. (Kakegawa et al. 1992). The structure of the examined compounds DSCG (**3**, disodium cromoglycate (Cox 1967)), traxanox (**6**), tranilast (**8**) (Koda et al. 1976), two chalcones **4** and **5** and three flavone derivatives **7**, **9**

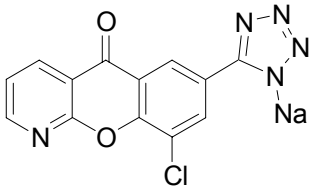
and **10** are shown in Fig. 3. The IC_{50} values of liquiritigenin (**10**), tranilast (**8**), baicalein (**7**), traxanox (**6**), isoliquiritigenin (**5**) and DSCG (**3**) were 0.74 mM, 0.35 mM, 0.165 mM, 85 µM, 64 µM and 29 µM respectively. The two glycosides **4** and **9** were not active on the bovine testicular hyaluronidase.



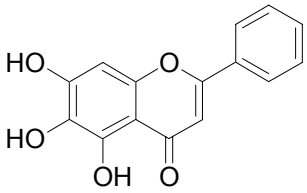
3 Disodium cromoglycate (DSCG)



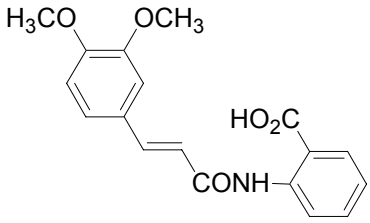
4 Isoliquiritin: R = glucose
5 Isoliquiritigenin: R = H



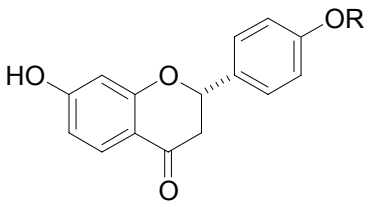
6 Traxanox



7 Baicalein



8 Tranilast



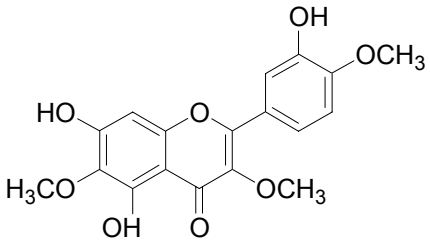
9 Liquiritin: R = glucose
10 Liquiritigenin: R = H

Fig. 3: Structures of the investigated natural products and synthetic anti-allergic drugs

The inhibitory effect of apigenin, kaempferol, luteolin, quercetin and tannic acid on the activity of venom hyaluronidase from *Crotalus adamenteus* (Kuppusamy et al. 1993) and on the activity of monkey sperm hyaluronidase (Li et al. 1997) were also investigated. The assayed compounds showed a very low inhibitory activity and never a complete inhibition of the enzymes.

The latest results on the inhibition of hyaluronidases by flavones and flavone related compounds were published by *Pessini et al.* in 2001 (Pessini et al.

2001). They investigated the effect of natural flavonoids (e. g. eupatorin, desmethoxycentaureidine, pinostrobin) on the activity of scorpion venom hyaluronidase. Among the examined compounds, only desmethoxycentaureidine **11**, a flavone derivative with three methoxy groups in the positions 3, 4' and 6 and three hydroxy groups in the positions 3', 5 and 7 reduced the activity of the scorpion venom hyaluronidase in a dose-dependent manner and induced an inhibition by 90 % at a concentration of 50 µM.



11

Fig. 4: Desmethoxycentaureidine

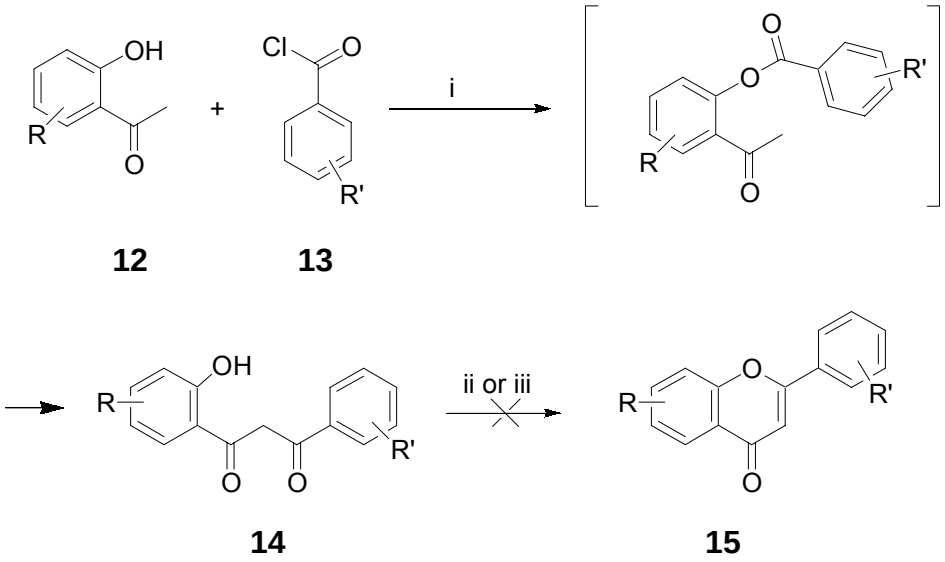
The published data, i.e. the inhibition given in % and the IC_{50} values of all aforementioned compounds, are not comparable with each other because of the differences in the applied test systems (e.g. incubation condition, enzymes and substrate concentrations).

Taken together the published results show that, in principle, flavones and related compounds are suitable to inhibit hyaluronidases. However, the inhibitory activities of the compounds described in literature are only in the millimolar range. This is also true for the widely accepted hyaluronidase inhibitors apigenin and kaempferol which have activities in the mM range, too.

2. Chemistry

As the flavonoid structure appeared to be a promising skeleton for the development of hyaluronidase inhibitors, new flavonoid compounds were synthesised and tested for their activity on the bovine testicular hyaluronidase.
To synthesise the desired flavone compounds we started with published procedures. *Costantino et al.* (Costantino et al. 1996) reported a one step synthesis of 5,7-dihydroxy-4'-methoxyflavone from 2,4,6-trihydroxyacetophenone and 4-methoxybenzaldehyde which were treated at first with lithium bis(trimethyl)silyl amide in THF at – 78 °C and then with glacial acetic acid and sulphuric acid at 100 °C. Following this procedure, the products could only be obtained in very low yields. Furthermore, the isolation of the product and the separation of the by-products was not possible by standard methods.

The next reaction tried was described by the group of *Jain* and *Saxena* (Jain et al. 1982; Saxena et al. 1985) (cf. Scheme 1). In this synthesis a substituted hydroxyacetophenone **12** was treated with an excess of an aroyl chloride **13** in a benzene/aqueous potassium carbonate biphase to get an O-aroylation. By the subsequent *Baker-Vankatarama* reaction with tetrabutylammonium hydrogensulphate a rearrangement to an o-hydroxydibenzoylmethane **14** takes place. The final cyclocondensation to the flavone **15** was achieved by using concentrated sulphuric acid or aqueous potassium carbonate followed by treatment with acetic acid. Following this synthesis, it was possible to synthesise the diketo derivatives but the final ring closure reaction failed. Different substituents at both phenyl rings may explain the failure of known synthesis.



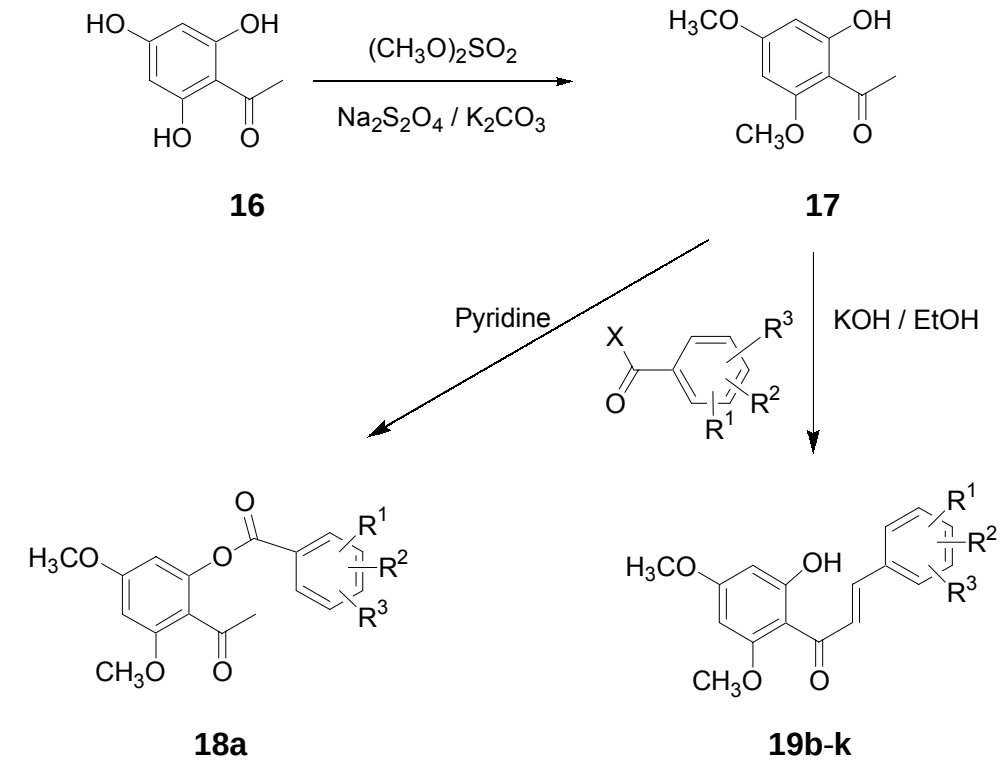
12 **13** **14** **15**

Scheme 1: Strategy of flavone synthesis reported by (Jain et al. 1982; Saxena et al. 1985); reagents used: i) 1. K_2CO_3, H_2O, C_6H_6, 60 °C; 2. $(n\text{-}C_4H_9)_4N^+$ HSO_4^+; ii) H_2SO_4, 0 °C; iii) 1. K_2CO_3, H_2O, ΔT, 2. AcOH

To synthesise the flavone derivatives a new synthetic strategy was developed which is illustrated in Scheme 2. In the first step 2,4,6-trihydroxyacetophenone (**16**) was protected by O-methylation in the positions 4 and 6, to increase the solubility of the compounds in organic solvents. The protection was prepared under alkaline conditions according to the method described by *Maurer* (Maurer 1976) with slight modifications.

Starting from the protected compound **17** the synthesis of the propan-1,3-dione and the chalcone derivatives as precursor of flavone compounds was performed according to two approaches. Following the first route, the esters **18** were synthesised from the protected hydroxyacetophenone **17** and the corresponding aroyl chloride under alkaline conditions as described by *Schnetzer* (Schnetzer 1991) and *Gratzfeld* (Gratzfeld 1978) with slight modifications. The final step, the rearrangement to the corresponding propan-1,3-dione **20** was accomplished via the *Baker-Vankatarama* reaction by a modified method described by *Maurer* (Maurer 1976). Following the second route, a one step reaction, the propenones **19** were synthesised from the acetophenone **17** and the corresponding benzaldehydes by an aldol condensation with potassium hydroxide in ethanol in good yields (cf. Scheme 2). The advantages of this synthetsis are the smaller number of reaction steps and the higher yields. Thus, we

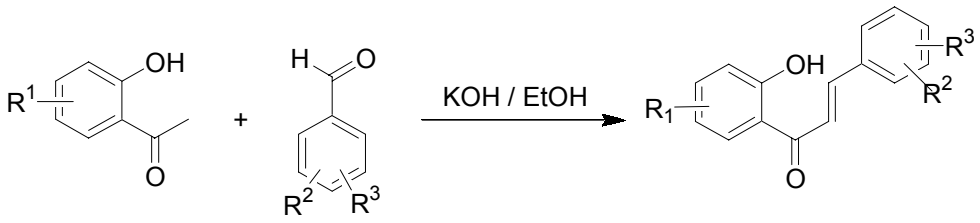
decided to synthesise our chalcone derivative as precursore of flavone compounds.



No	R^1	R^2	R^3
a	4'-CN	H	H
b	4'-OCH_3	H	H
c	3'-OCH_3	4'-OCH_3	H
d	4'-$N(CH_3)_2$	H	H
e	[3,4]-benzo-annelated	H	H
f	4'-CO_2H	H	H
g	2'-OSO_2H	4'-OSO_2H	H
h	4'-Cl	H	H
i	2'-Cl	3'-OCH_3	4'-OCH_3
j	2'-Cl	4'-Cl	6'-OCH_3
k	4'-F	H	H

Scheme 2: Synthesis of compounds **19b-k** and **20a**

The chalcone derivatives **23a**-**d** were also synthesised in one step, starting from the fluorinated 2-hydroxyacetophenones **21a** and **21b** under strong alkaline conditions (cf. Scheme 3).

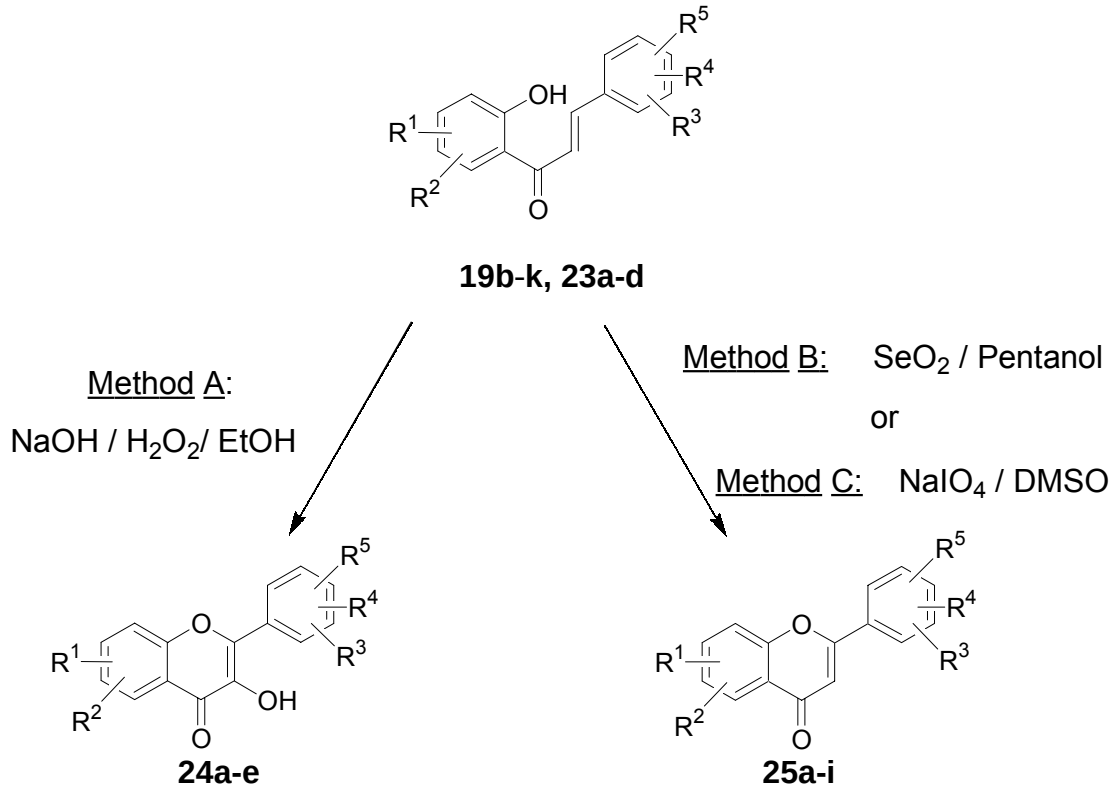


21a-**b** **22a**-**c** **23a**-**d**

No	R^1	R^2	R^3
a	4-F	3'-OCH_3	4'-OCH_3
b	4-F	4'-OCH_3	H
c	5-F	4'-OCH_3	H
d	5-F	4'-F	H

Scheme 3: Synthesis of the fluorinated chalcone derivatives **23a**-**d**

Starting from the synthesised chalcone derivatives **19b**-**k** and **23a**-**d** several published methods were tried to accomplished the ring closure. The cyclisation with copper (II) chloride in dimethylsulphoxide at high temperature described by *Saharabhuddhe et al*. (Sahasrabhuddhe et al. 1990) as well as the method using iodine in dimethylsulphoxide described by different authors (Doshi et al. 1986; Kitagawa et al. 1991; Silva et al. 1993; Silva et al. 1994; Silva et al. 1994; Pinto et al. 1996) did not lead to a successful synthesis of the flavone derivatives. Even modifications of these methods did not yield any flavone derivatives. Different substituents at both phenyl rings may explain the failure of known synthesis. Nevertheless, it was possible to synthesise the flavone derivatives **14a-e** and **25a-i** by following three different methods as shown in Scheme 4.



Scheme 4: Synthesis of the flavone derivatives **24a-e** and **25a-i**

Table 1: Substitution patterns of the synthesised flavone compounds

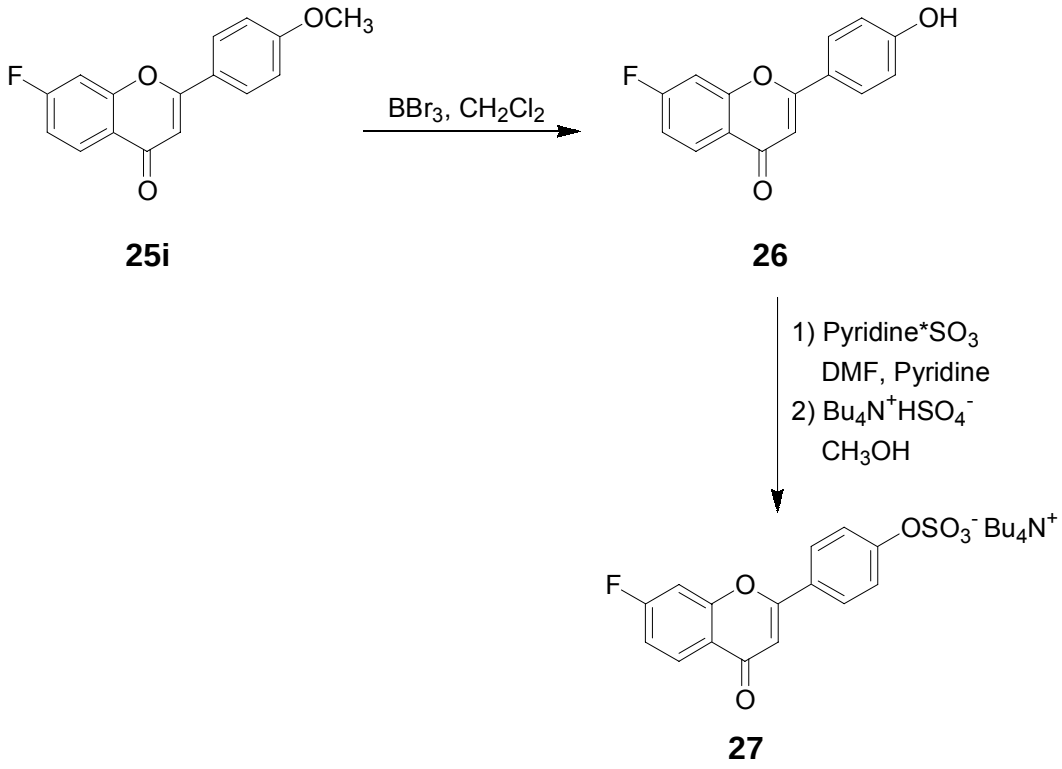
No	R^1	R^2	R^3	R^4	R^5
24a	5-OCH_3	7-OCH_3	4'-OCH_3	H	H
24b	5-OCH_3	7-OCH_3	2'-Cl	4'-Cl	6'-OCH_3
24c	5-OCH_3	7-OCH_3	4'-$N(CH_3)_2$	H	H
24d	5-OCH_3	7-OCH_3	4'-Cl	H	H
24e	5-OCH_3	7-OCH_3	2'-Cl	3'-OCH_3	4'-OCH_3
25a	5-OCH_3	7-OCH_3	4'-OCH_3	H	H
25b	5-OCH_3	7-OCH_3	3'-OCH_3	4'-OCH_3	H
25c	5-OCH_3	7-OCH_3	[3,4]-naphthyl	H	H
25d	5-OCH_3	7-OCH_3	4'-Cl	H	H
25e	5-OCH_3	7-OCH_3	4'-F	H	H
25f	6-F	H	4'-OCH_3	H	H
25g	6-F	H	4'-F	H	H
25g	7-F	H	3'-OCH_3	4'-OCH_3	H
25i	7-F	H	4'-OCH_3	H	H

The synthesis of the flavone derivatives **24a**-**e** (cf. Table 1) were carried out by treating the chalcone derivatives dissolved in ethanol with a sodium hydroxide solution and an aqueous solution of hydrogen peroxide at under reflux (cf. Method A in Scheme 4). According to this procedure an additional hydroxy group was introduced in position 3 – the obtained compounds are so-called flavonols. To synthesise the flavone derivatives via method A as described by different authors (De Meyer et al. 1991; Hariprasad et al. 1998; van Acker et al. 2000) some modifications were necessary. The problems of this synthesis were the low yield and the difficult separation of the product from by-products and starting material. To circumvent these problem, the flavone derivatives were prepared according to method B. This reaction was described by *Khan et al.* (Khan et al. 1990) and *Chang et al.* (Chang et al. 1961). The ring closure was achieved with selenium dioxide in isopropylalcohol and heated to reflux (cf. Method B in Scheme 4). The advantages were the facile practicability of the method, the short reaction time and the convenient purification of the product. Following this method most of the flavones **25a**-**c** and **25e**- **i** (cf. Table 1) could be obtained in good yields.

4'-Chloro-5,7-dimethoxyflavone (**25d**) could not be synthesised from the chalcone derivative **19h**, neither via method A nor by method B. Obviously, the reaction conditions of method A were too severe, as only decomposed products could be isolated. Following method B, the starting material was recovered completely; obviously, the reaction conditions were too mild to force the ring closure. Finally, it was possible to convert the chalcones to flavones by treatment with sodium periodate in dimethylsulphoxide according to a method described by *Naresh et al.* (Naresh et al. 1993) (cf. Method C in Scheme 4) with slight modifications.

Of all synthesised chalcone derivatives only the compounds **19f** and **19g** could not be converted into the corresponding flavone derivatives according to published methods with and without modifications.

To increase the solubility of the flavones the introduction of hydroxy and/or sulphate groups should be useful. Moreover, these polar substituents could interact with functional groups in the active site of the hyaluronidase thereby increasing the affinity of the potential enzyme inhibitors. To prove this hypothesis, one hydroxyflavone and its sulphated derivative were synthesised as shown in Scheme 5.

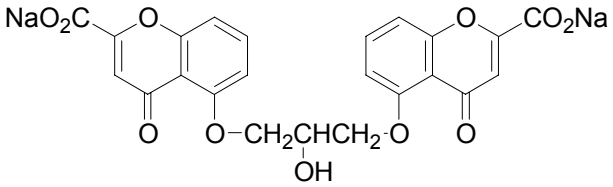


Scheme 5: Synthesis of the hydroxyflavone **26** and the sulphated derivative **27**

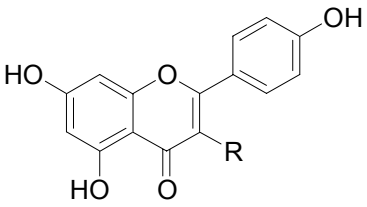
4'-Methoxy-7-fluoroflavone **25i** was deprotected with boron tribromide in anhydrous dichloromethane at room temperature to give the hydroxyflavone **26** (cf. Scheme 5). The subsequent sulphation of the compound **27** was carried out according to the method described by *Böddeker* (Böddeker 1995) with pyridine*SO_3 in dimethylformamide and pyridine at 100 °C. Disadvantages of this last step were the incomplete removal of the inorganic salt and the difficult separation of the product from the starting material by crystallisation and column chromatography.

3. Pharmacological investigations

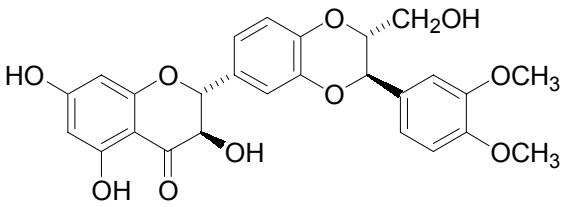
Due to the inconsistent published results, we investigated several assumed inhibitors like disodium cromoglycate (DSCG, **3**), apigenin (**28**), kaempferol (**29**) and silybinin (**30**) on the bovine testicular hyaluronidase to obtain comparable data determined under the same conditions (cf. Fig. 5).



3 DSCG



28 Apigenin R = H
29 Kaempferol R = OH



30 Silybinin

Fig. 5: Structure of several assumed hyaluronidase inhibitors

The inhibitory effects on the activities of hyaluronidases were determined in an optimised colorimetric assay (Morgan-Elson assay) as described in chapter 3. The compounds **28**-**30** could not be tested at concentrations above 1 mM or 10 mM due to poor solubility.

The widely accepted hyaluronidase inhibitors apigenin (**28**) and kaempferol (**29**) showed only weak inhibitory effects on the bovine testicular enzyme (inhibition by 56 % and 36 %, respectively, at a concentration of 1 mM). Moreover, the effects of these two compounds were not dose-dependent. The obtained

results are depicted in Fig. 6. Silybinin (**30**), a flavanol-related compound, induced an inhibition of the enzyme activity by 40 % at a concentration of 10 mM. From the four purported inhibitors only disodium cromoglycate (**3**) showed a dose-dependent and nearly complete inhibition of the enzyme. The calculated IC_{50} value of 1.24 mM for DSCG was higher those that reported in the literature (IC_{50} = 30 µM) (Sakamoto et al. 1980; Kakegawa et al. 1985; Kakegawa et al. 1992).

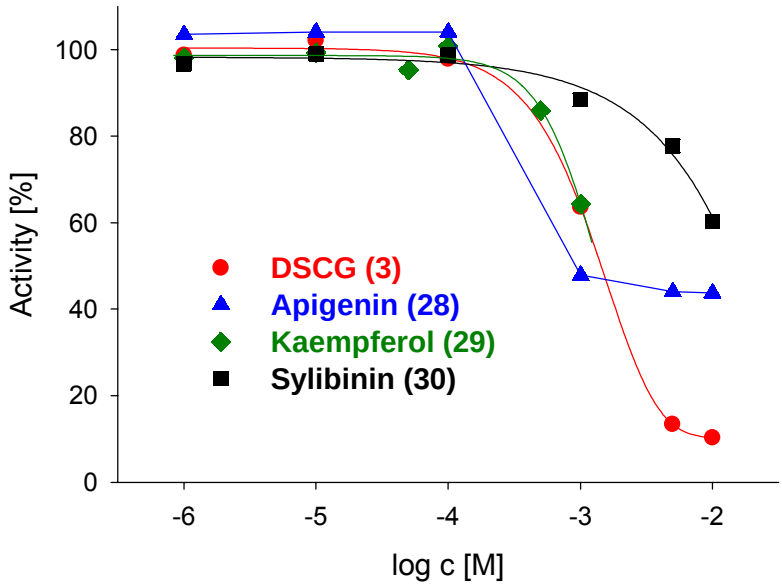
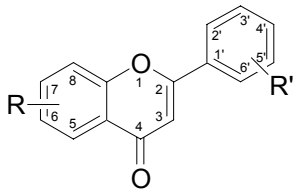


Fig. 6: Inhibitory effects of DSCG (**3**), apigenin (**28**), kaempferol (**29**) and sylibinin (**30**) on the activity of the bovine testicular hyaluronidase

To get more information about the structural requirements of flavone derivatives (e.g. substitution, functional groups, planarity) for hyaluronidase inhibitory activity, we investigated a series of flavones and related compound. The obtained results for the flavones **33**-**36**, the flavonols **37**-**41**, the flavanes **42**-**46**, the isoflavones **47** and **48**[1] are presented in the following (cf. Table 2 and Table 3).

[1] The compounds 33-48 were kindly provided from PD Dr. C. Paper, Institute of Pharmacy, University of Regensburg, Germany

Table 2: Inhibitory effects of the flavone derivatives **31-41**

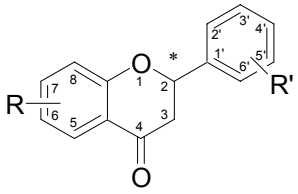


31-41

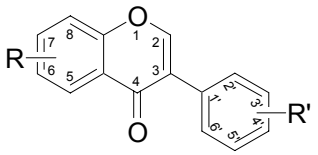
No	Substitution	% Inhibition [a] at	Concentration
31	—	11 %	10 mM
		inactive	≤ 2 mM
32	5-OH	inactive	≤10mM
33	6-OH	17 %	10 mM
		10 %	2 mM
34	7-OH	28 %	10 mM
		inactive	≤ 2 mM
35	[2',3']-benzo-annelated	21 %	5 mM
		inactive	≤ 2 mM
36	[3',4']-benzo-annelated	inactive	≤ 5 mM
37	3-OH	58 %	10 mM
		inactive	≤ 2 mM
38	3-OH, 6-OCH_3	inactive	≤ 5 mM
39	3-OH, 7-OCH_3	13 %	10 mM
		13 %	2 mM
40	3,3',4',5,5',7-hexy-OH	34 %	2 mM
41	3',4',5,7-tetra-OH, 3-*O*-rutinose	BTH: 15 % $hylB_{4755}$: 46 %	7.7 mM
		BTH: 11 % $hylB_{4755}$: inactive	2 mM

[a] % inhibition of BTH at an inhibitor concentration unless otherwise indicated

Table 3: Inhibitory effects of the compounds **42-48**



42-46



47-48

No	Substitution	% Inhibition [a] at	Concentration
42	4'-OH	32 %	10 mM
		21 %	2 mM
43	4',5,7-tri-OH	45 %	10 mM
		10 %	2 mM
44	3',4',5,7-tetra-OH	23 %	2 mM
45	4',5,7-tri-OH, 3'-OCH_3	17 %	2 mM
46	3,3',4',5,7-penta-OH	30 %	2 mM
47	4',5,7-tri-OH	BTH: inactive $hylB_{4755}$: 61 %	1.7 mM
48	3',4',7-tri-OH	BTH: inactive $hylB_{4755}$: 17 %	2 mM

[a] % inhibition of BTH at an inhibitor concentration unless otherwise indicated

The 5-hydroxyflavone **32** and the β-naphthoflavone **36**, two of the six flavone compounds, were ineffective as inhibitors of the bovine testicular hyaluronidase. The compounds **33-35** induced only very weak inhibition of the enzyme at millimolar concentrations (cf. Table 2). Compounds **33** and **34** which are hydroxylated in the positions 6 and 7, respectively, are more active than the unsubstituted flavone **31**. By contrast, a hydroxylation in position 5 (cf. compound **32**) results in a loss of the inhibitory activity.

Within the series of flavonols **37-41,** the compounds **37** and **39-41** showed a weak inhibitory activity, whereas 6-methoxyflavon-3-ol (**38**) was inactive on the bovine testicular hyaluronidase (cf. Table 2). The highest inhibitory activity was found for flavon-3-ol **37** (58 % inhibition at the concentration of 10 mM). A de-

crease in activity was induced by an additional methoxy group in position 7 (**39**), whereas the isomer with a methoxy group in position 6 (**38**) was completely inactive. Myricetin **40**, which is hydroxylated in the positions 3', 4', 5, 5' and 7 of the flavone skeleton, induced 34 % inhibition of the bovine testicular hyaluronidase at a concentration of 2 mM. The inhibitory effect of the glycosylated flavonol (+)-rutin (**41**) on BTH was similar to that reported in the literature (15 % inhibition at a concentration of 2 mM) (Kuppusamy et al. 1990). Compound **41** was also tested on the bacterial hyaluronidase from *S. agalactiae*. The inhibition oh the $hylB_{4755}$ by **41** at a concentration of 7.7 mM was 46 % corresponding to about 3-fold the percentual inhibition found on the bovine testicular hyaluronidase.

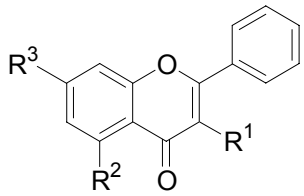
Furthermore, we investigated the flavanes **42-46** for bovine testicular hyaluronidase inhibitory activity (Table 3). The hydroxylated flavane **42** induced 32 % at a concentration of 10 mM. Additional hydroxylations in the positions 5 and 7 (**43**) led to an increase in activity (45 % inhibition at a concentration of 10 mM). Due to poor solubility, the compounds **44-46** were tested at a concentration of 2 mM, so that a comparison of the results with those found for the compound **42** and **43** are only reasonable at this lower concentration. Of all flavane derivatives, the penta hydroxylated compound (**46**) induced the highest inhibition of BTH by 30 %. Taxifolin **46** (flavan) as well as myricetin **40** (flavone) induced an inhibition of approximately 30 % at a concentration of 2 mM. Thus, the C=C double bond of the flavone seems to be less important than reported in the literature (Kuppusamy et al. 1990; Kuppusamy et al. 1991).

Isoflavones are a group of flavone analogues which have been neglected so far concerning investigation for inhibition of hyaluronidases. Only a few compounds like diadzein (4',7-dihydroxyisoflavone) and tectorigenin (4',7-dihydroxy-6-methoxyisoflavone) were tested on hyaluronidases (Matsuda 1985; Matsuda 1985; Kuppusamy et al. 1990). In this project, two isoflavones (**47** and **48**) were instigated for inhibition of BTH and $hylB_{4755}$. Both compounds were inactive on the bovine testicular enzyme but active on the bacterial hyaluronidase. Whereas compound **47** induced an inhibition of 61 % at a concentration of 1.7 mM, compound **48** was only weakly active (17 % at a concentration of

2 mM) (cf. Table 3). Obviously, a hydroxy group in position 5 is more favourable than a hydroxy group in position 3' of the phenyl ring.

In addition to the aforementioned flavones, the sulphated flavone derivatives **49-52**[2] were investigated (Table 4). The sulphate groups are suitable to improve the solubility of flavones. Moreover, it is conceivable that these polar substituents could interact similar to the carboxylates of the hyaluronic acid with amino acid residues in the active site of the hyaluronidase.
Whereas the flavone derivative **49** with the sulphate group in position 3 was inactive, compound **51** with an additional methoxy group in position 7 induced an inhibition of BTH by 37 % at a concentration of 2 mM.

Table 4: Inhibitory effect of the sulphated flavone derivatives **49-52**



No	R^1	R^2	R^3	IC_{50} [µM] or (% Inhibition) [a]
49	$OSO_3^-Na^+$	H	H	inactive[b]
50	H	H	$OSO_3^-Na^+$	**690**
51	$OSO_3^-Na^+$	H	OCH_3	(37 %)[c]
52	H	OH	$OSO_3^-Na^+$	**BTH: 190** **hylB$_{4755}$: 290**

[a] determination on BTH unless otherwise indicated
[b] at concentrations ≤ 2 mM
[c] % inhibition of BTH at an inhibitor concentration of 2 mM

The sulphated flavone **50** and chrysin-7-sulphate (**52**) induced a concentration-dependent inhibition of the bovine testicular hyaluronidase in the submillimolar range (IC_{50} values of 660 µM (**50**) and 190 µM (**52**)) (cf. Fig. 7). The introduc-

[2] The compounds 49-52 were kindly provided from PD Dr. C. Paper, Institute of Pharmacy, University of Regensburg, Germany

tion of an additional hydroxy group in position 5 (cf. compound **52**) led to a 3.5-fold increase in inhibitory potency. Additionally, on the *S. agalactiae* hyaluronate lyase an IC_{50} value of 290 µM was determined for chrysin-7-sulphate. The concentration-dependent inhibitory effect of compound **52** on $hylB_{4755}$ is depicted in Fig. 7, too.

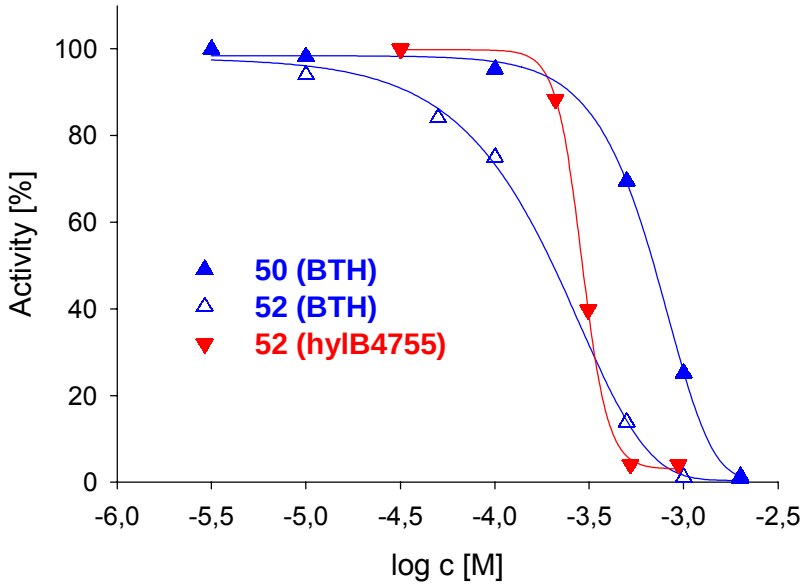
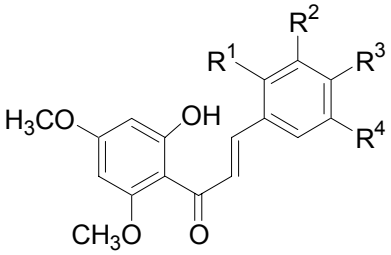


Fig. 7: Effect of the sulphated flavones **50** and **52** on the activity of BTH and $hylB_{4755}$ at optimum pH (BTH: pH 3.6, $hylB_{4755}$: pH 5.0)

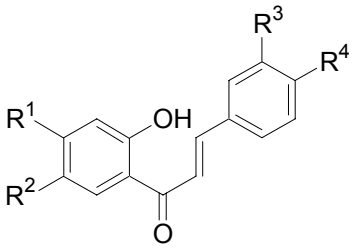
In summary, the first investigations revealed that flavone, flavonol and flavane derivatives have some inhibitory activity on the bovine testicular hyaluronidase, but only in the millimolar range. Furthermore, in the most cases the inhibitory effects were not concentration-dependent. The highest inhibitory activities were obtained with the sulphated flavones **50** and **52**, i.a. the introduction of an acidic group not only contributed to a better solubility of the compounds but also to an increase in potency.

Starting from these results a series of chalcones (**19b-f , 19h-k** and **23a-d**) and flavones (**25a-e**, **25f-i** and **26**) with different substituents, e.g. COO^- and OSO_3^-, OH, $N(CH_3)_2$, OCH_3, Cl and F was synthesised (cf. section 2 of this chapter) and tested for inhibition of BTH and $hylB_{4755}$. The inhibitory activities of these chalcone derivatives on the bovine testicular hyaluronidase (pH 3.6 and 7.4) and bacterial hyaluronidase (pH 5.0 and 7.4) are summarised in Table 5.

Table 5: Inhibitory activities of compounds **19b-f, 19h-k** and **23a-d** on BTH and $hylB_{4755}$



19b-f, 19h-k



23a-d

No	**Substitution**				**BTH**		**$hylB_{4755}$**	
					IC_{50} [µM] or (% Inhibition)			
	R^1	**R^2**	**R^3**	**R^4**	pH 3.6	pH 7.4	pH 5.0	pH 7.4
19b	H	H	OCH_3	H	**4000**	(70 %)[a]	(28 %)[a]	(66 %)[a]
19c	H	OCH_3	OCH_3	H	inactive[b]	inactive[b]	(44 %)[c]	**660**
19d	H	H	NMe_2	H	(71 %)[d]	(49 %)[d]	**310**	**380**
19e	H	benzo-annelated		H	inactive[b]	(15 %)[c]	(41 %)[c]	(80 %)[c]
19f	H	H	CO_2H	H	(55 %)[c]	(72 %)[c]	**110**	**220**
19h	H	H	Cl	H	**660**	**720**	(54 %)[c]	**70**
19i	Cl	OCH_3	OCH_3	H	inactive[e]	inactive[e]	(38 %)[f]	(44 %)[f]
19j	Cl	H	Cl	OCH_3	inactive[b]	(20 %)[c]	(24 %)[c]	(83 %)[c]
19k	H	H	F	H	(48 %)[c]	(40 %)[c]	**200**	**250**
23a	F	H	OCH_3	OCH_3	inactive[b]	inactive[b]	inactive[b]	**190**
23b	F	H	H	OCH_3	(21 %)[c]	(16 %)[c]	(34 %)[c]	**220**
23c	H	F	H	OCH_3	inactive[e]	inactive[e]	inactive[e]	inactive[e]
23d	H	F	H	F	(69 %)[c]	**1000**	**500**	(80 %)[c]

[a] % inhibition of the enzyme at an inhibitor concentration of 7 mM; [b] at concentrations ≤ 2 mM; [c] % inhibition of the enzyme at an inhibitor concentration of 2 mM; [d] % inhibition of the enzyme at an inhibitor concentration of 3.3 mM; [e] at concentrations of ≤100 µM; [f] % inhibition of the enzyme at an inhibitor concentration of 100 µM

Both methoxylated chalcones **19b** and **19c** induced different inhibition of the bovine testicular hyaluronidase. Whereas the IC_{50} value of compounds **19b** on BTH was 4 mM, the chalcone **19c** with an additional methoxy group in position 3' was inactive. By contrast, the inhibitory effect of the compound **19c** on the hyaluronate lyase was stronger than that of compound **19b**. The IC_{50} value determined for the chalcone **19c** on $hylB_{4755}$ was 660 µM at pH 7.4 (cf. Table5).

The chalcone derivative **19d** with a dimethylamino substituent in position 4' and two methoxy groups in the positions 4 and 6 inhibited both hyaluronidases in the millimolar range. The percentual inhibition of BTH by compound **19d** at a concentration of 3.3 mM was 71 % at optimum pH (3.6) and 49 % at physiological pH (7.4). The IC_{50} value of compound **19d** determined on $hylB_{4755}$ was 310 µM at pH 5.0 and 380 µM at pH 7.4. The concentration-dependent inhibition curves are depicted in Fig. 8.

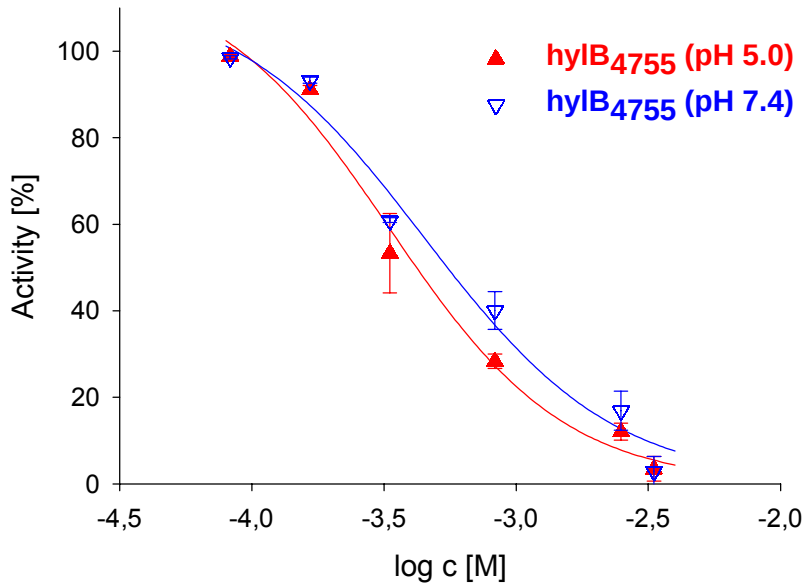


Fig. 8: Enzyme activity of hyaluronate lyase from *S. agalactiae* in the presence of the chalcone derivative **19d**

The chalcone derivative **19e** was almost inactive on the bovine testicular hyaluronidase and induced an inhibition of the bacterial hyaluronidase in the millimolar range (cf. Table 5).

Due to the results obtained with the sulphated flavones **50** and **52**, the 4'-carboxylated compound **19f** was expected to be also rather potent. However, the inhibition of BTH by the flavone **19f** was only 55 % at pH 3.6 and 72 % at pH 7.4 at a concentration of 2 mM. By contrast, the inhibitory effects on the bacterial hyaluronidase were concentration-dependent (cf. Fig. 9) and in the same order of magnitude as those of the sulphated flavones (IC_{50} values: 110 µM at pH 5.0 and 220 µM at pH 7.4).

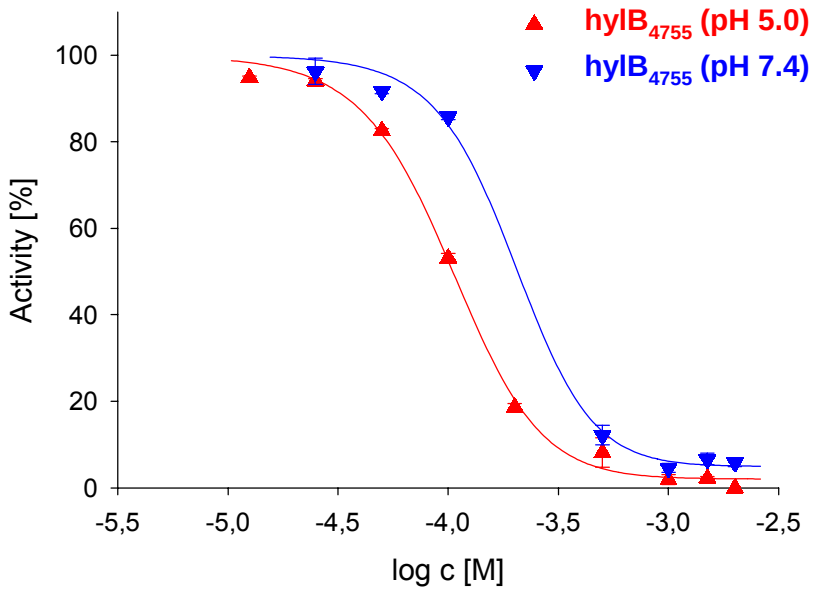


Fig. 9: Inhibitory effect of the chalcone derivative **19f** on the activity of the bacterial hyaluronidase from S. agalactiae at optimum and physiological pH

Within the series of chloro substituted derivatives **19h**, **19i** and **19j**, the monochlorinated compound **19i** (2'-Cl) was only active on the bacterial hyaluronidase (cf. Table 5), whereas the chalcone derivative **19j**, with two chloro substituents in the positions 2' and 4' inhibited both hyaluronidases. Surprisingly, the highest inhibitory effect was formed with compound **19h**, the chalcone derivative with p-chloro substituent and lacking the additional methoxy group(s) at the phenyl ring compared to the compounds **19i** and **19j** . The IC_{50} values of compound **19h** determined on BTH were 660 µM (pH 3.6) and 720 µM (pH 7.4). The potency of compound **19h** on $hylB_{4755}$ (IC_{50} of 70 µM) was 4-fold higher compared to chrysin-7-sulphat, the flavone derivative with a polar substituent. The concentration-dependent inhibition of BTH and $hylB_{4755}$ by compound **19h** are depicted in Fig. 10.

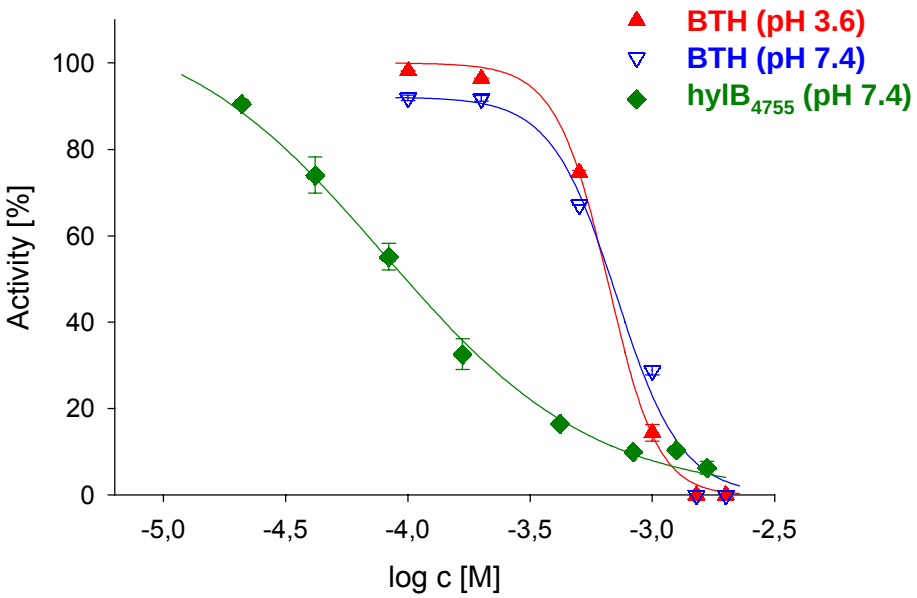


Fig. 10: Inhibition of the bovine testicular and the bacterial hyaluronidase by the chlorinated chalcone derivative **19h**

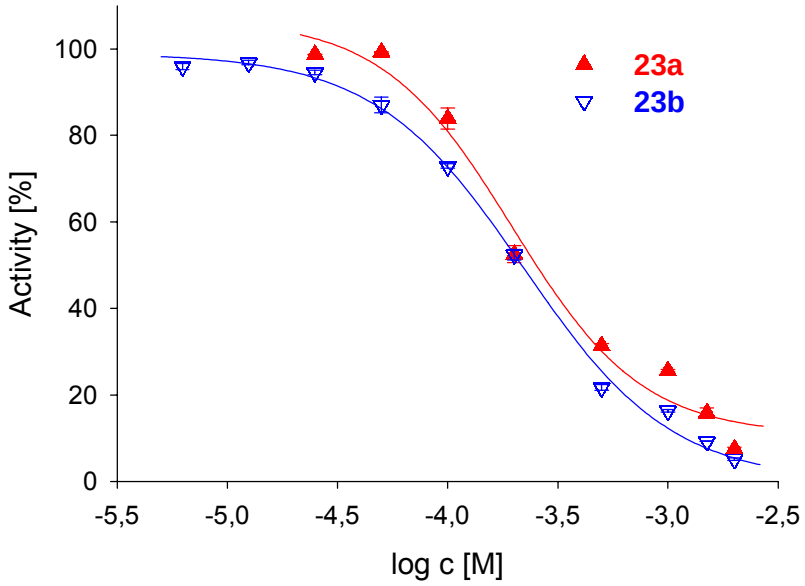


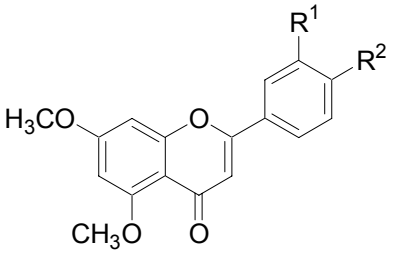
Fig. 11: Enzyme activity of $hylB_{4755}$ in the presence of the fluorinated chalcone derivatives **23a** and **23b** at physiological pH (7.4)

Of all fluorinated chalcone derivatives (**19k**, **23a**-**d**), the compounds **23c** and **23a** were inactive on the bovine testicular hyaluronidase and **23c** was inactive on the bacterial enzyme, too. The chalcone derivatives **19k**, **23b** and **23d** induced only a very weak inhibition of the BTH at millimolar concentration (cf. Table 5). Whereas the chalcone derivatives **23a** and **23b** showed no effect or a very weak effect on the bovine testicular hyaluronidase, the obtained inhibitory effects on the hyaluronate lyase at physiological pH were concentration-dependent (cf. Fig. 11). The calculated IC_{50} values of **23a** and **23b** were 190 µM and 200 µM, respectively.

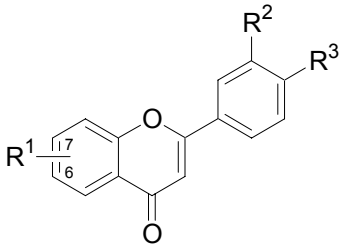
The IC_{50} values of compound **19k** determined on the bacterial enzyme were 200 µM at pH 5.0 and 250 µM at pH 7.4, whereas BTH was inhibited by only about 40 % at a concentration of 2 mM. In comparison to chrysin-7-sulphate (**52**), the fluorinated chalcone **19k** induced a lower inhibition of BTH, whereas the potencies on $hylB_{4755}$ were similar.

The inhibitory effects of the synthesised flavones **25a-i** and **26** on the bovine testicular hyaluronidase and the hyaluronate lyase from *S. agalactiae* at different pH values are summarised in Table 6.

Table 6: The effects of the synthesised flavone derivatives **25a-i** and **26** on the bovine testicular hyaluronidase and the *S. agalactiae* hyaluronate lyase



25a-e



25f-i, 26

No	**Substitution**			**BTH**		**hylB$_{4755}$**	
				IC_{50} [µM] or (% Inhibition)			
	R^1	**R^2**	**R^3**	pH 3.6	pH 7.4	pH 5.0	pH 7.4
25a	H	OCH_3	—	inactive[a]	inactive[a]	(13 %)[b]	(33 %)[b]
25b	OCH_3	OCH_3	—	inactive[a]	inactive[a]	inactive[a]	(45 %)[b]
25c	benzo-annelated		—	(26 %)[b]	inactive[a]	(26 %)[b]	(83 %)[b]
25d	H	Cl	—	(12 %)[b]	inactive[a]	(18 %)[b]	(66 %)[b]
25e	H	F	—	inactive[a]	inactive[a]	(64 %)[b]	(78 %)[b]
25f	6-F	H	OCH_3	(33 %)[b]	(36 %)[b]	(65 %)[b]	(77 %)[b]
25g	6-F	H	F	inactive[c]	inactive[c]	inactive[c]	inactive[c]
25h	7-F	OCH_3	OCH_3	inactive[a]	inactive[a]	(24 %)[b]	(56 %)[b]
25i	7-F	H	OCH_3	inactive[a]	inactive[a]	(63 %)[b]	**250**
26	7-F	H	OH	**970**	**1600**	**380**	**260**

[a] at concentrations ≤ 2 mM; [b] % inhibition of the enzyme at an inhibitor concentration of 2 mM; [c] at concentrations ≤ 100 µM

Comparing the inhibitory effects of the investigated flavone compounds **25a-i** and **26** on the bovine testicular enzyme, all compounds except 7-fluoro-4'-hydroxyflavone (**26**) showed either very weak inhibition or no effects at all. At millimolar concentrations the flavones **25c**, **25d** and **25f** induced about 12 % - 36 % inhibition of the bovine testicular hyaluronidase at (cf. Table 6). Only compound **26** was sufficiently active to determine IC_{50} values on both enzymes (BTH: 970 µM (pH 3.6) and 1.6 mM (pH 7.4) and $hylB_{4755}$: 380 µM (pH 5.0) and 260 µM (pH 7.4)). As expected the replacement of the p-methoxy group in compound **25i** with a hydroxy group (**26**) led to an increase in affinity on the bovine testicular hyaluronidase. The inhibition curves on BTH are depicted in Fig. 12.

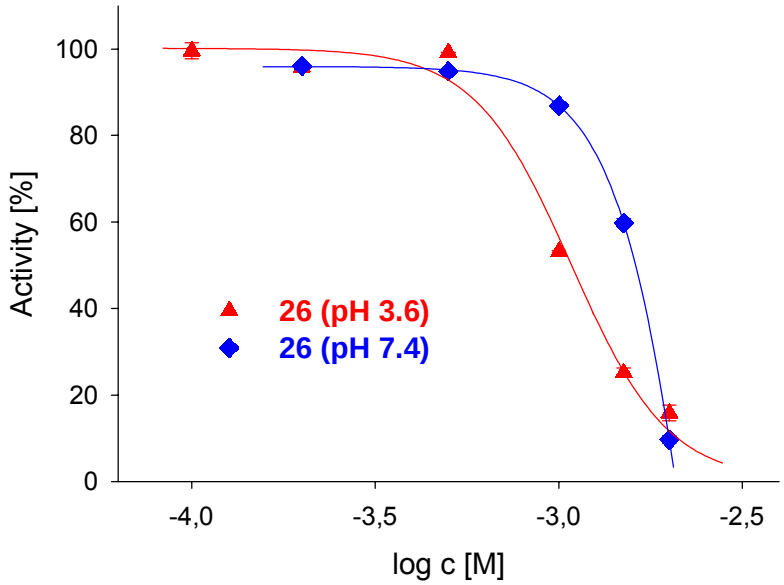


Fig. 12: Inhibitory effect of the 7-fluoro-4'-hydroxyflavone (**26**) on BTH at optimum pH (3.6) and physiological pH (7.4)

In contrast to the results for BTH, on the bacterial enzyme nine out of ten synthesised flavones induced inhibition at millimolar concentrations (cf. Table 6). The difluorinated flavone **25g** was inactive, whereas the compounds **25a-f** and **25h** induced partial inhibition of the hyaluronate lyase. The inhibitory effects on $hylB_{4755}$ at physiological pH were always stronger at pH 5.0, the optimum pH of the enzyme.

The highest inhibitory activities on the hyaluronate lyase were measured for the compounds **25i** and **26**. The IC_{50} values were 350 µM for **25i** at physiological pH, 380 µM and 260 µM for **26** at pH 5.0 and pH 7.4, respectively (cf. Table 6). The inhibition curves of the compounds **25i** and **26** are depicted in Fig. 13. Surprisingly, the replacement of the methoxy group by a hydroxy group did not increase the potency on the bacterial hyaluronidase.

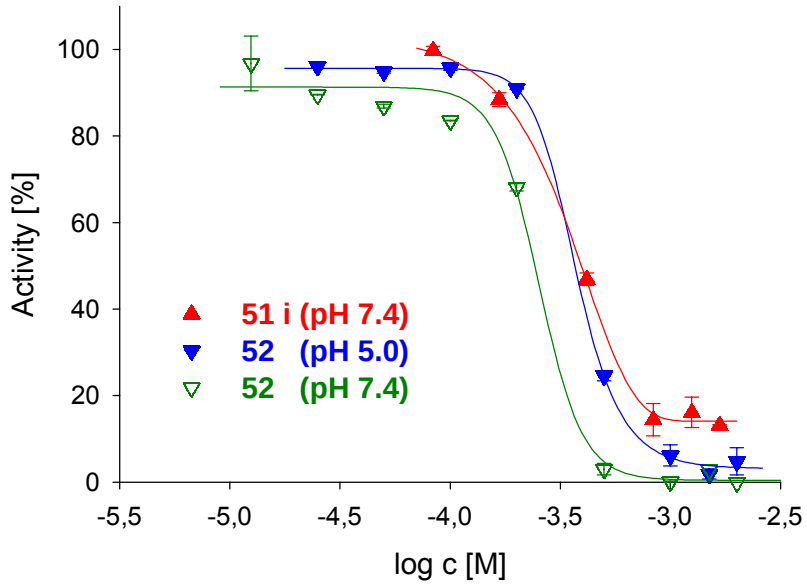


Fig. 13: Enzyme activity of the hyaluronate lyase $hylB_{4755}$ at physiological pH (7.4) in the presence of the fluorinated flavone derivatives **25i** and **26**

4. Summary

The inhibitory effects of some purported hyaluronidase inhibitors like apigenin, kaempferol and disodium chromoglycate (DSCG) on the bovine testicular hyaluronidase (BTH) were investigated. Of these compounds, disodium chromoglycate was the most potent inhibitor of BTH with an IC_{50} value of 1.24 mM. Furthermore, the inhibitory effects of some flavones (natural compounds) and sulphated flavones were measured. Sodium flavone-7-sulphate (**50**) and sodium 5-hydroxyflavone-7-sulphate (**52**) were more active BTH inhibitors (IC_{50}: 690 µM and 190 µM) than DSCG. Additionally, compound **52** was a hyaluronate lyase inhibitor with submillimolar activity (IC_{50}: 290 µM).

Starting from these results, a series of chalcone and flavone derivatives was synthesised and subsequently tested on the bovine testicular and the bacterial hyaluronidase. The chalcone derivatives 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-methoxyphenyl)propenone (**19b**) and 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-chlorophenyl)propenone (**19h**) and the flavone 7-fluoro-4'-hydroxyflavone (**26**) induced a concentration-dependent inhibition of the bovine testicular enzyme in the millimolar range. The IC_{50} values were comparable to those of the sulphated flavone **50** (690 µM), but higher than that of compound **52** (190 µM). In general, the increase in activity on BTH seems to be dependent on the presence of acidic functional groups.

Five chalcone derivatives and the two flavone derivatives were found to have inhibitory activity in the millimolar range on the bacterial hyaluronidase. The IC_{50} values of the most active compound were about 250 µM, that is comparable to that of the sulphated flavone **52**. Apparently, the presence of acidic groups is not that important for inhibition of hyaluronate lyase in comparison to the bovine testicular enzyme. 4'-Chloro-4,6-dimethoxychalcone (**19h**) was the most potent hyaluronate lyase inhibitor (IC_{50} = 70 µM) of all compounds described in this chapter.

5. Experimental section

5.1 General conditions

For a detailed description of the general procedures, equipments and chemicals used in the chemistry part, see section 4.1 of chapter 5.

5.2 Chemistry

1-(2-Hydroxy-4,6-dimethoxyphenyl)ethanone (**17**)

A solution of 1-(2,4,6-trihydroxyphenyl)ethanone (26.57 g, 142.52 mmol), potassium carbonate (59.17 g, 428.16 mmol), dimethyl sulphate (50.40 g, 399.62

mmol) and $Na_2S_2O_4$ (1.0 g, 5.74 mmol) in anhydrous acetone (800 ml) was stirred at room temperature for 2 h and then heated under reflux for two additional hours. After cooling to room temperature, the solution was diluted in small portion with water until a white solid precipitated. The product was collected and dried in vacuo at 55-60 °C.

Yield: 24.37 g (124.20 mmol, 87 %, white crystalline solid)

MP: 81 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 2.61 (3H, CC$\underline{H}_3$), 3.82 (s, 3H, OC$\underline{H}_3$), 3.86 (s, 3H, OC$\underline{H}_3$), 5.92 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.06 (d, 1H, ^{4}J = 2.4 Hz, H-5), 14.04 (s, 1H, O$\underline{H}$)

MS (PI-EIMS (70 eV)): m/z (%) = 196 ([$M^{\bullet+}$], 33), 181 ([M-CH_3]$^+$, 100), 166 ([M-2CH_3]$^+$, 8)

IR [cm^{-1}]: 3210 (O-H), 3000 (C-H) aromatic, 2927 (C-H) aliphatic, 1612 (C=O), 1422, 1366 (C-H), 1268 (C-H), 1202 (C-O), 1155, 1110, 940, 834, 804

$C_{10}H_8O_4$ (196.2)

1-[2-(4-Cyanobenzoyloxy)-4,6-dimethoxyphenyl]ethanone (18a)

A solution of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (2.0 g, 10.20 mmol) and p-cyanobenzoyl chloride (2.03 g, 12.24 mmol) in pyridine (20 ml) was stirred and heated under reflux for 90 min. Afterwards, the mixture was poured into diluted hydrochloric acid (40 ml, 5 %) and extracted three times with chloroform (50 ml). The combined extracts were washed with water (2 x 30 ml), dried over sodium sulphate and evaporated under reduced pressure to obtain a yellow solid.

Yield: 2.30 g (7.0 mmol, 67 %, yellow solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.39 (s, 3H, CC$\underline{H}_3$), 3.83 (s, 3H, OC$\underline{H}_3$), 3.90 (s, 3H, OC$\underline{H}_3$), 6.61 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.66 (d, 1H, ^{4}J = 2.3 Hz, H-5), 7.84-7.87 (m, 2H, H-3, H-5), 8.16-8.19 (m, 2H, H-2, H-6)

MS (PI-EIMS (70 eV)): m/z (%) =325 ([M$^{\bullet+}$], 27), 310 ([M-CH$_3$]$^+$, 61), 282 ([M-CH$_3$-CO]$^+$, 2), 130 ([CNC$_6$H$_4$CO]$^+$, 100), 102 ([CNC$_6$H$_4$]$^+$, 35)

IR [cm^{-1}]: 2989 (C-H) aromatic, 2861 (C-H) aliphatic, 2200 (C≡N), 1738-1657 (C=O), 1605 (C=C), 1407 (C-H), 1248 (C-O), 1154, 1105, 862, 838, 779, 686

$C_{18}H_{15}NO_5$ (325.32)

1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-cyanophenyl)propan-1,3-dione (20a)

To a solution of compound **18a** (1.0 g, 3.07 mmol) in ethanol (30 ml), a potassium hydroxide solution (15 ml, 60 %) was added dropwise and the mixture was stirred at room temperature for 24 h. After cooling with an ice bath, the reaction mixture was acidified with concentrated hydrochloric acid and diluted in small portion with water. The yellow precipitate was collected, washed with water and dried in vacuo over silica gel. The product was used without further purifications.

Yield: 0.96 g (yellow solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.81 (s, 3H, OC$\underline{H}_3$), 3.86 (s, 3H, OC$\underline{H}_3$), 6.08 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.11 (d, 1H, ^{4}J = 2.4 Hz, H-5), 7.97-8.01 (m, 4H, ph), 8.04-8.07 (m, 1H, =C$\underline{H}$), 13.35 (br, 1H, OH), 13.79 (s, 1H, OH)

$C_{18}H_{15}NO_5$ (325.32)

General procedure for the preparation of the chalcone derivatives 19b-k and 23a-d

To a solution of the pertinent substituted (2-hydroxyphenyl)ethanone and substituted benzaldehyde in ethanol (30-100 ml), an aqueous potassium hydroxide solution (15-30 ml, 60 %) was added dropwise and the mixture was stirred at room temperature for 24 h. After cooling with an ice bath, the reaction mixture was acidified with concentrated hydrochloric acid and diluted in small portion with water until a solid precipitated. The product was collected, dried over silica gel and recrystallised.

1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-methoxyphenyl)propenone (**20b)**

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (2.0 g, 10.19 mmol) and 4-methoxybenzaldehyde (3.06 g, 22.48 mmol); recrystallisation from DMSO;
Yield: 3.17 g (10.08 mmol, 99 %, orange crystalline solid)

Mp: 94 °C

^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.82 (s, 6H, OC$\underline{H}_3$), 3.90 (s, 3H, OC$\underline{H}_3$), 6.10 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.13 (d, 1H, ^{4}J = 2.3 Hz, H-5), 7.00 (d, 2H, ^{3}J = 8.8 Hz, H-3', H-5'), 7.67 (d, 2H, ^{3}J = 8.8 Hz, H-2', H-6'), 7.66-7.71 (m, 2H, =C$\underline{H}$), 13.61(s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 55.3 (+, O$\underline{C}H_3$), 55.6 (+, O$\underline{C}H_3$), 56.1 (+, O$\underline{C}H_3$), 91.1 (+, ph), 93.9 (+, ph), 106.3 (C_{quart}, ph), 114.5 (+, ph), 124.9 (+, =CH), 127.4 (C_{quart}, ph), 130.4 (+, ph), 142.6 (+, =CH), 161.3 (C_{quart}, ph), 161.9 (C_{quart}, ph), 165.4 (C_{quart}, ph), 165.6 (C_{quart}, ph), 192.2 (C_{quart}, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) = 314 ([M$^{\bullet+}$], 100), 313 ([M-H]$^+$, 72), 207 ([M-C$_6$H$_4$OMe]$^+$, 38), 134 ([MeOC$_6$H$_4$CH=CH$_2$]$^+$, 51), 121 ([MeOC$_6$H$_4$CH$_2$]$^+$, 58)

IR [cm^{-1}]: 3084 (C-H) aromatic, 2843 (C-H) aliphatic, 1580, 1512, 1439 (C-H), 1344 (C-H), 1255 (C-O), 1177, 1114, 972, 900, 821, 615

Analysis:

calculated	C: 68.78	H: 5.77
found	C: 68.40	H: 5.77

$C_{18}H_{18}O_5$ (314.34)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(3,4-dimethoxyphenyl)propenone (19c)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (3.0 g, 15.29 mmol) and 3,4-dimethoxybenzaldehyde (5.59 g, 33.64 mmol); recrystallisation from a 1:1 (v/v) mixture of toluene and acetone;
Yield: 5.08 g (14.75 mmol, 96 %, orange crystalline solid)

MP: 107-110°C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.82 (s, 3H, OC$\underline{H}_3$), 3.82 (s, 3H, OC$\underline{H}_3$), 3.83 (s, 3H, OC$\underline{H}_3$), 3.89 (s, 3H, OC$\underline{H}_3$), 6.12 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.16 (d, 1H, ^{4}J = 2.3 Hz, H-5), 7.00-7.04 (m, 1H, ph), 7.27-7.32 (m, 2H, ph), 7.60 (d, 1H, ^{3}J = 15.7 Hz, =C$\underline{H}$), 7.67 (d, 1H, ^{3}J = 15.7 Hz, =C$\underline{H}$), 13.41 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.5 (+, O$\underline{C}H_3$), 55.6 (+, O$\underline{C}H_3$), 55.6 (+, O$\underline{C}H_3$), 56.1 (+, O$\underline{C}H_3$), 91.0 (+, ph), 93.9 (+, ph), 106.5 (C_{quart}, ph), 110.8 (+, ph), 111.8 (+, ph), 122.8 (+, ph), 125,2 (+, =$\underline{C}$H), 127.6 (C_{quart}, ph), 143.0 (+, =$\underline{C}$H), 149.0 (C_{quart}, ph), 151.1 (C_{quart}, ph), 161.7 (C_{quart}, ph), 165.2 (C_{quart}, ph), 165.2 (C_{quart}, ph), 192.3 (C_{quart}, $\underline{C}$=O)

MS (PI EI 70 eV): m/z (%) = 344 ([M$^{\bullet+}$], 100), 164 ([MeO)$_2$C$_6$H$_4$CH=CH$_2$]$^{+}$, 73), 151 ([MeO)$_2$C$_6$H$_4$CH$_2$]$^{+}$, 93)

HR-MS: calculated: 344.12550 amu
found: 344.12599 amu

IR [cm^{-1}]: 3000 (C-H) aromatic, 2838 (C-H) aliphatic, 1618 (C=O), 1509, 1441 (C-H), 1303 (C-H), 1218 (C-O), 1145, 1110,1022, 815, 760, 615

$C_{19}H_{20}O_6$ (344.36)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-dimethylaminophenyl)propenone (19d)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (1.0 g, 5.10 mmol) and 4-*N,N*-dimethylaminobenzaldehyde (1.68 g, 11.22 mmol);
Yield: 1.59 g (4.87 mmol, 96 %, red solid)

Mp: 160 °C

^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.01 (s, 3H, NC$\underline{H}_3$), 3.05 (s, 3H, NC$\underline{H}_3$), 3.81 (s, 3H, OC$\underline{H}_3$), 3.90 (s, 3H, OC$\underline{H}_3$), 6.10 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.14 (d, 1H, ^{4}J = 2.4 Hz, H-5), 6.67-6.81 (m, 2H, H-3', H-5'), 7.54-7.72 (m, 2H, H-2', H-6'), 7.58 (d, 1H, ^{3}J = 15.5 Hz, =C$\underline{H}$), 7.67 (d, 1H, ^{3}J = 15.5 Hz, =C$\underline{H}$), 14.0 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 39.7 (+, N$\underline{C}$H$_3$), 39.7 (+, N$\underline{C}$H$_3$), 55.6 (+, O$\underline{C}$H$_3$), 56.1 (+, O$\underline{C}$H$_3$), 91.0 (+, ph), 93.9 (+, ph), 106.2 (C$_{quart}$, ph), 111.9 (+, ph), 121.2 (+, =CH), 122.0 (C$_{quart}$, ph), 130.5 (+, ph), 144.4 (+, =CH), 152.0 (C$_{quart}$, ph), 161.8 (C$_{quart}$, ph), 165.1 (C$_{quart}$, ph), 165.9 (C$_{quart}$, ph), 191.8 (C$_{quart}$, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) = 327 ([M$^{\bullet+}$], 52), 174 ([Me$_2$NC$_6$H$_4$CH=CHCO]$^+$, 7), 147 ([Me$_2$NC$_6$H$_4$CH=CH$_2$]$^+$, 83), 134 ([Me$_2$NC$_6$H$_4$CH$_2$]$^+$, 100)

IR [cm^{-1}]: 3000 (C-H) aromatic, 2832 (C-H) aliphatic, 1587, 1527, 1480 (C-H), 1346 (C-H), 1209 (C-O), 1153 (C-N), 1030, 985, 919, 808, 764, 615

Analysis:

calculated	C: 69.71	H: 6.47	N: 4.28
found	C: 69.34	H: 6.50	N: 4.44

$C_{19}H_{21}NO_4$ (327.38)

1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(naphth-2-yl)propenone (19e)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (3.0 g, 15.29 mmol) and naphthalene-2-carbaldehyde (5.25 g, 33.64 mmol); recrystallisation from dimethylsulphoxide

Yield: 3.20 g (9.57 mmol, 63 %, yellow solid)

Mp: 92 - 95 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.84 (s, 3H, OC$\underline{H}_3$), 3.93 (s, 3H, OC$\underline{H}_3$), 6.14 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.17 (d, 1H, ^{4}J = 2.3 Hz, H-5), 7.56-7.60 (m, 2H, naphthyl), 7.83 (s, 1H, =C$\underline{H}$), 8.23 (s, 1H, =C$\underline{H}$), 7.86-8.00 (m, 5H, naphthyl), 13.41 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.7 (+, O$\underline{C}H_3$), 56.3 (+, O$\underline{C}H_3$), 91.1 (+, ph), 93.91 (+, ph), 106.4 (C_{quart}, ph), 123.9 (+, naphthyl), 126.8 (+, naphthyl), 127.4 (+, naphthyl), 127.7 (+, naphthyl), 127.8 (+, naphthyl), 128.6 (+, naphthyl), 128.7 (+, naphthyl), 130.4 (+, =$\underline{C}$H), 132.4 (C_{quart}, naphthyl), 133.0 (C_{quart}, naphthyl), 133.8 (C_{quart}, naphthyl), 142.5 (+, =$\underline{C}$H), 161.9 (C_{quart}, ph), 165.4 (C_{quart}, ph), 165.5 (C_{quart}, ph), 192.3 (C_{quart}, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) =334 ([M$^{\bullet+}$], 100), 333 ([M-H]$^{+}$, 62), 207 ([M-naphthyl]$^{+}$, 58), 181 ([M-naphthyl-C_2H_2]$^{+}$, 26), 153 ([M- naphthyl-C_2H_2-CO]$^{+}$, 25)

IR [cm^{-1}]: 3399 (H-O), 3009 (C-H) aromatic, 2921 (C-H) aliphatic, 1619 (C=O), 1556, 1436 (C-H), 1361 (C-H), 1212 (C-O), 1157, 1112, 952, 856, 818, 761

$C_{21}H_{18}O_4$ (334.37)

(E) 4-[3-(2-Hydroxy-4,6-dimethoxyphenyl)-3-oxopropenyl]benzoic acid (19f)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (2.0 g, 10.19 mmol) and sodium 4-formylbenzoate (3.68 g, 22.42 mmol); recrystallisation from dimethylsulphoxide
Yield: 3.01 g (9.17 mmol, 90 %, orange solid)

Mp: 218 °C
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.84 (s, 3H, OC$\underline{H}_3$), 3.91 (s, 3H, OC$\underline{H}_3$), 6.13 (d, 1H, ^{4}J = 2.2 Hz, H-3), 6.15 (d, 1H, ^{4}J = 2.2 Hz, H-5), 7.66 (d, 1H, ^{3}J = 15.8 Hz, =C$\underline{H}$), 7.84 (d, 1H, ^{3}J = 15.8 Hz, =C$\underline{H}$), 7.70-7.88 (m, 2H, ph), 7.94-8.05 (m, 2H, ph), 11.50-13.30 (br, 1H, COO$\underline{H}$), 13.36 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 55.6 (+, O$\underline{C}H_3$), 56.2 (+, O$\underline{C}H_3$), 91.1 (+, ph), 93.8 (+, ph), 106.3 (C_{quart}, ph), 128.4 (+, ph), 129.6 (+, =$\underline{C}$H), 129.8 (+, ph), 131.8 (C_{quart}, ph), 138.9 (C_{quart}, ph), 140.5 (+, =$\underline{C}$H), 161.9 (C_{quart}, ph), 165.5 (C_{quart}, ph), 165.7 (C_{quart}, ph), 166.7 (C_{quart}, $\underline{C}$OOH), 192.0 (C_{quart}, $\underline{C}$=O)

MS (PI-EIMS (70 eV): m/z (%) = 328 ([M$^{\bullet+}$], 72), 329 ([M-H]$^+$, 52), 207 ([M-$C_6H_4CO_2H$]$^+$, 100), 181 ([M - CH=CH$C_6H_4CO_2H$]$^+$, 31)

IR [cm^{-1}]: 3008 (C-H) aromatic, 2832 (C-H) aliphatic, 1682 (C=O), 1556, 1408 (C-H), 1341 (C-H), 1211 (C-O), 1156, 1108, 935, 847, 751, 608

Analysis:

calculated	C: 65.49	H: 4.91
found	C: 65.16	H: 4.92

$C_{18}H_{16}O_6 \bullet 0.1\ H_2O$ (328.32)

(E) 4-[3-(2-Hydroxy-4,6-dimethoxyphenyl)-3-oxopropenyl]benzene-1,3-disulphonic acid (19g)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (1.0 g, 5.10 mmol) and disodium 4-formylbenzene-1,3-disulphonate monohydrate (3.68 g, 11.22 mmol); after filtration the solvent was removed under reduced pressure; The purification by standard methods failed.
Yield: 0.66 g (yellow solid)

^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.83 (s, 3H, OC<u>H</u>$_3$), 3.91 (s, 3H, OC<u>H</u>$_3$), 6.13 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.16 (d, 1H, ^{4}J = 2.4 Hz, H-5), 7.61 (m, 1H, H-5'), 7.68 (d, 1H, ^{3}J = 15.2 Hz, =C<u>H</u>), 7.74 (d, 1H, ^{3}J = 8.3 Hz, H-6'), 8.14 (d, 1H, ^{4}J = 2.0 Hz, H-3'), 8.74 (d, 1H, ^{3}J = 15.2 Hz, =C<u>H</u>), 13.77 (s, 1H, O<u>H</u>)

MS (ESI): m/z (%) = 443 ([M-H]$^-$, 48), 227 ([M-2TFA-H]$^-$, 100)

$C_{17}H_{16}O_{10}S_2$ (444.31)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-chlorophenyl)propenone (19h)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (3.0 g, 15.29 mmol) and 4-chlorobenzaldehyde (4.58 g, 33.64 mmol); recrystallisation from dimethylsulphoxide
Yield: 4.48 g (14.25 mmol, 93 %, yellow crystalline solid)

Mp: 153 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.83 (s, 3H, OC<u>H</u>$_3$), 3.90 (s, 3H, OC<u>H</u>$_3$), 6.14 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.16 (d, 1H, ^{4}J = 2.4 Hz, H-5), 7.48-7.53 (m, 2H, H-3', H-5'), 7.75-7.78 (m, 2H, H-2', H-6'), 7.62 (d, 1H, ^{3}J = 15.8 Hz, =C<u>H</u>), 7.76 (d, 1H, ^{3}J = 15.8 Hz, =C<u>H</u>), 14.00 (s, 1H, O<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.7 (+, O<u>C</u>H$_3$), 56.2 (+, O<u>C</u>H$_3$), 91.3 (+, ph), 93.9 (+, ph), 106.3 (C$_{quart}$, ph), 128.2 (+, =<u>C</u>H), 129.1 (+, ph), 130.1 (+, ph), 133.8 (C$_{quart}$, ph), 134.9 (C$_{quart}$, ph), 140.7 (+, =<u>C</u>H), 161.9 (C$_{quart}$, ph), 165.5 (C$_{quart}$, ph), 165.7 (C$_{quart}$, ph), 192.2 (C$_{quart}$, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 318 ([M$^{\bullet+}$], 76), 317 ([M-H]$^+$, 60), 207 ([(CH$_3$O)$_2$C$_6$H$_3$COCH=CH$_2$]$^+$, 100), 181 ([(CH$_3$)$_2$C$_6$H$_4$CO]$^+$, 52), 165 ([ClC$_6$H$_4$CH$_2$=CHCO]$^+$, 13), 138 ([ClC$_6$H$_4$CH$_2$=CH]$^+$, 16)

IR [cm^{-1}]: 3414 (O-H), 3021 (C-H) aromatic, 2947 (C-H) aliphatic, 1565, 1487, 1440 (C-H), 1337 (C-H), 1213 (C-O), 1024 (C-Cl), 970, 818, 788, 757

Analysis:

calculated	C: 64.06	H: 4.74
found	C: 63.65	H: 4.80

C$_{17}$H$_{15}$ClO$_4$ (318.76)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(2-chloro-3,4-dimethoxyphenyl)-propenone (19i)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (3.0 g, 15.29 mmol) and 2-chloro-3,4-dimethoxybenzaldehyde (6.72 g, 33.64 mmol); recrystallisation from dimethylsulphoxide

Yield: 4.38 g (11.56 mmol, 76 %, orange crystalline solid)

Mp: 152 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.77 (s, 3H, OC$\underline{H}_3$), 3.83 (s, 3H, OC$\underline{H}_3$), 3.89 (s, 3H, OC$\underline{H}_3$), 3.91 (s, 3H, OC$\underline{H}_3$), 6.13 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.15 (d. 1H, ^{4}J = 2.3 Hz, H-5), 7.15 (d, 1H, ^{3}J = 8.9 Hz, H-5'), 7.69 (d, 1H, ^{3}J = 15.6 Hz, =C$\underline{H}$), 7.70 (d, 1H, ^{3}J = 8.9 Hz, H-6'), 7.89 (d, 1H, ^{3}J = 15.6 Hz, =C$\underline{H}$), 13.33 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.7 (+, O$\underline{C}$H$_3$), 56.2 (+, O$\underline{C}$H$_3$), 56.3 (+, O$\underline{C}$H$_3$), 60.1 (+, O$\underline{C}$H$_3$), 91.1 (+, ph), 93.9 (+, ph), 106.3 (C$_{quart}$, ph), 112.0 (+, ph), 123.8 (+, ph), 125.3 (C$_{quart}$, ph), 128.1 (+, =$\underline{C}$H), 128.6 (C$_{quart}$, ph), 137.4 (+, =$\underline{C}$H), 145.0 (C$_{quart}$, ph), 155.0 (C$_{quart}$, ph), 161.9 (C$_{quart}$, ph), 165.4 (C$_{quart}$, ph), 165.6 (C$_{quart}$, ph), 191.9 (C$_{quart}$, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) = 378 ([M$^{\bullet+}$], 18), 343 ([M-Cl]$^{+}$, 100)

IR [cm^{-1}]: 3100 (C-H) aromatic, 2843 (C-H) aliphatic, 1626-1549 (C=O), 1489, 1421 (C-H), 1299 (C-H), 1209 (C-O), 1035 (C-Cl), 976, 884, 821, 796, 649

Analysis:

calculated	C: 60.24	H: 5.06
found	C: 60.00	H: 4.95

$C_{19}H_{19}ClO_6$ (378.80)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(2,4-dichloro-6-methoxyphenyl)-propenone (19j)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (5.0 g, 25.48 mmol) and 2,4-dichloro-6-methoxybenzaldehyde (11.49 g, 56.06 mmol); recrystallisation from dimethylsulphoxide;

Yield: 9.56 g (24.95 mmol, 98 %, yellow solid)

Mp: 145-147°C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.83 (s, 3H, OC$\underline{H}_3$), 3.89 (s, 3H, OC$\underline{H}_3$), 3.99 (s, 3H, OC$\underline{H}_3$), 6.12 (d, 1H, ^{4}J = 2.3 Hz, H-3), 6.14 (d, 1H, ^{4}J = 2.3 Hz, H-5), 7.26 (d, 1H, ^{4}J = 2.0 Hz, H-5'), 7.33 (d, 1H, ^{4}J = 2.0 Hz, H-3'), 7.88 (d, 1H, ^{3}J = 15.9 Hz, =C$\underline{H}$), 8.18 (d, 1H, ^{3}J = 15.9 Hz, =C$\underline{H}$), 13.50 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.7 (+, OC$\underline{H}_3$), 56.1 (+, OC$\underline{H}_3$), 56.9 (+, OC$\underline{H}_3$), 91.2 (+, ph), 93.9 (+, ph), 106.2 (C_{quart}, ph), 111.8 (+, ph), 120.2 (C_{quart}, ph), 121.7 (+, ph), 132.6 (+, =C$\underline{H}$), 133.2 (C_{quart}, ph), 135.5 (C_{quart}, ph), 135.9 (+, =C$\underline{H}$), 160.3 (C_{quart}, ph), 162.1 (C_{quart}, ph), 165.9 (C_{quart}, ph), 166.1 (C_{quart}, ph), 192.5 (C_{quart}, C$\underline{=O}$)

MS (PI-EI 70 eV): m/z (%) = 382 ([M$^{\bullet+}$], 76), 347 ([M-Cl]$^{+}$, 100)

HR-MS: calculated: 382.03754 amu
found: 382.03747 amu

IR [cm^{-1}]: 2988 (C-H) aromatic, 2860 (C-H) aliphatic, 1632 (C=O), 1548, 1448 (C-H), 1341 (C-H), 1220 (C-O), 1110-1037 (C-Cl), 979, 873, 833, 816, 621

$C_{19}H_{18}O_6$ (382,22)

(E) 1-(2-Hydroxy-4,6-dimethoxyphenyl)-3-(4-fluorophenyl)propenone (19k)

Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (3.0 g, 15.29 mmol) and 4-fluorobenzaldehyde (4.18 g, 33.68 mmol); recrystallisation from dimethylsulphoxide
Yield: 4.46 g (14.74 mmol, 96 %, orange solid)

Mp: 121 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.45 (s, 3H, OC$\underline{H}_3$), 3.83 (s, 3H, OC$\underline{H}_3$), 6.12 (d, 1H, ^{4}J = 2.4 Hz, H-3), 6.14 (d, 1H, ^{4}J = 2.4 Hz, H-5), 7.27 (d, 1H, ^{3}J = 8.9 Hz, H-3'), 7.29 (d, 1H, ^{3}J

= 8.9 Hz, H-5'), 7.62 (d, 1H, ^3J = 15.8 Hz, =CH), 7.71 (d, 1H, ^3J = 15.8 Hz, =CH), 7.77 (d, 1H, ^3J = 8.8 Hz, H-2'),7.80 (d, 1H, ^3J = 8.8 Hz, H-6'), 13.43 (s, 1H, O<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.7 (+, O<u>C</u>H$_3$), 56.2 (+, O<u>C</u>H$_3$), 91.1 (+, ph), 93.9 (+, ph), 106.3 (C$_{quart}$, ph), 115.9 (+, ph), 116.2 (+, ph), 127.3 (+, =<u>C</u>H), 130.7 (+, ph), 130.8 (+, ph), 131.5 (C$_{quart}$, $^4J_{C,F}$ = 3.2 Hz, C-F), 141.1 (+, =<u>C</u>H), 161.9 (C$_{quart}$, ph), 165.2 (C$_{quart}$, $^1J_{C,F}$ = 248.6 Hz, C-F), 165.5 (C$_{quart}$, ph), 165.6 (C$_{quart}$, ph), 192.2 (C$_{quart}$, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 302 ([M$^{\bullet+}$], 84), 301 ([M-H]$^+$, 77), 207 ([M- C$_6$H$_4$F]$^+$, 100), 181 ([MeO)$_2$C$_6$H$_4$(OH)CO]$^+$, 60)

IR [cm^{-1}]: 3012 (C-H) aromatic, 2944 (C-H) aliphatic, 1626 (C=O), 1505, 1443 (C-H), 1341 (C-H), 1276 (C-F), 1213 (C-O), 1154, 1112, 986, 822, 760, 614

Analysis:

calculated	C: 67.54	H: 5.00
found	C: 67.42	H: 4.88

C$_{17}$H$_{15}$FO$_4$ (302.30)

(E) 1-(4-Fluoro-2-hydroxyphenyl)-3-(3,4-dimethoxyphenyl)propenone (23a)

Reaction of 1-(4-fluoro-2-hydroxyphenyl)ethanone (0.70 g, 4.54 mmol) and 3,4-dimethoxybenzaldehyde (1.17 g, 7.14 mmol); recrystallisation from dimethylsulphoxide

Yield: 1.08 g (3.57 mmol, 79 %, orange crystalline solid)

Mp: 145 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.84 (s, 3H, OC$\underline{H}_3$), 3.88 (s, 3H, OC$\underline{H}_3$), 6.83-6.92 (m, 2H, ph), 7.03 (d, 1H, ^{3}J = 8.4 Hz, H-5'), 7.43 (dd, 1H, ^{4}J = 2.0 Hz, ^{3}J = 8.4 Hz, H-6'), 7.56 (d, 1H, ^{4}J = 2.0 Hz, H-2'), 7.81 (d, 1H, ^{3}J = 15.4 Hz, =C$\underline{H}$), 7.90 (d, 1H, ^{3}J = 15.4 Hz, =C$\underline{H}$), 8.40-8.46 (m, 1H, ph), 13.30 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.6 (+, O$\underline{C}$H$_3$), 55.7 (+, O$\underline{C}$H$_3$), 104.2 (+, $^{2}J_{C,F}$ = 23.5 Hz, C-F), 106.8 (+, $^{2}J_{C,F}$ = 22.3 Hz, C-F), 110.9 (+, ph), 111.5 (+, ph), 117.7 (C_{quart}, $^{4}J_{C,F}$ = 2.5 Hz, C-F), 118.6 (C_{quart}, ph), 124.7 (+, =$\underline{C}$H), 127.1 (C_{quart}, ph), 133.6 (+, $^{3}J_{C,F}$ = 12.1 Hz, C-F), 145.8 (+, =$\underline{C}$H), 148.9 (C_{quart}, ph), 151.7 (C_{quart}, ph), 164.6 (C_{quart}, $^{3}J_{C,F}$ = 13.9 Hz, C-F), 166.4 (C_{quart}, $^{1}J_{C,F}$ = 253.7 Hz, C-F), 192.5 (C_{quart}, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) = 302 ([M$^{\bullet+}$], 71), 301 ([M-H]$^{+}$, 23), 164 ([MeO)$_2$ C$_6$H$_4$CH=CH$_2$]$^{+}$, 47), 151 ([(MeO)$_2$C$_6$H$_4$CH$_2$]$^{+}$, 100), 139 ([FC$_6$H$_4$(OH)CO]$^{+}$, 26), 121 ([MeOC$_6$H$_4$CH$_2$]$^{+}$, 6)

IR [cm^{-1}]: 3017 (C-H) aromatic, 2841 (C-H) aliphatic, 1632-1560 (C=O), 1508, 1464 (C-H), 1359 (C-H), 1263 (C-F),1206 (C-O), 1140, 989, 974, 845, 798, 608

Analysis:

calculated	C: 67.54	H: 5.00
found	C: 67.59	H: 4.92

$C_{17}H_{15}FO_4$ (302.30)

1-(4-Fluoro-2-hydroxyphenyl)-3-(4-methoxyphenyl)propenone (23b)

Reaction of 1-(4-fluoro-2-hydroxyphenyl)ethanone (0.50 g, 3.25 mmol) and 4-methoxybenzaldehyde (0.97 g, 7.14 mmol); recrystallisation from dimethyl-sulphoxide;
Yield: 0.84 g (3.09 mmol, 95 %, yellow solid)

Mp: 126 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.84 (s, 3H, OC<u>H</u>$_3$), 6.83-6.91 (m, 2H, ph), 7.03-7.06 (m, 2H, ph), 7.81-7.88 (m, 2H, ph), 7.88-7.94 (m, 2H, =C<u>H</u>), 8.83-8.44 (m, 1H, ph), 13.23 (s, 1H, O<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.4 (+, O<u>C</u>H$_3$), 104.2 (+, $^{2}J_{C,F}$ = 23.5 Hz, C-F), 106.9 (+, $^{2}J_{C,F}$ = 22.3 Hz, C-F), 114.4 (+, ph), 117.7 (C_{quart}, $^{1}J_{C,F}$ = 2.5 Hz, C-F), 118.7 (C_{quart}, ph), 126.9 (+, =CH), 131.2 (+, ph), 133.6 (+, $^{3}J_{C,F}$ = 12.1 Hz, C-F), 145.2 (+, =CH), 161.7 (C_{quart}, ph), 164.5 (C_{quart}, $^{2}J_{C,F}$ = 13.9 Hz, C-F), 166.4 (C_{quart}, $^{1}J_{C,F}$ = 253.0 Hz, C-F), 192.4 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 272 ([M$^{\bullet+}$], 73), 271 ([M-H]$^+$, 53), 139 ([FC$_6$H$_4$(OH)CO]$^+$, 37), 134 ([MeOC$_6$H$_4$CH=CH$_2$]$^+$, 99), 121 ([MeOC$_6$H$_4$CH$_2$]$^+$, 100)

IR [cm^{-1}]: 3406 (O-H), 2949 (C-H) aromatic, 2848 (C-H) aliphatic, 1634 (C=O), 1548, 1416 (C-H), 1352 (C-H), 1280 (C-F),1255 (C-O), 1173, 1122, 1016, 843, 820, 609

Analysis:

calculated	C: 70.58	H: 4.81
found	C: 70.38	H: 4.88

$C_{16}H_{13}FO_3$ (272.27)

(E) 1-(5-Fluoro-2-hydroxyphenyl)-3-(4-methoxyphenyl)propenone (23c)

Reaction of 1-(5-fluoro-2-hydroxyphenyl)ethanone (2.32 g, 15.05 mmol) and 4-methoxybenzaldehyde (4.51 g, 33.11 mmol); recrystallisation from dimethyl-sulphoxide

Yield: 4.03 g (14.80 mmol, 98 %, orange crystalline solid)

Mp: 104 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.84 (s, 3H, OC<u>H</u>$_3$), 6.99-7.02 (m, 1H, H-3), 7.03-7.07 (m, 2H, ph), 7.41-7.49 (m, 1H, H-4), 7.81 (d, 1H, ^{3}J = 15.5 Hz, =C<u>H</u>), 7.88 (d, 1H, ^{3}J = 15.5 Hz, =C<u>H</u>), 8.11 (dd, ^{3}J = 3.12 Hz, $^{3}J_{H,F}$ = 9.8 Hz, H-F), 12.42 (s, 1H, O<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.4 (+, O<u>C</u>H$_3$), 114.4 (+, 2ph), 115.7 (+, $^{2}J_{C,F}$ = 23.8 Hz, C-F), 118.9 (+, =<u>C</u>H), 119.1 (+, $^{3}J_{C,F}$ = 7.6 Hz, C-F), 120.9 (C_{quart}, $^{3}J_{C,F}$ = 6.6 Hz, C-F), 123.3 (+, $^{2}J_{C,F}$ = 23.8 Hz, C-F), 126.9 (C_{quart}, ph), 131.3 (+, ph), 154.7 (C_{quart}, $^{1}J_{C,F}$ = 235.6 Hz, C-F), 158.0 (C_{quart}, $^{4}J_{C,F}$ = 1.4 Hz, C-F), 161.8 (C_{quart}, ph), 192.6 (C_{quart}, $^{2}J_{C,F}$ = 2.5 Hz, C-F)

MS (PI-EI 70 eV): m/z (%) = 272 ([M$^{\bullet+}$], 56), 271 ([M-H]$^+$, 35), 257 ([M-CH$_3$]$^+$, 5), 255 ([M-OH]$^+$, 7), 134 ([CH$_3$OC$_6$H$_4$CH=CH]$^+$, 100)

IR [cm^{-1}]: 2972 (C-H) aromatic, 2850 (C-H) aliphatic, 1640-1565 (C=O), 1510, 1416 (C-H), 1346 (C-H), 1247 (C-O), 1164 (C-F), 1023, 985, 819, 781, 674

Analysis:

calculated	C: 70.58	H: 4.81
found	C: 70.35	H: 4.54

$C_{16}H_{13}FO_3$ (272.27)

(E) 1-(5-Fluoro-2-hydroxyphenyl)-3-(4-fluorophenyl)propenone (23d)

Reaction of 1-(5-fluoro-2-hydroxyphenyl)ethanone (2.0 g, 12.98 mmol) and 4-fluorobenzaldehyde (3.54 g, 28.56 mmol); recrystallisation from dimethylsulphoxide

Yield: 3.29 g (12.64 mmol, 97 %, yellow crystalline solid)

Mp: 157 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 7.04 (dd, 1H, $^{4}J_{H,F}$ = 5.9 Hz, ^{3}J = 8.9 Hz, H-3,), 7.34 (m, 2H, ph), 7.46 (ddd, 1H, ^{4}J = 3.1 Hz, $^{3}J_{H,F}$ = 8.51 Hz, ^{3}J = 8.9 Hz, H-4,), 7.85 (d, ^{3}J = 15.6 Hz, =C<u>H</u>), 7.98 (d, 1H, ^{3}J = 15.6 Hz, =C<u>H</u>), 8.03 (m, 2H, ph), 8.11 (dd, 1H, ^{4}J = 3.1 Hz, $^{3}J_{C,F}$ = 9.7 Hz, H-6,), 12.20 (s, 1H, O<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 115.9 (+, ph), 115.9 (+, ph), 115.9 (+, ph), 119.2 (C_{quart}, ph), 121.04 (+, ph), 121.7 (+, =<u>C</u>H), 123.4 (+, ph), 131.1 (C_{quart}, ph), 131.7 (+, ph), 131.7 (+, ph), 144.1 (+, =<u>C</u>H), 154.7 (C_{quart}, $^{1}J_{F,C}$ = 235.5 Hz, C-F), 157.8 (C_{quart},, ph), 163.7 (C_{quart}, $^{1}J_{F,C}$ = 250.2 Hz, C-F), 192.6 (C_{quart}, $^{4}J_{C,F}$ = 2.5 Hz, C=O)

MS (PI-EI 70 eV): m/z (%) = 260 ([M$^{\bullet+}$], 100), 259 ([M-H]$^{+}$, 65), 243 ([M-OH]$^{+}$, 14), 241 ([M-F]$^{+}$, 5)

HR-MS: calculated: 260.064800 amu
found: 260.064886 amu

IR [cm^{-1}]: 3054 (C-H) aromatic, 1645-1578 (C=O), 1510-1484 (C=C), 1230 (C-O), 1165 (C-F), 1021, 943, 830, 782, 741, 674

$C_{15}H_{10}F_2O_2$ (260.24)

General procedures for the preparation of the flavone derivatives 24a-e and 25a-i

Method A

A solution of the chalcone derivative (1 eq), hydrogen peroxide (0.22 eq, 30 %), a sodium hydroxide solution (1.2 eq, 5-7 %) and ethanol (5-15 ml) was stirred and heated under reflux for 1-2 h. After cooling to ambient temperature a sodium hydroxide solution (6.6 %) was added dropwise and stirred at room temperature for two additional hours. The reaction mixture was diluted with water

and carbon dioxide was induced until a solid precipitated. The solid was filtered off, washed with water and dried in vacuo over silica gel.

Method B

A solution of the chalcone derivative, selenium dioxide and pentyl alcohol (20-100 ml) was stirred and heated under reflux for 10-16 h. Insoluble material was filtered off from the hot solution and washed with hot ethanol. Than ¾ of the solvent was removed under reduced pressure. After cooling in the refrigerator, the precipitated product was collected and dried in vacuo.

Method C

The chalcone derivative was dissolved in anhydrous dimethylsulphoxide and was stirred and heated at 100 - 120 °C with sodium periodate for 48 h. After cooling to room temperature, a solution of sodium thiosulphate (10 %) was added slowly to the reaction mixture. The precipitate was collected, washed with a solution of sodium thiosulphate and dried in vacuo over phosphorus pentoxide.

4',5,7-Trimethoxyflavon-3-ol (24a)

Method A: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-methoxyphenyl)propenone **19b** (0.50 g, 1.59 mmol), a sodium hydroxide solution (5.1 ml, 5.4 %) and a hydrogen peroxide solution (0.7 ml, 30 %). The obtained solid was a mixture of starting material and product; it was not possible to separate the two solids using standard methods.

^{1}H-NMR ([D_6]DMSO): [ppm] = 3.83 (s, 3H, OC$\underline{H}_3$), 3.89 (s, 3H, OC$\underline{H}_3$), 3.92 (s, 3H, OC$\underline{H}_3$), 6.34 (s, 1H, OH), 6.70 (s, 2H, ph), 7.04 (m, 2H, ph), 7.88 (m, 2H, ph)

$C_{18}H_{16}O_6$ (328.32)

2',4'-Dichloro-5,6',7-trimethoxyflavon-3-ol (24b)

Method A: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(2',4'-dichloro-6-methoxyphenyl)propenone **19j** (1.0 g, 2.62 mmol), a sodium hydroxide solution (4.5 ml, 17 %) and a hydrogen peroxide solution (24 ml, 6.6 %). The obtained solid was a mixture of the starting material and product, which was identified by MS analysis. The separation of the two solids by standard methods failed.

MS (PI-EI 70 eV): m/z (%) =292 ($[M^{\bullet+}]$, 3), 345 ($[M\text{-}Cl\text{-}H]^+$, 100)

$C_{19}H_{14}Cl_2O_6$ (396.21)

4'-Dimethylamino-3-hydroxy-5,7-dimethoxyflavone (24c)

Method A: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-dimethylamino-phenyl)propenone **19d** (1.0 g, 3.05 mmol), a hydrogen peroxide solution (4.40 ml, 30 %) and a sodium hydroxide solution (24 ml, 6.60 %). The obtained orange solid was a mixture of starting material and product; it was not possible to separate the two solids by standard methods.
Yield: 0.49 g (orange solid)

^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.35 (s, 6H, N($C\underline{H}_3$)$_2$), 3.82 (s, 3H, O$C\underline{H}_3$), 3.90 (s, 3H, O$C\underline{H}_3$), 6.48 (s, 1H, ph), 6.70-6.92 (m, 3H, chromen and ph), 8.00-8.15 (m, 2H, ph), 8.60 (s, 1H, OH)

$C_{19}H_{19}NO_5$ (341.36)

2'-Chloro-3-hydroxy-3',4',5,7-tetramethoxyflavone (24e)

Method A : Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(2-chloro-3,4-dimethoxyphenyl)propenone **19i** (1.0 g, 2.64 mmol), a hydrogen peroxide solution (4.5 ml, 17 %) and a sodium hydroxide solution (24 ml, 6.6 %). The obtained solid was a mixture of product, starting material and by-product. It was not possible to separate the product using standard methods. The product was identified by MS analysis.
Yield: 115 mg (0.29 mmol, 11 %, yellow solid)

MS (PI-EI 70 eV): m/z (%) =392 ($[M^{\bullet+}]$, 4), 341.5 ($[M\text{-}OH\text{-}Cl]^+$, 36), 181 ($[(CH_3O)_2Ph(OH)CO]^+$, 100)

$C_{19}H_{17}ClO_7$ (392.79)

4 ,5,7-Trimethoxyflavone (25a)

Method B: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-methoxyphenyl)propenone **19b** (3.0 g, 9.54 mmol) and selenium dioxide (2.59 g, 23.38 mmol). The product was purified by column chromatography on silica gel eluting with a 5:5:1 (v/v/v) mixture of petroleum ether 60-80 °C, ethyl acetate and methanol.
Yield: 1.92 g (6.15 mmol, 64 %, orange solid)

Mp: 143-145°C [Lit. 150-155 °C (Chauhan et al. 1996)]

^{1}H-NMR ([D_6]DMSO):
δ [ppm] = 3.87 (s, 3H, OC$\underline{H}_3$), 3.90 (s, 3H, OC$\underline{H}_3$), 3.95 (s, 3H, OC$\underline{H}_3$), 6.59 (s, 1H, H-3), 6.36 (d, 1H, ^{3}J = 2.3 Hz, H-6), 6.55 (d, 1H, ^{3}J = 2.3 Hz, H-8), 6.99 (d, 2H, ^{3}J = 8.9 Hz, H-2', H-6'), 7.81 (d, 2H, ^{3}J = 8.9 Hz, H-3', H-5')

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.5 (+, O<u>C</u>H$_3$), 55.7 (+, O<u>C</u>H$_3$), 56.4 (+, O<u>C</u>H$_3$), 92.8 (+, chromen), 96.1 (+, chromen), 107.6 (+, chromen), 109.2 (C_{quart}, chromen), 114.3 (+, ph), 123.8 (C_{quart}, ph), 127.6 (+, ph), 159.8 (C_{quart}, ph), 160.7 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 162.1 (C_{quart}, chromen), 163.9 (C_{quart}, chromen), 177.7 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 312 ([M$^{\bullet+}$], 100), 311 ([M-H]$^+$, 63), 283 ([M-CH$_3$-CH$_3$]$^+$, 41)

IR [cm^{-1}]: 2934 (C-H) aromatic, 2825 (C-H) aliphatic, 1639-1601 (C=O), 1510, 1466 (C-H), 1346 (C-H), 1253 (C-O), 1213, 1193, 1160, 1055, 828, 769

Analysis:

calculated	C: 68.83	H: 5.14
found	C: 68.78	H: 5.14

$C_{18}H_{16}O_5$ (312.12)

3',4',5,7-Tetramethoxyflavone (25b)

Method B: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3,4-dimethoxy-phenyl)propenone **19c** (1.0 g, 2.90 mmol) and selenium dioxide (0.79 g, 7.11 mmol). The product was purified by column chromatography on silica gel eluting with a 5:5:1 (v/v/v) mixture of petroleum ether 60-80 °C, ethyl acetate and methanol.
Yield: 0.26 g (0.76 mmol, 26 %, yellow solid)

Mp: 192-194°C [Lit. 190-191 °C (Nunez-Alarcon 1971)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.92 (s, 3H, OC<u>H</u>$_3$), 3.95 (s, 3H, OC<u>H</u>$_3$), 3.96 (s, 3H, OC<u>H</u>$_3$), 3.98 (s, 3H, OC<u>H</u>$_3$), 6.38 (d, 1H, ^{4}J = 2.0 Hz, H-8), 6.56 (d, 1H, ^{4}J = 2.3 Hz, H-2'), 6.62

(s, 1H, H-3), 6.96 (d, 1H, ^{3}J = 8.6 Hz, H-5'), 7.32 (d, 1H, ^{4}J = 2.0 Hz, H-6), 7.51 (dd, 1H, ^{4}J = 2.3 Hz, ^{3}J = 8.6 Hz, H-6')

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.8 (+, O<u>C</u>H$_3$), 56.1 (+, O<u>C</u>H$_3$), 56.1 (+, O<u>C</u>H$_3$), 56.5 (+, O<u>C</u>H$_3$), 92.8 (+, chromen), 96.1 (+, chromen), 107.9 (+, chromen), 108.6 (+, ph), 109.2 (C_{quart}, chromen), 111.1 (+, ph), 119.5 (+, ph), 124.1 (C_{quart}, ph), 149.2 (C_{quart}, ph), 151.7 (C_{quart}, ph), 159.9 (C_{quart}, chromen), 160.7 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 163.9 (C_{quart}, chromen), 177.7 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 342 ([M$^{\bullet+}$], 58), 296 ([M-OCH$_3$-CH$_3$]$^+$, 24), 57 ([M-PhOCH$_3$-OCH$_3$-PhOCH$_3$]$^+$,100)

IR [cm^{-1}]: 2924 (C-H) aromatic, 2839 (C-H) aliphatic, 1644-1600 (C=O), 1514, 1456 (C-H), 1354 (C-H), 1253 (C-O), 1157, 1138, 1016, 870, 831, 804, 766

Analysis:

calculated	C: 66.66	H: 5.30
found	C: 66.46	H: 5.64

$C_{19}H_{18}O_6$ (342.34)

5,7-Dimethoxy[3,4]benzoflavone (25c)

Method B: Reaction solution of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(naphthalen-2-yl)propenone **19e** (1.0 g, 2.99 mmol) and selenium dioxide (1.66 g, 14.95 mmol). The product was purified by column chromatography on silica gel eluting with a 5:5:1 (v/v/v) mixture of petroleum ether 60-80 °C, ethyl acetate and methanol.

Yield: 0.52 g (1.57 mmol, 52 %, orange solid)

Mp: 128-130°C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.95 (s, 3H, OC$\underline{H}_3$), 3.97 (s, 3H, OC$\underline{H}_3$), 6.39 (d, 1H, ^{4}J = 2.3 Hz, H-8), 6.65 (d, 1H, ^{4}J = 2.3 Hz, H-6), 6.91 (s, 1H, H-3), 7.54-7.60 (m, 2H, naphthyl), 7.84-7.96 (m, 4H, naphthyl), 8.42 (s, 1H, naphthyl H2)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 55.9 (+, O$\underline{C}H_3$), 56.5 (+, O$\underline{C}H_3$), 92.9 (+, chromen), 96.4 (+, chromen), 109.1 (+, chromen), 114.6 (C_{quart}, chromen), 122.4 (+, naphthyl), 126.5 (+, naphthyl), 127.0 (+, naphthyl), 127.9 (+, naphthyl), 128.6 (+, naphthyl), 128.9 (+, naphthyl), 128.1 (C_{quart}, naphthyl), 128.9 (+, naphthyl), 132.9 (C_{quart}, naphthyl), 134.6 (C_{quart}, naphthyl), 160.1 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 165.3 (C_{quart}, chromen), 177.7 (C_{quart}, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) =332 ([$M^{\bullet+}$], 100), 331 ([M-H]$^{+}$, 50), 303 ([M-H-CO]$^{+}$, 27), 152 ([M-naphthyl-C_2H_2-CO]$^{+}$, 38)

IR [cm^{-1}]: 2924 (C-H) aromatic, 2853 (C-H) aliphatic, 1641 (C=O), 1598 (C=C), 1461 (C-H), 1328 (C-H), 1206 (C-O), 1109, 870, 850, 823, 762, 669

Analysis:

calculated	C: 75.89	H: 4.85
found	C: 75.64	H: 4.84

$C_{21}H_{16}O_4$ (332.35)

4'-Chloro-5,7-dimethoxyflavone (25d)

Method C: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-chlorophenyl)-propenone **19h** (0.59 g, 1.84 mmol) and sodium periodate (2.0 g, 9.34 mmol).
Yield: 0.18 g (0.56 mmol, 30 %, light yellow solid)

Mp: 181 °C [Lit. 180-182 °C (Hsue 1959)]

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.91 (s, 3H, OC$\underline{H}_3$), 3.95 (s, 3H, OC$\underline{H}_3$), 6.36 (d, 1H, ^{4}J = 2.3 Hz, H-6), 6.54 (d, 1H, ^{4}J = 2.3 Hz, H-8), 6.65 (s, 1H, H-3), 7.44-7.46 (m, 2H, H-2', H-6'), 7.75-7.79 (m, 2H, H-3', H-5')

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 55.8 (+, O$\underline{C}H_3$), 56.4 (+, O$\underline{C}H_3$), 92.8 (+, chromen), 96.2 (+, chromen), 109.0 (+, chromen), 109.1 (C_{quart}, chromen), 127.1 (+, ph), 129.2 (+, ph), 120.9 (C_{quart}, ph), 137.4 (C_{quart}, ph), 159.5 (C_{quart}, chromen), 159.7 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 164.2 (C_{quart}, chromen), 177.3 (C_{quart}, $\underline{C}$=O)

MS (PI-EI 70 eV): m/z (%) = 316 ([$M^{\bullet+}$], 100), 287 ([(M-CH_3)-CH_3]$^{+}$, 55)

HR-MS: calculated: 316.050100 amu
found: 316.050237 amu

IR [cm^{-1}]: 3011 (C-H) aromatic, 2838 (C-H) aliphatic, 1639 (C=O), 1489, 1466 (C-H), 1339 (C-H), 1217 (C-O), 1104 (C-Cl), 1058, 952, 904, 837, 820

$C_{17}H_{13}ClO_4$ (316.74)

4'-Fluoro-5,7-dimethoxyflavone (25e)

Method B: Reaction of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(4-fluorophenyl)-propenone **19k** (2.11 g, 6.98 mmol) and selenium dioxide (1.90 g, 17.1 mmol).
Yield: 1.38 g (4.60 mmol, 66 %, yellow solid)

Mp: 165 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.91 (s, 3H, OC$\underline{H}_3$), 3.95 (s, 3H, OC$\underline{H}_3$), 6.36 (d, 1H, ^{4}J = 2.3 Hz, H-6), 6.54 (d, 1H, ^{4}J = 2.3 Hz, H-8), 6.64 (s, 1H, H-3), 7.13-7.27 (m, 2H, H-2', H-6'), 7.82-7.80 (m, 2H, H-3', H-5')

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 55.8 (+, O<u>C</u>H_3), 56.5 (+, O<u>C</u>H_3), 92.8 (+, chromen), 96.3 (+, chromen), 108.7 (+, chromen), 109.1 (C_{quart}, chromen), 116.0 (+, ph), 116.4 (+, ph), 127.72 (C_{quart}, $^4J_{C,F}$ = 3.2 Hz, C-1'), 128.1 (+, ph), 128.2 (+, ph), 159.8 (C_{quart}, chromen), 159.8 (C_{quart}, chromen), 160.9 (C_{quart}, chromen), 164.2 (C_{quart}, chromen), 164.5 (C_{quart}, $^1J_{C,F}$ = 252.4 Hz, C-F), 177.5 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 300 ([$M^{\bullet+}$], 100), 299 ([M-H]$^+$, 56), 271 ([(M-CH_3-CH_3]$^+$, 45)

IR [cm^{-1}]: 3000 (C-H) aromatic, 2960 (C-H) aliphatic, 1644-1604 (C=O), 1508, 1466 (C-H), 1344 (C-H), 1216 (C-O), 1162 (C-F), 1116, 873, 838, 686

Analysis:

calculated	C: 67.19	H: 4.38
found	C: 67.02	H: 4.44

$C_{17}H_{13}FO_4$ (300.88)

6-Fluoro-4'-methoxyflavone (25f)

Method B: Reaction of 1-(5-fluoro-2-hydroxyphenyl)-3-(4-methoxyphenyl)propenone **23c** (1.00 g, 3.67 mmol) and selenium dioxide (1.00 g, 8.99 mmol);
Yield: 0.52 g (1.92 mmol, 52 % (77 %), yellow solid)

Mp: 136-138 °C

^{1}H-NMR ([D_6]DMSO):

δ [ppm] = 3.92 (s, 3H, OC<u>H</u>$_3$), 6.74 (s, 1H, H-3), 7.00-7.05 (m, 2H, H-2', H-6'), 7.38-7.43 (m, 1H, chromen), 7.54-7.57 (m, 1H, chromen), 7.86-7.88 (m, 1H, chromen), 7.86-7.89 (m, 2H, H-3', H-5')

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 55.5 (+, O<u>C</u>H$_3$), 105.5 (+, C-3), 110.6 (+, $^2J_{C,F}$ = 23.7 Hz, C-5), 114.5 (+, ph), 120.0 (+, $^3J_{C,F}$ = 8.0 Hz, C-8), 121.7 (+, $^2J_{C,F}$ = 25.4 Hz, C-7), 123.7 (C_{quart}, ph), 125.2 (C_{quart}, $^3J_{C,F}$ = 7.4 Hz, C-9), 128.0 (+, ph), 152.4 (C_{quart}, $^4J_{C,F}$ = 1.8 Hz, C-9), 159.6 (C_{quart}, $^1J_{C,6}$ = 296.6 Hz, C-F), 162.6 (C_{quart}, ph), 163.7 (C_{quart}, C-2), 177.5 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 270 ([M$^{\bullet+}$], 100), 239 ([M-OCH$_3$]$^+$, 23), 138 ([FC$_6$H$_4$(=O)(=C=O]$^+$, 6), 132 ((CH$_3$OC$_6$H$_4$CCH]$^+$, 95)

IR [cm^{-1}]: 3076 (C-H) aromatic, 2928 (C-H) aliphatic, 1639-1604 (C=O), 1512, 1478 (C-H), 1362 (C-H), 1267 (C-O), 1176-1117 (C-F), 908, 895, 825, 777, 719

Analysis:

calculated	C: 71.11	H: 4.10
found	C: 71.26	H: 4.24

$C_{16}H_{11}FO_3$ (270.25)

4',6-Difluoroflavone (25g)

Method B: Reaction of 1-(5-fluoro-2-hydroxyphenyl)-3-(4-fluorophenyl)propen-one **23d** (1.0 g, 3.84 mmol) and selenium dioxide (1.05 g, 9.41 mmol).
Yield: 0.32 g (1.24 mmol, 32 %, yellow solid)

Mp: 149 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.78 (s, 1H, H-3), 7.20-7.28 (m, 2H, H-2', H-6'), 7.41-7.45 (m, 1H, chromen), 7.44-7.48 (m, 1H, chromen), 7.85-7.89 (m, 1H, chromen), 7.91-7.96 (m, 2H, H-3', H-5')

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 108.8 (+, C-3), 112.9 (+, $^{2}J_{C,F}$ = 23.8 Hz, H-5), 118.4 (+, ph), 118.7 (+, ph), 122.3 (+, $^{3}J_{C,F}$ = 8.1 Hz, C-8), 124.2 (+, $^{2}J_{c,F}$ = 25.6 Hz, C-7), 127.2 (C_{quart}, $^{3}J_{C,F}$ = 7.4 Hz, C-9), 129.9 (C_{quart}, $^{4}J_{C,F}$ = 3.3 Hz, C-1'), 130.7 (+, ph), 130.8 (+, ph), 154.5 (C_{quart}, $^{4}J_{C,F}$ = 1.8 Hz, C-10), 161.8 (C_{quart}, $^{1}J_{C,F}$ = 247.2 Hz, C-F), 164.9 (C_{quart}, chromen), 167.0 (C_{quart}, $^{1}J_{C,F}$ = 253.8 Hz, C-F), 179.6 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 258 ([$M^{\bullet+}$], 58), 138 ([$FC_6H_4(=O)(=C=O)]^+$, 100)

IR [cm^{-1}]: 3063 (C-H) aromatic, 1663 (C=O), 1593, 1348 (C-H), 1229 (C-O), 1166 (C-F), 1032, 907, 834, 814

Analysis:

calculated	C: 69.77	H: 3.12
found	C: 69.43	H: 3.13

$C_{15}H_8F_2O_2$ (258.22)

7-Fluoro-3',4'-dimethoxyflavone (25h)

Method B: Reaction of 1-(4-fluoro-2-hydroxyphenyl)-3-(3,4-dimethoxyphenyl)-propenone **23a** (0.50 g, 1.65 mmol) and selenium dioxide (0.45 g, 4.05).
Yield: 0.46 g (1.53 mmol, 93 %, green-yellow solid)

Mp: 159 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.97 (s, 3H, OC<u>H</u>$_3$), 3.99 (s, 3H, OC<u>H</u>$_3$), 6.76 (s, 1H, H-3), 6.99 (d, 1H, ^{3}J = 8.9 Hz, H-5'), 7.15 (ddd, 1H, ^{4}J = 2.4 Hz, $^{3}J_{H,F}$ = 8.2 Hz, ^{3}J = 8.9 Hz, H-6), 7.26 (ddd, 1H, ^{5}J = 0.4 Hz, ^{4}J = 2.4 Hz, $^{3}J_{H,F}$ = 9.1 Hz, H-8), 7.36 (d, 1H, ^{4}J = 2.2 Hz, H-2'), 7.55 (dd, 1H, ^{4}J = 2.2 Hz, ^{3}J = 8.6 Hz, H-6'), 8.24 (ddd, 1H, ^{5}J = 0.4 Hz, $^{4}J_{H,F}$ = 6.4 Hz, ^{3}J = 8.9 Hz, H-5)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 56.1 (+, O<u>C</u>H_3), 56.1 (+, O<u>C</u>H_3), 104.7 (+, $^2J_{C,F}$ = 25.4 Hz, C-8), 106.3 (+, ph), 108.7 (+, ph), 111.2 (+, ph), 113.9 (+, $^2J_{C,F}$ = 22.7 Hz, C-6), 120.1 (+, chromen), 120.6 (C_{quart}, $^4J_{C,F}$ = 2.4 Hz, C-10), 123.7 (C_{quart}, ph), 128.1 (+, $^3J_{C,F}$ = 10.5 Hz, C-5), 149.3 (C_{quart}, ph), 152.3 (+, ph), 157.1 (C_{quart}, $^3J_{C,F}$ = 13.4 Hz, C-9), 163.8 (C_{quart}, chromen), 165.6 (C_{quart}, $^1J_{C,F}$ = 254.8 Hz, C-7), 177.3 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 300 ([$M^{\bullet+}$], 100), 285 ([M-CH_3]$^+$, 14)

HR-MS: calculated: 300.079900 amu
found: 300.079787 amu

IR [cm^{-1}]: 3075 (C-H) aromatic, 2838 (C-H) aliphatic, 1653 (C=O), 1515, 1422 (C-H), 1322 (C-H), 1230 (C-O), 1143 (C-F), 1027, 974, 870, 799 748

$C_{17}H_{13}FO_4$ (300.28)

7-Fluoro-3'-methoxyflavone (25i)

Method B: Reaction of 1-(4-fluoro-2-hydroxyphenyl)-3-(4-methoxyphenyl)propenone **23b** (3.0 g, 11.02 mmol) and selenium dioxide (3.0 g, 27.0 mmol, 2.45 eq).
Yield: 1.15 g (4.26 mmol, 39 %, yellow-green solid)

Mp: 189 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.87 (s, 3H, OC<u>H</u>$_3$), 6.98 (s, 1H, H-3), 7.13 (d, 2H, ^3J = 9.1 Hz, H-2', H-6'), 7.14 (ddd, 1H, ^4J = 2.4 Hz, $^3J_{H,F}$ = 8.2 Hz, ^4J = 8.9 Hz, H-6), 7.24 (ddd, 1H, ^5J = 0.4 Hz, ^4J = 2.4 Hz, $^3J_{H,F}$ = 9.1 Hz, H-8), 8.07 (d, 2H, ^3J = 9.1 Hz, H-3', H-5'), 8.24 (ddd, 1H, ^5J = 0.4 Hz, $^4J_{H,F}$= 6.4 Hz, ^3J = 8.9 Hz, H-5)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 55.6 (+, O<u>C</u>H_3), 104.7 (+, $^2J_{C,F}$ = 25.4 Hz, C-8), 106.1 (+, chromen), 113.9 (+, $^2J_{C,F}$ = 22.9 Hz, C-6), 114.6 (+, ph), 123.6 (C_{quart}, chromen), 128.1 (+, ph), 128.3 (C_{quart}, ph), 129.8 (+, $^3J_{C,F}$ = 23.2 Hz, C-5), 157.2 (C_{quart}, $^3J_{C,F}$ = 13.4 Hz, C-9), 162.7 (C_{quart}, ph), 163.9 (C_{quart}, chromen), 163.7-167.7 (C_{quart}, $^1J_{C,7}$ = 254.9 Hz, C-F), 177.4 (C_{quart}, <u>C</u>=O)

MS (PI-EI 70 eV): m/z (%) = 270 ([$M^{\bullet+}$], 100), 255 ([M-CH_3]$^+$, 8)

HR-MS: calculated: 270.069300 amu
found: 270.069222 amu

IR [cm^{-1}]: 3087 (C-H) aromatic, 2844 (C-H) aliphatic, 1649 (C=O), 1510, 1439 (C-H), 1364 (C-H), 1312 (C-F), 1256 (C-O), 1186, 1013, 817, 757, 635

$C_{16}H_{11}FO_3$ (270.26)

7-Fluoro-4'-hydroxyflavone (26)

To a stirred solution of 7-fluoro-4'-methoxyflavone **25i** (0.50 g, 1.85 mmol) in anhydrous methylene chloride (40 ml), a solution of BBr_3 (2.32 g, 9.25 mmol, 5 eq) in methylene chloride (9.25 ml) was added dropwise at 0 °C under a nitrogen atmosphere. After stirring at room temperature for 24 h the reaction mixture was cooled again to 0°C and water and ice were added. The precipitated product was collected, washed with water, dried in vacuo over silica gel and purified by column chromathography on silica gel eluting with a 5:5:1 (v/v/v) mixture of petroleum ether 60-80 °C, ethyl acetate and methanol.
Yield: 0.46 g (1.80 mmol, 97 %, brown solid)

Mp: 280°C

^{1}H-NMR ([D_6]DMSO):

δ [ppm] = 4.32 (br, 1H, O<u>H</u>), 6.88 (s, 1H, H-3), 6.93 (d, 2H, ^{3}J = 8.9 Hz, H-2', H-6'), 7.36 (ddd, 1H, ^{4}J = 2.5 Hz, $^3J_{H,F}$ = 8.5 Hz, ^{3}J = 8.9 Hz, H-6), 7.72 (dd, 1H, ^{4}J

= 2.5 Hz, $^{3}J_{H,F}$ = 9.7 Hz, H-8), 7.94 (d, 2H, ^{3}J = 8.9 Hz, H-3', H-5'), 8.09 (dd, $^{3}J_{H,F}$ = 6.5 Hz, ^{3}J = 8.9 Hz, H-5)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 104.8 (+, chromen), 105.2 (+, $^{2}J_{C,F}$ = 25.8 Hz, C-8), 113.8 (+, $^{2}J_{C,F}$ = 23.1 Hz, C-6), 115.9 (+, ph), 120.5 (C_{quart}, $^{4}J_{C,F}$ = 2.2 Hz, chromen), 121.3 (C_{quart}, ph), 127.5 (+, $^{3}J_{C,F}$ = 10.9 Hz, C-5), 128.4 (+, ph), 156.6 (C_{quart}, $^{4}J_{C,F}$ = 14.0 Hz, chromen), 161.1 (C_{quart}, ph), 163.4 (C_{quart}, $^{5}J_{C,F}$ = 1.1 Hz, chromen), 164.9 (C_{quart}, $^{1}J_{C,7}$ = 251.3 Hz, C-F), 176.1 (C_{quart}, C=O)

MS (PI-EI 70 eV): m/z (%) = 256 ([M$^{\bullet+}$], 100), 239 ([M-OH]$^{+}$, 13)

IR [cm^{-1}]: 3137 (O-H), 3069 (C-H) aromatic, 2832 (C-H) aliphatic, 1576, 1502, 1443 (C-H), 1371 (C-H), 1254 (C-O), 1179-1151 (C-F), 908, 878, 823, 758, 661

Analysis:

calculated	C: 50.99	H: 2.85
found	C: 50.42	H: 3.06

$C_{15}H_9FO_3$ (256.23)

Tetrabutylammonium 7-fluoroflavon-4'-ylsulphate (27)

To a stirred solution of 7-fluoro-4'-hydroxyflavone **26** (200 mg, 0.78 mmol) and dimethylformamide (10 ml), pyridine*SO_3 (249 mg, 1.56 mmol) and pyridine (124 mg, 1.56 mmol) were added dropwise at room temperature under a nitrogen atmosphere. The reaction mixture was stirred and heated for 30 min at 100 °C. After cooling to room temperature, a saturated sodium hydrogencarbonate solution was added until the generation of gas ceased. Afterwards, the solvent was removed under reduced pressure and the oily residue was treated with an aqueous acetic acid solution (5 %) to adjust the pH 7 to and Bu_4N^{+} HSO_4^{-} (1.06 g, 3.12 mmol) was added. The obtained mixture was diluted with methanol, filtrated and reconcentrated. The residue was again diluted with a

mixture of 1:1 (v/v) methanol and water and concentrated in vacuo. The obtained yellow solid was analysed by MS spectroscopy.

MS (ESI (H_2O/MeOH + 1 % NH_4OH): m/z (%) =335 ([M-Bu_4N^+]$^-$, 58), 255 ([M-SO_3^-], 100)

$C_{16}H_{36}N \bullet C_{15}H_8FO_6S$ (577,76)

5.3 Pharmacological methods

The inhibitory effect of the examined compounds on the activity of hyaluronidases were determined by a colorimetric method (Muckenschnabel et al. 1998; Muckenschnabel et al. 1998) and a turbidimetric (Di Ferrante 1956) as described in chapter 3.

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Chapter 7

Structure-based design, synthesis and pharmacological investigations of hyaluronate lyase inhibitors

1. Introduction

Bacterial hyaluronan lyases (EC 4.2.2.1) differ from hyaluronidases from other sources by their mode of action. The two best known hyaluronate lyases, the *Streptococcus pneumoniae* and *Streptococcus agalactiae* hyaluronate lyase, cleave hyaluronan to yield 2-acetamido-2-deoxy-3-*O*-(β-*D*-gluco-4-enepyranosyluronic acid)-*D*-glucose (ΔDi-HA) as end product (for a review about hyaluronan and hyaluronidases cf. http://www.glycoforum.gr.jp). By cleaving hyaluronan, bacterial hyaluronate lyases, e. g. that of *S. agalactiae* ($hylB_{4755}$), facilitate the spreading of the microorganisms and their toxins in infectious diseases such as meningitis and septicaemia (Hynes et al. 2000). Potent inhibitors of hyaluronan lyases are not known so far. Such compounds could be useful in studying the role of hyaluronan and hyaluronidases in bacterial infections.

Some small compounds like arginine and guanidine derivatives and vitamin C with IC_{50} values in the range of 150 to 0.150 mM for inhibition of the bacterial hyaluronidase have been described to date (Li et al. 2001; Akhtar et al. 2003). For the development of a new class of bacterial hyaluronidases inhibitors, the primary goal of the present study was the identification of simple chemical structures as first lead compounds. A crucial issue for improving binding affinity, selectivity or bioavailability in an interactive approach is the quick synthetic accessibility of the target compounds, for example by modifications of the chemical lead. Until now, several computational methods like GROW (Moon et al. 1991), LEGEND (Nishibata et al. 1991) and LUDI (Böhm 1992; Böhm 1992) have been described enabling the rational design of new ligands using the 3D

structure of a target protein (Böhm 1996; Böhm 1996; Schneider et al. 2002). Most of these programmes perform a detailed analysis of the amino acid residues constituting the binding pocket and extract the spatial binding features which potential ligands have to fulfil. After this initial step, these favourable interaction sites are converted into a complex pharmacophore model of the active site of the enzyme. In consideration of this pharmacophore, large databases of small molecules can be computationally screened resulting in the identification of complementary ligands in terms of geometrical and physicochemical properties. Putative screening hits are selected with respect to a rough affinity prediction estimated by a scoring function. The scoring of the proposed ligand binding mode reflects the favourable interaction between the functional moieties of the ligand and the amino acid residues of the binding pocket (Klebe et al. 2000; Gohlke et al. 2002). Rational design includes several design cycles with appropriate structural modifications, synthesis and testing of the new candidate structures resulting in ligands with improved binding affinities.

As a pilot scheme for hyaluronidase inhibitor design in general, we started a structure-based ligand design programme to discover $hylB_{4755}$ inhibitors based on the crystal structures of both aforementioned streptococcal lyases using the *de novo* design software LUDI (Böhm 1992; Böhm 1992). The streptococcal hyaluronan lyase $hylB_{4755}$ shows extensive sequence identity to the homologous enzymes encoded by genes from group B *Streptococcus (S. agalactiae)* strain 3502 (98 % amino acid sequence identity) and *Streptococcus pneumoniae* (53 % amino acid sequence identity) characterised previously. The three-dimensional X-ray crystal structures of two hyaluronate lyases from *Streptococcus* species, one from *S. pneumonia* (hylSpn) and the other one from *S. agalactiae* strain 3502 ($hylB_{3502}$) were determined recently (Li et al. 2000; Li et al. 2001). Additionally, a complex of the hyaluronan hexasaccharide with $hylB_{3502}$ could be elucidated by means of X-ray crystallography. These results reveal the progressive mode of the catalytic process, including the identification of specific residues mechanistically involved in the degradation of hyaluronan by $hylB_{3502}$ (Mello et al. 2002). In this chapter the design, synthesis and identification of first promising leads as inhibitors of *S. agalactiae* strain 4755 hyaluronate lyase with micromolar activities are reported.

2. Structure-based lead discovery by LUDI calculations with the hylB$_{4755}$ model[1]

2.1 HylB$_{4755}$ model construction

Necessary conditions for comparative protein structure modelling are a detectable or significant similarity between the target sequence and the template structures and the construction of an accurate sequence alignment between them (Sanchez et al. 2000). The sequence identity of the crystallised hyaluronate lyases from *S. pneumoniae* (hylSpn, pdb code 1egu) (Li et al. 2000) and from *S. agalactiae* (hylB$_{3502}$, pdb code 1f1s) (Li et al. 2001) is 53 % whereas it is even 98 % between the streptococcal enzymes hylB$_{3502}$ and *S. agalactiae* strain 4755 hyaluronan lyase (hylB$_{4755}$). Thus, the crystal structures of the streptococcal hyaluronidases hylSpn and hylB$_{3502}$ have provided the first possibility to construct a reliable comparative model of the parent bacterial enzymes group B streptococcal hyaluronidase hylB$_{4755}$.

As a first step, a multiple sequence alignment of hylB$_{4755}$, hylB$_{3502}$ and hylSpn was generated using ClustalW (Higgins et al. 1988; Thompson et al. 1994). Due to the very high overall identity of the amino acid sequences of the streptococcal enzymes (85 % for hylB$_{4755}$ vs. hylSpn and 99 % for hylB$_{4755}$ vs. hylB$_{3502}$ with respect to the active sites), the homology modelling approach should result in a very reliable comparative model of hylB$_{4755}$. Additionally, the structurally conserved regions (SCR) of both crystal structures could easily be extracted since almost all insertions in the hylB$_{3502}$ sequence occur in the βII-domain surface loop areas (Li et al. 2001). Given that the primary structures of hylB$_{3502}$ and hylB$_{4755}$ are almost identical, the question arose whether to directly use the X-ray structure of *S. agalactiae* strain 3502 hyaluronan lyase as starting point for the structure-based ligand design instead of constructing a homology model of *S. agalactiae* strain 4755 hyaluronan lyase.

The crystal structure of the bacterial hyaluronan lyases from *S. pneumoniae* (hylSpn) was used as a template for model construction, since its conformation

is supposed to be catalytically active whereas the crystallised conformation of hylB$_{3502}$ is more open, therefore not optimal for catalysis (Li et al. 2001). Furthermore, it was observed that the α- and βII-domains were rotated by a small angle around the linker II in the hylB$_{3502}$ structure as compared to hylSpn. As a result, the hylB$_{3502}$ structure is more open than is the hylSpn structure. Consequently, corresponding subdomaines of hylSpn and hylB$_{3502}$ were superimposed using Sybyl 6.8 (Tripos Inc., St. Louis, MO). A full model of hylB$_{4755}$ was constructed by starting from the hylB$_{3502}$ α– and βII-domains with lowest root mean square (rms) deviation to hylSpn, inserting joining loops from hylB$_{3502}$, and mutating eight amino acids to achieve the hylB$_{4755}$ amino acid sequence. The resulting model was protonated and energetically minimised. The energy minimised model of hylB$_{4755}$ is shown in Fig. 1.

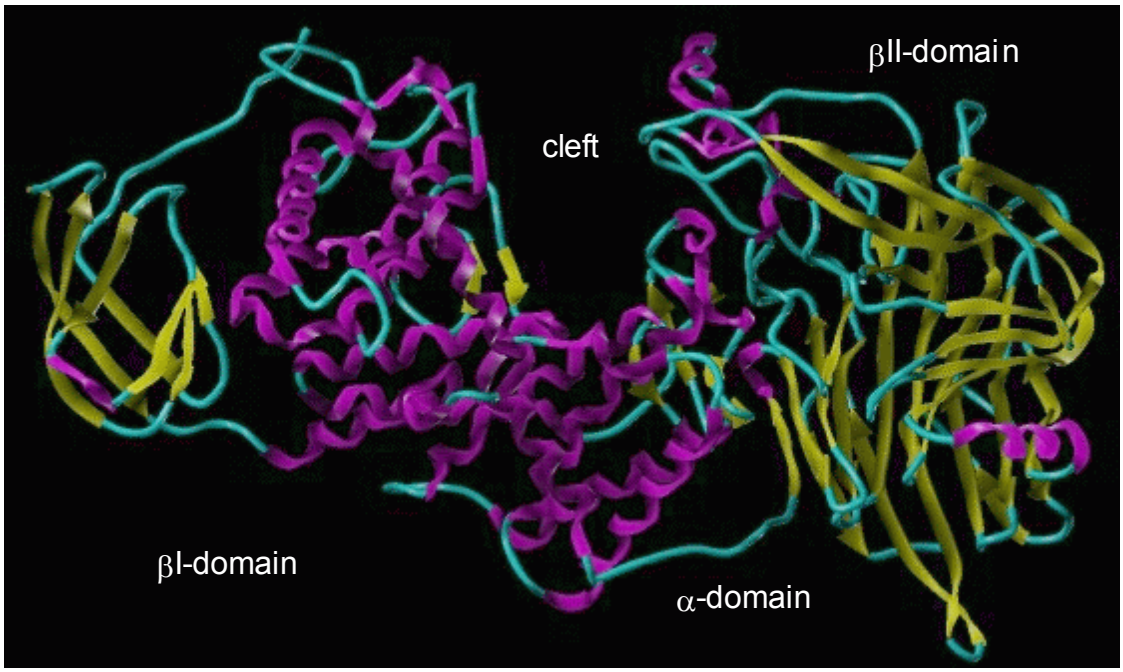


Fig. 1: Schematic representation of hylB$_{3502}$ model. α-helices, β-sheets and loop regions are coloured in purple, yellow and cyan, respectively (Botzki 2004).

[1] The construction of the hylB$_{4755}$ model and the LUDI calculations have been carried out by *Alexander Botzki* (Botzki 2004) as part of his PhD project.

2.2 Search for molecular fragments using the computer program LUDI

Using the *de novo* design program LUDI, small and fairly rigid molecules were retrieved from a 3D-structure database by positioning them into the protein-binding site. For each successfully docked ligand, LUDI estimates the expected binding affinity by an empirical scoring function (Böhm 1998). Based on the homology model of $hylB_{4755}$, we screened entries from the LeadQuest® Databases Vol. 1&2 (Tripos 2000) with LUDI. Prior to the search, the molecules from the LeadQuest® databases were processed using CONVERTER (Accelrys 2000) to generate reasonable 3D structures. Using standard parameters for LUDI calculations, all 3D structures are treated as rigid bodies independent of the existence of rotatable bonds on the molecules. The generated 3D database was reprocessed by GENFRA, a module of the LUDI programme suite, to classify all molecules in terms of their hydrogen-bonding and lipophilic properties and to calculate the fraction of solvent-accessible-surface of their functional groups. Appropriate fit centres were assigned to all functional moieties of the ligands to be screened and saved in a supplementary database by GENFRA. Subsequently, for all functional groups of the enzyme exposed to the active site, LUDI generates putative interaction sites in space according to rules derived from composite crystal-field environments compiled with appropriate small molecule crystal data (Cambridge Structural Database) (Böhm 1992). The programme tries to fit each database molecule onto the previously calculated interaction sites in the pre-defined binding pocket. The centre of this pocket was defined as the geometric mean of all atom positions of the active site amino acids Arg409, Trp460, Tyr576, Val579 and Arg634. All residues within a sphere of 5 Å around this centre were included into the LUDI calculation.

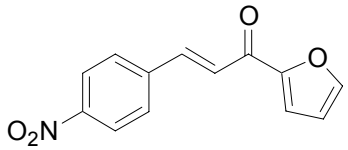
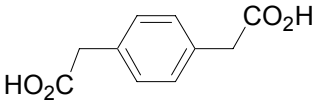
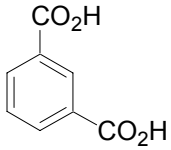
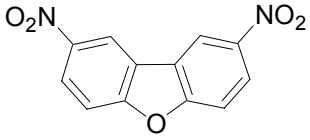
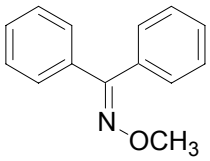
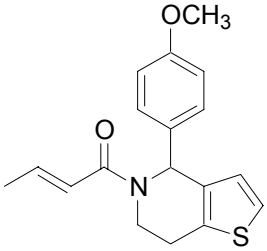
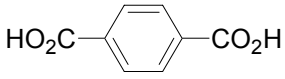
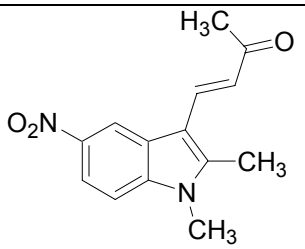
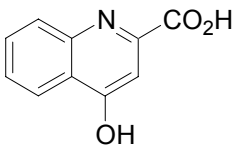
After four days calculation time, 122 hits were retrieved by the described procedure and were ranked on a relative scale in terms of their expected binding affinity using the scoring function described by *Böhm* (Böhm 1998). Since neither ligand nor protein flexibility is taken into account when using the parameters described above, a subsequent LUDI run with slightly altered parameters was accomplished. To allow for a larger search space and for more interaction possibilities, the radius of the sphere was enlarged to 8 Å and one

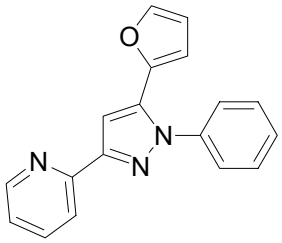
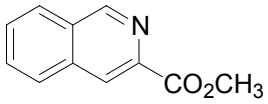
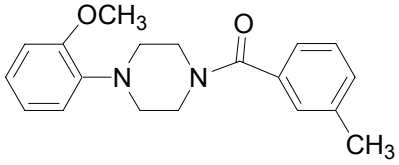
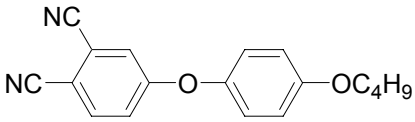
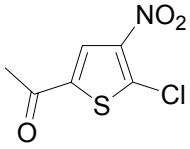
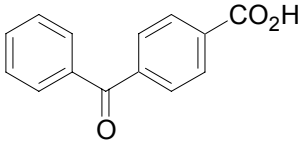
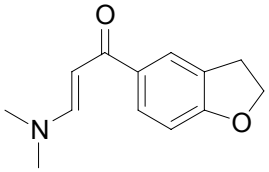
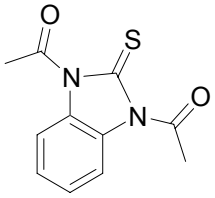
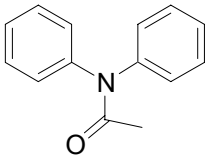
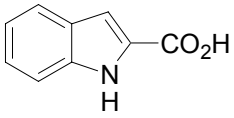
rotatable bond at a time was treated flexible. Besides, the hit database was combined with the original LUDI database as supplied by Accelrys because the size of the screened database should be enlarged in order to retrieve new compounds. A LUDI run with these adapted parameters on the whole LeadQuest database would have taken far too long to be completed in a reasonable time. 212 structures from this combined database were retrieved by LUDI and ranked in terms of their expected binding affinity with a predicted K_i value lower than 1 mM (LUDI score higher than 300).

In parallel to these investigations, an additional database with commercially available compounds has been constructed starting from the ChemACX database Version 5.5 from CambridgeSoft Corp. For the sake of reasonable calculation time, the applied parameters were altered with respect to the sphere radius (6 Å instead of 5 Å) and the density of lipophilic and polar interaction sites. Around 196908 compounds were screened in 5.5 days with LUDI resulting in 1063 hits.

Subsequently, both obtained hit lists were inspected visually. The final selection of compounds for purchase, synthesis and enzyme testing included the following criteria: (a) a predominantly high LUDI score until 325, (b) commercial availability, (c) efficient synthetic feasibility. All nineteen selected compounds are depicted with their calculated LUDI scores in Table 1.

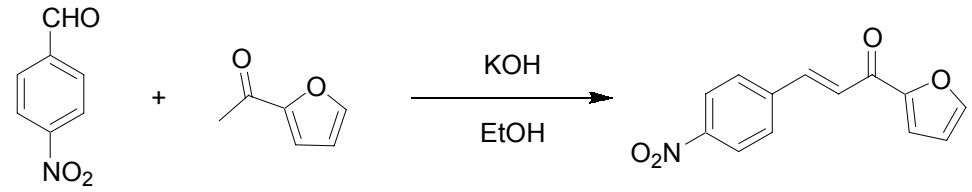
Table 1: Selected test compounds suggested by LUDI with calculated LUDI score and quoted position (A = Accelrys, C = ChemACX and LQ = LeadQuest® Database)

No	Structure	LUDI Score	Database
1		687	C
2		579	A
3		555	A
4		505	C
5		452	A
6		441	LQ
7		436	A
8		429	LQ
9		426	A

10		407	C
11		405	A
12		392	LQ
13		391	LQ
14		384	LQ
15		382	A
16		364	LQ
17		363	LQ
18		357	LQ
19		328	A

3. Chemistry

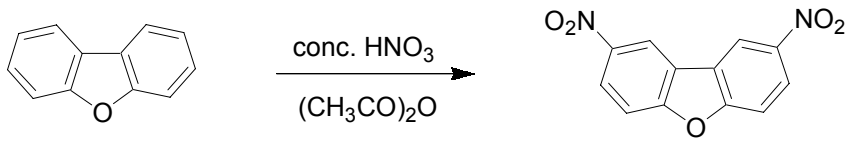
The furanyl derivative **1** was easily prepared in a one step reaction from 4-nitro-benzaldehyde (**20**) and 2-acetylfurane (**21**). The aldol condensation has been carried out in ethanol under strong basic conditions at ambient temperature (cf. Scheme 1).



20 **21** **1**

Scheme 1: Synthesis of 1-furan-2-yl-3-(4-nitrophenyl)propenone (**1**)

For the synthesis of 2,8-dinitro-dibenzofurane (**4**) different published procedures were tried. The most successful method was the method described by *Borsche et al.* (Borsche et al. 1908). Compound **4** was prepared by a nitration reaction of dibenzofuran (**22**) with a mixture of acetic anhydride and vitriolic acid (conc. HNO_3) at room temperature (cf. Scheme 2).

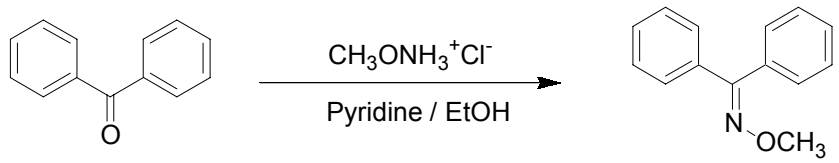


22 **4**

Scheme 2: Nitration of dibenzofuran

The *O*-methyl-oxime **5** was prepared in good yield from the commercially available benzophenone (**23**) with methoxyamine hydrochloride in ethanol and pyri-

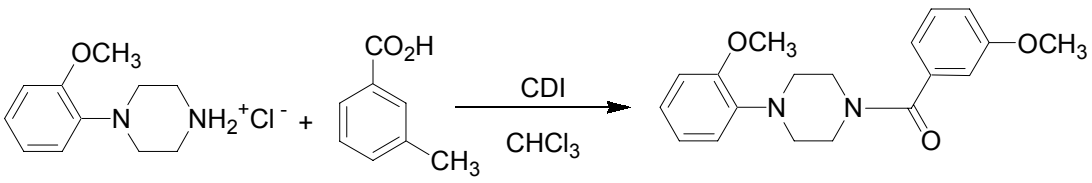
dine under reflux. The synthesis was based on a method described by *Kawase et al.* (Kawase et al. 1979) with some modifications (cf. Scheme 3).



23 **5**

Scheme 3: Synthesis of diphenylmethanone-*O*-methyl-oxime (**5**)

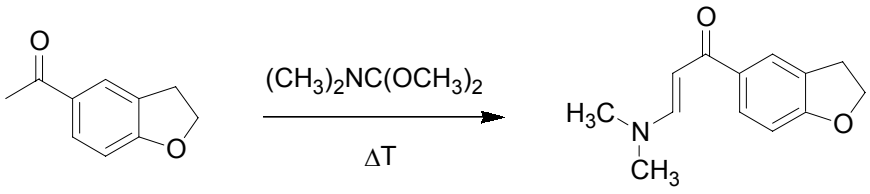
The piperazine derivative **12** can be obtained by a acetylation of piperazine **24** with 3-methylbenzoic acid **25**. For this synthesis, several standard methods are published. One very common method is the activation of the carboxylic acid with EDC and HOBt in chloroform and coupling of the amine with pyridine as base. This reaction was successful, but because of the low yield, we changed the method. The piperazine derivative **12** was prepared by activation of the acid with *N,N'*-carbonyldiimidazole (CDI) in anhydrous chloroform and 1-(2-methoxyphenyl)piperazine hydrochloride (**24**) in high yield (90 %) (cf. Scheme 4).



24 **25** **12**

Scheme 4: Synthesis of the piperazine derivative **12**

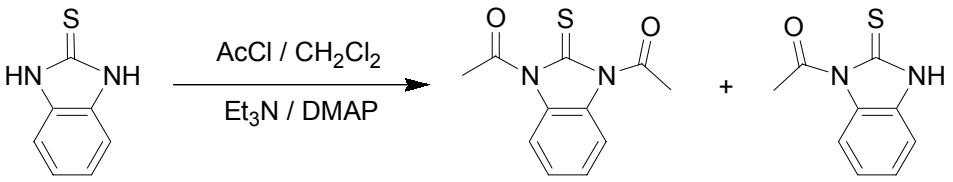
The vinylogous dimethylamine **16** was prepared from 5-acetyl-2,3-dihydrobenzo[b]furan (**26**) by a modified method described by *Tanaka et al.* (Tanaka et al. 1998) and *Wright et al.* (Wright et al. 1992). The aldol condensation was carried out at high temperature using *N,N*-dimethylformamide dimethyl acetal as reagent and as solvent (cf. Scheme 5).



26 **16**

Scheme 5: Aldol condensation of 5-acetyl-2,3-dihydrobenzo[b]furan (**26**) and *N,N*-dimethylformamide dimethyl acetal.

To synthesise the benzimidazol derivative **17** the method described by *Tittelbach et al*. (Tittelbach et al. 1988) was tried, that is benzimidazole-2-thione was treated with acetic anhydride at room temperature. However, it was not possible to get the desired product according to this method. All attempts to improve the reaction, e.g. increasing the reaction time or using the more reactive acetyl chloride instead of acetic anhydride were unsuccessful. Finally, compound **17** was obtained by the acetylation of the benzimidazole-2-thione (**27**) with triethylamine as base and *N,N*-dimethylaminopyridine (DMAP) as catalyst at room temperature. It was possible to isolate the mono- and the di-acetylated compounds **17** and **28** (cf. Scheme 6). Compound **28** is a by-product of this reaction and the product of hydrolytic cleavage of compound **17** at ambient temperature and moisture.



27 **17** **28**

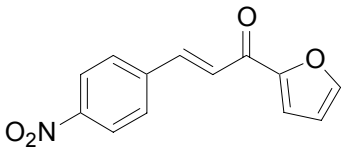
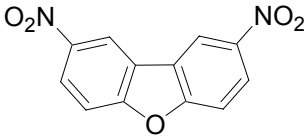
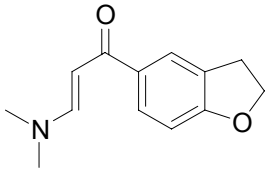
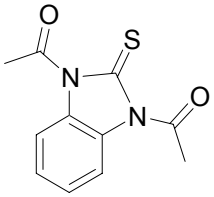
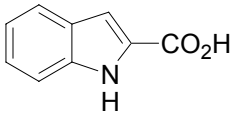
Scheme 6: *N*-Acetylation of benzimidazole-2-thione (**27**)

4. Pharmacological investigations

On the basis of our LUDI search, the proposed inhibitors compounds **1**-**19** were investigated for activity on the bovine testicular hyaluronidase (BTH) and the *S. agalactiae* hyaluronate lyase ($hylB_{4755}$) using an optimised colorimetric method (Muckenschnabel et al. 1998; Muckenschnabel et al. 1998) and a turbidimetric method (Di Ferrante 1956) as described in chapter 3. The inhibitory effects on BTH and $hylB_{4755}$ at optimum pH (BTH pH 3.6, $hylB_{4755}$ pH 5.0) and at physiological pH (7.4) are summarised in Table 2 and Table 3, respectively.

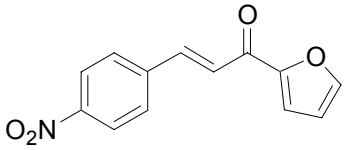
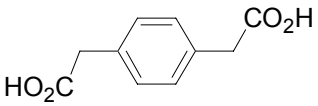
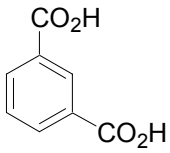
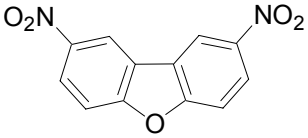
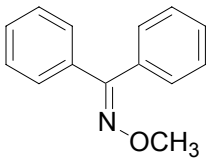
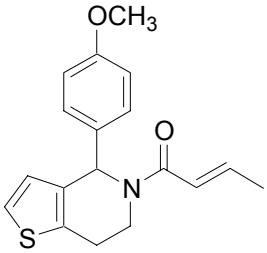
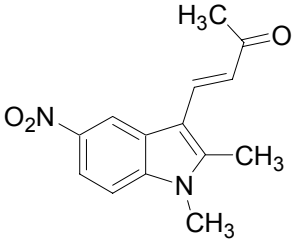
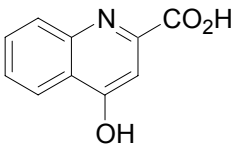
As aforementioned, the proposals of chemical structures resulting from LUDI calculations are based on a homology model of two hyaluronan lyases (*S. pneumoniae* and *S. agalactiae*) and are intended to be *S. agalactiae* $hylB_{4755}$ inhibitors. As expected the compounds **1**-**19** showed different inhibitory activities on the bovine testicular hyaluronidase and on the bacterial hyaluronidase. Surprisingly, within the series of tested compounds, we identified five compounds (**1**, **4**, **16**, **17** and **19**) with inhibitory activity on the bovine testicular hyaluronidase. The furan derivative **1** induced 77 % inhibition of BTH at a concentration of 10 mM at pH 3.6. At physiological pH (7.4) an IC_{50} value of 4 mM was determined. The inhibition of BTH by compound **4** was only 23 % at optimum pH (3.6) and 29 % at physiological pH (7.4), compound **16** produced 38 % inhibition at a concentration of 2 mM at pH 3.6 and the IC_{50} value of indole-2-carboxylic acid (**19**) on the bovine testicular enzyme (pH 3.6) was 7.1 mM. The benzimidazole-2-thione derivative **17** was the compound with the highest inhibitory activity (IC_{50} 250 µM) on the bovine testicular hyaluronidase (pH 7.4).

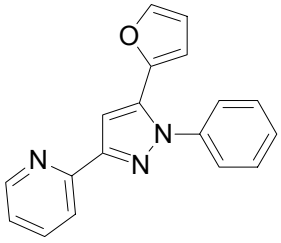
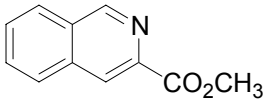
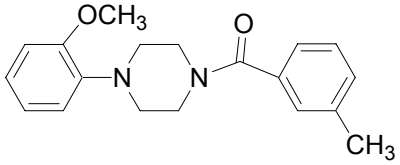
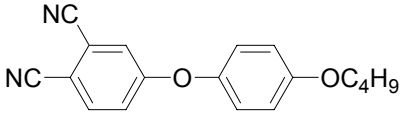
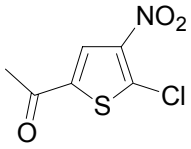
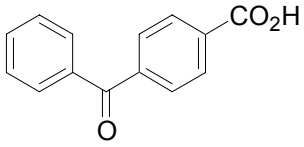
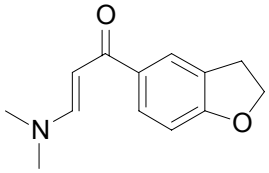
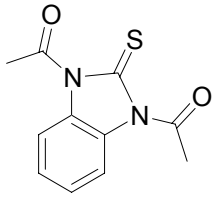
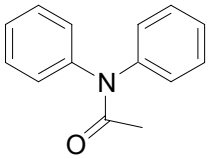
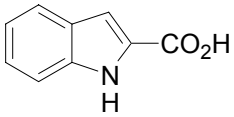
Table 2: Inhibitory activities of compounds **1**, **4**, **16**, **17** and **19** on BTH

No	**Structure**	**BTH** IC_{50} **[µM] or (% Inhibition)** pH 3.6	pH 7.4
1		78 %[a] (10 mM)	**4000**
4		23 %[a] (5 mM)	29 %[a] (5 mM)
16		38 %[a] (20 mM)	inactive (≤ 20 mM)
17		inactive (≤ 20 mM)	**250**
19		**7100**	inactive (≤ 20 mM)

[a] % inhibition of BTH at indicated inhibitor concentration

Table 3: Inhibitory activities of compounds **1**-**19** on hyaluronate lyase

No	Structure	LUDI Score	$hylB_{4755}$ IC_{50} [µM] or (% Inhibition)	
			pH 5.0	pH 7.4
1		687	**310**	**160**
2		579	**3710**	**900**
3		555	**2680**	inactive (≤ 20 mM)
4		505	**2900**	inactive (≤ 5 mM)
5		452	inactive (≤ 20 mM)	inactive (≤ 20 mM)
6		441	inactive (≤ 5.25 mM)	n.d
7		436	**2500**	inactive (≤ 20 mM)
8		429	inactive (≤ 2.5 mM)	n.d
9		426	**4470**	inactive (≤ 10 mM)

10		407	inactive (≤ 0.1 mM)	inactive (≤ 0.1 mM)
11		405	**11000**	**5000**
12		392	38 %[a] (20 mM)	83 %[a] (20 mM)
13		391	inactive (≤ 5 mM)	n.d.
14		384	20 %[a] (20 mM)	50 %[a] (20 mM)
15		382	50 %[a] (20 mM)	35 %[a] (20 mM)
16		364	**610**	46 %[a] (2 mM)
17		363	**160**	**5**
18		357	inactive (≤ 20 mM)	inactive (≤ 20 mM)
19		328	**3550**	44 %[a] (20 mM)

[a] % inhibition of $hylB_{4755}$ at indicated inhibitor concentration

Among the tested substances we identified six compounds **5**, **6**, **8**, **10**, **13** and **18** which were inactive on the hyaluronate lyase despite high or moderate LUDI scores (cf. Table 3).

The compound with the highest LUDI score of 687 corresponding to a predicted K_i value of about 1 µM, was the furan derivative **1**. The determined IC_{50} value of this compound was 310 µM at optimum pH (5.0) and 160 µM at physiological pH (7.4). The concentration-dependent inhibition of compound **1** on $hylB_{4755}$ is diagrammed in Fig. 2. Though the LUDI score of compound **1** did not reflect the obtained IC_{50} value, the measured and the predicted IC_{50} value are approximately in the same order of magnitude.

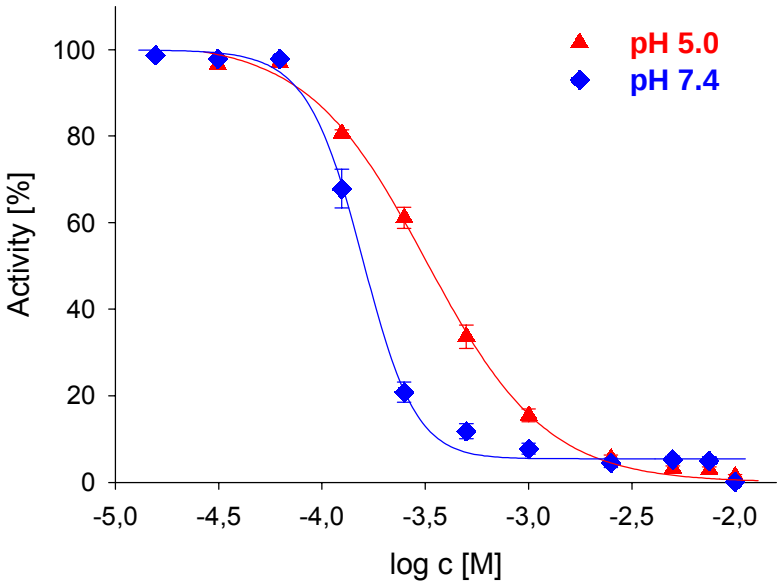


Fig. 2: Enzyme activity of hyaluronate lyase in the presence of 1-furan-2-yl-3-(4-nitrophenyl)propenone (**1**) at optimum (5.0) and physiological pH (7.4)

Compounds **2**, **3** and **7** with LUDI scores of 579, 555 and 436, respectively, are three related compounds, which showed a concentration dependent inhibitory activity on the hyaluronate lyase. The inhibition curves of the compounds **2**, **3** and **7** are depicted in Fig. 3. The IC_{50} values determined on the hyaluronate lyase at optimum pH (5.0) were 3.71 mM (**2**), 2.68 mM (**3**) and 2.5 mM (**5**) and were lower than expected from the LUDI score. Presumably, the LUDI scoring function did not perform well due to inaccurately predicted hydrogen bonding contribution (Ajay et al. 1995). Possibly, the LUDI scores just tend to overesti-

mate binding of these compounds at a highly charged (pH-dependent) active site. Other studies have shown predicted affinities to be accurate to about 1.3-1.5 lg units (Böhm 1998; Grüneberg et al. 2001). Additionally, depending on the enzyme, the inhibitory activity of the compounds were determined at different pH e.g. $hylB_{4755}$ at pH 5 and pH 7.4 corresponding to optimum and physiological pH. Although the protonation states of the molecules are dependent on the pH of the experimental measurement (pH 5 and 7.4), the LUDI scoring function consider only the protonation states of the amino acids residues inside the active site at neutral pH, a defined parameter of our constructed $hylB_{4755}$ model.

The IC_{50} value of 0.9 mM was determined for benzene-1,4-diacetic acid (**2**) at physiological pH, whereas isophthalic acid (**3**) and terephthalic acid (**7**) showed no inhibition of the enzyme at this pH. It is conspicuous that the inhibitors showed different activities at pH 5.0 and 7.4, and it may be speculated about the factors accounting for these differences. For example, the portion of charged test-compound depends on the pH value of the incubation mixture just like the protonation state of amino acids residues inside the active site of the enzyme.

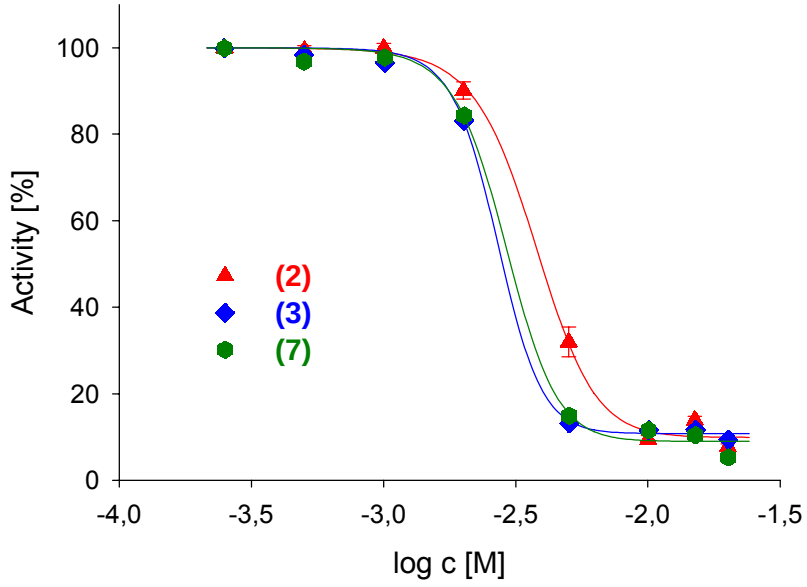


Fig. 3: Inhibitory effects of the three related acids **2**, **3** and **7** on the bacterial hyaluronidase at optimum pH (5.0)

The inhibitory activity of 2,8-dinitrodibenzofuran (**4**), kynurenic acid (**9**) and methylisoquinoline-3-carboxylate (**11**) on the bacterial hyaluronidase was in the millimolar range, whereas the calculated LUDI scores of 505, 426 and 405 predict an inhibitory activity in the submillimolar range. The determined IC_{50} values were 2.9 mM for compound **4,** 4.47 mM for compound **9** at pH 5.0, 11 mM and 5 mM for compound **11** at pH 5.0 and pH 7.4, respectively (cf. Table 3).

Weak and incomplete inhibition of the bacterial enzyme was produced by compounds **12**, **14** and **15**. The inhibitory effects of these three substances ranged from 20 % to 83 % at concentration of 15 mM and 20 mM (cf. Table 3).

One of the most potent inhibitors of the hyaluronate lyase at optimum pH was found for the 1-(2,3-dihydrobenzo[b]furan-5-yl)-3-(dimethylamino)propenone (**16**) (cf. Table 3). An IC_{50} value of 0.61 mM was determined on the bacterial enzyme at pH 5.0, whereas the percentual inhibition at pH 7.4 was only 46 % at a concentration of 2 mM (cf. Fig. 4). In the case of compound **16** the determined IC_{50} value and the predicted LUDI score of 364 are in good agreement.

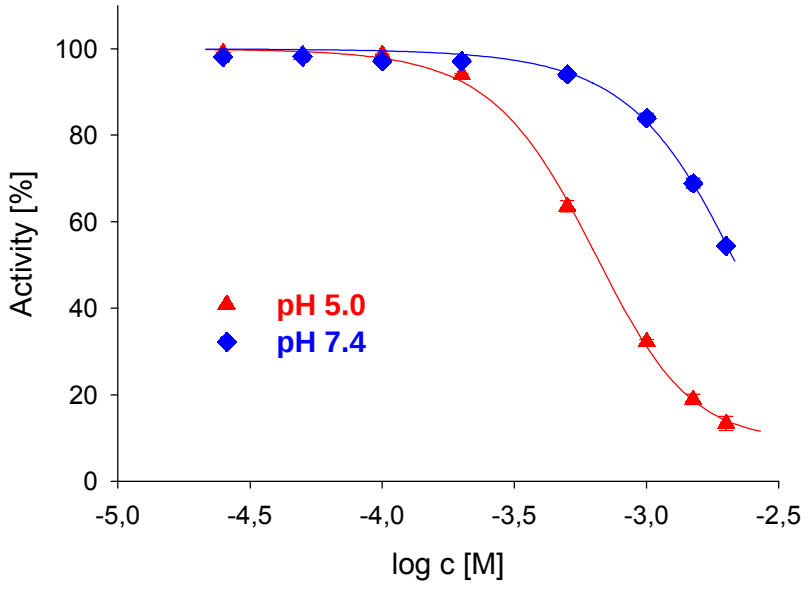


Fig. 4: Enzyme activity of hyaluronate lyase from *S. agalactiae* in the presence of 1-(2,3-dihydrobenzo[b]furan-5-yl)-3-(dimethylamino)propenone (**16**) at optimum pH (5.0) and physiological pH (7.4)

The LUDI score of indole-2-carboxylic acid (**19**) was calculated to be 328 corresponding to a predicted K_i value of approximately 1 mM for the bacterial enzyme. The measured IC_{50} values of the indole derivative **19** were 7.1 mM on the bovine testicular hyaluronidase and 3.55 mM on the bacterial hyaluronidase at optimum pH (5.0). The concentration-dependent inhibitory effect of indole-2-carboxylic acid on BTH and on $hylB_{4755}$ are diagrammed in Fig. 5. The IC_{50} value of compound **19** determined on the hyaluronate lyase is in agreement with the predicted value from the LUDI score.

The indole-2-carboxylic acid was a first lead with millimolar enzyme inhibitory activity which seemed to be sufficiently promising for further investigation and structural optimisation. The chemical work and the pharmacological investigations based on the results achieved for this indole derivative are described in chapter 9.

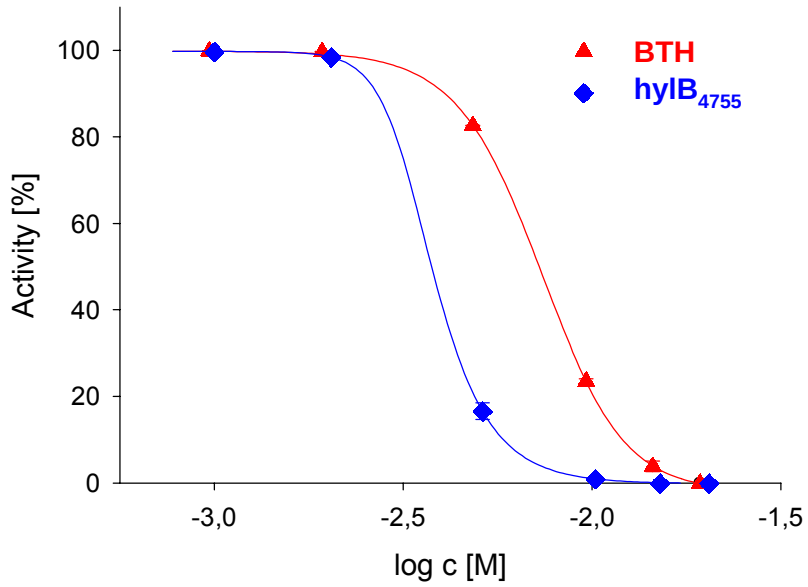


Fig. 5: Inhibitory effect of indole-2-carboxylic acid (**19**) on the bovine testicular hyaluronidase (pH 3.6) and the hyaluronate lyase (pH 5.0)

Within the series of tested compounds the highest inhibitory activity on the hyaluronate lyase was found for 1,3-diacetylbenzimidazole-2-thione (**17**) with an IC_{50} value of 160 µM at optimum pH (5.0) and 5 µM at physiological pH (7.4) (cf. Fig 6). Surprisingly, despite a LUDI score of 363 (predicted K_i value of about

1 mM), the determined IC_{50} values were in the micromolar range. In particular at pH 7.4 the compound was almost one order of magnitude more active than expected.

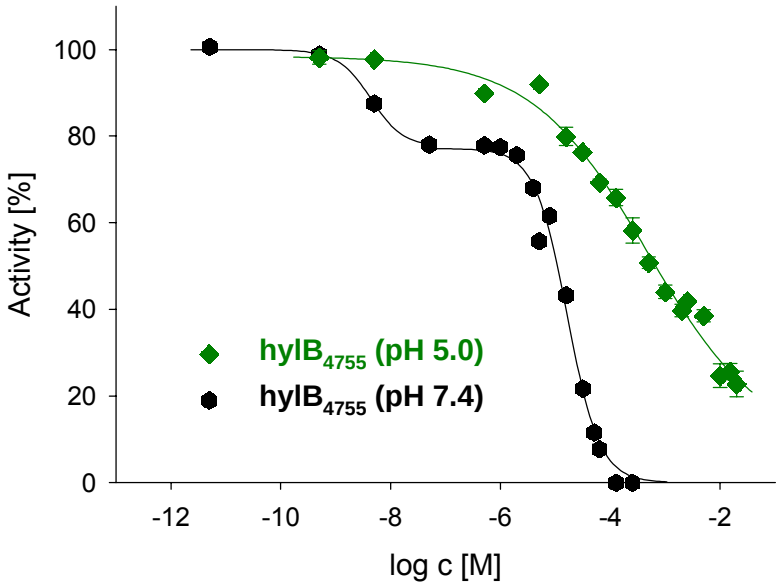


Fig. 6: Enzyme activity of the *S. agalactiae* hyaluronate lyase in the presence of 1,3-diacetylbenzimidazole-2-thione (**17**) at pH 5.0 and pH 7.4

The predicted binding mode of compound **17** inside the active site of the $hylB_{4755}$ model is depicted in Fig. 7. Different hydrophobic interactions as well as hydrogen bonds are probably responsible for the inhibition of the hyaluronate lyase by 1,3-diacetylbenzimidazole-2-thione. The interaction of the sulphur atom of compound **17** with the indole moiety of the amino acid Trp460 is of hydrophobic nature. Further hydrophobic interactions can be postulated with the amino acids Arg468, Arg630, Val579, and Tyr576, whereby the amino acid residue Val579 lies perpendicular to the phenyl moiety of the inhibitor. The two important hydrogen bonds are formed between the oxygen atom of the two acetyl groups of the inhibitor and the amino acid residues Arg409 and Arg634 (cf. Fig. 7).

To verify the predicted binding mode of compound **17** inside the active site of the $hylB_{4755}$ model, co-crystallisation experiments were carried out. Unfortunately, due to solubility problems and degradation of the inhibitor by moisture it

was not possible to co-crystallise the inhibitor inside the active site of the hyaluronate lyase, so that the binding mode predicted by LUDI calculations could not be confirmed.

The inhibition curve of the compound **17** determined on $hylB_{4755}$ at physiological pH suggests two different binding modes with different affinity. It is conceivable that the inhibitor binds inside the active site of the enzyme and additionally at other regions of the enzyme.

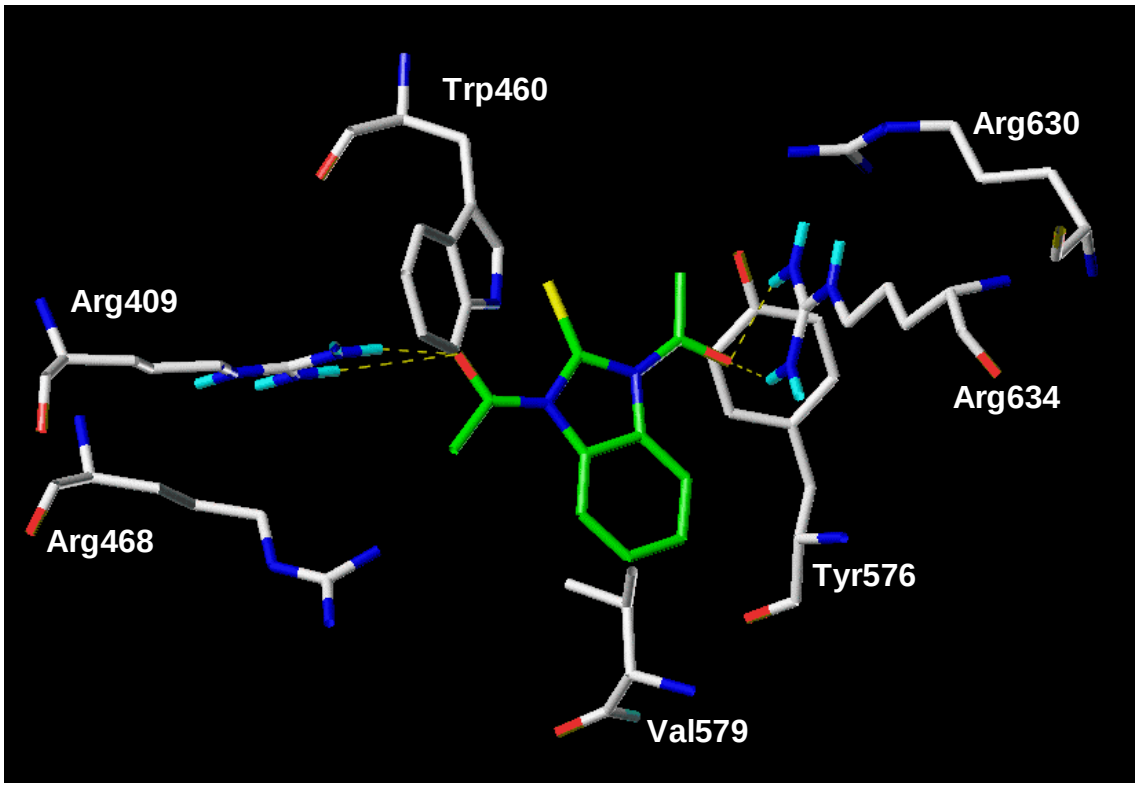
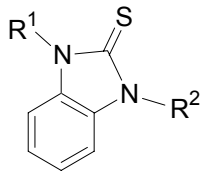


Fig. 7: Predicted binding mode of compound **17** inside the active site of the $hylB_{4755}$ model; O: red; N: blue; S: yellow; H-bonds: dotted lines (Botzki 2004).

In addition to compound **17**, both benzimidazole-2-thione derivatives **27** and **28** were tested for inhibition of BTH and $hylB_{4755}$. The unsubstituted benzimidazole-2-thione (**27**) was the starting material for the synthesis of compound **17**. Compound **28** is a synthetic intermediate and the product of hydrolytic cleavage of compound **17**. The activities of the three compounds determined on the bovine testicular hyaluronidase as well as on the bacterial enzyme are summarised in Table 4.

Table 4: Inhibitory activity of the benzimidazole derivatives **17**, **27** and **28** on the bovine testicular hyaluronidase and the *S. agalactiae* hyaluronate lyase



Compound			**BTH** IC_{50} **[µM]**		**$hylB_{4755}$** IC_{50} **[µM]**	
	R^1	R^2	pH 3.6	pH 7.4	pH 5.0	pH 7.4
17	Ac	Ac	inactive (≤ 20 mM)	**390**	**160**	**5**
27	H	H	**4100**	**3200**	**2210**	**1280**
28	Ac	H	inactive (≤ 2 mM)	**110**	**660**	**40**

The inhibitory activities of the non-substituted benzimidazole derivative **27** were in the millimolar range for both hyaluronidases (cf. Table 4). The concentration-dependent effects of compound **27** are illustrated in Fig. 8. In comparison to compound **17**, the absence of both acetyl groups at the nitrogen atoms led to an 8-fold decrease in inhibitory activity on BTH (pH 7.4) and a 14- and 256-fold decrease in activity on $hylB_{4755}$ at optimum and physiological pH, respectively.

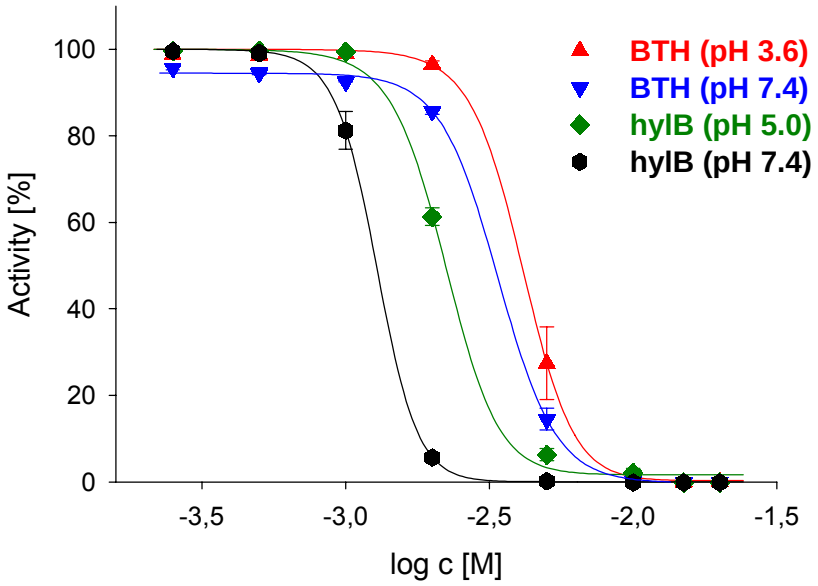


Fig. 8: Effect of benzimidazole-2-thione (**27**) on the activity of BTH and $hylB_{4755}$ at different pH values.

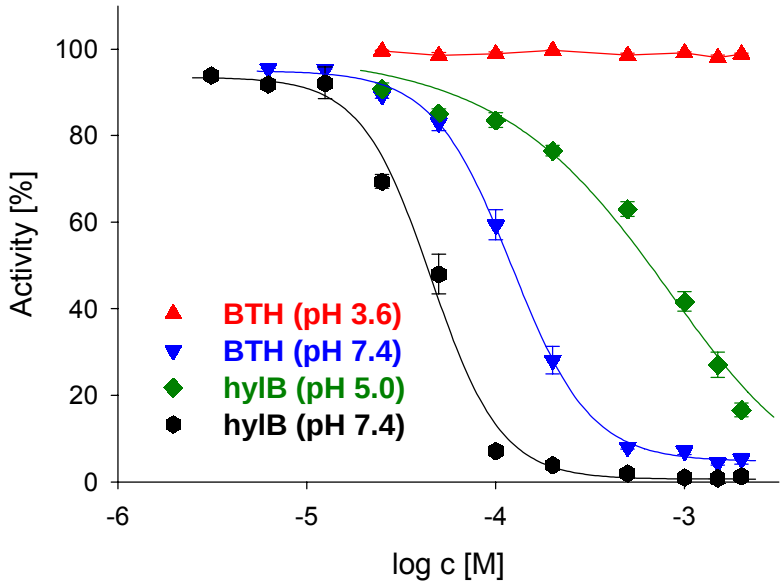


Fig. 9: Inhibitory effects of 1-acetylbenzimidazole-2-thione (**28**) on the activity of the bovine testicular hyaluronidase and the bacterial hyaluronidase at optimum and physiological pH.

The mono-acetylated benzimidazole-2-thione derivative **28** showed concentration dependent inhibitory effects on BTH and on $hylB_{4755}$, except for BTH at optimum pH (3.6). The enzyme inhibition curves are depicted in Fig. 9. The determined IC_{50} values were 110 µM on the bovine testicular enzyme at physiological pH, 660 µM and 40 µM on $hylB_{4755}$ at pH 5.0 and pH 7.4, respectively.
Summarising the results of the three related compounds, 1-acetylbenzimidazole-2-thione (**28**) was the most potent BTH inhibitor, whereas 1,3-diacetylbenzimidazole-2-thione (**17**) was the most potent *S. agalactiae* hyaluronate lyase inhibitor. The dependence of the potency on the number of acetyl groups could be interpreted as a hint that the inhibitors bind actually inside the active site of the enzymes.

5. Summary

In this chapter, a *de novo* design approach is presented, starting from the X-ray structure of bacterial hyaluronate lyases and molecular modelling investigations. The aim was to identify promising leads for the development of hyaluronidase inhibitors. Based on the constructed $hylB_{4755}$ model, 29717 compounds from the LeadQuest® and the Accelrys database and 196908 compounds from the ChemACX database were virtually screened with LUDI resulting in 212 and 1063 hits, respectively. The final selection of compounds for purchase, synthesis and enzyme testing included the following criteria: (a) a predominantly high LUDI score above 325, (b) commercial availability, (c) efficient synthetic feasibility. 19 compounds of all 1275 hits were selected for pharmacological investigations, 13 compounds therefrom revealed inhibitory activity on the bacterial hyaluronidase. One of the 13 active compounds proved to have IC_{50} value in the micromolar range (IC_{50}(**17**) 5 µM). Additionally, 5 of the investigated compounds showed inhibitory activities in the millimolar range on the bovine testicular hyaluronidase.
The results demonstrate that the chosen strategy based on a homology model of the hyaluronan lyase $hylB_{4755}$ is useful to identify promising leads despite partly non-correlating LUDI scores.

Starting from the results obtained by a *de novo* design, different projects were envisaged:

- Inspired by the structures of the compound **5**, **15** and **18**, diphenylpropionic acids and diphenylacrylic acids have been investigated (cf. chapter 8).
- Due to the inhibitory activity of indole-2-carboxylic acid (**19**), a series of indole derivative and phenylindole derivatives was investigated. This led to the discovery of a new class of indole-type hyaluronidase inhibitors. Design, synthesis and pharmacological investigations of this class of compounds as well as a X-ray analysis of an inhibitor-enzyme complex are reported in chapter 9.
- The most promising lead discovered by virtual screening was 1,3-diacetylbenzimidazole-2-thione (**17**) with an IC_{50} value in the micromolar range. Structural modifications of this compound are subject of ongoing work.

6. Experimental section

6.1 General conditions

For a detailed description of the general procedures, equipments and chemicals used in the chemistry part, see section 4.1 of chapter 5.

6.2 Chemistry

1-Furan-2-yl-3-(4-nitrophenyl)propenone (1)

To a solution of 4-nitrobenzaldehyde (5.0 g, 33.10 mmol), 2-acetylfurane (3.65 g, 33.10 mmol) and ethanol (300 ml) an aqueous solution (60 %) of potassium hydroxide (25 ml) was slowly added. After stirring at room temperature for 16 h, the reaction mixture was diluted with water and acidified with concentrated hydrochloric acid. The precipitated product was collected, washed with

water and recrystallised from dimethylsulphoxide to give a brown crystalline solid.

Yield: 4.21 g (17.31 mmol, 52 %, brown crystalline solid)

Mp: 205-210 °C [Lit.: 228-232 °C (Kabli et al. 1991)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.83 (dd, 1H, ^{3}J = 1.7 Hz, ^{3}J = 3.6 Hz, furyl H-4), 7.82 (d, 1H, ^{3}J = 15.8 Hz, =C<u>H</u>), 7.90 (d, 1H, ^{3}J = 15.8 Hz, =C<u>H</u>), 7.91 (dd, 1H, ^{4}J = 0.7 Hz, ^{3}J = 3.6 Hz, furyl H-3), 8.11 (dd, 1H, ^{4}J = 0.7 Hz, ^{3}J = 1.7 Hz, furyl H-5), 8.12-8.16 (m, 2H, C_6H_4), 8.27-8.31 (m, 2H, C_6H_4)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 112.8 (+, furyl), 120.4 (+, furyl), 123.9 (+, C_6H_4), 125.9 (+, =<u>C</u>H), 129.7 (+, C_6H_4), 139.9 (+, =<u>C</u>H), 140.8 (C_{quart}, C_6H_4), 148.0 (C_{quart}, C_6H_4), 148.9 (+, furyl), 152.6 (C_{quart}, furyl), 176.1 (C_{quart}, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 243 ([$M^{\bullet+}$], 100), 242 ([M-H]$^{+}$, 28)

IR [cm^{-1}]: 3157 (C-H) michael system, 3115 (C-H) aromatic, 1663 (C=O), 1613 (C=C), 1511 (N-O), 1336 (N-O), 1167 (C-O), 883, 837, 769, 754

Analysis:

calculated	C: 64.20	H: 3.73	N: 5.76
found	C: 64.12	H: 3.55	N: 5.73

$C_{13}H_9NO_4$ (243.22)

2,8-Dinitrodibenzofuran (4)

To a solution of dibenzofuran (5.0 g, 29.73 mmol) and acetic anhydride (30 ml) concentrated HNO_3 (10 ml) was carefully added dropwise. After stirring at room temperature for 16 h, the reaction mixture was poured into ice water and diluted

with water. The precipitated product was collected, washed with water and re-crystallised from dimethylsulphoxide to give a light pink solid.
Yield: 0.90 g (3.49 mmol, 12 %, light pink solid)

Mp: 305-307 °C [Lit. 329-330 °C (Yamashiro 1938)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 8.08 (d, 2H, ^{3}J = 9.1 Hz, H-4, H-6), 8.53 (dd, 2H, ^{4}J = 2.5 Hz, ^{3}J = 9.1 Hz, H-3, H-7), 9.51 (d, 2H, ^{4}J = 2.5 Hz, H-1, H-9)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 113.1 (+, ar), 119.2 (+, ar), 123.7 (C_{quart}, ar), 124.6 (+, ar), 144.2 (C_{quart}, $\underline{C}O$), 159.7 (C_{quart}, $\underline{C}NO_2$)

MS (PI-EIMS (70 eV)): m/z (%) = 258 ([M$^{\bullet+}$], 100), 228 ([M-NO]$^+$, 16), 212 ([M-NO_2]$^+$, 20), 138 ([M-2NO_2-CO]$^+$, 26)

IR [cm^{-1}]: 3101 (C-H) aromatic, 1517 (N-O), 1466 (C-H), 1343 (N-O), 1200 (C-O), 1018, 783, 748, 664

Analysis:

calculated	C: 55.82	H: 2.34	N: 10.85
found	C: 55.73	H: 2.31	N: 10.82

$C_{12}H_6NO_5$ (258.19)

Diphenylmethanone-*O*-methyloxime (5)

A solution of benzophenone (5.70 g, 31.30 mmol), methoxylamine hydrochloride (2.67 g, 32.40 mmol), pyridine (100 ml) and ethanol (100 ml) was stirred under reflux for 24 h. After evaporation of the solvent, the residue was diluted with water (100 ml) and extracted three times with ethyl acetate (50 ml). The organic solution was washed with water (3 x 50 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was puri-

fied by column chromatography on silica gel eluting with a 15:1 (v/v) mixture containing ethyl acetate and petroleum ether 60 - 80 °C to give a white solid.
Yield: 4.68 g (27.6 mmol, 88 %, white solid)

Mp: 43-45 °C [Lit. 49-51 °C (Kerr et al. 1970)]
^{1}H-NMR ($CDCl_3$):
δ [ppm] = 3.86 (s, 3H, OC<u>H</u>$_3$), 7.21-7.31 (m, 2H, C_6H_5), 7.33-7.41 (m, 5H, C_6H_5), 7.41-7.50 (m, 3H, C_6H_5)

^{13}C-NMR ($CDCl_3$):
δ [ppm] = 61.9 (+, O<u>C</u>H$_3$), 127.2 (+, C_6H_5), 128.3 (+, C_6H_5), 128.4 (+, C_6H_5), 128.6 (+, C_6H_5), 128.8 (+, C_6H_5), 129.5 (+, C_6H_5), 132.9 (C_{quart}, C_6H_5), 135.7 (C_{quart}, C_6H_5), 156.1 (C_{quart}, <u>C</u>=N)

MS (PI-EIMS (70eV)): m/z (%) = 211 ([$M^{\bullet+}$], 61), 180 ([M - OCH_3]$^+$, 100)

IR [cm^{-1}]: 2939 (C-H) aromatic, 2900 (C-H) aliphatic, 1579 (C=N), 1442 (C-H), 1048, 983, 878, 771, 692, 649

Analysis:

calculated	C: 79.60	H: 6.20	N: 6.63
found	C: 79.53	H: 6.45	N: 6.65

$C_{14}H_{13}NO$ (211.26)

1-(2-Methoxyphenyl)-4-(3-methylbenzoyl)piperazine (12)

A solution of 1-(2-methoxyphenyl)piperazine hydrochloride (1.0 g, 4.37 mmol), 1,1'-carbonyldiimidazole (0.71 g, 4.37 mmol) and anhydrous chloroform (20 ml) was stirred at room temperature for 30 min. Afterwards, m-toluene sulphonic acid (0.60 g, 4.37 mmol) was added and the reaction mixture was stirred at ambient temperature for 16 h. After evaporation of the chloroform the residue was diluted with water (100 ml) and extracted three times with ethyl acetate

(50 ml). The combined organic layer was washed with water (2 x 50 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 2:1 (v/v) mixture of ethyl acetate and petroleum ether 60 - 80 °C to give a white solid.

Yield: 1.22 g (3.93 mmol, 90 %, white solid)

Mp: 82-84 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 2.38 (s, 3H, C$\underline{H}_3$), 2.86-3.35 (m, 4H, C$\underline{H}_2$), 3.48-3.83 (m, 2H, C$\underline{H}_2$), 3.87 (s, 3H, OC$\underline{H}_3$), 3.91-4.15 (m, 2H, C$\underline{H}_2$), 6.85-7.42 (m, 8H, C_6H_4)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 21.4 (+, $\underline{C}H_3$), 42.3 (-, $NCH_2\underline{C}H_2NCO$), 47.9 (-, $NCH_2\underline{C}H_2NCO$), 50.9 (-, $N\underline{C}H_2CH_2NCO$), 51.2 (-, $N\underline{C}H_2CH_2NCO$), 55.5 (+, $O\underline{C}H_3$), 111.5 (+, C_6H_4), 118.7 (C_{quart}, C_6H_4), 121.1 (+, C_6H_4), 123.8 (+, C_6H_4), 124.0 (+, C_6H_4), 127.2 (+, C_6H_4), 127.7 (+, C_6H_4), 128.3 (+, C_6H_4), 130.4 (+, C_6H_4), 135.8 (C_{quart}, C_6H_4), 138.5 (C_{quart}, C_6H_4), 152.3 (C_{quart}, C_6H_4), 170.6 (C_{quart}, $\underline{C}$=O)

MS (PI-DCIMS (NH_3)): m/z (%) = 311 ([MH^+], 100)

IR [cm^{-1}]: 3057 (C-H) aromatic, 2860 (C-H) aliphatic, 2815 (C-H) aliphatic, 1632 (C=O), 1499 (C-H), 1431 (C-H), 1242 (C-O), 1023, 795, 745

Analysis:

calculated	C: 73.53	H: 7.14	N: 9.03
found	C: 73.44	H: 7.65	N: 8.56

$C_{19}H_{22}N_2O_2$ (310.38)

1-(2,3-Dihydrobenzo[b]furan-5-yl)-3-dimethylaminopropenone (16)

A solution of 5-acetyl-2,3-dihydrobenzo[b]furan (0.45 g, 2.77 mmol) and *N,N*-dimethylformamide dimethyl acetale (10 ml) was stirred and heated under reflux for 14 h. The solvent was removed under reduced pressure and the residue was recrystallised from diethyl ether to give the pure product as a yellow solid.
Yield: 0.19 g (0.87 mmol, 31 %, yellow solid)

Mp: 102-104 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.88 (br, 3H, NC$\underline{H}_3$), 3.11 (br, 3H, NC$\underline{H}_3$), 3.20 (t, 2H, ^{3}J = 8.8 Hz, H-3), 4.58 (t, 2H, ^{3}J = 8.8 Hz, H-2), 5.79 (d, 1H, ^{3}J = 12.4 Hz, =C$\underline{H}$CO), 6.77 (d, 1H, ^{3}J = 8.5 Hz, H-7), 7.65 (d, 1H, ^{3}J = 12.4 Hz, =C$\underline{H}$CN), 7.72 (dd, 1H, ^{4}J = 1.9 Hz, J = 8.5 Hz, H-6), 7.82 (d, 1H, ^{4}J = 1.9 Hz, H-4)

^{13}C-NMR ([D$_6$]DMSO) / HMQC-NMR:

δ [ppm] = 28.5 (-, C-3), 36.9 (+, N$\underline{C}$H$_3$), 44.3 (+, N$\underline{C}$H$_3$), 71.5 (-, C-2), 90.6 (+, =$\underline{C}$HCO), 108.1 (+, C-7), 124.4 (+, C-4), 127.2 (C$_{quart}$, C-9), 128.1 (+, C-6), 132.9 (C$_{quart}$, C-5), 153.5 (+, =$\underline{C}$HN), 162.0 (C$_{quart}$, C-8), 184.7 (C$_{quart}$, $\underline{C}$=O)

MS (PI-CIMS (NH$_3$)): m/z (%) = 218 ([MH$^+$], 100)

IR [cm^{-1}]: 3000 (C-H) aromatic, 2888 (C-H) aliphatic, 1637 (C=O), 1601 (C=C), 1546 (C=C), 1428 (C-H), 1352 (C-H), 1239 (C-O), 1102, 925, 740

Analysis:

calculated	C: 71.78	H: 6.96	N: 6.45
found	C: 71.42	H: 7.37	N: 6.19

$C_{13}H_{15}NO_2$ (217.27)

1,3-Diacetylbenzimidazole-2-thione (17)

A solution of benzimidazole-2-thione (1.50 g, 10.0 mmol), triethylamine (2.88 g, 28.50 mmol), catalytic amount (100 mg) of DMAP and anhydrous dichloromethane was stirred at room temperature for 10 min. A solution of acetyl chloride (2.83 g, 36.0 mmol) and anhydrous dichloromethane was added dropwise to the mixture and stirring was continued for 16 h. After diluting with water (100 ml) and dichloromethane (50 ml), the organic phase was separated, washed three times with an aqueous solution of potassium hydrogen sulphate (1 M, 50 ml) and water (50 ml), dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 1:8 (v/v) mixture containing ethyl acetate and petroleum ether 60 - 80 °C to give a white solid. The product decompose at ambient temperature and moisture to 1-acetylbenzimidazole-2-thione (**28**).
Yield: 1.30 g (5.55 mmol, 56 %, white solid)

Mp: 100-102 °C [Lit. 102-103°C (Tittelbach et al. 1988)]
^{1}H-NMR ($CDCl_3$):
δ [ppm] = 3.05 (s, 6H, OC$\underline{H}_3$), 7.31 (dd, 2H, ^{4}J = 3.4 Hz, ^{3}J = 6,3 Hz, H-5 and H-6), 7.97 (dd, 2H, ^{4}J = 3.4 Hz, ^{3}J = 6,3 Hz, H-4 and H-7)

MS (PI-EIMS (70eV)): m/z (%) = 234 ([$M^{\bullet+}$], 11), 192 ([M-CH_2=C=O]$^{+}$, 18), 150 ([M-2CH_2=C=O]$^{+}$, 100)

IR [cm^{-1}]: 3018 (C-H) aromatic, 2900 (C-H) aliphatic, 1719 (C=O), 1467 δ(C-H), 1366 (C-H), 1153 (C=S), 987, 743, 67

$C_{11}H_{10}N_2O_2S$ (234.20)

1-Acetylbenzimidazole-2-thione (**28**)

The product was isolated as by-product of the aforementioned reaction.
Yield: 0.51g (2.60 mmol, 26 %, white solid)

Mp: 177-180 °C [Lit.: 191-192 °C (Gosselin et al. 1978)]
^{1}H-NMR ($CDCl_3$):
δ [ppm] = 3.01 (s, 3H, $COC\underline{H}_3$), 7.16-7.35 (m, 3H, H-4, H-5, H-6), 8.00 (d, 1H, ^{3}J = 7.4 Hz, H-7), 13.34 (s, 1H, N$\underline{H}$)

^{13}C-NMR ($CDCl_3$):
δ [ppm] = 27.9 (+, $CO\underline{C}H_3$), 109.4 (+, C_6H_4), 115.3 (+, C_6H_4), 123.3 (+, C_6H_4), 125.3 (+, C_6H_4), 130.8 (C_{quart}, C_6H_4), 130.9 (C_{quart}, C_6H_4), 169.5 (C_{quart}, $\underline{C}$=O), 172.0 (C_{quart}, $\underline{C}$=S)

IR [cm^{-1}]: 3110 (N-H), 3060 (C-H) aromatic, 2931 (C-H) aliphatic, 1714 (C=O), 1512 (C=C), 1454 (C-H), 1362 (C-H), 1204 (C-O), 1152 (C=S), 985, 742, 687, 632

$C_9H_8N_2O_1S$ (192.24)

6.3 Theoretical methods

The construction of the $hylB_{4755}$ model and the LUDI calculations have been carried out by *Alexander Botzki* (Institute of Pharmacy, University of Regensburg, 93040 Regensburg, Germany) and are subject of his PhD project. More detailed information about the used enzyme model, the calculation methods, databases and LUDI will be given in the thesis of *A. Botzki* (Botzki 2004).

6.3.1 Database preparation

The LeadQuest® databases Vol. 1&2 (Tripos 1998), the ChemACX database (CambrigeSoft, Cambrige, UK) and the Accelrys database (Accelrys Inc., San Diego, CA, USA) were converted into 3D using the CONVERTER module from Insight 2000 (Accelrys Inc., San Diego, CA, USA). To allow for energy minimum conformations of the resulting 3D structures, all-*trans* conformations for the chain portions of the molecules and chair conformations for 6-membered rings consisting of all sp^3 atoms were applied. For each molecule of the original 2D databases two stereoisomeres (if possible) were generated. The resulting database was prepared for use with the LUDI programme (Accelrys Inc.) by means of the LUDI module genfra 5 (Böhm 1992; Böhm 1992) constructing a structure and a target database with types of each target site (e.g. acceptor or donor atom) in the fragments.

6.3.2 LUDI calculations with the $hylB_{4755}$ model

A LUDI approach was set up with a sphere of 5 Å radius in the space of the active site of $hylB_{4755}$. The centre of this sphere was determined as the geometric mean of six amino acid residues inside the active site of the model (Arg409, Trp460, Tyr576, Val579 and Arg634). The values of the most important LUDI parameters for design of $hylB_{4755}$ ligands were as follows: the maximal RMSD of the fit between the fragment and the interaction sites was 0.45 Å, the density of lipophilic and polar interaction sites per protein atom was set to 35 and the minimal contact surface between ligand and protein was set to 30 %. The retrieved candidate molecules were ranked with respect to their expected binding affinity using the empirical scoring function developed by Böhm (Böhm 1994) with a minimal scoring value of 300 (predicted K_i value of 1mM). All other parameters were set to default values. Conducting a LUDI run with the constructed LeadQuest® Database resulted in 122 hits. To enable a larger sphere (r = 8 Å) and to consider rotatable bonds in reasonable CPU time, these hits were combined with 1020 compounds from the Accelrys database as supplied within the LUDI module. Recalculation with this combined hit database pro-

posed 212 new hits. Sixteen of the proposed 212 hits were selected for testing hyaluronan lyase inhibition according to high LUDI scores, availability and efficient synthetic feasibility (cf. Table 1).
The LUDI run with the ChemACX database resulted in 1063 hits from which 3 of them were selected for testing hyaluronan lyase inhibition also according to high LUDI score, availability and efficient synthetic feasibility (cf. compounds **1**, **4** and **10** of Table 1).

6.4 Pharmacological methods

The inhibitory effect of the compounds on the activity hyaluronidases were determined by the method of *Reissig (Reissig et al. 1955)* based on the Morgan-Elson reaction and by a turbidimetric assay according to the description of *Di Ferrante* (Di Ferrante 1956), as described in chapter 3.

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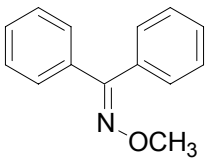
Chapter 8

Diphenylacrylic acids and diphenylpropionic acids as potential inhibitors of hyaluronidases: synthesis and pharmacological investigations

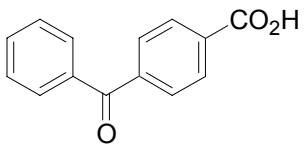
1. Introduction

For a *de novo* design of enzyme inhibitors we followed a structure-based strategy. Initially, a model of $hylB_{4755}$ hyaluronidase was constructed by partially superimposing corresponding domains of the crystal structures of *S. agalactiae* ($hylB_{3502}$) (Li et al. 2001) and *S. pneumoniae* hyaluronate lyases (Li et al. 2000) using Sybyl 6.8 (Tripos Inc., St. Louis). Subsequent LUDI calculations based on this model resulted in different proposals. Nineteen compounds were selected for biological investigations. The results are described in chapter 7.

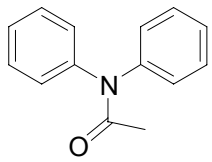
The compounds **1-3** (cf. Fig. 1) were among the structure suggested by LUDI. The structures of theses compounds, the arrangement of the phenyl rings and the predicted binding modes at the active site of $hylB_{4755}$ are similar. According to the LUDI scores (LS) of 452 (**1**), 382 (**2**) and 357 (**3**), the compounds should have inhibitory activities in the low millimolar range.



1 (LS 452)



2 (LS 382)



3 (LS 357)

Fig. 1: Three of the "LUDI proposals" and their respective LUDI score (LUDI scores give a rough estimate of the inhibition constant K_i value, i.e. LS of 600 and 300 correspond to K_i value of 1 µM and 1 mM, respectively.)

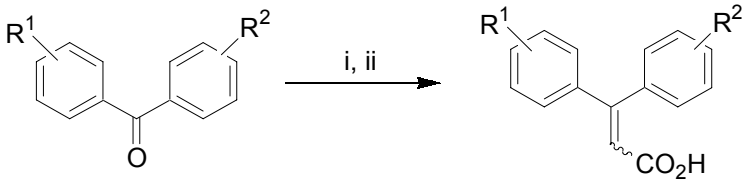
However, the results of the pharmacological investigations of these three compounds were disappointing because only compound **2** showed an inhibitory effect on hyaluronidases. The inhibition by compound **2** at a concentration of 20 mM was only 50 % on BTH and 35 % on $hylB_{4755}$ (cf. chapter 7).

As the carboxylic acid **2** was the only active substance among the compounds **1-3** structural modifications were made: We decided to keep the two phenyl rings and to introduce an acid functionality by incorporating acrylic acid or propionic acid. Comparing the flexibility of the two compounds, the acrylic acid derivatives are more rigid whereas the propionic acid derivatives are more flexible (cf. Fig. 2). Furthermore, by introducing different substituents at the phenyl rings a variety of compounds can be synthesised and pharmacological investigated.

Fig. 2: New proposals for hyaluronidases inhibitors

2. Chemistry

The acrylic acids **5a-f** were prepared by the *Horner-Wadsworth-Emmons* reaction described in Scheme 1. The commercially available benzophenones **4a-f** were dissolved in *tert*-butanol and treated with triethyl phosphonoacetate and ^{t}BuOK under reflux. The prepared esters were cleaved under basic conditions with sodium hydroxide in methanol to get the corresponding acrylic acids **5a-f**. All unsymmetrical substituted compounds were obtained as mixtures of E/Z-isomers.

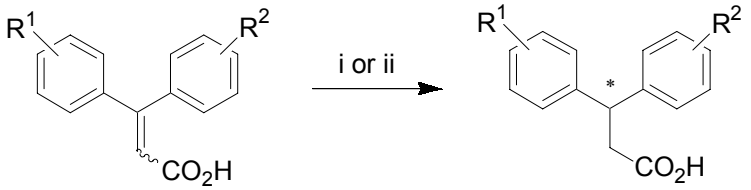


4a-f **5a-f**

No	R^1	R^2
a	F	H
b	F	F
c	CH_3	H
d	CF_3	H
e	Cl	H
f	Br	Br

Scheme 1: Synthesis of the diphenylacrylic acids **5a-f**; used reagents: i) ^{t}BuOK, ^{t}BUOH, $(EtO)_2P(O)CH_2CO_2Et$, ΔT; ii) NaOH, MeOH, ΔT;

The subsequent hydrogenation was carried out according to standard procedures using either palladium on charcoal (Pd/C), 5 bar hydrogen pressure, room temperature or Lindlar catalyst ($Pd/CaCO_3$) and 1 bar hydrogen pressure (Scheme 2). All unsymmetrical substituted compounds were obtained as racemates.



5a-d **6a-d**

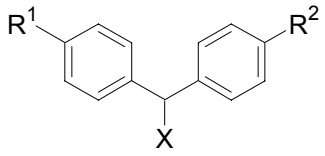
No	R^1	R^2
a	F	H
b	F	F
c	CH_3	H
d	CF_3	H

Scheme 2: Synthesis of the diphenylpropionic acid derivatives **6a-d**; used reagents: i) Pd/C, MeOH, H_2 5 bar, RT; ii) $Pd/CaCO_3$, H_2, RT;

3. Pharmacological investigations

First, a series of diphenylpropionic acids **7-11** with hydroxy-, bromo and chloro substituents at the phenyl rings and (4,4'-dichlorodiphenyl)acetic acid (**12**) were examined. Their inhibitory effects on the bovine testicular hyaluronidase and on the hyaluronate lyase at optimum pH (BTH 3.6, $hylB_{4755}$ 5.0) and physiological pH (7.4) are summarised in Table 1.

Table 1: Inhibitory activities of the diphenylalkanoic acids **7-12**[1] determined on BTH and $hylB_{4755}$ at different pH



7-12

No	Substituents			BTH IC$_{50}$ [µM] or (% Inhibition)		$hylB_{4755}$ IC$_{50}$ [µM] or (% Inhibition)	
	X	R^1	R^2	pH 3.6	pH 7.4	pH 5.0	pH 7.4
7	CH_2COOH	H	H	**3200**	inactive[a]	**2200**	**8900**
8	CH_2COOH	OH	H	inactive[a]	inactive[a]	inactive[a]	inactive[a]
9	CH_2COOH	OH	OH	inactive[a]	inactive[a]	inactive[a]	inactive[a]
10	CH_2COOH	Br	H	**200**	**5100**	**330**	**740**
11	CH_2COOH	Cl	Cl	inactive[b]	inactive[a]	**560**	(79 %)[c]
12	COOH	Cl	Cl	**400**	**8300**	**400**	**1600**

[a] at concentrations ≤ 15 mM; [b] at concentrations ≤ 1.8 mM
[c] % inhibition of the enzyme at an inhibitor concentration of 2 mM

[1] The compounds 7-12 were kindly provided from Dr. Ch. Hutzler, Institute of Pharmacy, University of Regenbsurg, Germany

The IC_{50} values of the unsubstituted diphenylpropionic acid **7** were 3.2 mM for the bovine testicular and 2.2 mM for the bacterial enzyme at optimum pH (BTH pH 3.6, $hylB_{4755}$ pH 5.0) and 8.9 mM for the hyaluronate lyases at physiological pH. The introduction of one or two hydroxy groups at the phenyl rings (compounds **8** and **9**) led to a complete loss of the inhibitory activity. This observation was surprising, because earlier investigations of diverse compounds suggested hat hydroxy groups may substantially contribute to an increase in inhibitory activity.

In contrast to hydroxylation (**8, 9**), the introduction of one bromo substituent at the phenyl ring (compound **10**) led to a more potent inhibitor for both hyaluronidases. The IC_{50} values determined for compound **10** were 200 µM (BTH at pH 3.6), 5.1 mM (BTH at pH 7.4), 330 µM ($hylB_{4755}$ at pH 5.0) and 740 µM ($hylB_{4755}$ at pH 7.4) (cf. Table 1, Fig. 3). In comparison to the unsubstituted diphenylpropionic acid **7**, the brominated derivative **10** showed a 7- to 16-fold higher inhibition of the bacterial and the bovine testicular enzyme.

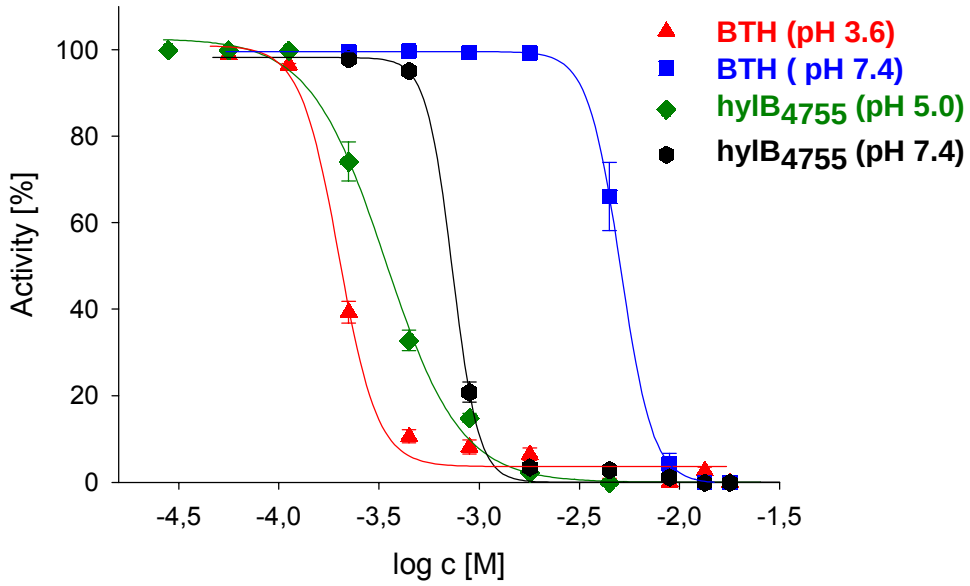


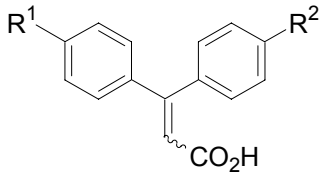
Fig. 3: Inhibitory effect of 3-(4-bromophenyl)-3-phenylpropionic acid (**10**) on the activity of BTH and $hylB_{4755}$ at optimum and physiological pH

The 4,4'-dichloro substitution of the phenyl rings (compound **11**), led to a complete loss of activity on the bovine testicular enzyme, whereas the IC_{50} value for inhibition of the hyaluronate lyase at optimum pH (5.0) was 4-fold lower than for compound **7**. The inhibition of the bacterial enzyme at physiological pH was 79 % at a concentration of 0.89 mM, unfortunately, the IC_{50} value could not be determined due to poor solubility at higher concentration.

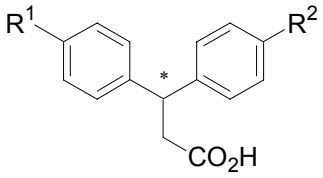
The inhibitory effects of 4,4'-dichlorodiphenylacetic acid (**12**) are comparable with the effects of the brominated diphenylpropionic acid (**10**). Compound **12** inhibited the hyaluronidases with IC_{50} values of 400 µM (BTH at pH 3.6), 8.3 mM (BTH at pH 7.4), 400 µM ($hylB_{4755}$ at pH 5.0) and 1.6 mM ($hylB_{4755}$ at pH 7.4).
This results suggested that this class of substances was promising and worthwhile to be investigated in more detail. Apart from the importance of the conformational flexibility between C-2 and C-3, the influence of fluoro, trifluoromethyl and methyl substituents at the phenyl rings should be examined. The conformational flexibility can be constrained for example, by double bond between C-2 and C-3. Therefore, we synthesised diphenylacrylic acids and diphenylpropionic acids, with different substitution pattern.

The synthesised diphenylacrylic acids **5a-f** and propionic acids **6a-d** were tested for inhibition of the bovine testicular hyaluronidase and the bacterial hyaluronidase using an optimised turbidimetric assay. The inhibitory activities of the enzymes at optimum pH (BTH 3.6, $hylB_{4755}$ 5.0) and at physiological pH (7.4) are summarised in Table 2.

Table 2: IC_{50} values of the diphenylacrylic acids **5a-f** and the diphenylpropionic acids **6a-d** for the inhibition of BTH and $hylB_{4755}$



5a-f



6a-d

No	**Substituents**		**BTH** IC_{50} **[µM] or (% Inhibition)**		**$hylB_{4755}$**	
	R^1	**R^2**	pH 3.6	pH 7.4	pH 5.0	pH 7.4
5a	F	H	inactive[a]	inactive[a]	**1600**	inactive[a]
5b	F	F	**1580**	inactive[b]	**1410**	**3550**
5c	CH_3	H	**3160**	(50 %)[c]	**1120**	**7080**
5d	CF_3	H	(55 %)[c]	(80 %)[c]	(80 %)[c]	**2000**
5e	Cl	H	**11200**	**14000**	**890**	**2200**
5f	Br	Br	**150**	**2660**	**260**	**330**
6a	F	H	**2820**	inactive[b]	**1780**	**4470**
6b	F	F	**1600**	inactive[a]	**1200**	(32 %)[d]
6c	CH_3	H	inactive[a]	inactive[a]	**1260**	(20 %)[d]
6d	CF_3	H	**600**	inactive[a]	**380**	**780**

[a] at concentrations ≤ 2 mM; [b] at concentrations ≤ 20 mM; [c] % inhibition of the enzyme at an inhibitor concentration of 20 mM; [d] % inhibition of the enzyme at an inhibitor concentration of 2 mM

The diphenylacrylic acids **5a-e** were more active inhibitors of the bacterial enzyme than of the bovine testicular hyaluronidase. Whereas the monofluorinated acrylic acid **5a** inhibited only the bacterial enzyme (IC_{50} = 1.6 mM), the difluorinated compound **5b** showed inhibition of both hyaluronidases with IC_{50} values of 1.58 mM (BTH at pH 3.6), 1.41 mM ($hylB_{4755}$ at pH 5.0) and 3.55 mM ($hylB_{4755}$ at pH 7.4). Compared to **5b**, the trifluoromethylsubstituted diphenylpropionic acid **5d** showed a weaker inhibition of the BTH (at pH 3.6 and 7.4) and of the $hylB_{4755}$ at pH 5.0 but a stronger inhibitory activity of $hylB_{4755}$ at physiological pH (IC_{50} value 2 mM). The inhibitory activities of **5c** on the bovine testicular and the bacterial hyaluronidase were similar to those of **5b**. For the chloro substituted compound **5e**, IC_{50} values of 11.2 mM (BTH at pH 3.6), 14 mM (BTH at pH 7.4), 0.89 mM ($hylB_{4755}$ at pH 5.0) and 2.2 mM ($hylB_{4755}$ at pH 7.4) were determined (cf. Table 2).

Within the series of the substituted diphenylacrylic acids, the highest inhibitory activity on both the bovine testicular and the bacterial enzyme was found for the dibrominated compound **5f**. The IC_{50} values were 150 µM (BTH at pH 3.6), 2.66 mM (BTH at pH 7.4), 260 µM ($hylB_{4755}$ at pH 5.0) and 330 µM ($hylB_{4755}$ at pH 7.4) (cf. Table. 2, Fig. 4). The acrylic acid **5f** was a 1.3 - 2.25-fold more active than the monobrominated compound acid **10**. The introduction of the C=C double bond and the introduction of a second bromo substituent on the phenyl ring seem to enhance potency.

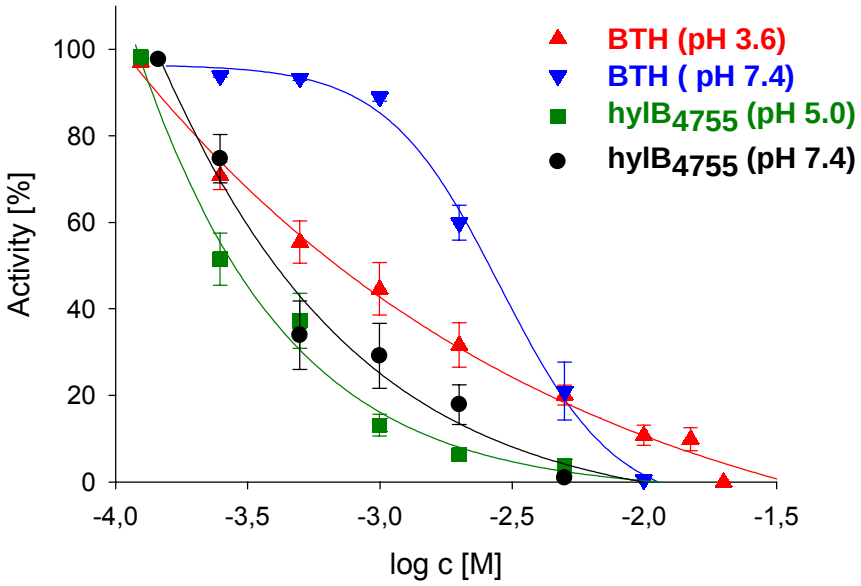


Fig. 4: Effect of 4,4'-dibromodiphenylacrylic acid (**5f**) on the activities of the bovine testicular and the bacterial hyaluronidase at their optimum pH and at the physiological pH (7.4).

The inhibitory activities of the synthesised propionic acids **6a-c** (cf. Table 2) were in the same range for the bacterial hyaluronidase but different for the bovine testicular enzyme. Whereas compound **6c** was inactive on the BTH, the compounds **6a** and **6b** showed inhibition in the millimolar range at optimum pH (3.6). The determined IC_{50} values were 2.82 mM (**6a**) and 1.6 mM (**6b**). The IC_{50} values for the inhibition of the bacterial hyaluronidase at optimum pH (5.0) were 1.78 mM (**14 a**), 1.2 mM (**6b**) and 1.26 mM (**6c**). The inhibitory activity seems to be independent of monofluor, difluor or methy substitution of the parent compound.

Within the series of ring-substituted diphenylpropionic acids, compound **6d** (3-(4-trifluoromethylphenyl)-3-phenyl-propionic acid) was the most potent inhibitor of the bacterial enzyme at optimum (5.0) and physiological pH and of BTH at optimum pH (3.6) (cf. Table 2). The IC_{50} values were 600 µM (BTH at pH 3.6), 380 µM ($hylB_{4755}$ at pH 5.0) and 780 µM ($hylB_{4755}$ at pH 7.4). Interestingly, the hydrogenation of the C=C double bond (**5d** vs. **6d**) led to a consid-

erably more potent compound on both the bovine testicular and the bacterial enzyme. The inhibition curves of compound **6d** are depicted in Fig. 5.

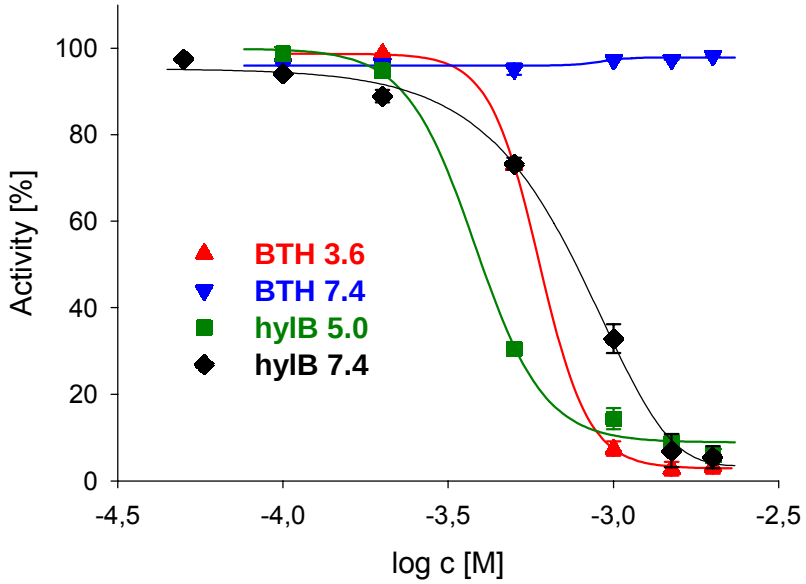
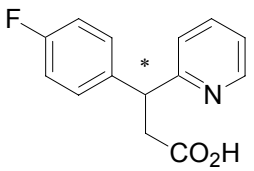


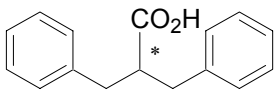
Fig. 5: Inhibitory effect of **14 d** on the activity of BTH and $hylB_{4755}$ at different pH values

In addition to the aforementioned diphenylalkanoic acids and diphenylalkenoic acids, 3-(4-fluorophenyl)-3-pyridin-2-yl-propionic acid (**15**) and two symmetric dibenzylacetic acids **14** and **15** were tested (cf. Table 3). The propionic acid **13** is a heterocyclic analogue of the synthesised derivative **6a**. The replacement of phenyl with pyridyl (compound **13**) led to complete loss of activity on BTH at pH 3.6 and pH 7.4 (cf. Table 3) and to a decrease in inhibitory activity on $hylB_{4755}$ at pH 5.0 and a complete loss of activity at the physiological pH (7.4).

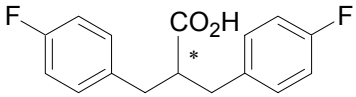
Table 3: Inhibition of **13-15** on the bovine testicular hyaluronidase and the hyaluronate lyase at optimum and physiological pH.



13



14



15

No	**BTH**		**hylB$_{4755}$**	
	IC$_{50}$ [µM] or (% Inhibition)			
	pH 3.6	pH 7.4	pH 5.0	pH 7.4
13	inactive[a]	inactive[a]	(66 %)[a]	inactive[a]
14	(79 %)[b]	inactive[c]	(82 %)[b]	inactive[c]
15	**1000**	inactive[c]	**1600**	inactive[c]

[a] at concentrations ≤ 20 mM
[b] % inhibition of the enzyme at an inhibitor concentration of 2 mM
[c] at concentrations ≤ 2 mM

2-Benzyl-3-phenylpropionic acid (**14**) and 2-fluorobenzyl-3-fluorophenylpropionic acid (**15**), structurally similar compounds to 3,3-diphenylpropionic acid (**7**) and 3,3-bis(4-fluorophenyl)propionic acid (**5b**), showed weak or no inhibition of the enzyme activity (cf. Table 3). Compound **14** at a concentration of 2 mM inhibited the bovine testicular hyaluronidase (pH 3.6) and the bacterial enzyme (pH 5.0) by about 80 %. This compound was inactive on both enzymes at physiological pH (7.4).

The obtained IC_{50} values of compound **15** were 1 mM for the bovine testicular hyaluronidase at pH 3.6 and 1.6 mM for the bacterial enzyme at pH 5.0. The inhibitory effect of compound **15** on BTH and $hylB_{4755}$ was comparable to those of the fluorinated diphenyl propionic acid **5b**.

4. Summary

A *de novo* design approach starting from the x-ray structures of bacterial hyaluronate lyase and molecular modelling investigation, led to the discovery of first lead structures with enzyme inhibitory activities in the millimolar range. The structural modification of these compounds resulted in new hyaluronidase inhibitors: the diphenylacrylic and diphenylpropionic acids. Within the series of the examined diphenylacrylic and diphenylpropionic acids, 3,3-bis-(4-bromophenyl)-acrylic acid (**5f**), 3-(4-bromophenyl)-3-phenylpropionic acid (**10**) and 3-(4-trifluoromethyl-phenyl)-3-phenylpropionic acid (**6d**) were the most potent inhibitors on the bovine testicular hyaluronidase and the bacterial enzyme (IC_{50} values (BTH/hylB$_{4755}$): 150 µM / 260 µM, 200 µM / 330 µM and 660 µM / 380 µM, respectively).

Since the inhibitory activities of the new compounds were only in the low millimolar range additional structural modifications are necessary. Such modification might be an introduction of e.g. alkyl chains, which increase the lipophilicity of the compounds, or inserting carbon chains between C-1 and C-3. Earlier examinations indicated that carboxylic acids and hydroxy groups are important for the solubility but also for better inhibitory activities, so that the introduction of a second carboxylic group or/and a hydroxy group might increase the potency. Nevertheless, additional molecular modelling studies with respect to the binding mode could be helpful for further investigations.

5. Experimental section

5.1 General conditions

For a detailed description of the general procedures, equipments and chemicals used in the chemistry part, see section 4.1 of chapter 5.

5.2 Chemistry

5.2.1 Synthesis of the diphenylacrylic acids 5a-f

General procedure for the preparation of the acrylic acids

A solution of potassium *tert*-butylate (2 eq), triethyl phosphonoacetate (1 eq) and the corresponding benzophenone (1 eq) in *tert*-butanol (100 ml) was stirred and heated under reflux for 24 h. After evaporation of the solvent the residue was treated with methanol (100 ml), water (50 ml) and sodium hydroxide (5 eq), stirred and heated under reflux for 24 h again. The solvent was removed under reduced pressure and the solid was diluted with water. After several extractions with chloroform the aqueous solution was acidified with concentrated hydrochloric acid and extracted again with chloroform (4 x 70 ml). The last organic layers were combined, dried over magnesium sulphate and the solvent was removed under reduced pressure.

(EZ)-3-(4-Fluorophenyl)-3-phenylacrylic acid (5a)

Reaction of 4-fluorobenzophenone (1.00 g, 5.00 mmol) with triethyl phosphonoacetate (1.12 g, 5.00 mmol); purification of the product by column chromatography on silica gel eluting with a 1:4 (v/v) mixture of ethyl acetate and petroleum ether 60-80 °C;
Yield: 1.99 g (8.22 mmol, 82 %, white solid)

Mp: 127-129 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.36 / 6.37 (s, 1H, =C<u>H</u>CO$_2$H), 7.13-7.23 (m, 4H, ph), 7.25-7.33 (m, 2H, ph), 7.35-7.41 (m, 3H, ph), 12.21 (s, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 114.6 / 114.9 (+, ph), 115.2 / 115.5 (+, ph), 118.8 / 119.0 (+, =<u>C</u>HCO$_2$H), 127.8 / 127.9 (+, ph), 128.5 / 128.8 (+, ph), 129.3 (+, ph), 129.9 / 130.1 (+, ph), 131.0 / 131.1 (+, ph), 134.9 / 135.0 (C$_{quart}$, ph), 136.8 /136.9 (C$_{quart}$, ph), 152.4 / 152.8 (C$_{quart}$, <u>C</u>=CHCO$_2$H), 161.8 (C$_{quart}$, $^{1}J_{C,F}$ = 244.7 Hz, =<u>C</u>F), 162.5 (C$_{quart}$, $^{1}J_{C,F}$ = 247.9 Hz, =<u>C</u>F), 166.6 / 166.6 (C$_{quart}$, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 242 ([M$^{\bullet+}$], 100), 241 ([M-H]$^+$, 77), 225 ([M-OH]$^+$, 17), 197 ([M-CO$_2$H]$^+$, 35), 196 ([M-CO$_2$H-H]$^+$, 67)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 2930 (C-H) aromatic, 1690 (C=O), 1599 (C=C), 1415 δ(C-H), 1212 (C-O), 1157 (C-F), 837, 775, 697, 617

Analysis:

calculated	C: 73.29	H: 4.58
found	C: 73.28	H: 4.23

C$_{15}$H$_{11}$FO$_2$ • 0.2 H$_2$O (245.83)

3,3-Bis(4-fluorophenyl)acrylic acid (5b)

Reaction of 4,4'-difluorobenzophenone (2.18 g, 10.00 mmol) and triethyl phosphonoacetate (2.24 g, 10.00 mmol); recrystallisation of the product from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane;
Yield: 1.48 g (5.69 mmol, 57 %, white crystalline solid)

Mp: 124-127 °C [Li.: 146 °C (Bergmann et al. 1948)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.26 (s, 1H, =C<u>H</u>CO$_2$H), 6.98-7.10 (m, 4H, ph), 7.13-7.20 (m, 2H, ph), 7.21-7.29 (m, 2H, ph), 7.33-8.36 (br, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 115.0 (+, =<u>C</u>HCO$_2$H), 115.3 (+, ph), 115.5 (+, ph), 115.7 (+, ph), 116.3 (+, ph), 130.4 (+, ph), 130.5 (+, ph), 131.2 (+, ph), 131.3 (+, ph), 134.0 (C_{quart}, $^{4}J_{C,F}$ = 3.7 Hz, C-1), 137.0 (C_{quart}, $^{4}J_{C,F}$ = 3.0 Hz, C-1´), 157.1 (C_{quart}, <u>C</u>=CHCO$_2$H), 163.0 (C_{quart}, $^{1}J_{C,F}$ = 248.3 Hz, C-F), 163.8 (C_{quart}, $^{1}J_{C,F}$ = 251.2 Hz, C-F), 170.7 (C_{quart}, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 260 ([M$^{\bullet+}$], 100), 259 ([M-H]$^+$, 36), 215 ([M-CO$_2$H]$^+$, 28), 214 ([M-CO$_2$H-H]$^+$, 47)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 2934 (C-H) aromatic, 1691 (C=O), 1598 (C=C), 1418 (C-H), 1209 (C-O), 1156 (C-F), 900, 837, 804, 683

Analysis:

calculated	C: 69.23	H: 3.87
found	C: 69.28	H: 4.17

$C_{15}H_{10}F_2O_2$ (260.23)

(EZ)-3-(4-Methylphenyl)-3-phenylacrylic acid (5c)

Reaction of 4-methylbenzophenone (1.96 g, 10.00 mmol) and triethyl phosphonoacetate (2.24 g, 10.00 mmol); recrystallisation of the product from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane;
Yield: 2.16 g (9.06 mmol, 91 %, white solid)

Mp: 120-123 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.30 / 2.34 (s, 3H, C<u>H</u>$_3$), 6.30 / 6.33 (s, 1H, =C<u>H</u>CO$_2$H), 7.00-7.07 (m, 1H, ph), 7.09-7.21 (m, 4H, ph), 7.22-7.30 (m, 1H, ph), 7.32-7.43 (m, 3H, ph), 12.12 (s, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 20.7 / 20.8 (+, C<u>H</u>$_3$), 117.7 / 118.5 (+, =<u>C</u>HCO$_2$H), 127.7 (+, ph), 127.8 (+, ph), 127.9 (+, ph), 128.8 (+, ph), 128.8 (+, ph), 128.9 (+, ph), 128.9 (+, ph), 129.0 (+, ph), 129.1 (+, ph), 135.8 (C_{quart}, ph), 137.2 / 137.6 (C_{quart}, ph), 138.9 / 140.8 (C_{quart}, ph), 153.7 / 153.8 (C_{quart}, <u>C</u>=CHCO$_2$H), 166.7 / 166.8 (C_{quart}, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 238 ([M$^{\bullet+}$], 100), 237 ([M-H]$^+$, 50), 221 ([M-OH]$^+$, 15), 193 ([M-CO$_2$H]$^+$, 20), 178 ([M-CO$_2$H-CH$_3$]$^+$, 34)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 2916 (C-H) aromatic, 1692 (C=O), 1594 (C=C), 1493 (C-H), 1351 (C-H), 1205 (C-O), 877, 822, 775, 731, 698,685

Analysis:

calculated	C: 80.65	H: 5.92
found	C: 80.15	H: 5.84

$C_{16}H_{14}O_2$ (238.29)

(EZ)-3-(4-Trifluoromethylphenyl)-3-phenylacrylic acid (5d)

Reaction of 4-trifluoromethylbenzophenone (2.50 g, 10.00 mmol) and triethyl phosphonoacetate (2.24 g, 10.00 mmol); recrystallisation of the product from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane;
Yield: 2.75 g (9.41 mmol, 94 %, white solid)

Mp: 153-155 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.48 (s, 1H, =C<u>H</u>CO$_2$H), 7.24-7.32 (m, 2H, ph), 7.34-7.43 (m, 5H, ph), 7.72-7.79 (m, 2H, ph), 12.32 (s, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 119.6 (+, C=<u>C</u>HCO$_2$H), 121.1 (+, ph), 124.2 (C$_{quart}$, $^{1}J_{C,F}$ = 272.1 Hz, <u>C</u>F$_3$), 124.7 (+, $^{3}J_{C,F}$ = 3.8 Hz, <u>C</u>CH(CF$_3$), 125.3 (+, ph), 127.7 (+, ph), 128.1 (C$_{quart}$, $^{2}J_{C,F}$ = 31.8 Hz, <u>C</u>(CF$_3$)), 128.6 (+, ph), 128.9 (+, ph), 129.5 (+, ph), 129.7 (+, ph), 139.4 (C$_{quart}$, ph), 143.3 (C$_{quart}$, ph), 152.5 (C$_{quart}$, <u>C</u>=CHCO$_2$H), 166.3 (C$_{quart}$, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 292 ([M$^{\bullet+}$], 100), 291 ([M-H]$^+$, 81), 275 ([M-OH]$^+$, 15), 247 ([M-CO$_2$H]$^+$, 23), 178 ([M-CO$_2$H-CF$_3$]$^+$, 27)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 2981 (C-H) aromatic, 1681 (C=O), 1624 (C=C), 1408 (C-H), 1330 (C-F), 1206 (C-O), 1117, 1067, 835, 774, 694, 641

Analysis:

calculated	C: 65.76	H: 3.79
found	C: 65.96	H: 4.10

C$_{16}$H$_{11}$F$_3$O$_2$ (292.26)

(EZ)-3-(4-Chlorophenyl)-3-phenylacrylic acid (5e)

Reaction of 4-chlorobenzophenone (3.40 g, 10.00 mmol) and triethyl phosphonoacetate (2.24 g, 10.00 mmol); recrystallisation of the product from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane;
Yield: 2.25 g (8.70 mmol, 87 %, white solid)

Mp: 139-140 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.39 (s, 1H, =C<u>H</u>CO$_2$H), 7.12-7.20 (m, 2H, ph), 7.24-7.31 (m, 2H, ph), 7.33-7.42 (m, 3H, ph), 7.42-7.48 (m, 2H, ph), 12.27 (s, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 119.2 / 119.4 (+, =<u>C</u>HCO$_2$H), 127.8 (+, ph), 127.9 (+, ph), 127.9 (+, ph), 128.5 (+, ph), 128.5 (+, ph), 128.8 (+, ph), 129.3 (+, ph), 129.6 (+, ph), 130.8 (+, ph), 132.5 / 133.9 (C_{quart}, ph), 137.6 / 138.3 (C_{quart}, ph), 139.3 / 139.9 (C_{quart}, ph), 152.0 / 152.6 (C_{quart}, <u>C</u>=CHCO$_2$H), 166.4 / 166.5 (C_{quart}, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 258 ([M$^{\bullet+}$], 100), 257 ([M-H]$^+$, 56), 241 ([M-OH]$^+$, 15)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3006 (C-H) aromatic, 1690 (C=O), 1610 (C=C), 1408 (C-H), 1212 (C-O), 1085 (C-Cl), 833, 768, 723, 696, 612

Analysis:

calculated	C: 69.64	H: 4.29
found	C: 69.38	H: 4.14

$C_{21}H_{16}O_4$ (332.35)

3,3-Bis(4-bromophenyl)acrylic acid (5f)

Reaction of 4,4'-dibromobenzophenone (3.40 g, 10.00 mmol) and triethyl phosphonoacetate (2.24 g, 10.00 mmol); recrystallisation of the product from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane;
Yield: 3.00 g (7.85 mmol, 79 %, white solid)

Mp: 170-171 °C [Lit.: 190 - 191 °C (Bergmann et al. 1948)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 6.43 (s, 1H, =C<u>H</u>CO$_2$H), 7.09-7.13 (m, 2H, phenyl), 7.19-7.24 (m, 2H, ph), 7.55-7.61 (m, 4H, ph), 12.34 (s, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 119.7 (+, =<u>C</u>HCO$_2$H), 121.4 (C$_{quart}$, ph), 122.8 (C$_{quart}$, ph), 129.8 (+, ph), 130.9 (+, ph), 131.0 (+, ph), 131.5 (+, ph), 137.5 (C$_{quart}$, ph), 139.0 (C$_{quart}$, ph), 151.3 (C$_{quart}$, <u>C</u>=CHCO$_2$H), 166.3 (C$_{quart}$, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 381 ([M$^{\bullet+}$], 100), 364 ([M-OH]$^+$, 8), 303 ([M-Br]$^+$, 3), 256 ([M-Br-CO$_2$H]$^+$, 20)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 2921 (C-H) aromatic, 1692 (C=O), 1610 (C=C), 1581 (C=C), 1413 (C-H), 1210 (C-O), 1073 (C-Br), 1010, 827, 765, 714

Analysis:

calculated	C: 47.15	H: 2.64
found	C: 46.94	H: 2.60

$C_{15}H_{10}Br_2O_2$ (382,07)

6.2.2 Synthesis of the diphenylpropionic acids 6a-d

(RS)-3-(4-Fluorophenyl)-3-phenylpropionic acid (6a)

3-(4-Fluorophenyl)-3-phenylacrylic acid (1.0 g, 4.13 mmol) was dissolved in methanol (40 ml), treated with a catalytic amount (75 mg) of palladium on charcoal (10 % Pd/C) and stirred at room temperature under a hydrogen atmosphere (5 bar) for 24 h. Afterwards, the reaction mixture was filtrated and the solvent was removed under reduced pressure. The product was recrystallised from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane.
Yield: 0.9 g (3.68 mmol, 89 %, white crystalline solid)
Mp: 92-94 °C

^{1}H-NMR ($CDCl_3$):

δ [ppm] = 3.02 (dd, 1H, ^{3}J = 8.1 Hz, ^{2}J = 16.0 Hz, -CHC$\underline{H}_2$-), 3.08 (dd, 1H, ^{3}J = 7.7 Hz, ^{2}J = 16.0 Hz, -C$\underline{H}_2$-), 4.50 (dd, 1H, ^{3}J = 7.7 Hz, ^{3}J = 8.1 Hz, -C$\underline{H}CH_2$), 6.91-7.00 (m, 2H, ph), 7.14-7.30 (m, 7H, ph), 7.52 (br, 1H, COO$\underline{H}$)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 40.5 (-, -CH$\underline{C}H_2$-), 45.9 (+, -$\underline{C}HCH_2$-), 115.3 (+, ph), 115.6 (+, ph), 126.8 (+, ph), 127.5 (+, ph), 128.7 (+, ph), 129.1 (+, ph), 129.2 (+, ph), 138.9 (C_{quart}, $^{4}J_{C,F}$ = 3.7 Hz, C-1), 143.0 (C_{quart}, ph), 161.6 (C_{quart}, $^{1}J_{C,F}$= 245.1 Hz, C-4), 177.4 (C_{quart}, $\underline{C}$=O)

MS (PI-EIMS (70 eV)): m/z (%) = 244 ([$M^{\bullet+}$], 29), 185 ([M-CH_2CO_2H]$^{+}$, 100)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3033 (C-H) aromatic, 2910 (C-H) aliphatic, 1702 (C=O), 1413 (C-H), 1307 (C-H), 1210 (C-O), 1162 (C-F), 914, 837, 799, 745, 715, 698

Analysis:

calculated	C: 73.76	H: 5,36
found	C: 73.68	H: 5.35

$C_{15}H_{13}FO_2$ (244.27)

3,3-Bis(4-fluorophenyl)propionic acid (6b)

3,3-Bis(4-fluorophenyl)acrylic acid (1.59 g, 6.11 mmol) was dissolved in ethanol (40 ml), treated with a catalytic amount (100 mg) of Lindlar catalyst (5 % Pd/$CaCO_3$) and stirred at room temperature under a hydrogen atmosphere for 24 h. After filtration, the solvent was removed under reduced pressure and the product was purified first by column chromatography on silica gel eluting with a 4:1 (v/v) mixture containing petroleum ether 60-80 °C and chloroform and then by recrystallisation from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane.

Yield: 1.46 g (5.57 mmol, 92 %, white crystalline solid)

Mp: 103-105°C [Lit.: 107 - 108 °C (Bergmann et al. 1948)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.02 (d, 2H, ^{3}J = 8.0 Hz, CHC<u>H</u>$_2$CO$_2$H), 4.46 Hz (t, 1H, ^{3}J = 8.0 Hz, C<u>H</u>CH$_2$CO$_2$H), 7.05-7.15 (m, 4H, ph), 7.32-7.41 (m, 4H, ph), 12.17 (s, 1H, CO$_2$<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 39.7 (-,CH<u>C</u>H$_2$CO$_2$H), 45.0 (+, <u>C</u>HCH$_2$CO$_2$H), 114.9 (+, ph), 115.1 (+, ph), 129.2 (+, ph), 129.3 (+, ph), 140.2 (C$_{quart}$, ph), 140.2 (C$_{quart}$, ph), 160.6 (C$_{quart}$, $^{1}J_{C,F}$ = 242.5 Hz, C-F), 172.5 (C$_{quart}$, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 262 ([M$^{\bullet+}$], 20), 203 ([M-CO$_2$H]$^+$, 100), 201 ([M-2H]$^+$, 125), 183 ([M-H-F]$^+$, 33)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3064 (C-H) aromatic, 2907 (C-H) aliphatic, 1700 (C=O), 1410 (C-H), 1266-1214 (C-O), 1159, 1104, 828, 791, 756, 717, 671

Analysis:

calculated	C: 66.41	H: 4.64
found	C: 66.54	H: 4.65

$C_{15}H_{12}F_2O_2$ (262.25)

(RS)-3-(4-Methylphenyl)-3-phenylpropionic acid (6c)

3-(4-Methylphenyl)-3-phenylacrylic acid (0.50 g, 2.08 mmol) in ethanol (40 ml) was treated with a catalytic amount (50 mg) of Lindlar catalyst (5 % Pd/CaCO$_3$) and stirred at room temperature under a hydrogen atmosphere for 24 h. Afterwards, the reaction mixture was filtrated, the solvent was removed under reduced pressure and the pure product was dried in vacuo.

Yield: 0.48 g (2.00 mmol, 96 %, white solid)

Mp: 123-125 °C

^{1}H-NMR ([D$_6$]DMSO):

[ppm] = 2.22 (s, 3H, C$\underline{H}_3$), 2.98 (d, 2H, J = 7.96 Hz, -CHC$\underline{H}_2$-), 4.37 (t, 1H, J = 7.96 Hz, -C$\underline{H}$CH$_2$-), 7.03-7.10 (m, 2H, ph), 7.11-7.32 (m, 7H, ph), 12.12 (br, 1H, COO$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

[ppm] = 20.4 (+, $\underline{C}H_3$), 40.2 (-, -CH$\underline{C}H_2$-), 46.2 (+, -$\underline{C}$HCH$_2$-), 126.0 (+, ph), 127.3 (+, ph), 127.3 (+, ph), 128.2 (+, ph), 128.8 (+, ph), 135.1 (C_{quart}, ph), 141.1 (C_{quart}, ph), 144.3 (C_{quart}, ph), 172.6 (C_{quart}, $\underline{C}$=O)

MS (PI-EIMS (70 eV)): m/z (%) = 240 ([M$^{\bullet+}$], 34), 181 ([M-CH$_2$CO$_2$H]$^+$, 100), 166 ([M-CH$_2$CO$_2$H-CH$_3$]$^+$, 16), 165 ([M-CH$_2$CO$_2$H-CH$_3$-H]$^+$, 20)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3026 (C-H) aromatic, 2913 (C-H) aliphatic, 2622 (C-H) aliphatic, 1695 (C=O), 1417 δ(C-H), 1223 (C-O), 944, 821, 741, 698, 612

Analysis:

calculated	C: 79.97	H: 6.71
found	C: 79.95	H: 6.91

$C_{16}H_{16}O_2$ (240.31)

(RS)-(-3-(4-Trifluoromethylphenyl)-3-phenyl-propionic acid (6d)

3-(4-Trifluoromethylphenyl)-3-phenylacrylic acid (0.25 g, 0.86 mmol) in ethanol (20 ml) was treated with a catalytic amount (50 mg) of Lindlar catalyst (5 % Pd/$CaCO_3$) and stirred at room temperature under a hydrogen atmosphere for 24 h. Afterwards, the reaction mixture was filtrated, the solvent was removed under reduced pressure and the pure product was dried in vacuo.

Yield: 0.24 g (0.82 mmol, 95 %, white solid)

Mp: 79-81 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 3.06 (m, 1H, CHC<u>H</u>$_2$), 3.13 (m, 1H, CHC<u>H</u>$_2$), 4.54 (m, 1H, C<u>H</u>CH$_2$), 7.14-7.24 (m, 1H, ph), 7.24-7.39 (m, 4H, ph), 7.53-7.68 (m, 4H, ph), 12.23 (br, 1H, COO<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 40.2 (-, CH<u>C</u>H$_2$), 46.3 (+, <u>C</u>HCH$_2$), 124.2 (C$_{quart}$, $^{1}J_{C,F}$ = 272.0 Hz, <u>C</u>F$_3$), 125.2 (+, $^{4}J_{C,F}$ = 3.7 Hz, <u>C</u>=C(CF$_3$), 126.4 (+, ph), 126.9 (C$_{quart}$, $^{3}J_{C,F}$ = 31.7 Hz, <u>C</u>(CF$_3$)), 127.5 (+, ph), 128.3 (+, ph), 128.5 (+, ph), 143.2 (C$_{quart}$, ph), 148.9 (C$_{quart}$, ph), 172.4 (C$_{quart}$, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 294 ([M$^{\bullet+}$], 33), 275 ([M-F]$^+$, 5), 235 ([M-CH$_2$CO$_2$H]$^+$, 100)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3028 (C-H) aromatic, 2873 (C-H) aliphatic, 2595 (C-H) aliphatic, 1707 (C=O), 1421 (C-H), 1326 (C-F), 1208 (C-O), 1017, 959, 865, 741, 699, 615

Analysis:

calculated	C: 65.30	H: 4.45
found	C: 65.59	H: 4.78

C$_{16}$H$_{13}$F$_3$O$_2$ (294.28)

2-Benzyl-3-phenylpropionic acid (14)

Crude 2-benzyl-3-phenylpropionic acid was kindly provided by Dr. Fabien Leurquin. The compound was purified by recrystallisation from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane.

Mp: 68-70 °C

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.73-2.90 (m, 5H, C$\underline{H}_2$C$\underline{H}$C$\underline{H}_2$), 7.16-7.21 (m, 6H, ph), 7.25-7.31 (m, 4H, ph), 12.12 (s, 1H, COO$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 37.6 (-, $\underline{C}$H$_2$CH$\underline{C}$H$_2$), 48.6 (+, CH$_2\underline{C}$HCH$_2$), 126.1 (+, ph), 128.1 (+, ph), 128.7 (+, ph), 139.2 (C$_{quart}$, ph), 175.4 (C$_{quart}$, $\underline{C}$=O)

MS (PI-EIMS (70 eV)): m/z (%) = 240 ([M$^{\bullet+}$], 50), 149 ([M-C$_7$H$_7$]$^+$, 79), 131 ([M-C$_7$H$_7$-H$_2$O]$^+$, 44), 91 ([C$_7$H$_7$]$^+$, 100)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3020 (C-H) aromatic, 2962 (C-H) aliphatic, 1700 (C=O), 1420 (C-H), 1259 (C-O), 1017, 797, 745, 694

Analysis:

calculated	C: 79.97	H: 6.71
found	C: 79.88	H: 7.09

C$_{16}$H$_{16}$O$_2$ (240.30)

2-(4-Fluorobenzyl)-3-(4-fluorophenyl)propionic acid (15)

Crude 2-(4-fluorobenzyl)-3-(4-fluorophenyl)propionic acid was kindly provided by Dr. Fabien Leurquin. The compound was purified by recrystallisation from a 2:1 (v/v) mixture of petroleum ether 60-80 °C and dichloromethane.

Mp: 92-94 °C

^{1}H-NMR (CDCl$_3$):

δ [ppm] = 2.67-2.84 (m, 2H, CHC$\underline{H}_2$), 2.85-2.92 (m, 2H, CHC$\underline{H}_2$), 2.93-3.03 (m, 1H, C$\underline{H}$CH$_2$), 6.91-7.07 (m, 4H, ph), 7.06-7.12 (m, 4H, ph), 7.36 (br, 1H, COO$\underline{H}$)

^{13}C-NMR ($CDCl_3$):

δ [ppm] = 36.9 (-, CH<u>C</u>H_2), 50.00 (+, <u>C</u>HCH_2), 115.2 (+, ph), 115.5 (+, ph), 130.3 (+, ph), 130.4 (+, ph), 134.1 (C_{quart}, ph), 134.2 (C_{quart}, ph), 161.7 (C_{quart}, $^1J_{C,F}$ = 244.7 Hz, C-F), 180.3 (C_{quart}, <u>C</u>=O)

MS (PI-EIMS (70 eV)): m/z (%) = 276 ([$M^{\bullet+}$], 27), 109 ([$FC_6H_4CH]^+$, 100)

IR [cm^{-1}]: 3100 (O-H) carboxylic acid, 3015 (C-H) aromatic, 2900 (C-H) aliphatic, 1713 (C=O), 1506 (C=C), 1454 (C-H), 1210 (C-O), 1175 (C-F), 834, 734

Analysis:

calculated	C: 69.56	H: 5.11
found	C: 69.52	H: 5.28

$C_{16}H_{14}F_2O_2$ (276.28)

5.3 Pharmacological methods

The inhibitory effect of the diphenyl derivatives on the activities of hyaluronidases were determined by a turbidimetric assay according to the method *of Di Ferrante* (Di Ferrante 1956) described in chapter 3.

7. References

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Bergmann, F., M. Weizmann, E. Dimant, J. Patai, J. Szmuszkowicz (1948). b,b-Diarylacrylic acids. I. Synthesis and properties of symmetrical and unsymmetrical b,b-diarylacrylic acids. *J Am Chem Soc* **70**: 1612-17.

Di Ferrante, N. (1956). Turbidimetric measurement of acid mucopolysaccharides and hyaluronidase activity. *J Biol Chem* **220**: 303-6.

Li, S., M. J. Jedrzejas (2001). Hyaluronan binding and degradation by Streptococcus agalactiae hyaluronate lyase. *J Biol Chem* **276**: 41407-16.

Li, S., S. J. Kelly, E. Lamani, M. Ferraroni, M. J. Jedrzejas (2000). Structural basis of hyaluronan degradation by Streptococcus pneumoniae hyaluronate lyase. *Embo J* **19**: 1228-40.

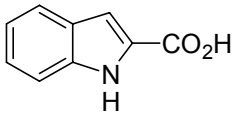
Chapter 9

Indole derivatives as hyaluronidase inhibitors synthesis, pharmacology and binding mode elucidation by X-ray analysis of an enzyme-inhibitor complex

1. Introduction

Starting from crystal structures of two homologous bacterial lyases (*Streptococcus pneumoniae* (Ponnuraj et al. 2000) and *S. agalactiae 3502* $hylB_{3502}$ (Li et al. 2001); sequence identity: 70 %), a structure-based strategy for designing inhibitors of bacterial hyaluronan lyases (*S. agalactiae*, $hylB_{4755}$) was followed. From the results of LUDI calculations (Accelrys Inc. (Böhm 1992)), using the LeadQuest® databases Vol. 1&2 (Tripos 2000), promising compounds were selected and pharmacological investigations were carried out (cf. chapter 7)**.**

One of the compounds suggested by LUDI was indole-2-carboxylic acid (**1**) (cf. Fig. 1). A LUDI score of 328 was calculated for compound **1** corresponding to a K_i value of about 1 mM.



1

Fig. 1: Structure of indole-2-carboxylic acid

The measured IC_{50} values of the indole derivative **1** were 7.1 mM on the bovine testicular enzyme at optimum pH (3.6) and 3.55 mM on the bacterial hyaluronidase at optimum pH (5.0). The concentration-dependent effect of indole-2-carboxylic acid on BTH and on $hylB_{4755}$ are depicted in Fig. 2. The IC_{50} value of compound **1** determined on the hyaluronate lyase is in agreement with the predicted value (LUDI score).

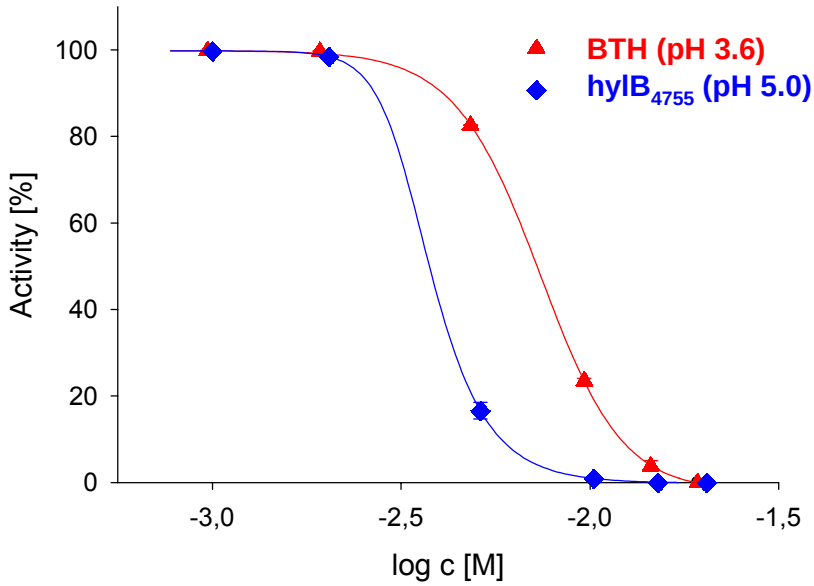


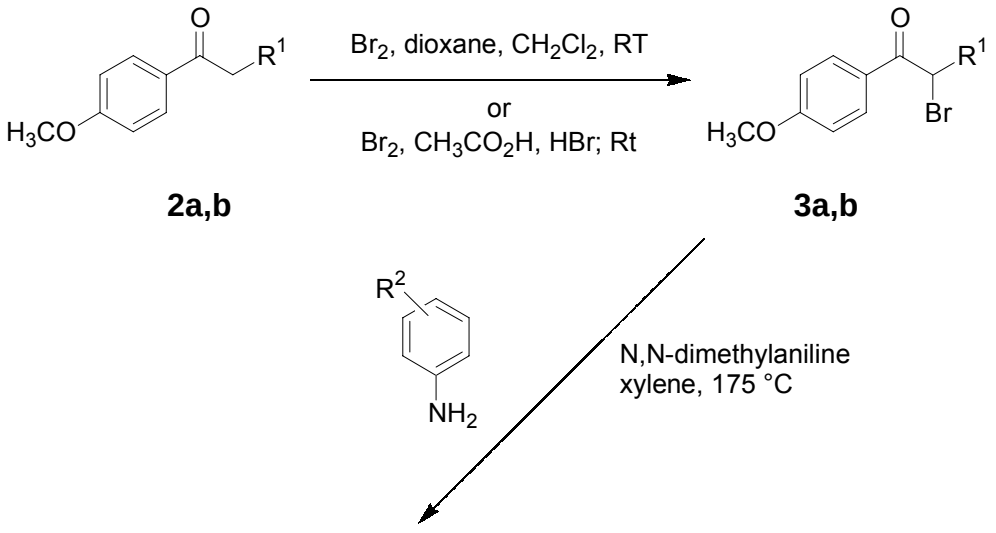
Fig. 2: Inhibitory effect of indole-2-carboxylic acid (**1**) on the bovine testicular hyaluronidase and on the hyaluronate lyase from S. *agalactiae* at optimum pH

Indole-2-carboxylic acid was a first lead with millimolar enzyme inhibitory activity predicted by a *de novo* design approach and molecular modelling investigations. Starting from these results structurally related indole derivatives were investigated.

2. Chemistry

All 2-phenylindole derivatives were synthesised according to the method of *Bischler* and *Brown* (Bischler 1892; Brown 1972). Hydroxy substituted compounds were expected to have higher inhibitory potential. Because of their high reactivity, these compounds were liberated by cleavage of corresponding

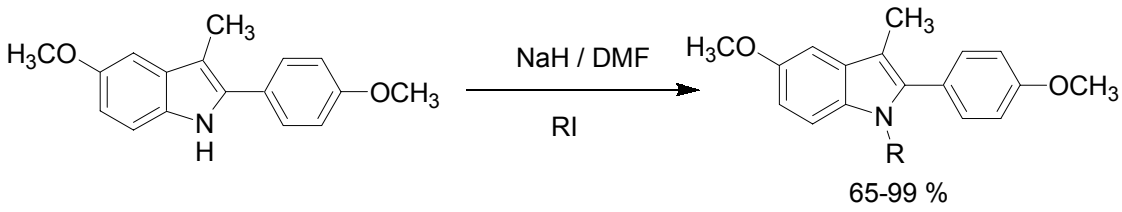
methyl ethers at the end of the synthetic route. Starting materials for the indole synthesis were 4'-methoxypropiophenone (**2a**) or 4'-methoxyacetophenone (**2b**). Both compounds were converted into the corresponding bromo compounds **3a** and **3b** by direct bromination. Acid catalysis prevented a multiple bromination at the aliphatic carbon atom and bromination at the phenyl ring (Rival et al. 1992). The final ring closure was achieved at high temperature (175 °C) with the corresponding ring-substituted aniline in presence of *N,N*-dimethylaniline (cf. Scheme 1). The use of a two-fold excess of aniline increased the yields up to 34 % - 66 %. The mechanism was described by *Bischler* and *Borsche* (Bischler 1893; Borsche et al. 1908) as a multi-step reaction. In the first step, the brominated phenone reacts with one aniline molecule via a nucleophilic substitution to an α-aminoketone. This α-aminoketone is converted to a Schiff's base after nucleophilic addition of a second aniline molecule to the carbonyl group and subsequent condensation reaction. The final cyclisation of the endiamine takes place under displacement of the "first" aniline molecule after 1,3-H-shift to give the corresponding 2-phenylindole.



No	R^1	R^2
a	H	5-OCH_3
b	CH_3	5-OCH_3
c	CH_3	6-OCH_3

Scheme 1: Synthesis of the phenylindole derivatives **4a-c**

The N-substitution of the indoles were carried out according to standard procedures. The 2-phenylindoles **5a-d** were prepared by treating the 2-phenylindole **4b** with sodium hydride in dimethylformamide and the corresponding alkyl halogen via a nucleophilic substitution (cf. Scheme 2).

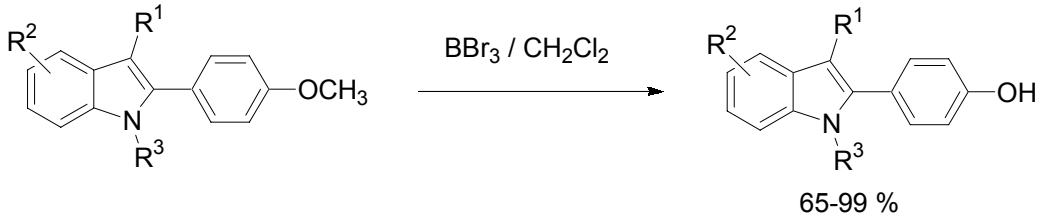


4b **5a-d**

No	5a	5b	5c	5d
R	CH_3	C_3H_7	C_5H_{11}	C_7H_{15}

Scheme 2: Alkylation of the 2-phenylindole derivative **4b**

The cleavage of the methyl ether was carried out with BBr_3 in anhydrous dichloromethane. Boron tribromide as a Lewis acid forms a complex with the ether oxygen, which is hydrolysed to the desired hydroxy compound, boronic acid and two molecules of hydrogen bromide (McOmie et al. 1968) (Scheme 3).



4a-c, 5a-d **6a-g**

No	R^1	R^2	R^3
6a	H	5-OH	H
6b	CH_3	6-OH	H
6c	CH_3	5-OH	H
6d	CH_3	5-OH	CH_3
6e	CH_3	5-OH	C_3H_7
6f	CH_3	5-OH	C_5H_{11}
6g	CH_3	5-OH	C_7H_{15}

Scheme 3: Cleavage of the methyl ether with boron tribromide

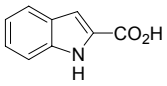
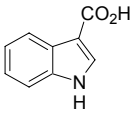
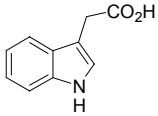
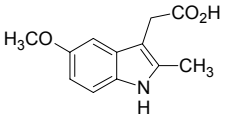
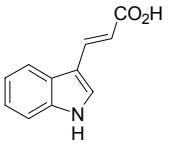
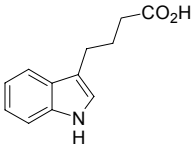
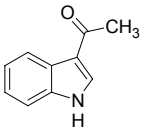
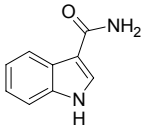
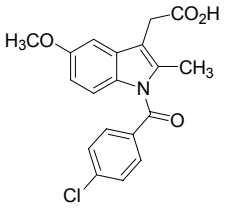
3. Pharmacological investigations

The widely used method for the determination of an inhibitory activity on hyaluronidases is the Morgan-Elson assay. This colorimetric assay is based on the reaction of *N*-acetyl-D-glucosamine at the reducing ends with *N,N*-dimethylaminobenzaldehyde (Ehrlich reagent) to give a red coloured product (Hynes et al. 1994; Muckenschnabel et al. 1998). This method could not be used for the examination of the indole derivatives because the Ehrlich's reagent is well known to react with indole rings (cf. for example Van-Urk reaction (Dibbern et al. 1963)). *N,N*-Dimethylaminobenzaldehyde reacts with the indole moiety either in position 2 or in position 3 to the corresponding alcohol as intermediate. After elimination of water under acidic conditions a coloured product (cyanine) is formed. This product is also detectable at 590 nm like the coloured product of the Morgan-Elson reaction. As the colorimetric assay could not be applied, all indole derivatives were investigated for hyaluronidase inhibition with a turbidimetric assay according to the method of *Di Ferrante* described in chapter 3.

Starting from the results of compound **1**, the structurally related compounds **1**, **7-15** were investigated. The inhibitory effect of these compounds are summarised in Table 1.

The inhibitory effect of unsubstituted indole (**7**) on the bovine testicular and on the bacterial hyaluronidase was lower than the inhibition by indole-2-carboxylic (**1**) acid. The IC_{50} values of compound **7** determined on BTH and $hylB_{4755}$ were in the millimolar range (cf. Table 1). The shift of the carboxylic acid from position 2 to position 3 (cf. compound **8**) led to a complete loss of activity for the bovine testicular enzyme. The IC_{50} value of compound **8** determined on the bacterial hyaluronidase at optimum pH (5.0) was comparable to that of compound **1**, namely 3 mM (cf. Table 1). Obviously, the presence and the position of the carboxylic group is not crucial for the inhibition of the hyaluronate lyase, since the inhibitory effects of all three compounds were similar. By contrast, concerning inhibition of the bovine testicular hyaluronidase, the shift of the carboxylic acid from position 2 to 3 led to a complete loss of activity, whereas the lack of the carboxylic group resulted only in about two-fold decrease in activity.

Table 1: Inhibitory activities of the indole derivatives **1** and **7-15** determined on the bovine testicular hyaluronidase (BTH) and the bacterial enzyme ($hylB_{4755}$)

No	Structure	BTH IC$_{50}$ [µM] or (% Inhibition)		$hylB_{4755}$ IC$_{50}$ [µM] or (% Inhibition)	
		pH 3.6	pH 7.4	pH 5.0	pH 7.4
1		**7000**	inactive [a]	**4000**	inactive [a]
7		**14100**	**22400**	**7100**	**5600**
8		inactive [a]	inactive [a]	**3000**	inactive [a]
9		inactive [a]	inactive [a]	**4000**	inactive [a]
10		inactive [b]	inactive [b]	inactive [b]	inactive [b]
11		inactive [a]	inactive [a]	**3500**	(76 %) [c]
12		inactive [a]	inactive [a]	**2200**	(28 %) [c]
13		inactive [a]	inactive [a]	**24000**	**15800**
14		inactive [a]	inactive [a]	(45 %) [c]	(30 %) [c]
15		**540**	**5490**	**350**	**1140**

[a] at concentrations ≤ 20 mM; [b] at concentrations ≤ 2 mM; [c] % inhibition of the enzyme at an inhibitor concentration of 2 mM;

The introduction of an additional methylene group between the carboxylic acid as in compound **9** did not lead to significant differences in inhibitory activity compared to the lower homologue **8**. Additional methyl and methoxy substitution in the positions 2 and 5 (cf. compound **10**) led to a complete loss of activity. The compounds **11** and **12** were inactive on the bovine testicular enzyme at optimum and physiological pH, whereas the IC_{50} values on the bacterial hyaluronidase at optimum pH (5.0) were similar to that of compound **1**: 3.5 mM (**11**) and 2.2 mM (**12**) (cf. Table 1), i.e. homologisation of the side chain to 3 or 4 carbon atoms (cf. compounds **11** and **12**) did not lead to increase of the inhibitory activity.

3-Acetylindole (**13**) induced a concentration-dependent inhibition of the hyaluronate lyase. The calculated IC_{50} values were 24 mM at pH 5.0 and 15.8 mM at pH 7.4 (cf. Table 1). Whereas 3-acetylindole showed a complete inhibition of the bacterial hyaluronidase, indole-3-carboxamide (**14**) induced only a weak inhibition of $hylB_{4755}$ by 45 % at pH 5.0 and 30 % at pH 7.4 at a concentration of 2 mM. Both indole derivatives were inactive on the bovine testicular hyaluronidase at both pH values.

The *N*-benzoyl derivative of compound **10**, indomethacin (**15**) proved to be the most potent inhibitor of this series. The IC_{50} values of compound **15** on the bovine testicular enzyme were 540 µM at optimum pH (3.6) and 5.49 mM at physiological pH. The IC_{50} values for the inhibition of the bacterial hyaluronidase were 350 µM at optimum pH (5.0) and 1.14 mM at physiological pH (7.4) (cf. Table 1). The inhibition curves of indomethacin are depicted in Fig. 3.

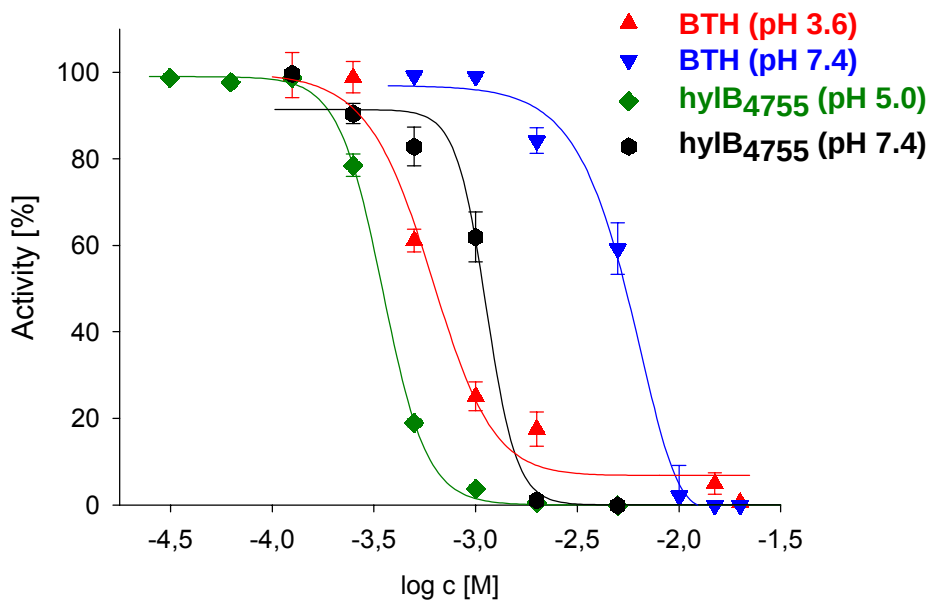


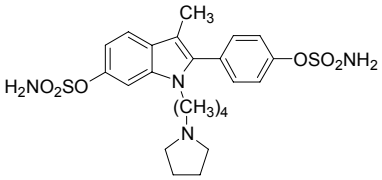
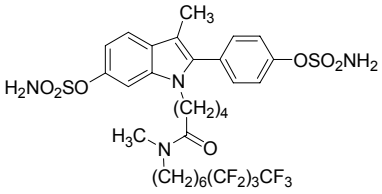
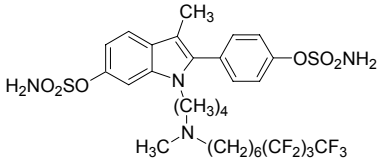
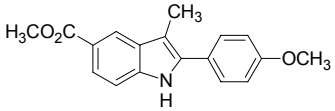
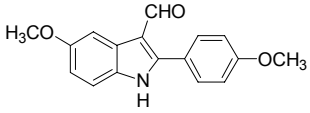
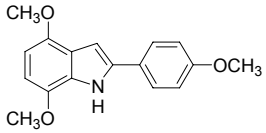
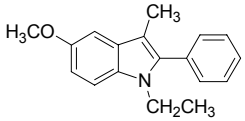
Fig. 3: Inhibitory effects of indomethacin (**15**) on BTH and hylB$_{4755}$ at optimum pH (3.6 and 5.0, respectively) and physiological pH (7.4).

Compared to the structure suggested by LUDI calculations, compound **1**, the analagues with lipophilic substituents like phenyl and methyl were 13- and 11-fold more potent inhibitors of the bovine testicular hyaluronidase and the hyaluronate lyase, respectively.

In parallel to the compounds **1** and **7-15**, the inhibitory effects of the 2-phenylindole derivatives **16-27**[1] and **4b** on the bovine testicular and the bacterial hyaluronidases were investigated. The tested 2-phenylindole derivatives **16-27** and **4b** possess various functional groups like hydroxy, methoxy, methyl and sulphamoyloxy groups in positions 3, 4', 5 and 6 and diverse *N*-substituents. The structures of the examined phenylindole derivatives and their inhibitory effects on BTH and hylB$_{4755}$ are summarised in Table 2 and Table 3.

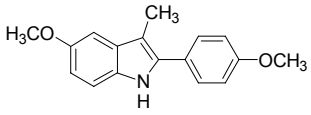
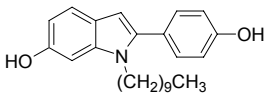
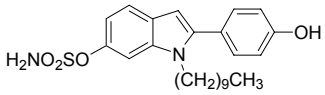
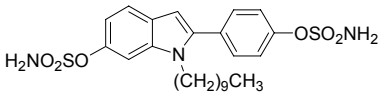
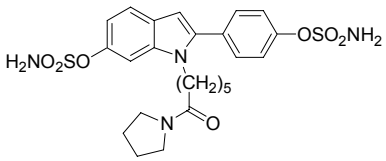
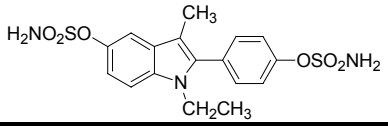
[1] The compounds 16-25 were kindly provided from Prof. Dr. E. von Angerer, Institute of Pharmacy, University of Regensburg, Germany

Table 2: Inhibitory effects of 2-phenylindole derivatives **16-22** on BTH and $hylB_{4755}$ at optimum and physiological pH.

No	Structure	BTH % Inhibition at Concentration [mM][a]		$hylB_{4755}$ % Inhibition at Concentration [mM][a]	
		pH 3.6	pH 7.4	pH 5.0	pH 7.4
16		inactive (≤ 1)	inactive (≤ 1)	inactive (≤ 1)	inactive (≤ 1)
17		inactive (≤ 1)	inactive (≤ 1)	inactive (≤ 1)	inactive (≤ 1)
18		inactive (≤ 2)	12 % (2)	inactive (≤ 2)	19 % (2)
19		inactive (≤ 2)	inactive (≤ 2)	inactive (≤ 2)	inactive (≤ 2)
20		inactive (≤ 0.2)	inactive (≤ 0.2)	inactive (≤ 0.2)	30 % (0.2)
21		inactive (≤ 0.2)	inactive (≤ 0.2)	inactive (≤ 0.2)	inactive (≤ 0.2)
22		inactive (≤ 0.1)	inactive (≤ 0.1)	inactive (≤ 0.1)	inactive (≤ 0.1)

[a] concentration [mM] given in paranthese

Table 3: Effects of the 2-phenylindole derivatives **4b** and **23-27** on the bovine testicular hyaluronidase and *S. agalactiae* hyaluronate lyase

No	Structure	BTH IC_{50} [µM]		$hylB_{4755}$ IC_{50} [µM]	
		pH 3.6	pH 7.4	pH 5.0	pH 7.4
4b		**1590**	**2840**	**880**	**93**
23		inactive[a]	inactive[a]	**13**	**17**
24		inactive[b]	inactive[b]	**8**	**13**
25		inactive[a]	inactive[a]	**11**	**16**
26		**1000**	inactive[c]	(77 %)[d]	**12600**
27		(45 %)[d]	**18000**	(47 %)[d]	**4000**

[a] at concentrations ≤ 3.6 m; [b] at concentrations ≤ 1.8 mM; [c] at concentrations ≤ 2 mM;
[d] % inhibition of the enzyme at an inhibitor concentration of 2 mM

Within this series the phenylindoles **16**, **17**, **19**, **21** and **22** were inactive on the bovine testicular hyaluronidase and the hyaluronate lyase at optimum pH and at physiological pH. The phenylindole **18** with a methyl group in position 3, a sulphamoyloxy group in position 4' and 6 and a 15-member side chain at the nitrogen atom induced a weak inhibition of BTH and $hylB_{4755}$ by about 10-20 % at a concentration of 1.8 mM at pH 7.4. This compound was inactive on both hyaluronidases at pH 3.6 or 5.0, respectively. Compound **20**, the 4',5-pheny-

lindole-3-carbaldehyde, induced only an inhibition of $hylB_{4755}$ at physiological pH by 30 % at a concentration of 200 µM. Due to poor solubility the tested compounds could not be investigated at higher concentration than denoted.

Among the investigated 2-phenylindole derivatives, the compounds **4b** and **23-25** proved to be the most interesting compounds. The inhibitory effects on BTH and $hylB_{4755}$ are summarised in Table 3.

The dimethoxy indole derivative **4b** was active on both hyaluronidases at millimolar concentration (IC_{50} values: at BTH 1.59 mM (pH 3.6) / 2.84 mM (pH 7.4), at $hylB_{4755}$ 0.88 mM (pH 5.0) / 93 µM (pH 7.4)) (Table 3).

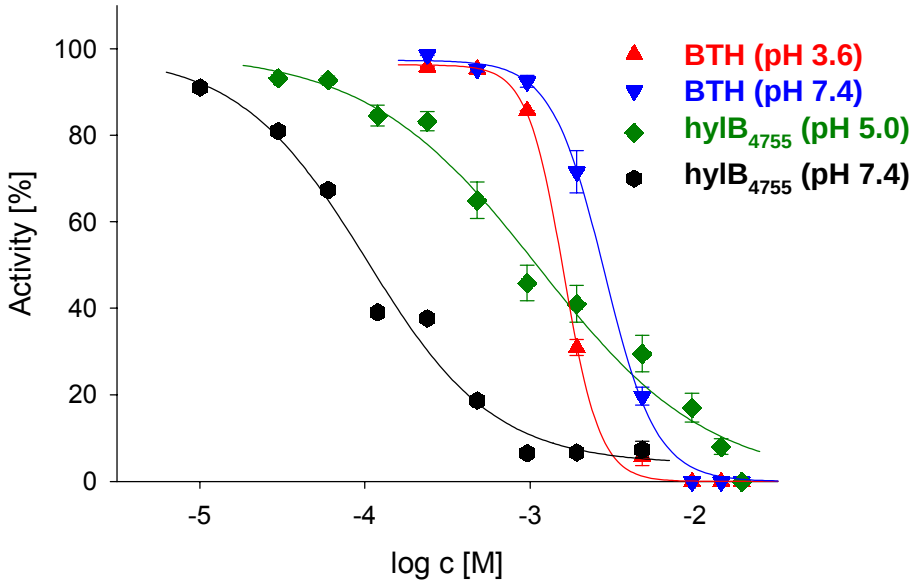


Fig. 4: Inhibitory effects of 5-methoxy-2-(4'-methoxyphenyl)-3-methylindole (**4b**) on the bovine testicular hyaluronidase and the bacterial hyaluronidase from *S. agalactiae* at optimum and physiological pH.

Whereas compound **4b** inhibited both hyaluronidases, the compounds **23-25** were selective for hyaluronate lyase (Table 3). The calculated IC_{50} values on $hylB_{4755}$ at optimum pH (5.0) were 13 µM (**23**), 8µM (**24**) and 11 µM (**25**). The inhibitory effects were concentration dependent for all three compounds, although a residual enzyme activity of 18-27 % could not be blocked by increasing the inhibition concentration as shown in Fig. 5.

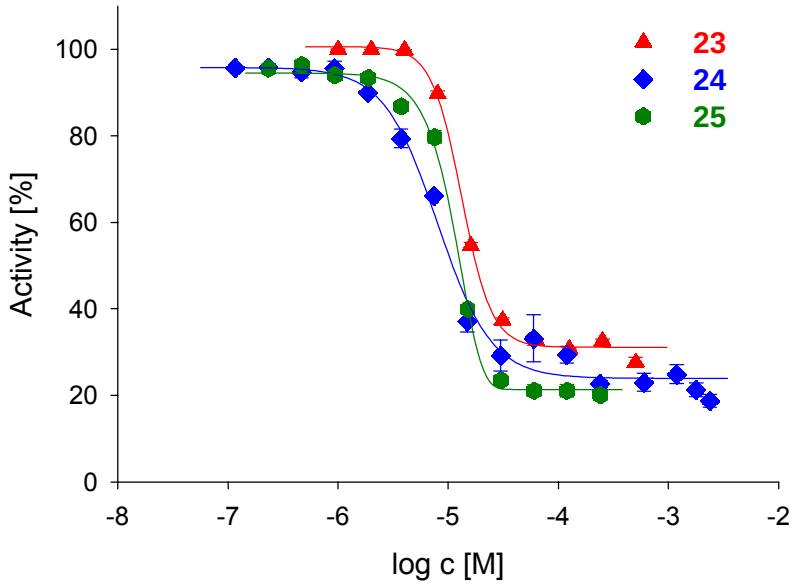


Fig. 5: Inhibitory effects of the phenylindole derivatives **23-25** determined on the hyaluronate lyase from *S. agalactiae* at pH 5.0

The inhibitory effects of the 2-phenylindole derivatives **23-25** determined on the hyaluronate lyase at pH 7.4 were in the micromolar range, too. The calculated IC_{50} values were 17 µM (**23**), 13.3 µM (**24**) and 16 µM (**25**) (cf. Table 3). For all three compounds a flattening of the curves at lower concentration range could be observed as shown in Fig. 6, suggesting two different binding modes with different affinities.

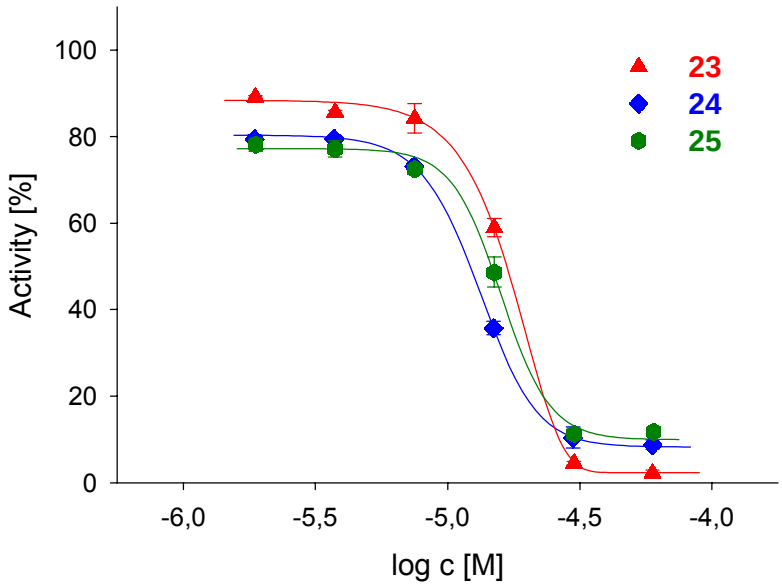


Fig. 6: Inhibitory effects of the compound **23-25** on $hylB_{4755}$

Compared to the *N*-unsubstituted compound **4b**, the analogues with 10-membered alkyl chain at the indole-*N* were about 6-fold and 70-fold more potent inhibitors of the bacterial hyaluronidase at physiological pH and at optimum pH, respectively. Moreover, different H-bonding patterns of **4b** vs. **23-25** are conceivable, as the substituents in position 4' and 5 in compounds **23-25** may act as donors at the active site of $hylB_{4755}$. Nevertheless, the increase in affinity and selectivity appears to be mainly dependent on the presence of a large lipophilic substituent at the nitrogen atom.
To determine the binding mode of hyaluronate lyase inhibitors and thereby to verify the $hylB_{4755}$ model an X-ray analysis of the enzyme-inhibitor complex was performed in co-operation with *M. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, USA). It was possible to crystallise the inhibitor **25** inside the active site of *S. pneumoniae* hyaluronate lyase (cf. section 4 of this chapter).

The phenylindole derivatives **26** and **27** which have two sulphamoyloxy substituents in position 4' and 5 or 6 were only weakly active at both the bovine testicular hyaluronidase and the *S. agalactiae* hyaluronate lyase (Table 3). The phenylindole derivative **26** with a 6-oxo-6-pyrrolidin-1-yl-hexyl side chain was active on the bovine testicular enzyme at the optimum pH (IC_{50} = 1 mM) but inactive at physiological pH. Compound **26** induced inhibition of the bacterial hyaluronidase at millimolar concentration, too. The lower inhibitory potency of compound **26** on $hylB_{4755}$ compared to the co-crystallised inhibitor **25** may result from the larger space required for the binding of the hydrophobic tail of the compound **26** than for the decyl substituent of compound **25**. Even if the oxygen atom of the carbonyl function could interact with the arginine residue Arg366, the pyrrolidine moiety is too bulky for the small crevice between the amino acid residues His399 and Phe343.

The phenylindole **27** excited our interest because of the structural similarity to the potent indole derivative **25** possessing both sulphamoyloxy groups in position 4' and 5 and an ethyl group at the nitrogen instead of a decyl group. The inhibitory activities of compound **27** on BTH and $hylB_{4755}$ at optimum pH were in the millimolar range. At pH 7.4, compound **27** induced an inhibition in the millimolar range (IC_{50} = 1.8 mM (BTH) and 4 mM ($hylB_{4755}$). On the bovine testicu-

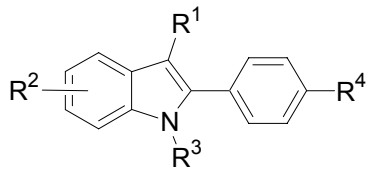
lar enzyme an increase in inhibitory potency was achieved by shortening the alkyl chain from 10 to 2 carbon atoms (compound **25** vs. compound **27**), whereas the potency on the hyaluronate lyase was 250-times lower. Concidering the results obtained for the alkyl substituted hydroxylated phenylindole derivatives **6c**-**g**, IC_{50} values in the range of 200 µM should be expected. One explanation for the discrepancy between the expected and the found results could be a negative influence on the binding mode by the methyl group in position 3 together with the bulky sulphamoyloxy group in position 5 of the indole ring.

To explore the structure-activity relationships concerning the role of a methyl substituent in position 3, the importance of the methoxy group in position 5 vs. 6 and the optimal *N*-alkyl chain length the phenylindole derivatives **4a-b, 5a-d,** and **6a-g** were synthesised and tested for inhibition of bovine testicular hyaluronidase and bacterial hyaluronidase in an optimised turbidimetric assay (cf. chapter 3). The inhibitory effects on both hyaluronidases are summarised in Table 4.

Whereas the phenylindole derivatives **4b** induced an inhibition of the bovine testicular and the bacterial hyaluronidase in the millimolar range its structural isomer **4c** was inactive at concentrations $\leq$ 100 µM. Nevertheless, this difference should not be over-interpreted as the solubility of compound **4c** is extremely poor, so that partial precipitation in the course of the pharmacological assay cannot be precluded. The inhibition of BTH and $hylB_{4755}$ by compound **4a** which is lacking the 3-methyl group ranged between 13 % and 27 % at a concentration of 500 µM.

The *N*-substituted indoles **5a-d** were inactive at the concentration tested (50µM - 250 µM) due to poor solubility.

Table 4: IC_{50} values of the synthesised 2-phenylindole derivatives (**4a-c, 5a-d, 6a-g**) determined on the bovine testicular hyaluronidase and the hyaluronate lyase



No	Subtitution				BTH		hylB$_{4755}$	
					IC_{50} [µM] or (% Inhibition)[a]			
	R^1	R^2	R^3	R^4	pH 3.6	pH 7.4	pH 5.0	pH 7.4
4a	H	5-OCH_3	H	OCH_3	inactive[b]	(13 %)[b]	(27 %)[b]	(21 %)[b]
4b	CH_3	5-OCH_3	H	OCH_3	**1590**	**2840**	**880**	**93**
4c	CH_3	6-OCH_3	H	OCH_3	inactive	inactive	inactive	inactive
5a	CH_3	5-OCH_3	CH_3	OCH_3	inactive	inactive	inactive	inactive
5b	CH_3	5-OCH_3	C_3H_7	OCH_3	inactive[c]	inactive[c]	inactive[c]	inactive[c]
5c	CH_3	5-OCH_3	C_5H_{11}	OCH_3	inactive	inactive	inactive	inactive
5d	CH_3	5-OCH_3	C_7H_{15}	OCH_3	inactive[d]	inactive[d]	inactive[d]	inactive[d]
6a	H	5-OH	H	OH	(15 %)[e]	(31 %)[e]	(75 %)[e]	**160**
6b	CH_3	6-OH	H	OH	(50 %)[e]	(46 %)[e]	**330**	**120**
6c	CH_3	5-OH	H	OH	inactive[f]	(45 %)[f]	**740**	**280**
6d	CH_3	5-OH	CH_3	OH	inactive[e]	inactive[e]	**480**	**220**
6e	CH_3	5-OH	C_3H_7	OH	inactive[b]	inactive[b]	**220**	**160**
6f	CH_3	5-OH	C_5H_{11}	OH	inactive	inactive	**23**	**36**
6g	CH_3	5-OH	C_7H_{15}	OH	inactive	inactive	**26**	**12**

[a] at concentrations ≤ 100 µM unless otherwise indicated
[b] 500 µM; [c] 200 µM; [d] 50 µM; [e] 1000 µM; [f] 2000 µM

To increase the solubility of the indoles the introduction of polar groups such as hydroxy groups should be useful. Moreover, this polar substituent could interact with functional groups in the active site of the enzyme thereby increasing the affinity of the inhibitor. Therefore, the hydroxylated phenylindole derivatives **6a-g** were synthesised. The inhibition of the bovine testicular hyaluronidase induced by the compounds **6a-c** ranged from 15 % to 50 % (cf. Table 4). Comparing the inhibitory effect of the phenylindole derivative **6a** with **6b** on BTH, the compound with an additional methyl group in position 3 (compound **6b**) was the more potent one.

Generally, the phenylindole derivatives **6a**-**g** were more active on the hyaluronate lyase than on the bovine testicular hyaluronidase. For instance, the IC_{50} values determined on $hylB_{4755}$ at physiological pH were between 100 µM and 300 µM for **6a-e**, and the inhibitory potency could be further increased to IC_{50} values of 36 µM (**6f**) and 12 µM (**6g**) by extending the chain length of the N-substituent (cf. Table 4). Exemplarily, the concentration inhibition curves of the compounds **6a**-**c** on the hyaluronate lyase at optimum pH (5.0) are depicted in Fig. 7.

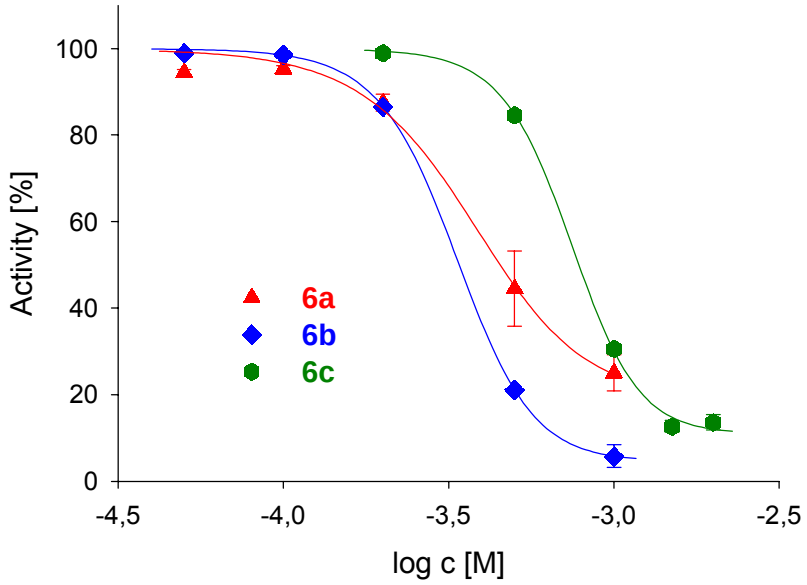


Fig. 7: Effects of the hydroxylated phenylindole derivatives **6a**-**c** on $hylB_{4755}$ at optimum pH (5.0)

Obviously, the methyl group in position 3 is not essential for inhibitory activity although it appeares to contribute to the increase in potency observed in the series of compounds listed in Table 4. By the introduction of a methyl group in position 3 the binding modes described in section 4 are no longer possible. Assuming the same binding mode for a 3-methylindole derivative as for **25** in the crystal structure, the additional methyl group would lead to sterical clashes with the amino acid residue Asn290 (cf. Fig. 13 at section 4 of this chapter). The distance between the nitrogen atom of the asparagin residue and the carbon atom C-3 of the indole ring is just about 3 Å, so that the required space for a methyl group would be too small. Nevertheless, the methylated indole derivative appear to bind to the active site of the enzyme. One possible explanation is, that the asparagin residue Asn290 possesses the ability to rotate so that the sterical hindrance could be repealed. In addition, by the introduction of the methyl group a coplanar orientation of the phenyl and the indole ring as formed for **25** in the crystal structure could be prevented. Thereby, the spatial requirement of the 2-phenylindole moiety is higher, so that the inhibitor should be directed to the more open part of the binding cleft.

The indole derivative **6b** with the hydroxy group in position 6 is about 2-fold more potent than the 5-hydroxy isomer **6c**. In consideration of the crystal structure of the enzyme-inhibitor complex (cf. section 4 of this chapter) one explanation for the higher inhibition of $hylB_{4755}$ by compound **6b** may be the stronger possible hydrogen bonds to the amino acids Arg466/Ser463 and Trp292/-Asp352/Asn349 of the protein. Comparing the inhibitory effect of the indole derivatives **6a**-**c**, the optimal structure seems to be a phenylindole derivative with a methyl group in position 3 and two hydroxy groups in the positions 4' and 6.

The increase in activity and selectivity for the bacterial enzyme achieved by N-substitution (**6d-g**) is in accordance with the results obtained with the compounds **23-25**. For these investigations the indole substitution pattern (**6c-g**) different from that of the compounds **23-25** was used (5-OH instead of 6-OH) due to low yield and difficult purification of 6-hydroxy-2-(4'-hydroxyphenyl)-indole.

The concentration inhibition curves obtained with **6c-g** on bacterial hyaluronidase are depicted in Fig. 8 and Fig. 9. At pH 5.0, the phenylindole **6c** without

any alkyl substituent at the nitrogen atom induced the weakest inhibition of $hylB_{4755}$ (IC_{50} = 740 µM), whereas the compounds with the chain length of 5 (cf. compound **6f**) and 7 carbon atoms (cf. compound **6g**) were the most active hyaluronate lyase inhibitors (IC_{50} = 23 µM and 26 µM, respectively).

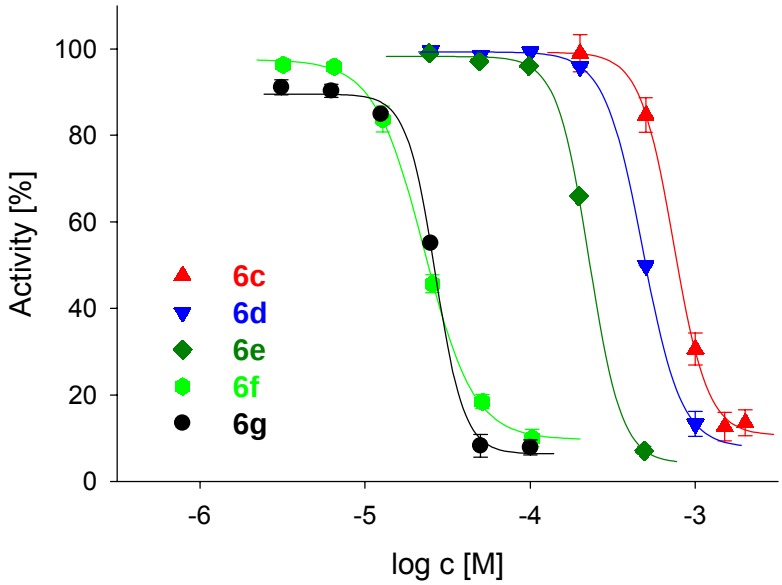


Fig. 8: Inhibitory effects of the hydroxy phenylindole derivatives **6c-g** on the activity of the bacterial hyaluronidase at optimum pH (5.0)

At pH 7.4, the enhancement of the potency by enlarging the alkyl chain length could be observed, too. The phenylindole **6g** with the heptyl group was the strongest inhibitor of the hyaluronate lyase and achieved about 3-fold higher potency than compound **6f** with a pentyl substituent (IC_{50} = 12 µM and 36 µM, respectively). For the phenylindoles **6c**-**e** a flattening of the inhibition curves at lower concentrations could be observed. This might result from different binding modes of the indole derivatives with different affinities.

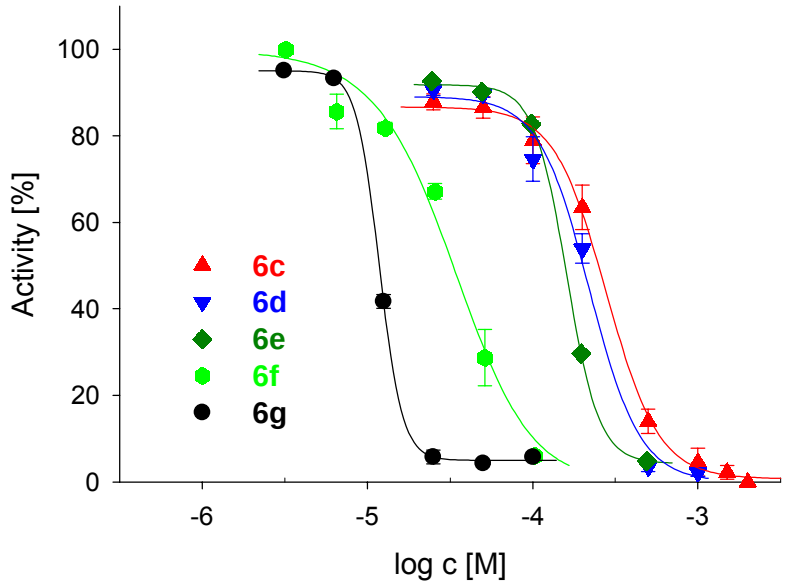


Fig. 9: Effects of the phenylindole derivatives **6c**-**g** determined on the activity of *S. agalactiae* hyaluronate lyase at physiological pH (7.4)

The dependency of the potency on the length of the *N*-alkyl chain is an additional hint that the 2-phenylindole derivatives with a methyl group in position 3 bind to the catalytic site of the enzyme in a similar way as found for compound **25** (cf. crystal structure of the enzyme-inhibitor complex section 4 of this chapter). Even if the position of the phenylindole moiety is slightly changed by introducing a methyl group in comparison to the inhibitor **25** (Fig. 10–12, section 4 of this chapter), the hydrophobic alkyl chain may bind in the same cleft of the active site as the hydrophobic tail of compound **25**.

4. X-ray analysis of the enzyme-inhibitor complex of compound 25 inside the active site of *S. pneumoniae* hyaluronate lyase

To determine the binding mode of the 2-phenylindole derivatives inside the active site of the bacterial hyaluronidase an X-ray analysis of the enzyme-inhibitor complex would be helpful. Therefore, we started co-crystallisation experiments for the indole derivatives **24** and **25** in co-operation with *Mark J. Jedrzejas*

(Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and *Daniel J. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil). The co-crystallisation experiments were successful for the phenylindole derivative **25** inside the active site of *S. pneumoniae* hyaluronate lyase. The crystals of the enzyme-inhibitor complex were analysed by X-ray diffraction and the structure was solved by rigid bond refinement (cf. section 6.3 of this chapter)

Binding mode of 6-sulphamoyloxy-2-(4-sulphamoyloxyphenyl)-1-decyl-indole (25) inside the active site of *Streptococcus pneumoniae* hyaluronate lyase

X-ray crystallography was used for the determination of the binding mode of the phenylindole derivative **25** inside the active site of the bacterial hyaluronidase from *S. pneumoniae*. The density at the catalytic site allowed for a satisfactory modelling of compound **25** as shown in Fig. 10.

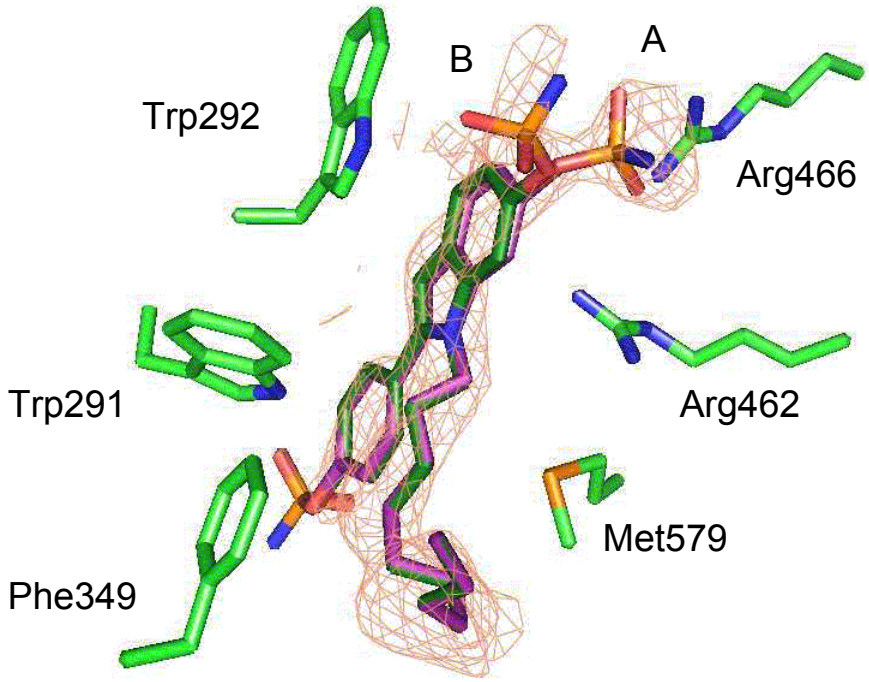


Fig. 10: Schematic representation of the electron density map $|2F_o\text{-}F_c|$ (contoured at 0.65 σ) of the phenylindole **25** inside the active site of *S. pneumoniae* hyaluronate lyase [adapted from *D. J. Rigden* (personal communication)].

In general, the inhibitor was reasonably well converted by electron density in the final map, but the clear exception was the sulphonamide group carried by the phenyl moiety of the inhibitor, for which the density was completely lacking (cf. Fig. 10). Presumably, findings reflect solvent exposure and suggest free rotation about the sulphonamide-phenyl bond. The sulphonamide group linked by the phenyl moiety was included in the final model only for the sake of completeness. During structure solution and refinement it became clear that the second sulphonamide group carried by the indole moiety can adopt two different conformations A and B (cf. Fig 10 and Fig. 11). As shown in both figures the existence of these alternative binding modes leads to small shifts in position of the indole ring. Whereas the position of the sulphonamide group in mode A is well-defined by the electron density, the position of the sulphonamide group in mode B is less well-defined. But, if this second mode were omitted a clear positive difference electron density would remain. Thus, the inclusion of the second mode in the model is justifiable. Presumably, mode A is favoured and is more highly occupied than mode B, but a specification is not justifiable by the available resolution. In the final model, an additional difference density was evident at the catalytic site, which suggested an availability of alternative modes of inhibitor binding. However, only the conformations shown before were well supported by electron density. The likely existence of other binding modes and/or the incomplete occupancy of the binding modes in the final model are also apparent in the elevated B-factors for the inhibitor in the final structure (cf. Table 5, section 6.4 of this chapter).

As shown in Fig. 10-12, the binding of the indole derivative **25** involves hydrophobic interaction of the rings and the aliphatic tail and hydrogen bonds between the indole linked sulphonamide group and the protein. The aliphatic tail of the substituted phenylindole **25** lies in a surface crevice. The alkyl chain is lined mainly by hydrophobic residues (Met579, Trp291 and Phe343), but also by the amino acids Arg336, Glu388 and His399 (cf. Fig. 12). The phenyl moiety of the inhibitor is sandwiched between the amino acids Trp291 and Asn580, whereby the interaction with Trp292 involves an approximately perpendicular ring arrangement (cf. Fig. 10, 12). The indole ring of the inhibitor is bound on one side by Trp292 in an energetically favourable manner, thereby the two ring systems lie parallel. The other side of the indole ring is in contact with the

guanidinium part of the amino acid Arg462 (cf. Fig 10-12). Such cation-π interaction can be highly energetically favourable (Gallivan et al. 1999).

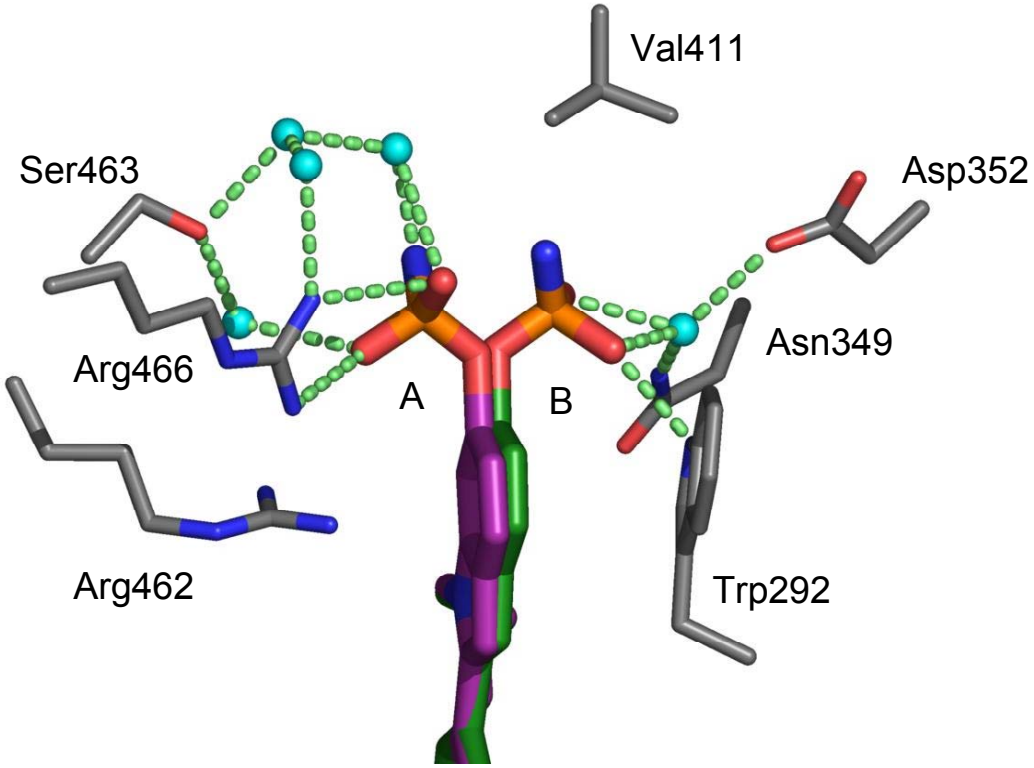


Fig. 11: Comparison of the modelled alternative binding modes for phenylindole derivative **25** in the vicinity of the indole sulphonamide group. The cyan spheres represent water molecules, possible hydrogen bonds are shown as light green dotted lines. Alternative modes A and B are drawn and labelled in purple and green, respectively [adapted from *D. J. Rigden* (personal communication)].

Each alternative binding mode of the sulphonamide group carried by the indole ring forms a network of hydrogen bonding interaction with protein and solvent (cf. Fig. 12). The interactions of the sulphonamide group in binding mode A seem particularly favourable, with a twin interaction between the amino acid Arg466 and two sulphonamide oxygen atoms. Both oxygen atoms together with the amide nitrogen form additional interaction with the protein (Ser463) involving water molecules (cf. Fig. 12). The interaction in the binding mode B are different from those of mode A. There is a single direct hydrogen bond between

one sulphonamide oxygen and the amino acid Trp292. In addition, the sulphonamide oxygen can interact via water hydrogen bonds with the amino acids Asp352 and Asn349 of the protein.

Successive structural determinations (Li et al. 2000; Ponnuraj et al. 2000; Li et al. 2001; Jedrzejas 2002; Jedrzejas et al. 2002; Mello et al. 2002; Nukui et al. 2003) and mutagenesis studies (Li et al. 2000; Kelly et al. 2001; Nukui et al. 2003) have enabled the proposal of a firmly supported catalytic mechanism (Jedrzejas 2001; Jedrzejas 2002; Jedrzejas et al. 2002; Jedrzejas 2003). The catalysis occurs at the most constricted part of the catalytic cleft. The basic amino acids involved in catalysis are Asn349, His399 and Tyr408. In addition, a hydrophobic patch composed by the amino acids Trp291, Trp292 and Phe343 are implicated in the precise positioning of the substrate.

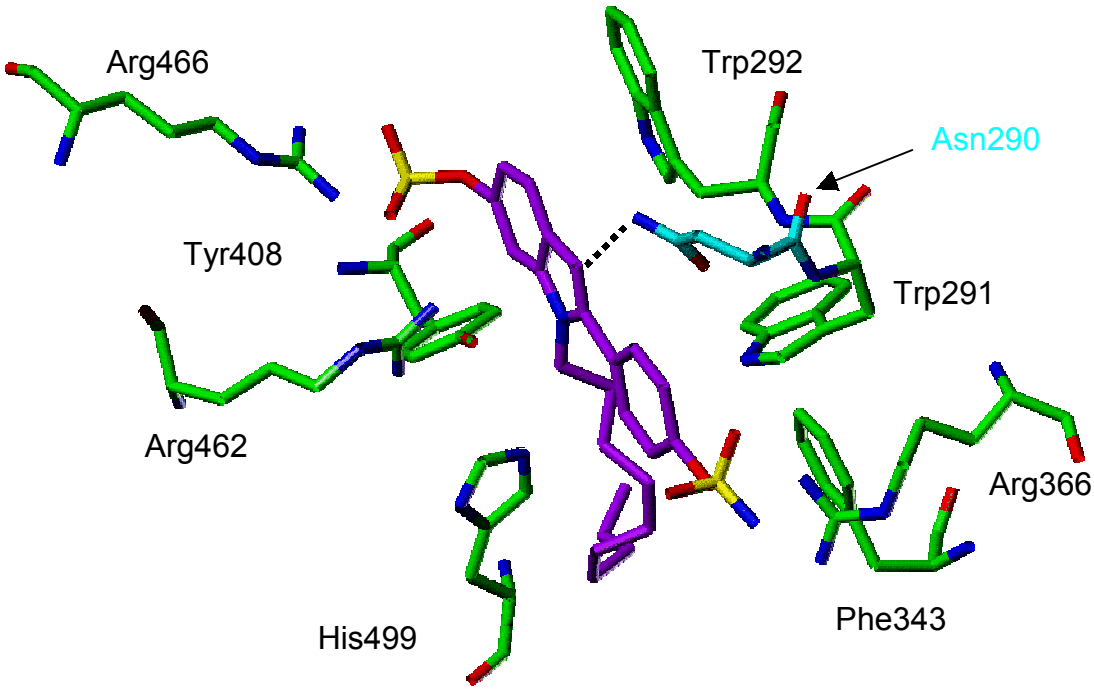


Fig. 12: Schematic representation of the alternative binding mode A of the phenylindole **25** inside the catalytic site of *S. pneumoniae* hyaluronate lyase (Botzki 2004)

The ring system of the phenylindole compound **25** binds within the catalytic site of the enzyme (cf. Fig. 10-12) which explains the inhibition of the hyaluronidase activity. Atoms of the inhibitor lie within 3.0 Å and 3.1 Å of the catalytic key residues Tyr408 and His399, respectively. In addition, the inhibitor is also linked to the protein by water mediated hydrogen bonds between the nitrogen atom of Asn349 and the sulphonamide group of the indole moiety in binding mode B. Furthermore, all amino acids of the hydrophobic patch are involved in binding of the inhibitor. The tryptophan residues Trp291 and Trp292 are important, because they contribute to the strong hydrophobic interactions with the phenyl and the indole rings of the inhibitor.
Previously, the binding mode of palmitoyl vitamin C was determined (*Rigden et al.*, unpublished results). The results obtained for the vitamin C derivative agree with those of compound **25**. The binding of the two different inhibitors causes no significant changes in the protein structure. The vitamin C structure binds in exactly the same portion of the catalytic site as the indole ring of the compound **25**. Even the binding of the aliphatic tails are generally similar.

5. Summary and future perspectives

5.1 Summary

A *de novo* design approach starting from X-ray structure of bacterial hyaluronate lyases and molecular modelling investigations led to the discovery of indole-2-carboxylic acid (**1**) as a first lead with millimolar enzyme inhibitory activity (IC_{50} of 7.1 mM (BTH) and 3.55 mM ($hylB_{4755}$)).
The investigation of further indole compounds with different substituents like acetic acid, acetyl, carboxamide, butyric acid, methyl etc. at the indole moiety (cf. compounds **7**-**15**) did not lead to more potent inhibitors. The first improvement was obtained with indomethacin (**15**), which was 11-times more potent than compound **1** on the *S. agalactiae* hyaluronate lyase (IC_{50} = 350 µM) and 13-times more potent on the bovine testicular hyaluronidase (IC_{50} = 540 µM) at optimum pH. Furthermore, in contrast to **1** indomethacin also showed inhibitory

activities on both enzymes at physiological pH (IC_{50} = 5.49 mM (BTH) and 1.14 mM ($hylB_{4755}$.

In addition to the compounds **7-15**, we investigated a series of phenylindole derivatives **4a** and **16-27** with hydroxy, methoxy, methyl and/or sulphamoyloxy substituents at the phenyl and indole rings and different substituents at the nitrogen atom. Generally, the phenylindoles were more active on the bacterial than on the bovine testicular hyaluronidase. Highest hyaluronan lyase inhibitory potency resides in the phenylindole derivatives **23-25** with hydroxy and/or sulphamoyl groups in the positions 4' and 6 and with a 10-membered alkyl chain at the nitrogen atom (IC_{50} values ranging from 8 µM to 17 µM). These phenylindole derivatives are 27-fold and 67-fold more potent on $hylB_{4755}$ at optimum pH (5.0) and physiological pH (7.4), respectively, than indomethacin and around 300-times more active than our first lead **1**.

Structure-activity relationship studies revealed that a hydroxy group in position 6 is more favourable than in position 5. Furthermore, the introduction a methyl group in position 3 and an alkyl chain in position1 leads to an increase in activity and selectivity for the bacterial vs. the bovine enzyme. Of all synthesised indole derivatives (**4a-c**, **5a-d**, **6a-g**) only the compounds without N-substituents were active on the bovine testicular hyaluronidase.

In conclusion, compared to the first lead, indole-2-carboxylic acid (**1**), the increase of lipophilicity by introducing phenyl and alkyl substituents and the introduction of hydroxy and/or sulphamoyloxy groups resulted in about 500-fold more potent inhibitor with remarkable selectivity for the bacterial vs. the bovine testicular hyaluronidase.

On the basis of the X-ray analysis[2] of the enzyme-inhibitor complex, *S. pneumoniae* hyaluronate lyase-6-sulphamoyloxy-2-(4-sulphamoyloxyphenyl)-1-decyl-1-indole (**25**) we were able to determine the binding mode of the phenylindole compound. The inhibitor **25** binds within the catalytic site of the enzyme whereby hydrophobic interactions with the amino acids Trp292, Trp291, His399 and Met579 and hydrogen bonds with Asn466, Ser463, Asp352, Asn349 and Trp292 are involved.

[2] in co-operation with *Mark J. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and *Daniel J. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

5.2 Future perspectives

Based on the previously described pharmacological results and the X-ray analysis of an enzyme-inhibitor complex, phenylindole derivative **25** bound at the active site of *S. pneumoniae* hyaluronidase, further new phenylindole-type hyaluronidase inhibitors can be suggested.

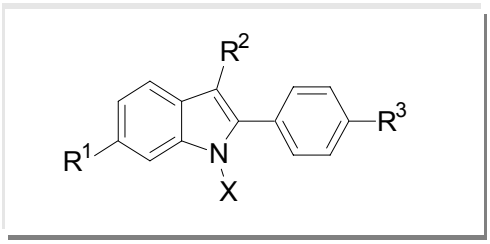


Fig. 13: General structure of phenylindole-type hyaluronidase inhibitors

Based on the structure-activity relationships compounds having the substitution patter R^1, R^3 = OH, R^2 = CH_3 and X = C_5H_{11} or C_7H_{15} are expected to be more potent inhibitors of the hyaluronate lyase that the compounds discussed above.

Based on the X-ray structure of the enzyme-inhibitor complex **25** the following structural modifications outlined below are reasonable concerning the residues R^1 and R^2 as well as the substituents X at the nitrogen atom. In addition to the potency the solubility of the compounds could be taken into account.

- By introducing acidic groups in the positions 4' (R^3) and 6 (R^1) the solubility of the compounds would probably increase. At position 4', different solvent exposed substituents which do not interact with the protein might be introduced. The ideal substituents at position 6 should be carboxylic acid (-CO_2H), acetic acid (-CH_2CO_2H) and acetyl (-$C(O)CH_3$) moieties because the oxygen atoms of these substituents could form hydrogen bonds with the amino acid residues Arg466 and Ser463 at the active site of the enzyme. This analysis should be carried out without changing the substituent X for better comparison with the phenylindole derivative **25**.

- Based on the results obtained with compound **26**, 5- to 7-membered alkyl chain with a terminal amide, amino or alcohol function would be interesting; the alkyl chain would act as a spacer and the new functionality as a possible donnor/acceptor of hydrogen bonds with the amino acids His399 or Arg366.

- The introduction of a phenyl ring or a 2-pyridyl ring linked to indole-*N* via a 4-membered carbon chain should be investigated as hydrophobic interactions of the phenyl/2-pyridyl ring with the amino acids His399 and Phe343 appear possible. In addition, the nitrogen atom of the 2-pyridyl ring could form hydrogen bond with Arg366.

- The superposition of crystal structures of a substrate-like hexasaccharide and the co-crystallised phenylindole **25** both with *S. pneumoniae* hyaluronidase may lead to suggestions of new inhibitor structures combining the phenylindole moiety and a carbohydrate portion. As scaffold the phenylindole with an hydroxy group in the position 4', the 10-membered *N*-alkyl substituent (X = $C_{10}H_{21}$) appears promissing, whereas glucuronic acid (GlAc) could be used as a carbohydrate building block linked via a spacer with the indole moiety in position 6 (Fig. 14).

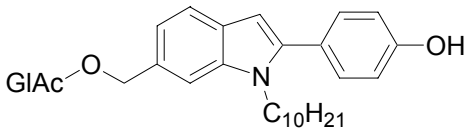


Fig. 14: Proposal of a new hyaluronate lyase inhibitor

6. Experimental section

6.1 General conditions

For a detailed description of the general procedures, equipments and chemicals used in the chemistry part, see section 4.1 of chapter 5.

6.2 Chemistry

6.2.1 Synthesis of the brominated compounds 3a-b

2-Bromo-4'-methoxyacetophenone (3a)

To a solution of 4-methoxyacetophenone (8.41 g, 56.00 mmol), dioxane (200 ml) and dichloromethane (100 ml), bromine (8.95 g, 56.00 mmol) was slowly added so that the brown colour always disappeared. Afterwards the reaction mixture was stirred at room temperature for 1 h. After washing with water (2 x 200 ml) the aqueous solution was extracted several times with ethyl acetate. All combined organic layer were dried over sodium sulphate and the solvent was removed under reduced pressure. The product was purified by recrystallisation from ethanol to give a white crystalline solid. (von Angerer et al. 1984)
Yield: 10.84 g (47.32 mmol, 85 %, white crystalline solid)

^{1}H-NMR ([D_6]DMSO):
δ [ppm] = 3.89 (s, 3H, OC$\underline{H}_3$), 4.40 (s, 2H, -C$\underline{H}_2$Br), 6.94-6.99 (m, 2H, ph), 7.95-8.00 (m, 2H, ph)

$C_9H_9BrO_2$ (229.07)

2-Bromo-4'-methoxypropiophenone (3b)

To a solution of 4'-methoxypropiophenone (31.86 g, 194.0 mmol), a few drops of aqueous hydrobromic acid (48 %) and glacial acetic acid (150 ml), bromine (31.00 g, 194.00 mmol) was slowly added so that the temperature did not exceed 20 °C. The reaction mixture was stirred at room temperature for 30 min. After cooling, the mixture was poured into ice water, then the precipitated product was collected and washed with water. The product was purified by recrystallisation from ethanol to give a white solid. (von Angerer et al. 1984)
Yield: 36.73 g (151.10 mmol, 79 %, white solid)

^{1}H-NMR ($CDCl_3$):
δ [ppm] = 1.89 (d, 3H, ^3J = 6.7 Hz, CHBrC<u>H</u>$_3$), 3.89 (s, 3H, OC<u>H</u>$_3$), 5.27 (q, 1H, ^3J = 6.7 Hz, C<u>H</u>BrCH$_3$), 6.93-6.99 (m, 2H, ph), 7.95-8.04 (m, 2H, ph)

$C_{10}H_{11}BrO_2$ (243.10)

6.2.2 Synthesis of the phenylindole derivatives 4a-c

All three methoxylated phenylindoles **4a-c** were synthesised by the following general procedure:
A solution of m- or p-anisidine (2.11 eq) and *N,N*-dimethylaniline was stirred at 175 °C. To this solution, a solution of the pertinent α-bromophenone (1 eq) dissolved in xylene was added dropwise and stirring was continued for 3 h. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate (200 ml) and aqueous hydrochloric acid (2 N, 200 ml) and the layers were separated. The water phase was extracted three times with ethyl acetate (100 ml). Afterwards the combined organic layers were washed with water (100 ml) and dried over magnesium sulphate, and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with dichloromethane and recrystallised from ethanol to give a white solid.

5-Methoxy-2-(4-methoxyphenyl)indole (4a)

Reaction of p-anisidine (4.56 g, 37.0 mmol), *N,N*-dimethylaniline (15 ml) and 2-bromo-4'-methoxyacetophenone (4,01 g, 17.50 mmol);
Yield: 1.56 g (6.16 mmol, 34 %, white solid)

Mp: 196-198° [Lit. 206-208 °C (von Angerer et al. 1984)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 3.75 (s, 3H, OC$\underline{H}_3$), 3.80 (s, 3H, OC$\underline{H}_3$), 6.66 (d, 1H, ^{4}J = 1.4 Hz, H-3), 6.71 (dd, 1H, ^{4}J = 2.5 Hz, ^{3}J = 8.8 Hz, H-6), 6.99 (d, 1H, ^{4}J = 2.5 Hz, H-4), 7.00-7.04 (m, 2H, ph), 7.25 (d, 1H, ^{3}J = 8.8 Hz, H-7), 7.73-7.77 (m, 2H, ph), 11.21 (s, 1H, N$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 55.1 (+, O$\underline{C}$H$_3$), 55.2 (+, O$\underline{C}$H$_3$), 97.2 (+, indole), 101.4 (+, indole), 110.9 (+, indole), 111.6 (+, indole), 114.2 (+, ph), 124.9 (C_{quart}, indole), 126.1 (+, ph), 129.2 (C_{quart}, ph), 132.0 (C_{quart}, indole), 138.2 (C_{quart}, indole), 153.5 (C_{quart}, indole), 158.6 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 253 ([$M^{\bullet+}$], 100), 237 ([M-CH_3]$^+$, 24), 209 ([M-CH_3-CO]$^+$, 22)

IR [cm^{-1}]: 3441 (N-H), 2999 (C-H) aromatic, 2900 (C-H) aliphatic, 1606 (C=C), 1543 (N-H), 1478 (C-H), 1394 (C-H), 1215 (C-O), 1154 (C-N), 1118, 1022, 831, 779, 752

Analysis:

calculated	C: 75.57	H: 5.97	N: 5.53
found	C: 75.07	H: 5.86	N: 5.41

$C_{16}H_{15}NO_2$ (253.30)

5-Methoxy-2-(4-methoxyphenyl)-3-methylindole (4b)

Reaction of p-anisidine (4.56 g, 37.0 mmol), *N,N*-dimethylaniline (15 ml) and 2-bromo-4'-methoxypropiophenone (4.25 g, 17.50 mmol)
Yield: 3.10 g (11.60 mmol, 66 %, white crystalline solid)

Mp: 128-130 °C [Lit. 135-137 °C (von Angerer et al. 1984)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 2.35 (s, 3H, 3-C$\underline{H}_3$), 3.78 (s, 3H, OC$\underline{H}_3$), 3.81 (s, 3H, OC$\underline{H}_3$), 6.72 (dd, 1H, ^{4}J = 2.4 Hz, ^{3}J = 8.6 Hz, H-6), 6.97 (d, 1H, ^{4}J = 2.4 Hz, H-4), 7.05-7.08 (m, 2H, ph), 7.22 (d, 1H, ^{3}J = 8.80 Hz, H-7), 7.56-7.59 (m, 2H, ph), 10.84 (s, 1H, N$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 9.8 (+, CH_3), 55.1 (+, O$\underline{C}H_3$), 55.3 (+, O$\underline{C}H_3$), 100.0 (+, indole), 105.4 (C_{quart}, indole), 111.0 (+, indole), 111.4 (+, indole), 114.1 (+, ph), 125.7 (C_{quart}, indole), 128.6 (+, ph), 129.8 (C_{quart}, ph), 130.8 (C_{quart}, indole), 134.4 (C_{quart}, indole), 153.1 (C_{quart}, indole), 158.2 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 267 ([$M^{\bullet+}$], 100)

IR [cm^{-1}]: 3382 (N-H), 2990 (C-H) aromatic, 2900 (C-H) aliphatic, 1608 (C=C), 1550 (N-H), 1449 (C-H), 1217 (C-O), 1178 (C-N), 1062, 1027, 839, 802, 622

Analysis:

calculated	C: 76.38	H: 6.41	N: 5.24
found	C: 76.20	H: 6.27	N: 5.20

$C_{17}H_{17}NO_2$ (267.33)

6-Methoxy-2-(4-methoxyphenyl)-3-methylindole (4c)

Reaction of m-anisidine (4.56 g, 37.0 mmol), *N,N*-dimethylaniline (15 ml) and 2-bromo-4'-methoxypropiophenone (4,25 g, 17.50 mmol).
Yield: 1.63 g (6.10 mmol, 35 %, white solid)

Mp: 125-127 °C [Lit. 133-135 °C (von Angerer et al. 1984)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 2.34 (s, 3H, 3-C$\underline{H}_3$), 3.77 (s, 3H, OC$\underline{H}_3$), 3.81 (s, 3H, OC$\underline{H}_3$), 6.66 (dd, 1H, ^{4}J = 2.3 Hz, ^{3}J = 8.6 Hz, H-5), 6.83 (d, 1H, ^{4}J = 2.3 Hz, H-7), 7.36 (d, 1H, ^{3}J = 8.6 Hz, H-4), 7.04-7.09 (m, 2H, ph), 7.53-7.57 (m, 2H, ph)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 9.8 (+, $\underline{C}H_3$), 55.1 (+, O$\underline{C}H_3$), 55.1 (+, O$\underline{C}H_3$), 94.1 (+, indole), 105.5 (C_{quart}, indole), 108.4 (+, indole), 114.1 (+, ph), 118.7 (+, indole), 123.9 (C_{quart}, indole), 125.8 (C_{quart}, ph), 128.3 (+, ph), 132.4 (C_{quart}, indole), 136.4 (C_{quart}, indole), 155.6 (C_{quart}, indole), 158.0 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 267 ([$M^{\bullet+}$], 79), 252 ([M-CH_3]$^{+}$, 100)

IR [cm^{-1}]: 3441 (N-H), 3000 (C-H) aromatic, 2900 (C-H) aliphatic, 1573 (C=C), 1513 (N-H), 1454 (C-H), 1327 (C-H), 1257 (C-O), 1185 (C-N), 1125, 1018, 823, 806

Analysis:

calculated	C: 74.62	H: 6.39	N: 5.12
found	C: 74.47	H: 6.17	N: 5.07

$C_{17}H_{17}NO_2$ (267.33)

6.2.3 Synthesis of the *N*-alkylated phenylindole derivatives 5a-d

The N-alkylated 2-phenylindoles **5a-d** were synthesised from the 2-phenylindole **4b** by alkylation reaction at the nitrogen atom.
To an ice-cold solution of sodium hydride (1.60 eq) in DMF (10 - 15 ml) stirred under a nitrogen atmosphere, a solution of the 2-phenylindole **4b** (1 eq) in DMF (10 - 15 ml) was slowly added and stirring was continued, until the gas generation ceased. Afterwards, a solution of the pertinent iodoalkane (1 eq) in DMF (10- 15 ml) was added dropwise. The reaction mixture was allowed to warm up to room temperature and stirring was continued for 2 h. The products were purified by colomn chromatography.

5-Methoxy-2-(4-methoxyphenyl)-3-methyl-1-methylindole (5a)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**4b**) (0.50 g, 1.87 mmol), sodium hydride (0.12 g, 3.0 mmol) and methyl iodide (0.27 g, 1.87 mmol). After dilution with water (100 ml) the precipitate was collected and washed with large amount of water. The product was purified by column chromatography on silica gel eluting with a 5:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate.
Yield: 0.50 g (1.78 mmol, 95 %, white solid)

Mp: 129-131 °C [Lit. 139-141 °C (von Angerer et al. 1984)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 2.15 (s, 3H, C$\underline{H}_3$), 3.34 (s, 3H, NC$\underline{H}_3$), 3.78 (s, 3H, OC$\underline{H}_3$), 3.82 (s,3H, OC$\underline{H}_3$), 6.79 (dd, 1H, ^{4}J = 2.4 Hz, ^{3}J = 8.7 Hz, H-6), 6.99 (d, 1H,^{4}J = 2.4 Hz, H-4), 7.30 (d, 1H, ^{3}J = 8.7 Hz, H-7), 7.04-7.11 (m, 2H, ph), 7.33-7.39 (m, 2H, ph)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 9.4 (+, $\underline{C}H_3$), 30.8 (+, N$\underline{C}H_3$), 55.2 (+, O$\underline{C}H_3$), 55.4 (+, O$\underline{C}H_3$), 100.2 (+, indole), 106.6 (C_{quart}, indole), 110.3 (+, indole), 111.1 (+, indole), 113.9 (+, ph), 123.7 (C_{quart}, indole), 128.2 (C_{quart}, ph), 131.5 (+, ph), 132.0 (C_{quart}, indole), 137.7 (C_{quart}, indole), 153.4 (C_{quart}, indole), 158.8 (C_{quart}, indole)

MS (PI-EIMS (70 eV)): m/z (%) = 281 ([$M^{\bullet+}$], 100), 266 ([$M-CH_3$]$^+$, 30)

IR [cm^{-1}]: 2921 (C-H) aromatic, 2846 (C-H) aliphatic, 1600 (C=C), 1486 (C-H), 1239 (C-O), 1176 (C-N), 1066, 1031, 906, 840, 795, 609

Analysis:

calculated	C: 76.84	H: 6.81	N: 4.98
found	C: 76.59	H: 6.98	N: 4.77

$C_{18}H_{19}NO_2$ (281.35)

5-Methoxy-2-(4-methoxyphenyl)-3-methyl-1-propylindole (5b)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**4b**) (1.0 g, 3.74 mmol), sodium hydride (0.24 g, 6.0 mmol) and propyl iodide (0.46 g, 3.74 mmol). After diluting with water (100 ml) the reaction mixture was extracted three times with ethyl acetate (100 ml). The combined organic layers were dried over magnesium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 10:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate.

Yield: 1.03 g (3.33 mmol, 89 %, white solid)

Mp: 113-115 °C [Lit. 119-121 °C (von Angerer et al. 1984)]

^{1}H-NMR ([D_6]DMSO):

δ [ppm] = 0.61 (t, 3H, ^3J = 7.4 Hz, $NCH_2CH_2C\underline{H}_3$), 1.46 (m, 2H, $NCH_2C\underline{H}_2CH_3$), 2.12 (s, 3H, $C\underline{H}_3$), 3.79 (s, 3H, $OC\underline{H}_3$), 3.88 (s, 3H, $OC\underline{H}_3$), 3.95 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2CH_2CH_3$), 6.78 (dd, 1H, ^4J = 2.5 Hz, ^3J = 8.8 Hz, H-6), 6.99 (d, 1H, ^4J = 2.5 Hz, H-4), 7.06-7.10 (m, 2H, ph), 7.30-7.35 (m, 3H, H-7 and ph)

^{13}C-NMR ([D_6]DMSO):

δ [ppm] = 9.2 (+, $\underline{C}H_3$), 10.9 (+, $NCH_2CH_2\underline{C}H_3$), 22.8 (-, $NCH_2\underline{C}H_2CH_3$), 44.7 (-, $N\underline{C}H_2CH_2CH_3$), 55.1 (+, $O\underline{C}H_3$), 55.4 (+, $O\underline{C}H_3$), 100.4 (+, indole), 106.9 (C_{quart},

indole), 110.6 (+, indole), 110.9 (+, indole), 113.9 (+, ph), 123.9 (C_{quart}, indole), 128.3 (C_{quart}, ph), 131.2 (C_{quart}, indole), 131.3 (+, ph), 137.5 (C_{quart}, indole), 153.3 (C_{quart}, indole), 158.8 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 309 ([$M^{\bullet+}$], 91), 280 ([$M-C_2H_5$]$^+$, 100)

IR [cm^{-1}]: 2922 (C-H) aromatic, 2849 (C-H) aliphatic, 1609 (C=C), 1450 (C-H), 1223 (C-O), 1176 (C-N), 1039, 908, 842, 800, 621

Analysis:

calculated	C: 77.64	H: 7.49	N: 4.53
found	C: 77.80	H: 7.61	N: 4.27

$C_{20}H_{23}NO_2$ (309.40)

5-Methoxy-2-(4-methoxyphenyl)-3-methyl-1-pentylindole (5c)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**4b**) 1.0 g, 3.74 mmol), sodium hydride (0.24 g, 6.0 mmol) and pentyl iodide (0.57 g, 3.74 mmol). After diluting with water (100 ml) the mixture was extracted three times with ethyl acetate (100 ml). The combined organic layer were dried over magnesium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 10:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate.
Yield: 1.21 g (3.59 mmol, 96 %, white solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 0.70 (t, 3H, ^3J = 7.2 Hz, $CH_2C\underline{H}_3$), 0.97-1.11 (m, 4H, $C\underline{H}_2C\underline{H}_2$), 1.43 (q, 2H, ^3J = 7.2 Hz, $NCH_2C\underline{H}_2$), 2.12 (s, 3H, $C\underline{H}_3$), 3.79 (s, 3H, $OC\underline{H}_3$), 3.83 (s, 3H, $OC\underline{H}_3$), 3.98 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2CH_2$), 6.78 (dd, 1H, ^4J = 2.5 Hz, ^3J = 8.8 Hz, H-6), 6.99 (d, 1H, ^4J = 2.5 Hz, H-4), 7.06-7.10 (m, 2H, ph), 7.30-7.33 (m, 3H, indole and ph)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.2 (+, $\underline{C}H_3$), 13.6 (+, $CH_2\underline{C}H_3$), 21.5 (-, -$\underline{C}H_2CH_3$), 28.1 (-, -$\underline{C}H_2$-), 29.1 (-, -$\underline{C}H_2$-), 43.0 (-, N$\underline{C}H_2$-), 55.1 (+, O$\underline{C}H_3$), 55.4 (+, O$\underline{C}H_3$), 100.4 (+, indole), 106.9 (C_{quart}, indole), 110.5 (+, indole), 110.9 (+, indole), 113.9 (+, ph), 124.0 (C_{quart}, indole), 128.4 (C_{quart}, ph), 131.2 (C_{quart}, indole), 131.4 (+, ph), 137.5 (C_{quart}, indole), 153.3 (C_{quart}, indole), 158.8 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 337 ([$M^{\bullet+}$], 89), 280 ([M-C_4H_9]$^+$, 100), 265 ([M-C_4H_9-CH_3]$^+$, 19), 249 ([M-C_4H_9-CH_3-CO]$^+$, 19)

IR [cm^{-1}]: 2956 (C-H) aromatic, 2864 (C-H) aliphatic, 1611 (C=C), 1480 (C-H), 1457 (C-H), 1242 (C-O), 1168 (C-N), 1035, 905, 834, 797

Analysis:

calculated	C: 78.30	H: 8.06	N: 4.15
found	C: 78.11	H: 7.98	N: 4.01

$C_{22}H_{27}NO_2$ (337.46)

5-Methoxy-2-(4-methoxyphenyl)-3-methyl-1-heptylindole (5d)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**4b**) (0.50 g, 1.87 mmol), sodium hydride (0.12 g, 3.0 mmol) and heptyl iodide (0.37 g, 1.87 mmol). After diluting with water (100 ml) the mixture was extracted with ethyl acetate (3 x 100 ml). The combined organic layers were dried over magnesium sulphate and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel eluting with a 5:1 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate.
Yield: 0.65 g (1.78 mmol, 95 %, white solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 0.78 (t, 3H, ^{3}J = 6.9 Hz, -$CH_2C\underline{H}_3$), 1.00-1.16 (m, 6H, -$C\underline{H}_2$-), 1-18-1.26 (m, 2H, -$C\underline{H}_2$-), 1.35-1.46 (m, 2H, -$C\underline{H}_2$-), 2.11 (s, 3H, $C\underline{H}_3$), 3.79 (s, 3H,

$OC\underline{H}_3$), 3.83 (s, 3H, $OC\underline{H}_3$), 3.98 (t, 2H, ^3J = 7.1 Hz, $NC\underline{H}_2CH_2$-), 6.78 (dd, 1H, ^4J = 2.5 Hz, ^3J = 7.9 Hz, H-6), 6.99 (d, 1H, ^4J = 2.5 Hz, H-4), 7.07-7.08 (m, 2H, ph), 7.30-7.33 (m, 3H, indole and ph)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.3 (+, $\underline{C}H_3$), 13.8 (+, $-CH_2\underline{C}H_3$), 21.9 (-, $-\underline{C}H_2CH_3$), 25.9 (-, $-\underline{C}H_2$-), 27.9 (-, $-\underline{C}H_2$-), 30.9 (-, $-\underline{C}H_2$-), 42.9 (-, $N\underline{C}H_2$-), 55.1 (+, $O\underline{C}H_3$), 55.4 (+, $O\underline{C}H_3$), 100.4 (+, indole), 106.9 (C_{quart}, indole), 110.9 (+, indole), 110.9 (+, indole), 113.9 (+, ph), 124.0 (C_{quart}, indole), 128.4 (C_{quart}, ph), 131.2 (C_{quart}, indole), 131.4 (+, ph), 137.5 (C_{quart}, indole), 153.3 (C_{quart}, indole), 158.8 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 365 ($[M^{\bullet+}]$, 98), 280 ($[M-C_6H_{13}]^+$, 100), 265 ($[M-C_4H_9-CH_3]^+$, 17), 249 ($[M-C_4H_9-CH_3-CO]^+$, 17)

IR [cm^{-1}]: 2925 (C-H) aromatic, 2849 (C-H) aliphatic, 1611 (C=C), 1483 (C-H), 1457 (C-H), 1241 (C-O), 1172 (C-N), 1034, 907, 834, 795, 611

Analysis:

calculated	C: 78.86	H: 8.55	N: 3.83
found	C: 78.40	H: 8.68	N: 3.67

$C_{24}H_{31}NO_2$ (365.51)

6.2.4 Cleavage of the methyl ether

To a solution of BBr_3 (10 eq) in anhydrous dichloromethane (10 ml), a solution of the corresponding methoxy-substituted phenylindole (1 eq) in anhydrous dichloromethane (100 ml) was added under a nitrogen atmosphere at –10 °C. The reaction mixture was stirred for 1 h at -10 °C and 3 days at room temperature, then it was diluted with a saturated solution of sodium hydrogen carbonate (100 - 150 ml) and ethyl acetate (150 - 200 ml) and stirred for additional 30 min. After separation, the aqueous phase was extracted three times with ethyl acetate (50 ml). The combined organic layers were dried over magnesium sulphate and the solvent was removed under reduced pressure.

5-Hydroxy-2-(4-hydroxyphenyl)indole (6a)

Reaction of 5-methoxy-2-(4-methoxyphenyl)indole (**4a**) (1.0 g, 3.95 mmol) and BBr_3 (39.50 mmol). The product was purified by column chromatography on silica gel eluting with a 1:2 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a green solid.
Yield: 0.23 g (0.96 mmol, 24 %, green solid)

Mp: 265-267 °C [Lit. 265-268 °C (von Angerer et al. 1984)]
^{1}H-NMR ([D$_6$]DMSO):
δ [ppm] = 6.50 (d, 1H, ^4J = 1.7 Hz, H-3), 6.56 (dd, 1H, ^4J = 2.2 Hz, ^3J = 8.5 Hz, H-6), 6.79 (d, 1H, ^4J = 2.2 Hz, H-4), 6.82 (d, 2H, ^3J = 8.8 Hz, H-2', H-6'), 7.13 (d, 1H, ^3J = 8.5 Hz, H-7), 7.61 (d, 2H, ^3J = 8.8 Hz, H-3', H-5'), 8.59 (s, 1H, O<u>H</u>), 9.56 (s, 1H, O<u>H</u>), 10.98 (s, 1H, N<u>H</u>)

^{13}C-NMR ([D$_6$]DMSO):
δ [ppm] = 96.1 (+, indole), 103.4 (+, indole), 110.9 (+, indole), 111.1 (+, indole), 115.5 (+, ph), 123.6 (C_{quart}, indole), 126.1 (+, ph), 129.6 (C_{quart}, ph), 131.3 (C_{quart}, indole), 138.5 (C_{quart}, indole), 150.7 (C_{quart}, indole), 156.8 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 225 ([$M^{\bullet+}$], 100)

IR [cm^{-1}]: 3427 (N-H), 3309 (O-H), 1596 (C=C), 1441 (C-H), 1376 (C-H), 1260 (C-O), 1138 (C-N), 952, 831, 778

Analysis:

calculated	C: 74.06	H: 4.92	N: 6.16
found	C: 73.86	H: 5.28	N: 5.55

$C_{14}H_{11}NO_2$ (225.24)

6-Hydroxy-2-(4-hydroxyphenyl)-3-methylindole (6b)

Reaction of 6-methoxy-2-(4-methoxyphenyl)indole (**4c**) (0.54 g, 2.0 mmol) and BBr_3 (20 mmol). The product was purified by column chromatography on silica gel eluting with a 3:2 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a light green solid.

Yield: 0.31 g (1.30 mmol, 65 %, light green solid)

Mp: 234-238 °C [Lit. 250-253 °C (von Angerer et al. 1984)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.29 (s, 3H, C$\underline{H}_3$), 6.51 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.4 Hz, H-5), 6.69 (d, 1H, ^4J = 2.0 Hz, H-7), 6.85-6.89 (m, 2H, ph), 7.23 (d, 1H, ^3J = 8.4 Hz, H-4), 7.40-7.42 (m, 2H, ph), 8.85 (br, 1H, O$\underline{H}$), 9.52 (br, 1H, O$\underline{H}$), 10.56 (s, 1H, N$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 10.1 (+, $\underline{C}H_3$), 96.6 (+, indole), 105.4 (C_{quart}, indole), 109.3 (+, indole), 115.9 (+, ph), 118.9 (+, indole), 123.7 (C_{quart}, indole), 124.8 (C_{quart}, ph), 128.8 (+, ph), 132.2 (C_{quart}, indole), 137.2 (C_{quart}, indole), 153.5 (C_{quart}, indole), 156.6 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 239 ([$M^{\bullet+}$], 100), 238 ([M-H]$^+$, 80)

IR [cm^{-1}]: 3397 (N-H), 3254 (O-H), 1514 (C=C), 1435 (C-H), 1378 (C-H), 1223 (C-O), 1150 (C-N), 956, 841, 829, 801, 652, 627

Analysis:

calculated	C: 73.36	H: 5.45	N: 5.70
found	C: 73.59	H: 5.36	N: 5.44

$C_{15}H_{13}NO_2 \bullet 0.35\ H_2O$ (245.59)

5-Hydroxy-2-(4-hydroxyphenyl)-3-methylindole (6c)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methylindole (**4b**) (0.54 g, 2.0 mmol) and BBr_3 (20 mmol). The product was purified by column chromatography on silica gel eluting with a 3:2 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate to give a green solid.

Yield: 0.34 g (1.42 mmol, 71 %, green solid)

Mp: 188-190 °C [Lit. 201-204 °C (von Angerer et al. 1984)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.27 (s, 3H, C$\underline{H}_3$), 6.60 (dd, 1H, ^4J = 2.2 Hz, ^3J = 8.5 Hz, H-6), 6.77 (d, 1H, ^4J = 2.2 Hz, H-4), 6.88 (d, 2H, ^3J = 8.8 Hz, H-2', H-6'), 7.10 (d, 1H, ^3J = 8.5 Hz, H-7), 7.44 (d, 2H, ^3J = 8.8 Hz, H-3', H-5'), 8.60 (s, 1H, O$\underline{H}$), 9.58 (s, 1H, O$\underline{H}$), 10.62 (s, 1H, N$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 10.4 (+, $\underline{C}H_3$), 102.5 (+, indole), 104.7 (C_{quart}, indole), 111.5 (+, indole), 111.6 (+, indole), 115.9 (+, ph), 124.8 (C_{quart}, indole), 129.1 (+, ph), 130.6 (C_{quart}, ph), 130.7 (C_{quart}, indole), 135.3 (C_{quart}, indole), 150.8 (C_{quart}, indole), 156.9 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 239 ([$M^{\bullet+}$], 100), 238 ([M-H]$^+$, 85)

IR [cm^{-1}]: 3441 (N-H), 3355 (O-H), 1592 (C=C), 1449 (C-H), 1354 (C-H), 1230 (C-O), 1194 (C-N), 939, 830, 791

Analysis:

calculated	C: 73.63	H: 5.48	N: 5.75
found	C: 73.87	H: 5.47	N: 5.55

$C_{15}H_{13}NO_2 \bullet 0.3\ H_2O$ (244.68)

5-Hydroxy-2-(4-hydroxyphenyl)-1-methyl-3-methylindole (6d)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-methylindole (**5a**) (0.53 g, 1.89 mmol) and BBr_3 (18.90 mmol). The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of dichloromethane and ethyl acetate to give a light brown solid.

Yield: 0.41 g (1.62 mmol, 86 %, light brown solid)

Mp: 192-195 °C [Lit. 199-200 °C (von Angerer et al. 1984)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 2.09 (s, 3H, C$\underline{H}_3$), 3.49 (s, 3H, NC$\underline{H}_3$), 6.66 (dd, 1H, ^{4}J = 2.3 Hz, ^{3}J = 8.6 Hz, H-6), 6.8 (d, 1H, ^{4}J = 2.3 Hz, H-4), 6.90 (d, 2H, ^{3}J = 8.5 Hz, H-2', H-6'), 7.18 (d, 1H, ^{3}J = 8.6 Hz, H-7), 7.22 (d, 2H, ^{3}J = 8.5 Hz, H-3', H-5'), 8.68 (s, 1H, O$\underline{H}$), 9.67 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.9 (+, $\underline{C}H_3$), 14.6 (+, N$\underline{C}H_3$), 102.7 (+, indole), 105.9 (C_{quart}, indole), 110.4 (+, indole), 111.6 (+, indole), 115.7 (+, ph), 122.6 (C_{quart}, indole), 129.1 (C_{quart}, ph), 131.9 (C_{quart}, indole), 131.9 (+, ph), 138.4 (C_{quart}, indole), 151.1 (C_{quart}, indole), 157.5 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 253 ([$M^{\bullet+}$], 100), 238 ([M-H]$^+$, 76)

IR [cm^{-1}]: 3276 (O-H), 2926 (C-H) aliphatic, 1600 (C=C), 1468 (C-H), 1378 (C-H), 1226 (C-O), 1186 (C-N), 1064, 917, 842, 786

Analysis:

calculated	C: 74.81	H: 5.96	N: 5.43
found	C: 74.49	H: 6.14	N: 5.07

$C_{16}H_{15}NO_2 \bullet 0.2\ H_2O$ (256.90)

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-propylindole (6e)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-propylindole (**5b**) (0.50 g, 1.62 mmol) and BBr_3 (16.20 mmol); The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of dichloromethane and ethyl acetate to give a brown solid.

Yield: 0.45 g (1.60 mmol, 99 %, brown solid)

Mp: 147-149 °C [Lit. 153-154 °C (von Angerer et al. 1984)]

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 0.62 (t, 3H, ^3J = 7.5 Hz, $NCH_2CH_2C\underline{H}_3$), 1.45 (m, 2H, $NCH_2C\underline{H}_2CH_3$), 2.05 (s, 3H, $C\underline{H}_3$), 3.89 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2CH_2CH_3$), 6.64 (dd, 1H, ^4J = 2.3 Hz, ^3J = 8.6 Hz, H-6), 6.78 (d, 1H, ^4J = 2.3 Hz, H-4), 6.89 (m, 2H, ph), 7.16-7.22 (m, 3H, ph and H-7), 8.66 (s, 1H, O$\underline{H}$), 9.66 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.8 (+, $\underline{C}H_3$), 11.6 (+, $NCH_2CH_2\underline{C}H_3$), 23.3 (-, $NCH_2\underline{C}H_2CH_3$), 45.2 (-, $N\underline{C}H_2CH_2CH_3$), 102.8 (+, indole), 106.4 (C_{quart}, indole), 110.7 (+, indole), 111.5 (+, indole), 115.8 (+, ph), 122.9 (C_{quart}, indole), 129.3 (C_{quart}, ph), 131.1 (C_{quart}, indole), 131.8 (+, ph), 138.3 (C_{quart}, indole), 151.0 (C_{quart}, indole), 157.5 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 281 ([$M^{\bullet+}$], 69), 252 ([$M-C_2H_5$]$^+$, 100)

IR [cm^{-1}]: 3400 (O-H), 2921 (C-H) aliphatic, 1600 (C=C), 1464 (C-H), 1345 (C-H), 1232 (C-O), 1176 (C-N), 914, 839, 795, 613

Analysis:

calculated	C: 75.87	H: 6.79	N: 4.91
found	C: 75.88	H: 7.15	N: 4.57

$C_{18}H_{19}NO_2 \bullet 0.2\ H_2O$ (284.95)

5-Hydroxy-2-(4-hydroxyphenyl)-3-methyl-1-pentylindole (6f)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-pentylindole (**5c**) (0.68 g, 2.0 mmol) and BBr_3 (20 mmol). The product was purified by column chromatography on silica gel eluting with a 1:2 (v/v) mixture of petroleum ether 60-80 °C and ethyl acetate.

Yield: 0.60 g (1.94 mmol, 97 %, dark green crystalline solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 0.79 (t, 3H, ^3J = 7.0 Hz, -$CH_2C\underline{H}_3$), 1.01-1.10 (m, 4H, -$C\underline{H}_2$-), 1.40-1.47 (m, 2H, -$C\underline{H}_2$-), 2.05 (s, 3H, $C\underline{H}_3$), 3.92 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2CH_2$-), 6.64 (dd, 1H, ^4J = 2.2 Hz, ^3J = 8.6 Hz, H-6), 6.78 (d, 1H, ^4J = 2.2 Hz, H-4), 6.87-6.91 (m, 2H, ph), 7.15-7.20 (m, 3H, ph and H-7), 8.61 (s, 1H, O$\underline{H}$), 9.60 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.2 (+, $\underline{C}H_3$), 13.7 (+, -$CH_2\underline{C}H_3$), 21.8 (-, -$\underline{C}H_2$-),), 27.9 (-, -$\underline{C}H_2$-), 29.2 (-, -$\underline{C}H_2$-), 42.9 (-, N$\underline{C}H_2$-), 102.3 (+, indole), 105.8 (C_{quart}, indole), 110.0 (+, indole), 110.9 (+, indole), 115.2 (+, ph), 122.4 (C_{quart}, indole), 128.7 (C_{quart}, ph), 130.4 (C_{quart}, indole), 131.2 (+, ph), 137.7 (C_{quart}, indole), 150.4 (C_{quart}, indole), 156.9 (C_{quart}, ph)

IR [cm^{-1}]: 3349 (O-H), 2925 (C-H) aliphatic, 1613 (C=C), 1461 (C-H), 1357 (C-H), 1230 (C-O), 1169 (C-N), 918, 836, 791, 716

$C_{20}H_{23}NO_2$ (309.2)

5-Hydroxy-2-(4-hydroxyphenyl)-1-heptyl-3-methylindole (6g)

Reaction of 5-methoxy-2-(4-methoxyphenyl)-3-methyl-1-heptylindole (**5d**) (0.73 g, 2.0 mmol) and BBr_3 (20 mmol). The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of dichloromethane and ethyl acetate to give a green solid.

Yield: 0.65 g (1.94 mmol, 97 %, green solid)

^{1}H-NMR ([D$_6$]DMSO):

δ [ppm] = 0.79 (t, 3H, ^{3}J = 7.2 Hz, -$CH_2\underline{C}H_3$), 1.01-1.10 (m, 4H, -$C\underline{H}_2$-), 1.11-1.23 (m, 2H, -$C\underline{H}_2$-), 1.39-1.47 (m, 2H, -$C\underline{H}_2$-), 2.05 (s, 3H, $C\underline{H}_3$), 3.92 (t, 2H, ^{3}J = 7.2 Hz, $NC\underline{H}_2CH_2$-), 6.64 (dd, 1H, ^{4}J = 2.2 Hz, ^{3}J = 8.4 Hz, H-6), 6.79 (d, 1H, ^{4}J = 2.2 Hz, H-4), 6.88-6.98 (m, 2H, ph), 7.15-7.21 (m, 3H, ph and H-7), 8.62 (s, 1H, O$\underline{H}$), 9.62 (s, 1H, O$\underline{H}$)

^{13}C-NMR ([D$_6$]DMSO):

δ [ppm] = 9.2 (+, =$C\underline{C}H_3$), 13.7 (+, -$CH_2\underline{C}H_3$), 21.8 (-, -$\underline{C}H_2$-), 25.8 (-, -$\underline{C}H_2$-), 27.9 (-, -$\underline{C}H_2$-), 29.2 (-, -$\underline{C}H_2$-), 30.9 (-, -$\underline{C}H_2$-), 42.9 (-, $N\underline{C}H_2$-), 102.3 (+, indole), 105.8 (C_{quart}, indole), 110.0 (+, indole), 110.9 (+, indole), 115.2 (+, ph), 122.4 (C_{quart}, indole), 128.7 (C_{quart}, ph), 130.4 (C_{quart}, indole), 131.2 (+, ph), 137.7 (C_{quart}, indole), 150.4 (C_{quart}, indole), 156.9 (C_{quart}, ph)

MS (PI-EIMS (70 eV)): m/z (%) = 337 ([$M^{\bullet+}$], 100), 294 ([M-C_3H_7]$^+$, 3), 252 ([M-C_6H_{13}]$^+$, 84)

IR [cm^{-1}]: 3381 (O-H), 2928 (C-H) aliphatic, 1603 (C=C), 1433 (C-H), 1373 (C-H), 1260 (C-O), 1169 (C-N), 1042, 944, 836

Analysis:

calculated	C: 74.72	H: 7.75	N: 3.96
found	C: 74.54	H: 7.92	N: 3.54

$C_{22}H_{27}NO_2$ (337.46)

6.3 X-ray crystallography

The co-crystallisation experiments have been carried out by *Mark J. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and *Daniel J. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

6.3.1 Enzyme production

The hyaluronate lyase from S. pneumoniae (Jedrzejas et al. 1998; Jedrzejas et al. 2000; Li et al. 2000; Jedrzejas 2001) was produced as described previously by *Jedrzejas et al.* (Jedrzejas et al. 1998). The enzyme was concentrated to 5 mg/ml in 10 mm Tris-HCl buffer (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol (DTT) using centrifugal spin devices with 50 kDa molecular weight cutoff (millipore). The hyaluronate lyase was used for the production of crystals with the inhibitor; the enzyme concentration was determined by UV absorption at 280 nm as described elsewhere (Pace et al. 1995; Jedrzejas et al. 1998).

6.3.2 Crystallisation of the complex

To grow the crystals of the enzyme-inhibitor complex the hanging drop vapour diffusion (McPherson 1999) using Linbro culture plates (HamptonResearch 2003) at room temperature was used. Equal volumes of protein, reservoir solution (1 µl each) and various amounts of inhibitor solutions (0.1, 0.5 and 1.0 µl) were mixed and equilibrated against 1 ml of the reservoir solution. The reservoir solution was prepared as described by *Jedrzejas et al.* (Jedrzejas et al. 1998) and contained 0.2 M NaCl, 2 % dioxane, 60 % - 65 % saturated ammonium sulphate and 0.1 M sodium citrate buffer (pH 6.0).

6.3.3 X-ray diffraction

The crystals of the enzyme-inhibitor complex were cryoprotected using 30 % xylitol (w/v), 3.5 M ammonium sulphate and 0.1 M sodium citrate buffer (pH 6.0) as reported for the native crystals (Li et al. 2000) and frozen in liquid nitrogen. To pick up and mount the frozen crystals under a nitrogen flow at -180 °C standard fiber loops (HamptonResearch 2003) of a suitable size were used. The X-ray diffraction for the inhibitor complex data was collected using rotation (oscillation) photography and Quantum 4u CCD detector. The crystallographic set-up of beamline 5.0.1 of the Berkeley Centre for Structural Biology, Advanced Light Source, Lawrence Berkeley National Laboratory was used. By using the HKL2000 software package (Otwinowski et al. 1997) the collected data were analysed, indexed, integrated, and scaled. The crystals were isomorphous to the native *S. pneumoniae* hyaluronate lyase crystals (Jedrzejas et al. 1998). The statistics of the native diffraction data were analysed.

6.3.4 Structure solution and refinement

The structure was solved by rigid body refinement with the programm CNS (Brunger et al. 1998) using the crystal structure of *S. pneumoniae* hyaluronate lyase in complex with palmitoyl vitamine C (Rigden et al., unpublished results) as a search model. Refinement proceeded with alternating rounds of computational refinement with CNS (Brunger et al. 1998) and manual rebuilding using the programm “O” (Jones et al. 1991). No intensity or sigma-based cut-offs were applied to the data. Sigma-A-weighted map coefficients (Read 1986) were used all the time. To monitor the progress of refinement the R_{free} value (Brunger 1992), calculated for a test set of 5 % of reflection, was used. Difference density at the active site was apparent even in initial maps. Whereas the aliphatic portion of the inhibitor was relatively easily placed, different orientations of the residual of the inhibitor had to be tested. The possibilities were evaluated by using the quality of electron density maps, B-factors and the R_{free} value as criteria. Sulphate and xylitol molecules, deriving from the crystallisation and cryo-

cooling solutions respectively, were modelled into suitably shaped regions of electron density. Final statistics for the model are shown in Table 5.

Programs of the CCP4 package (Collaborative Computational Project 1994) were used for manipulations and structural superpositions were made with LSQMAN (Kleywegt 1999).

Table 5: Crystallographic and refinement statistics

Space group		$P2_12_12_1$
Unit cell	a (Å)	84.20
	b (Å)	103.27
	c (Å)	103.26
Low resolution diffraction limit (Å)		44.0
High resolution diffraction limit (Å)		2/0
Non-hydrogen protein atoms		5824
Sulphate atoms		25
Non-hydrogen xylitol atoms		30
Non-hydrogen w249b atoms		70
Non-hydrogen solvent atoms		560
Number of reflections		27035 (621)
R (%)		17.8 (30.2)
*R*free (%)		20.2 (23.9)
Mean temperature factor *B* (Å)	All atoms	27.9
	Protein	26.3
	Protein main chain	25.8
	Protein side chain	27.4
	Sulphate	59.9
	Xylitol	52.3
	Inhibitor	81.6
	Solvent	33.5
r.m.s. deviation from ideal values	Bond lengths (Å)	0.006
	Bond angles (º)	1.3

6.4 Pharmacological methods

The inhibitory effect of the indole derivatives on the activity of hyaluronidases were determined by a turbidimetric assay according to the description of *Di Ferrante* (Di Ferrante 1956), as described in chapter 3.

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Chapter 10

Summary

Hyaluronan and hyaluronidases have been used in several medical fields for many years. For example, sodium hyaluronate is frequently used in the treatment of osteoarthritis; preparations of bovine testicular hyaluronidase have been applied therapeutically in the fields of internal medicine, ophthalmology and orthopaedia. Furthermore, it has been reported that some hyaluronidases play a role in of a variety of diseases, e.g. meningitis, septicaemia, arthroses or cancer. To prove the role and the importance of hyaluronic acid and hyaluronidases in physiological and pathophysiological processes, selective and potent hyaluronidase inhibitors are required. As such compounds are not known so far, the goal of this thesis was to identify and to synthesise lead-like compounds as inhibitors of bovine testicular hyaluronidase and *S. agalactiae* hyaluronate lyase, to optimise the structures and to study the structure-activity relationships. Additionally, a crystal structure of an enzyme-inhibitor complex was envisaged to determine the binding mode of hyaluronate lyase inhibitors.

Starting from the report that heparin and heparan, structurally similar to hyaluronic acid, are hyaluronidase inhibitors, a set of sulphated and non-sulphated oligosaccharides with three to eight sugar monomers as well as two sugar units and a phenyl ring were investigated. The results obtained in the present study suggest that sulphated sugar derivatives are useful as inhibitors of hyaluronidase, whereas the basic structures lacking sulphate groups are inactive. Furthermore, the inhibitory potency strongly depends on the degree of sulphation: the higher the degree of sulphation the higher the inhibitory activity. As inhibitors of the bovine testicular and the bee venom hyaluronidase sulphated tri-, tetra-, pentasaccharides and/or sulphated disaccharides with a phenyl moiety were the most active compounds. By contrast, in case of the hyaluronate lyase, the highest activity was found for the sulphated octasaccharide.

Based on mechanistical studies of hyaluronan degradation by hyaluronate lyases, we designed a substrate analogue as potential inhibitor consisting of three carbohydrate units including a fluorinated glucuronic acid as central building block. The synthesis of the pertinent protected *N*-acetylglucosamine was successful, whereas the attempts to synthesise the fluorinated glucuronic acid failed. As the problems with the fluorination of hex-4-enopyranoside and hex-5-enopyranoside derivatives were not conquerable an alternative strategy should be envisaged, for instance, the synthesis of non-carbohydrate hyaluronidase inhibitors.

Due to inconsistent results published by different authors, we investigated several assumed inhibitors like disodium cromoglycate, apigenin, kaempferol and silybinin on the bovine testicular hyaluronidase to obtain comparable data determined under the same conditions. To further elaborate the structure-activity relationships some flavones (natural compounds) and sulphated flavones were measured. Subsequently, a variety of new chalcone and flavone derivatives with different substituents and substitution patterns was synthesised and pharmacologically investigated. Three chalcone and one flavone derivative induced inhibition of the bovine testicular hyaluronidase at millimolar concentrations. Sodium flavone-7-sulphate and sodium 5-hydroxyflavone-7-sulphate were the most potent bovine testicular hyaluronidase inhibitors with micromolar inhibitory activity. As hyaluronate lyase inhibitors five chalcone and four flavone derivatives were identified, whereby 4'-chloro-4,6-dimethoxychalcone was the most potent hyaluronate lyase inhibitor achieving micromolar inhibitory activity.

As part of the hyaluronate lyase project, the main focus of this thesis was the identification and evaluation of promising lead-like compounds based on a *de novo* design approach. The virtual screening of three different databases with the computer program LUDI resulted in 1275 hit. Of all 19 selected compounds, 13 molecules revealed inhibitory activity on the bacterial hyaluronidase and 5 compounds on the bovine testicular hyaluronidase. 1,3-Diacetylbenzimidazole-2-thione has an IC_{50} value in the micromolar range and is therefore the most potent hyaluronate lyase inhibitor. The results demonstrate that the chosen

strategy based on a homology model of the hyaluronan lyase $hylB_{4755}$ is useful to identify promising leads.

Structural modifications of *de novo* design lead-like structures resulted in new hyaluronidase inhibitors: the diphenylacrylic and diphenylpropionic acids. Within the series of the examined compounds the brominated diphenylacrylic and diphenylpropionic acid as well as 3-(4-trifluoromethyl-phenyl)-3-phenylpropionic acid were the most potent inhibitors on the bovine testicular hyaluronidase and the bacterial enzyme.

As indole-2-carboxylic acid was identified as a compound with millimolar enzyme inhibitory activity a set of indoles with different substituents like acetic acid, acetyl, carboxamide, butyric acid, methyl etc. as well as a series of 2-phenylindole derivatives with hydroxy, methoxy, methyl and/or sulphamoyloxy substituents at the phenyl and indole rings and different substituents at the nitrogen atom was investigated. Generally, the phenylindoles were more active on the bacterial enzyme than on the bovine testicular hyaluronidase. Only the compounds lacking the N-substituent exhibited weak activity on the bovine testicular enzyme. Structure-activity relationship studies revealed that a hydroxy group in position 6 is more favourable than in position 5. Furthermore, the introduction of a methyl group in position 3 and a 5 to 10-membered alkyl chain in position 1 leads to an increase in activity and selectivity for the bacterial vs. the bovine enzyme. Compared to the first lead-like compound, indole-2-carboxylic acid, the increase in lipophilicity by introducing phenyl and alkyl substituents and the introduction of hydroxy and/or sulphamoyloxy groups at the phenyl and indole ring resulted in about 500-fold more potent inhibitors with remarkable selectivity for hyaluronan lyases.

On the basis of the X-ray analysis[1] of *S. pneumoniae* hyaluronate lyase in complex with 6-sulphamoyloxy-2-(4-sulphamoyloxyphenyl)-1-decylindole we were able to determine the binding mode of the inhibitor. The phenylindole binds within the catalytic site of the enzyme whereby hydrophobic interactions

[1] in co-operation with *Mark J. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and *Daniel J. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

with the amino acids Trp292, Trp291, His399 and Met579 and hydrogen bonds with Asn466, Ser463, Asp352, Asn349 and Trp292 are involved.

In summary, ligand-based and structure-based approaches led to the identification of hyaluronidase inhibitors with micromolar inhibitory activity. Especially, by *de novo* design we achieved potent hyaluronate lyase inhibitors which can be used as pharmacological tools to study the role of hyaluronic acid and hyaluronidases in physiological and pathophysiological processes.

List of abbreviations

Ac	acetyl
Bn	benzyl
BSA	bovine serum albumin
BTH	bovine testicular hyaluronidase
BVH	bee venom hyaluronidase
CDI	*N,N'*-carbonyldiimidazole
CTAB	cetyltrimethylammonium bromide
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMAP	p-dimethylaminobenzaldehyd
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulphoxide
GluNAc	*N*-acetyl-D-glucosamine
h	hour
HA	hyaluronic acid
HMPT	hexamethylphosphoric triamide
$hylB_{3502}$	*S. agalactiae* hyaluronate lyases strain 3502
$hylB_{4755}$	*S. agalactiae* hyaluronate lyases strain 4755
hylSpn	*S. pneumoniae* hyaluronate lyases
IC_{50}	concentration of inhibitor giving a 50% decrease of the enzyme activity
IU	international units
min	minutes
Mp	melting point
MS	mass spectrometry
MW	molecular weight
n.d.	not determined
NMR	nuclear magnetic resonance
PG	protecting group
pH	negative logarithm of the hydrogen ion concentration
Ph	phenyl
ppm	parts per million
RT	room temperature
THF	tetrahydrofuran
TMS	(nuclear magnetic resonance) tetramethyl silane

Ts	tosyl (4-methylphenylsulfonyl)
UV	ultra violet

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