Synthesis and structure-activity relationships of inhibitors of bacterial hyaluronidase: An approach to obtain compounds with drug-like properties

Dissertation

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Für meinen Vater Stefan Meyer (1945 – † 2002)*

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Contents

1 Introduction

1.1 Hyaluronic acid

1.1.1 Structure and physicochemical properties

Hyaluronic acid (hyaluronan) was discovered in the vitreous humor of the eye by Meyer and Palmer in 1934 and subsequently found in most parts of the body, including the synovial fluid of joints and the skin. The proposed name “hyaluronic acid” derives from hyaloid (vitreous) and the constituent uronic acid.[1] The structure of hyaluronan was also determined by Meyer and coworkers, but not before the 1950s.[2] Hyaluronan is a linear high molecular weight polysaccharide and belongs to the family of glycosaminoglycans (GAG). It consists of repeating disaccharides comprised of β-1,3 linked *N*-acetyl-D-glucosamine (GlcNAc) and glucuronic acid (GlcUA) which are connected by β-1,4 glycosidic bonds.[3] The structure of hyaluronic acid is displayed in Figure 1.1.

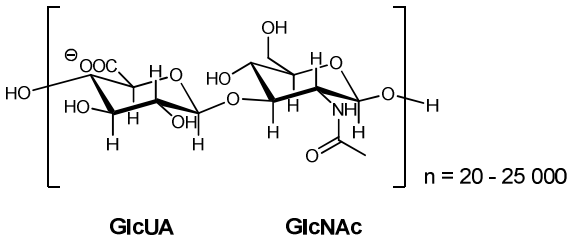

Figure 1.1 Chemical structure of hyaluronic acid (HA).

Hyaluronan consists of 20-25,000 disaccharide units, corresponding to a polymer length of 2-25 µm. The hyaluronic acid (HA) polymers are among the largest matrix molecules with molecular weights between 10^6 to 10^7 Da.[4] The apparent size of hyaluronan is even greater because the polymer is able to incorporate a large volume of water into its solvent domain that is 1000 times greater than the original volume.[5] In the body, hyaluronan occurs in salt form and of highly negative charge because the carboxylic groups of the glucuronic acid moieties are deprotonated under physiological conditions (pK_a 3-4).[6] Hence, this macromolecule is most frequently referred to as “hyaluronan”.

X-ray diffraction and NMR experiments in dimethyl sulfoxide (DMSO) confirmed that the secondary structure of hyaluronan is a tape-like, two-fold helix.[7-10] Recent NMR and computer modeling studies by Almondet al. suggest a dynamic local confirmation, which is on average a contracted 4-fold helix.[11] In general, the backbone of the hyaluronan molecule is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interaction with the solvent.[12] Therefore, in solution, the hyaluronan polymer

adopts the shape of an expanded, random coil with reversible formation and breakdown of interactions between the hyaluronan strains.[6,13,14]

1.1.2 Hyaluronan metabolism and physiological functions

Compared to other members of the glycosaminoglycan family, hyaluronan differs in several respects: it is the only GAG that is non-sulfated, it is synthesized at the cytoplasmic surface of the plasma membrane as a free linear polymer, not in the Golgi apparatus and, therefore, is not covalently linked to a protein core.[3,4,15,16] Hyaluronan is synthesized at the inner face of the plasma membrane by one of the three membrane-bound hyaluronan synthases, namely HAS1, HAS2 or HAS3.[17] The cytoplasmic product is extruded through the plasma membrane into the extracellular matrix (ECM) permitting unconstrained polymer growth.[18,19]

Hyaluronic acid is a major constituent of the extracellular matrix (ECM) of most tissues. It is found at high concentrations in rooster comb and several soft connective tissues, including skin, umbilical cord, synovial fluid, and vitreous humor.[3,20] Hyaluronan can form highly viscous solutions and, thereby, influence the properties of this matrix.[21] It plays an important role in various physiological and pathophysiological processes, such as embryonic development and morphogenesis[22], wound healing[23-25], repair and regeneration, inflammation[26-28] and tumorigenesis[29-31]. Moreover, hyaluronan interacts with cell-surface receptors, such as CD44 (cluster of differentiation 44) and RHAMM (receptor for hyaluronic-acid-mediated motility) that are associated with signal transduction and may help with cell motility and adhesion.[32,33]

1.2 Hyaluronidases

1.2.1 Occurrence and classification

Hyaluronidases are a group of enzymes that degrade hyaluronan. They were first discovered in an extract of mammalian testes and other tissues by Duran-Reynals at the beginning of the last century. This so called “spreading factor”, which facilitated the diffusion of dyes and antiviral vaccines, was later named hyaluronidase by Karl Meyer to denote enzymes that degrade hyaluronan.[34,35] The hyaluronidases are widely distributed in the animal kingdom and can be found in mammals, invertebrate animals (crustaceans, leeches, and insects),

animal venoms, bacteria (e.g. *Streptomyces*) and bacteriophages, and in pathogenic fungi (e.g. *Candida*).[21,35]

Based on their catalytic mechanism, the hyaluronidases can be divided into three groups (Figure 1.2).[36,37]

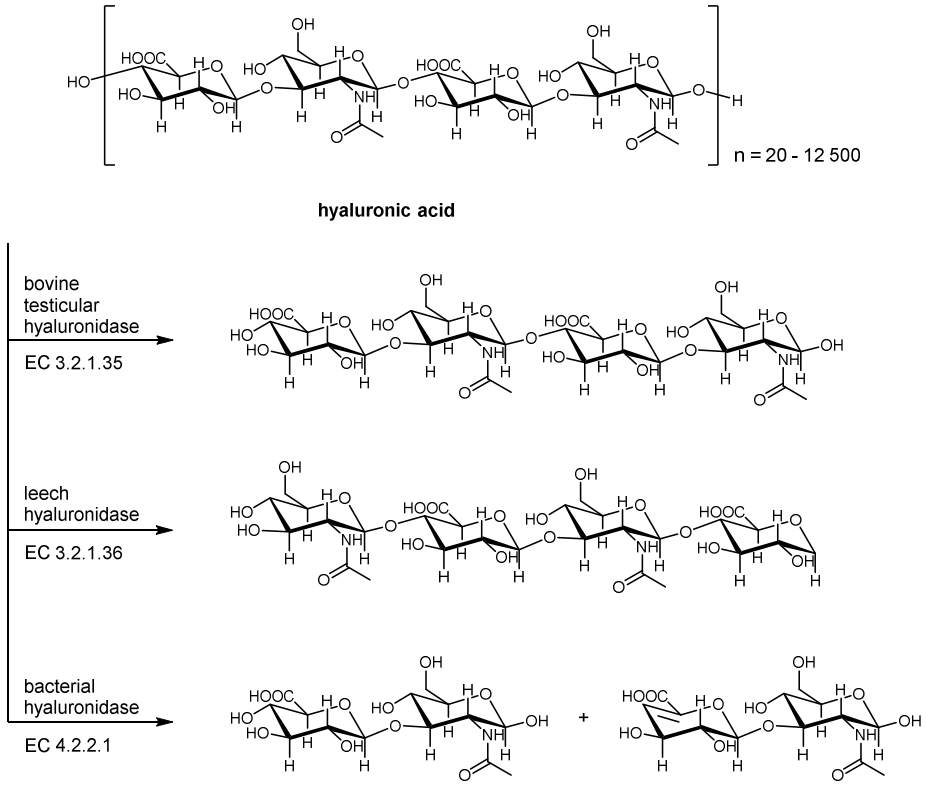

Figure 1.2 Classification of hyaluronidase according to Meyer.[36] Adopted from Muckenschnabel.[38]

The hyaluronidases from the first group (EC 3.2.1.35) are endo-β-*N*-acetyl-D-hexosaminidases that cleave the β-1,4-glycosidic linkages in hyaluronan to yield tetra- and hexasaccharides as the major final products with *N*-acetylglucosamine at the reducing end. Enzymes of this class can be found in mammalian spermatozoa, lysosomes, and the venoms of snakes and hymenoptera.[21,39,40] The second group (EC 3.2.1.36) includes the endo-β-glucuronidases, which are present in leeches and in certain crustaceans.[41,42] These enzymes cleave the β-1,3-glycosidic bond in hyaluronan and yield tetra- and hexasaccharides as the main end-products with glucuronic acid at the reducing end.[35] The third group (EC 4.2.2.1) includes the microbial hyaluronidases, which act as endo-*N*-acetyl-D-hexosaminidases. These enzymes cleave the β-1,4-glycosidic linkage by a β-elimination resulting in unsaturated disaccharides as products of exhaustive degradation of hyaluronan. Based on their catalytic mechanism, these enzymes were also termed hyaluronate lyases. According to

their catalytic mechanism, the latter must be clearly distinguished from the other two groups.[35,43-45]

1.2.2 Hyaluronidases from eukaryotes

In humans, there are six hyaluronidase-like sequences, five of them encoding functionally active enzymes, the hyaluronidases Hyal-1, Hyal-2, Hyal-3, Hyal-4 and PH-20 (SPAM1), whereas the pseudogene *hyalp1* is transcribed, but not translated.[46,47] The sequence similarity between the six subtypes varies from 33 % to 42 %.[37] Hyal-1 was detected in mammalian plasma and urine, as well as at high levels in the liver, kidney, spleen, and heart and is localized in the lysosomes.[48] Hyal-2 is present in many tissues, except the adult brain. It is either localized in the lysosome or anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) link.[49] Hyal-1 and Hyal-2 are the major hyaluronidases and have a pronounced activity optimum at pH 4. [48,50,51] However, the expression of Hyal-2 is extremely low and difficult to purify.[52] The GPI-anchored PH-20 protein (SPAM1 sperm adhesion molecule) is located on the surface of mammalian sperm to facilitate penetration of the sperm through the hyaluronan-rich matrix of the oocyte.[53]

The major soluble hyaluronidase present in bull testes extract, the bovine testicular hyaluronidase (BTH), which has been therapeutically applied as a spreading factor in several medical fields for many years, has been shown to be a fragment of the membrane bound PH-20 enzyme.[54,55]

Hyaluronidase activity has been detected in the venom of many animals, such as snakes[56,57], stonefish[58], scorpions[59,60], spiders[61], lizards,[62] and caterpillars[63], in which they predominantly serve as “spreading factor” but have no intrinsic toxic activity.[45] By degrading the hyaluronan of the extracellular matrix of the prey, local tissue damage is caused, which facilitates the distribution of other venom constituents. The hyaluronidase from bee venom was the first to be characterized in detail.[64] Hyaluronidases in snake venom have been recognized as possible targets for the treatment of snake bites. Therefore, the venoms from two different snake species will be characterized in chapter 7.

1.2.3 Hyaluronidases from prokaryotes

Hyaluronidases are produced by many different genera of bacteria. Proteins or enzymes found on the surface of gram-positive organisms significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens. Human infections caused by gram-positive bacteria are increasingly difficult to treat, predominantly due to the emergence of antibiotic-resistant strains not only against penicillin but also against novel antibiotics such as vancomycin.[65,66] The HA lyases produced by gram-positive bacteria are considered to act as virulence factors facilitating the spread of pathogens or toxins by breaking down the components of the extracellular matrix of the host.[67] The enhanced tissue-permeability, caused by hyaluronidase-mediated degradation of HA, appears to play a role in many health problems such as gangrene, meningitis, synovitis, hyperplasia, nephritis, mycoplasmosis, periodontal disease, mastitis, pneumonia, septicemia, syphilis, toxic shock syndrome, and wound infections.[68-72]

Gram-positive microorganisms capable of producing HA lyases include various species of *Spreptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium*, *Streptomyces,* and *Clostridium*.[73-77] The HA lyases produced by gram-negative bacteria are not excreted to the extracellular matrix and, therefore, are less likely to play a role in pathogenesis.[35,67,78]

By reducing the spreading of the pathogen, the inhibition of the hyaluronate lyases could be an approach to the treatment of bacterial infections.[79] In this context, the HA lyases from *S. agalactiae* strain 4755 (*Sag*Hyal$_{4755}$) and *S. pneumoniae* (*Spn*Hyl) are the best characterized among the microbial hyaluronidases.[80-82] *S. pneumoniae* predominantly colonizes the upper respiratory tract of humans and is a major human pathogenic bacterium. It causes life-threatening diseases, examples of which are pneumonia, bacteremia, and meningitis, as well as less threatening diseases, such as otitis media and sinusitis. Young children and the elderly are particularly prone to these diseases.[82] The currently licensed pneumococcal vaccine is only moderately effective and it is not prescribed for children younger than two years.[65,83] The bacterium *S. agalactiae* (group B streptococcus, GBS) is part of the normal human flora but has been an important cause of infection in newborn and young infants.[84,85] Human infections by this gram-positive bacterium are meningitis and septicemia, which result in considerable morbidity and mortality.[86] In cultures of *S. pneumoniae* and *S. agalactiae* the HA lyase is found both in the culture and in cell-associated fractions, which suggests that at least part of the enzyme is released by the pathogen to surrounding host tissues during infection in order to facilitate bacterial invasion.[74,81]

The native hyaluronidases from both *S. agalactiae* and *S. pneumoniae* have molecular masses of 111 kDa and 107 kDa, respectively, but undergo autocatalytic conversion to smaller enzymatically active 92 kDa and 82 kDa forms.[81,87] X-ray crystallography revealed that the structures of the HA lyases from *S. agalactiae* and *S. pneumoniae* are similar regarding the architecture of the entire enzyme as well as the geometry of the active site, sharing a similarity in the range of 65 % to 80 %.[37,44,65] The active site of the lyases are composed of two main parts, a catalytic group responsible for substrate degradation and an aromatic patch responsible for the selection of cleavage sites on the substrate chains.[44] In the catalytic cleft three catalytic residues, Asn[429], His[476], and Tyr[488] (*Sag*Hyal$_{4755}$ numbering[88]), are located, which degrade the substrate through a proton acceptance and donation (PAD) mechanism (Figure 1.3).[44,71,82]

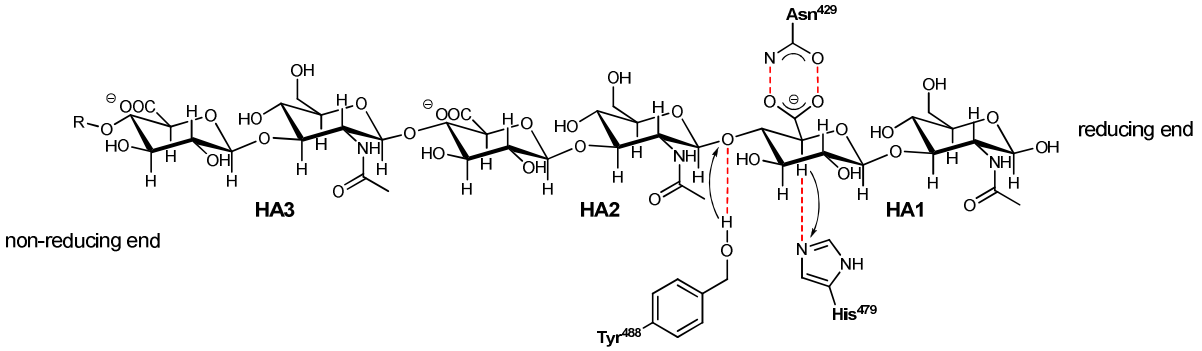

Figure 1.3 Mechanism of hyaluronan degradation by hyaluronate lyase from *S. agalactiae* (*Sag*Hyal$_{4755}$); schematic presentation of hyaluronan with HA1, HA2 and HA3 as disaccharide units and the position of the side chains Tyr[488], His[479] and Asn[429] relative to the substrate (modified from Li and Jedrzejas).[71,82]

The degradation process includes five different steps: (1) enzyme binding to negatively charged hyaluronan; (2) cleaving of β-1,4-glycosidic bond; (3) hydrogen exchange between enzyme and microenvironment; (4) release of disaccharide product; (5) translocation of the remaining HA by one disaccharide unit towards the reducing end.[65,89,90] The truncated polymeric hyaluronan stays in the cleft after the release of the disaccharide and undergoes a processive mode of action until the chain is fully degraded into disaccharides.[91] However, Kühn et al. proposed a nonprocessive mode of action for *Sag*Hyal$_{4755}$, based upon the observation that hyaluronan degradation by *Sag*Hyal$_{4755}$ yields a mixture of oligosaccharides and hyaluronan fragments of different sizes.[92]

1.2.4 Hyaluronidase inhibitors

In general, the role of hyaluronan and hyaluronan degrading enzymes, the hyaluronidases, is far from being understood. Potent and selective inhibitors for the hyaluronidases are required as pharmacological tools to further characterize and understand the physiological and pathophysiological role of hyaluronidases. Furthermore, from a therapeutic point of view, inhibitors might be useful in developing anti-bacterial[67], anti-venom/toxin[35], anti-tumor[93], anti-allergic agents,[93] or even as contraceptives.[94] An overview of all previously reported putative inhibitors of hyaluronidases was given by Girish in 2009.[77] Documented hyaluronidase inhibitors are of different chemical nature. The presence of proteins as hyaluronidase inhibitors in human serum was thought to be a defense mechanism against venoms and pathogenic bacteria.[95-97] A purified high molecular mass, thermolabile magnesium dependent glycoprotein from mouse serum, probably belonging to the inter-α-inhibitor family, was reported to be an inhibitor of bovine, testicular, snake and bee venom hyaluronidase.[98] The inhibitory activity of diverse metal ions, among them being iron, cadmium, copper, or zinc salts was reported long ago.[99] Based on substrate similarity, several glycosaminoglycans (GAGs) and polysaccharides were identified as hyaluronidase inhibitors. Heparin and heparan sulfate were observed to inhibit hyaluronidase, but only at concentrations much higher than physiological levels and in a non-competitive manner.[100-102] Other described structurally related compounds were oxygen-sulfated GAGs and oxygen-sulfated fragments of hyaluronan.[103] Toida et al. discovered that the *O*-sulfated hyaluronan fragments showed stronger inhibition than other *O*-sulfated GAGs. Furthermore, not only did the inhibition increase with the chain length of the hyaluronan oligomer, but these compounds also showed both competitive and non-competitive inhibition, in contrast to heparin.[104] Chitosan was also characterized as an inhibitor with direct correlation between molecular weight and inhibitory activity.[105] Alginic acid, which is also structurally related to hyaluronan, consists of L-glucuronic and D-mannuronic acid and shows molecular weight dependent inhibition of hyaluronidase.[106] The synthetic condensation polymer of mandelic acid is reported to inhibit hyaluronidase.[94]

A lot of low molecular weight natural products, such as: alkaloids[107,108], flavones and flavone-analogs[60,109-114], and terpenes[115,116] have been investigated for hyaluronidase inhibition. Other substances reported to show weak inhibition of hyaluronidase activity are antioxidants[79,117] and polyphenols[118], non-saturated fatty acids[119], antibiotics[120], and lanostanoids.[121] Moreover, anti-allergic drugs (e.g. disodium cromoglycate, DSCG)[111,122] and anti-inflammatory drugs (cf. chapter 3.1) are claimed to possess hyaluronidase inhibitory activities. The research in our laboratories has led to the discovery and validation of potent inhibitors of hyaluronidases. For example, lipophilic derivatives of L-ascorbic acid (vitamin C) were developed, which are

among the most potent inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ known today.[123-126] A variety of heterocycles, such as benzimidazoles, benzoxazoles, indoles, phenylindoles and gluconolactones have been identified as potent hyaluronidase inhibitors.[127-136] The aforementioned compounds are not drug-like mainly due to very high plasma protein binding, high molecular weights (> 500 g/mol), low bioavailability, or bioincompatibility (e.g. heparin). Because of the important role of hyaluronan in biological systems, potent inhibitors of hyaluronidases are needed to further investigate the role of hyaluronan and hyaluronidases in physiological and pathophysiological processes.

1.3 Methods for the determination of hyaluronidase activity

Since the discovery of the hyaluronidases by Duran-Reynals in 1928[137], various methods for the determination of the enzymatic activity have been described. In 1994 Hynes and Ferretti[138] summarized previously described test systems and divided them into groups based upon the assay performed, namely spectrophotometric[139,140], radiochemical[141], fluorogenic[142], enzymoimmunological[143], plate (solid media)[144], chemical[145,146], physicochemical[145,147,148], and zymographic[137,149] methods. Although many different methods for the determination of hyaluronidase activity can be found in the literature, only few can be deployed for routine analyses. Many of the early developed assays lack sensitivity or the workup procedures are rather laborious and time-consuming. M. Stern and R. Stern[150] discussed the advantages and limitations of the conventional methods in an article about a new ELISA-like assay for hyaluronidase and hyaluronidase inhibitors. A lot of assay techniques are based upon changes in viscosity[151] and turbidity[152]. In 1944 the turbidimetric assay was described by Kass and Seastone[153], based upon the observation of Meyer and Palmer[154] that hyaluronan forms insoluble complexes with acidified blood serum. It was further developed by Dorfman and Ott[152], as well as Di Ferrante.[148] In this thesis, a colorimetric assay, which was developed by Reissig et al.[146], and the turbidimetric assay are routinely exploited to determine the inhibitory effect of the synthesized compounds. Inhibitory activity was determined for the bacterial hyaluronate lyases from *S. agalactiae* strain 4755 (*Sag*Hyal$_{4755}$), *S. pneumoniae* (*Spn*Hyl), and the bovine testicular enzyme BTH (formerly an approved drug; e.g. under the trade name Neopermease®). Both assay systems are briefly explained in the following sections.

1.3.1 Morgan-Elson assay

The Morgan-Elson assay is a colorimetric assay and has been classified as a chemical assay by Hynes and Ferretti.[138] The reducing ends of *N*-acetylhexosamine moieties form a red colored product after reaction with *p*-(dimethylamino)benzaldehyde (Ehrlich's reagent; DMAB), as described by Reissig[146] and Gacessa.[155] This so called Morgan-Elson reaction is often used to determine the activity of hyaluronidases that liberate hyaluronan fragments with *N*-acetyl-D-glucosamine at the reducing end. The photometrical detection of the red-colored product at a wavelength of 586 nm enables the determination of the enzymatic activity. The classes of hyaluronidases that can be assayed by the Morgan-Elson reaction are the hyaluronate-4-glycanohydrolases (EC 3.2.35) and hyaluronate lyases (EC 4.2.2.1), as described in section 1.2.

The reaction, which was investigated and described in detail by Muckenschnabel et al.,[140] is shown in Figure 1.4. After incubation of the hyaluronidase at 37 °C the cleaved hyaluronan is heated to 100 °C under basic conditions (pH 9). The furanose form of *N*-acetyl-D-glucosamine residues is converted to the chromogens I (*α*-configuration) and II (*β*-configuration) after elimination of ROH (cf. Figure 1.4).[156,157] Subsequent treatment with concentrated hydrochloric acid (HCl) and glacial acetic acid results in elimination of water yielding chromogen III. The final red colored product, mesomeric forms of N^3-protonated 3-acetylimino-2-(4-dimethylaminophenyl)-methylidene-5-(1,2-dihydroxyethyl)furane, is obtained after the chromogen III reacts with *p*-(dimethylamino)benzaldehyde (Ehrlich's reagent).

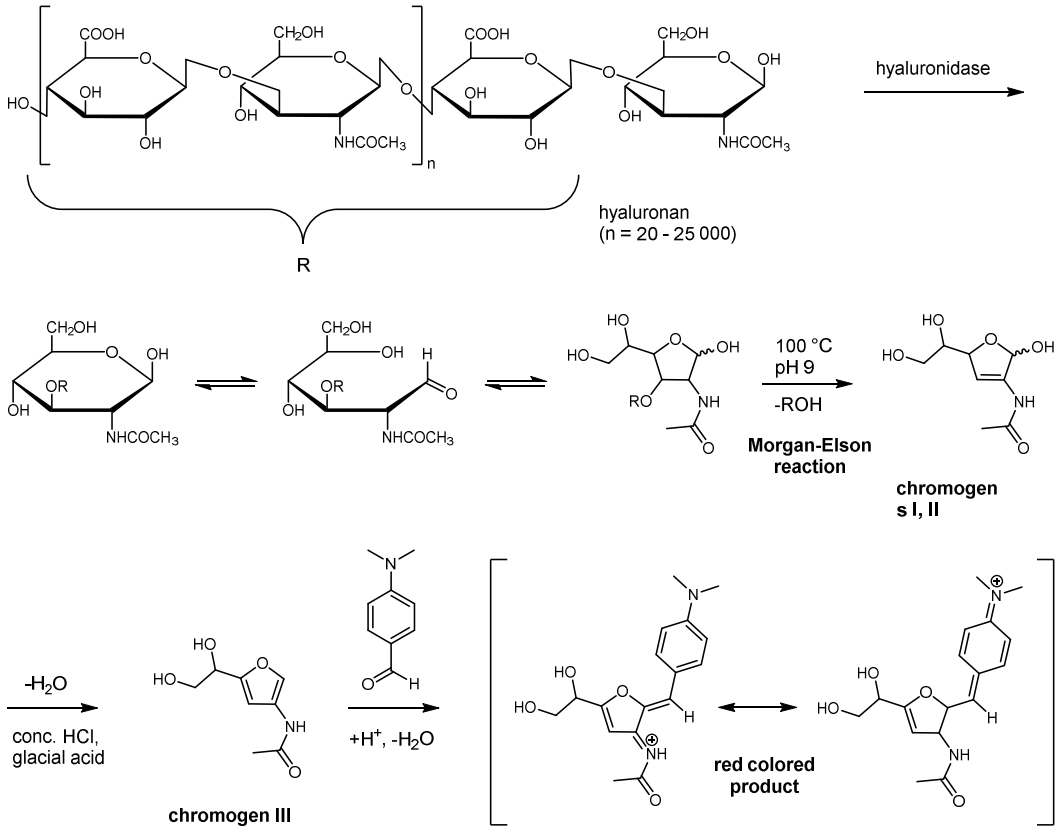

Figure 1.4 Mechanism of the Morgan-Elson reaction resulting in a red colored product according to Muckenschnabel et al.[140]

The Morgan-Elson assay represents a useful, widely applicable and well reproducible method for the determination of hyaluronidase activity in the presence of inhibitors. However, in some cases the Morgan-Elson assay cannot be operated. If the putative inhibitor is colored or undergoes side-reactions with DMAB the photometric detection produces false-negative results. Heterocycles, such as indoles or phenylindoles, which were identified to be potent inhibitors of *Sag*Hyal$_{4755}$, often form colored products under assay conditions. Pindur and coworkers extensively investigated the reaction of 3-methyl-1*H*-indole with *p*-(dimethylamino)benzaldehyde. They were able to show that the reaction follows the mechanism proposed by van Urk, resulting in intensively colored products.[136,158,159] The yellow-colored indole derivative indomethacin, which is a non-steroidal anti-inflammatory drug, is also known as an inhibitor of *Sag*Hyal$_{4755}$. It undergoes the van Urk-reaction providing colored products, rendering the application of the Morgan-Elson assay impossible.

The Morgan-Elson assay can be used to quantify hyaluronidase activity according to the definition of the International Union of Biochemistry by defining 1 unit (U) as the amount of enzyme that catalyzes the liberation of 1 µmol *N*-acetyl-D-glucosamine at the reducing ends of sugars per minute under specified conditions.[160]

1.3.2 Turbidimetric assay

According to the classification by Hynes and Ferretti[138], the turbidimetric assay represents a physicochemical assay. This method, as described by Di Ferrante[148], is based upon the formation of insoluble complexes between non-degraded, high-molecular hyaluronan (mw > 8 kDa) and cetrimonium bromide (cetyltrimethylammonium bromide, CTAB). In contrast, a solution of oligosaccharides and smaller hyaluronan fragments (mw < 6-8 kDa) remains clear under the same conditions. Since the formed turbidity is proportional to the amount of high molecular weight hyaluronan fragments, the enzymatic activity can be quantified by photometric detection at 580 nm and 600 nm by means of reference samples (Figure 1.5).

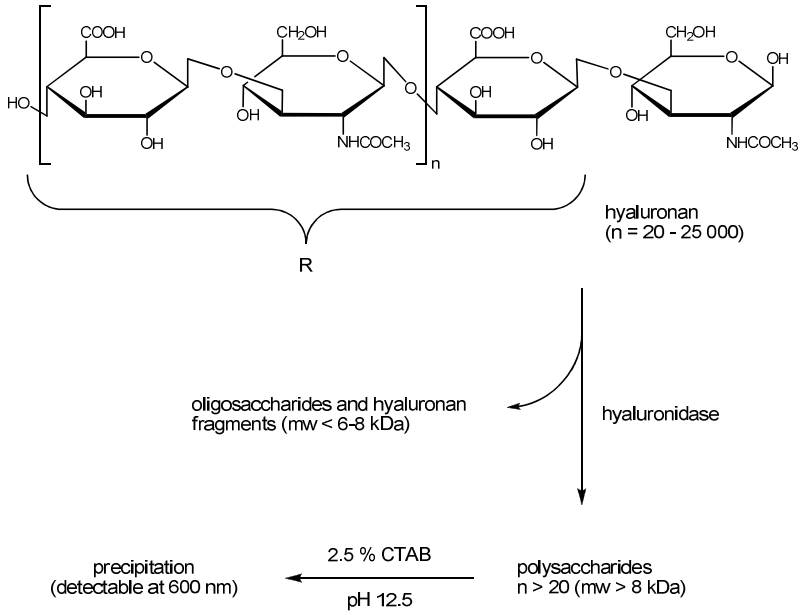

Figure 1.5 Principle of the turbidimetric assay based on the method of Di Ferrante[148]. Figure adopted from Braun.[131]

This assay is fast, highly reproducible, easy to perform in cuvettes or microtiter plates and can be considered as the standard method for the determination of hyaluronidase activity and enzyme inhibition of the synthesized compounds in this thesis.

1.4 Influence of the pH value on enzymatic activity

The activities of the different hyaluronidases are dependent on the pH value. Hoechstetter[160] and Hofinger[161] studied the pH dependent activity profiles of BTH and *Sag*Hyal$_{4755}$. It is not possible to find a pH value, where all investigated enzymes show high activity. The bacterial

hyaluronate lyase shows highest activity at pH 5.0. Jedrzejas[87] reported a pH 6 as the optimum for *Spn*Hyl. Figure 1.6 shows the pH dependent activity of *Spn*Hyl as determined in the turbidimetric and Morgan-Elson assay.

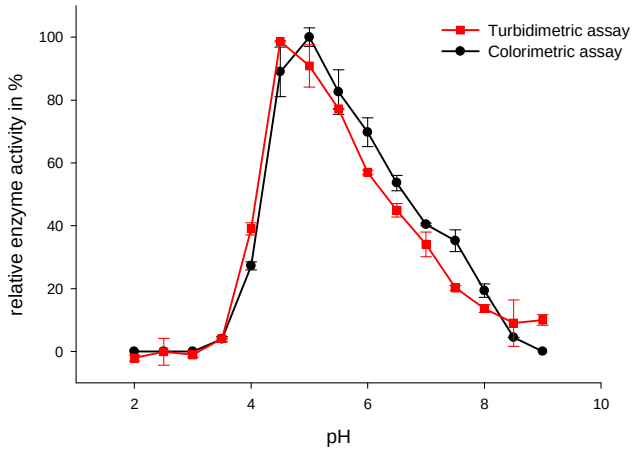

Figure 1.6 pH dependent activity of bacterial hyaluronate lyase from *Streptococcus pneumoniae* (*Spn*Hyl) as determined in the turbidimetric assay (red) and the Morgan-Elson assay (black).

The results differ from those reported by Jedrzejas. The pH values between 4.5 and 5.0 were determined to be the optimum for hyaluronidase activity. To obtain comparable IC_{50} values on BTH and *Spn*Hyl the inhibitors were investigated at pH 5.0 as these enzymes show considerably high activity under these conditions in the turbidimetric assay. Thus, the obtained IC_{50} values are comparable to previously gained data from our workgroup (cf. investigations by Binder[126], Braun[131], Salmen[127], Spickenreither[124], and Textor[136]).

Hyaluronan and hyaluronan degrading enzymes, the hyaluronidases, are widespread in nature and were both described in the middle of the last century. However, the role of both is far from being understood and the academic research remains a challenging field.[1,147] The hyaluronidases have been a group of less extensively studied glycosidases.[21,77] Inhibitors of hyaluronidases are required as pharmacological tools to investigate their complex functions in hyaluronan metabolism. Moreover, such substances are of potential therapeutic value, e. g. for the treatment of bacterial infections or snake bites.[79,108]

The research in our laboratory has led to the discovery of potent inhibitors with a preference for the bacterial enzymes. For example, lipophilic derivatives of L-ascorbic acid (vitamin C) were developed, which are among the most potent inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ known today.[123-126] Moreover, potent hyaluronidase inhibitors were identified among a variety of heterocycles (cf. section 0).[127-136] Generally, the potency of hyaluronidase

inhibition correlates with the lipophilicity of the compounds, which strongly affects the drug-like properties, due to very high plasma protein binding. Although (weak) inhibitors of the bacterial hyaluronidases have been discovered among natural products and synthetic molecules, small drug-like molecules with sufficient inhibitory potency are not available at present.[77,162,163] To cope with this problem, the focus of this thesis was set on the design, synthesis and characterization of inhibitors for the bacterial hyaluronidase *Sag*Hyal$_{4755}$ with drug-like properties.

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2 Scope and Objectives

Hyaluronidases can be ubiquitously found in animals and microorganisms. They play an important role as a spreading factor in animal venoms and are thought to act as virulence factors of pathogenic bacteria. Potent and selective inhibitors of hyaluronidases are required as pharmacological tools to study the physiological and pathophysiological role of hyaluronan and hyaluronidases in detail. Furthermore, such substances are of potential therapeutic value for the treatment of a variety of diseases, such as cancer, bacterial infections, or bites of venomous animals, for example snake bites.

Hyaluronan has amphiphilic properties due to the axial hydrogen atoms that contribute to the hydrophobic patch and the negatively charged carboxylic groups of glucuronic acid, which form a hydrophilic face. Compounds bearing acidic functional groups and long, lipophilic alkyl residues have been identified among the most potent inhibitors of bacterial hyaluronidase from *Streptococcus agalactiae* (*Sag*Hyal$_{4755}$) with IC_{50} values in the lower micromolar range (cf. vitamin C palmitate: IC_{50} = 4 µM). However, the high degree of lipophilicity, which is characteristic of these compounds, is unfavorable in terms of drug-like properties and with respect to *in vivo* investigations, for example due to a high degree of plasma protein binding.

The aim of this thesis was the design, synthesis, and characterization of novel inhibitors for the bacterial hyaluronate lyase from *Streptococcus agalactiae* strain 4755 (*Sag*Hyal$_{4755}$) with focus on drug-like properties of these substances. The search for inhibitors was extended on approved, commercially available, non-steroidal, anti-inflammatory drugs (NSAIDs), as several NSAIDs were purported to have some hyaluronidase inhibiting activities.

Indoles, in particular *N*-alkylated and hydroxylated 2-phenylindoles, were previously identified as potent inhibitors of bacterial hyaluronidases in our laboratory (cf. 4-{[6,7-dichloro-2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-1-yl]methyl}benzoic acid (UR-CT619) IC_{50} = 9 µM). Therefore, the indole scaffold was further exploited for the development of novel inhibitors. In addition, the synthesis and the investigation of the inhibitory activity of a series of oxadiazol-2-yl- and 2-carboxy-substituted indoles were objectives of this thesis.

Bioisosteric approaches represent fundamental strategies in medicinal chemistry for the design of new lead compounds. Based upon a computer-assisted prediction of indolizines as inhibitors of the bacterial hyaluronidase, another project of this thesis is focused on the synthesis and investigation of a small library of 2-phenylindolizines.

Snake venom hyaluronidases represent a possible therapeutic target in the treatment of snake bites. Therefore, the final part of this thesis deals with the characterization of snake venom from two different snake species with regard to hyaluronidase activity.

3 Screening of commercially available drugs for hyaluronidase inhibition

3.1 Introduction

Recently, Girish et al. (2009) published a comprehensive though uncritical review on the biological and therapeutic perspective of inhibitors of hyaluronidases.[1] A comparison of the published data from various sources (percent inhibition and IC_{50} values) is difficult due to differences in the applied test systems and assay protocols (e.g. incubation conditions, enzymes, enzyme concentrations, and substrate concentrations). However, according to this survey, inhibitors of bacterial, mammalian, and snake venom hyaluronidases can be found among proteins, glycosaminoglycans, polysaccharides, fatty acids, lanostanoids, antibiotics, antinematodal, synthetic, organic, and plant derived bioactive compounds, such as alkaloids, antioxidants, polyphenols, flavonoids, terpenoids, and anti-inflammatory drugs (cf. chapter 1.2.4). In this context, in search for drug-like hyaluronidase inhibitors, commercially available non-steroidal anti-inflammatory drugs (NSAIDs) were selected and tested as potential inhibitors of *Sag*Hyal$_{4755}$ and bovine testis hyaluronidase (BTH) under standardized conditions. The selection of the compounds was inspired by previous experiences with hyaluronidase inhibitors in our workgroup.[2-8]

Non-steroidal anti-inflammatory drugs (NSAIDs) are drugs that provide analgesic, antipyretic, and anti-inflammatory effects by inhibiting arachidonic acid metabolism and are used to treat inflammation, pain, and fever. They are among the most commonly prescribed drugs worldwide. Acidic NSAIDs, which carry a lipophilic and hydrophilic part in the molecule, are often used for the symptomatic relief of pain, fever, or rheumatic arthritis. NSAIDs are traditionally grouped according to their chemical structure and are classified into different groups, such as salicylates, acetic acid derivatives, propionic acid derivatives, anthranilic acid derivatives, oxicams, and coxibs.[9,10]

The typical anti-rheumatic drug sodium salicylate was reported to inhibit hyaluronidases from bacterial origin and testicular extracts by reducing the spreading effect in connective tissues, when injected into the skin by Guerra.[11,12] Several reports confirmed the observation that salicylate and its derivatives, e. g. gentisic acid, inhibit hyaluronidases *in vivo*.[13-16] In 1975 the anti-inflammatory drug indomethacin was reported by Szarzy, et al., to be an inhibitor for the bacterial hyaluronidase *Sag*Hyal$_{4755}$.[17] Moreover, phenylbutazone and oxyphenbutazone were stated to be potent hyaluronidase inhibitors.[18] Suleyman, et al., evaluated the inhibitory effect of selected COX-2 inhibitors, such as rofecoxib, celecoxib, and nimesulide, on hyaluronidases by determining the vascular permeability.[19]

All selected commercially available drugs were small molecules. The aim was to identify substances, which might serve as a basis for the development of inhibitors with drug-like properties in terms of enhanced water solubility and reduced plasma protein binding. Moreover, compounds that are considered drug-like have sufficient ADME properties. Hence, good absorption and permeability are more likely for compounds that fulfill Lipinski's "rule of five" ($0 \leq \log P \geq 5$, molecular weight ≤ 500 Da, number of H-bond donors ≤ 5, number of H-bond acceptors ≤ 10).[20,21] The inhibitory activities of the selected compounds on the bacterial hyaluronidase *Sag*Hyal$_{4755}$ and the bovine testis hyaluronidase (BTH) are described in this chapter.

3.2 Materials and methods

3.2.1 Materials and methods

Hyaluronan (hyaluronic acid) from *Streptococcus zooepidemicus* was purchased from Aqua Biochem (Dessau, Germany). Bovine serum albumin (BSA) was obtained from Serva (Heidelberg, Germany). The investigated hyaluronidases were enzyme preparations from different sources. Stabilized lyophilized hyaluronate lyase, i.e. 200,000 units (according to the supplier; 0.572 mg of enzyme from *S. agalactiae* strain 4755 plus 2.2 mg of BSA and 37 mg of tris/HCl per vial) was kindly provided by id-Pharma (Jena, Germany). Lyophilized hyaluronidase from bovine testis (Neopermease®) (200,000 units, according to the supplier; 4 mg enzyme plus 25 mg of gelatin per vial) was a gift from Sanabo (Vienna, Austria). Ascorbic acid 6-palmitate (Vcpal), diclofenac, indomethacin, and *p*-(dimethylamino)-benzaldehyde (DMAB) were purchased from Sigma-Aldrich (Munich, Germany). If not otherwise indicated, all chemicals were of analytical HPLC grade and obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Eschborn, Germany).

3.2.2 Morgan-Elson assay

3.2.2.1 General procedures

The following reagents and solutions were prepared immediately before usage. McIlvaine's buffer was prepared from solution 1 (0.2 M Na_2HPO_4, 0.1 M NaCl) and solution 2 (0.1 M citric acid, 0.1 M NaCl) by mixing appropriate portions to adjust the required pH value (pH 5.0).

The alkaline borate solution was prepared from a borate carbonate solution (solution A: 17.3 g H_3BO_4 and 7.8 g KOH in 100 mL water) and a potassium carbonate solution (solution B: 8.0 g K_2CO_3 in 10 mL water) by combining 10 volumes of solution A and 1 volume of solution B. For the solution of Ehrlich's reagent, 1 volume of concentrated solution (20.0 g *p*-(dimethylamino)benzaldehyde dissolved in 25 mL of concentrated HCl and 75 mL of glacial acetic acid; storage conditions: 4 °C, light protection) was diluted with 4 volumes of glacial acetic acid. Stock solutions containing 5 mg/mL hyaluronic acid in water and 0.2 mg/mL BSA in water were prepared and stored at 4 °C.

For the incubation mixture test compounds, dissolved in 18 µL of DMSO (final DMSO concentration: 3.9 % (v/v)), were incubated at 37 °C in the assay mixture containing 200 µL of buffer, 50 µL of BSA solution (0.2 mg/mL in water), 50 µL of HA solution (5 mg/mL in water), 82 µL of H_2O and 50 µL of enzyme solution. The enzymatic reaction was stopped after 60 min by addition of 110 µL of alkaline borate solution and subsequent for 4.5 min on a heating block (100 °C). After cooling on ice for at least 2 minutes, 1250 µL of Ehrlich's reagent were added, and the mixture was incubated at 37 °C for 20 minutes. The samples were transferred to acryl cuvettes, and the absorbance of the colored product was measured photometrically at a wavelength of 586 nm using a Cary 100 UV-Vis spectrometer (Varian, Darmstadt, Germany). As reference for 100 % enzyme activity, the formation of the red colored product of a sample without inhibitor (replaced by 18 µL DMSO) was quantified. In the absence of both, enzyme and inhibitor, (replaced by 50 µL of BSA solution and 18 µL DMSO, respectively), a reference for 0 % enzyme activity was determined.

3.2.2.2 Calculation of enzyme inhibition and IC_{50} values

The highest concentration of the test compounds was adjusted to 1 mM. Regularly, a dilution series, covering a concentration range from 0.5 µM to 1 mM, was used for the determination of IC_{50} values. The effect of the test compounds on the enzymatic activity was calculated according to Equation 3.1.

$$relative\ activity\ (\%) = \frac{(E_{sample} - E_{blank})}{(E_{max} - E_{min})} \cdot 100 \qquad \text{Equation 3.1}$$

E_{sample}: mean extinction of the incubation mixture containing inhibitor (containing enzyme)

E_{blank}: mean extinction of the incubation mixture containing inhibitor (without enzyme)

E_{max}: mean extinction of the incubation mixture without inhibitor (containing enzyme)

E_{min}: mean extinction of the incubation mixture without inhibitor (without enzyme)

3.2.3 Turbidimetric assay

3.2.3.1 General procedures

The following dilutions were prepared immediately before use. Measurements were performed in McIlvaine's incubation buffer, unless otherwise indicated. For BTH, *Sag*Hyal$_{4755}$ and *Spn*Hyl, the pH value was 5.0. The stopping reagent was prepared from a solution of 2.5 % cetrimonium bromide (cetyltrimethylammonium bromide, CTAB) in 0.5 N NaOH (w/v). Stock solutions containing 2 mg/mL of hyaluronan in water and 0.2 mg/mL of BSA in water were prepared and stored at 4 °C. Table 3.1 summarizes the enzymatic activities, pH values, and incubation periods in the two different assay formats in cuvettes and 96-well plates, respectively.

Table 3.1 Conditions the turbidimetric assay, depending on the format. Modified from Textor.[4]

assay format	enzyme	enzyme solution prepared (mU/mL)	enzyme solution added to incubation mixture (total volume) (µL)	final enzymatic activity in the incubation mixture (mU)	pH	incubation time (min)
cuvette	BTH	9.0	30 µL (270 µL)	0.27	5.0	30
cuvette	*Sag*Hyal$_{4755}$	3.3	30 µL (270 µL)	0.1	5.0	30
96-well	*Spn*Hyl	10.0	10 µL (75 µL)	0.1	5.0	30

3.2.3.2 Cuvette assay

The IC_{50} values for BTH (Neopermease®) and *Sag*Hyal$_{4755}$ were generally determined in the turbidimetric assay performed in acryl cuvettes according to the following procedure.[6] For the incubation mixture, 120 µL of incubation buffer, 30 µL of BSA solution (0.2 mg/mL in water), 30 µL of HA (2 mg/mL in water), and 50 µL of H_2O were dispensed to Eppendorf reaction vessels. Then, 10 µL of inhibitor solution (inhibitor dissolved in 100 % DMSO) were added. The final DMSO concentration in the incubation mixture was 4 %, an amount, which is well tolerated, as it had been previously demonstrated in our workgroup.[7,8] After addition of 30 µL of enzyme solution, the mixture was immediately incubated at 37 °C for 30 minutes (cf. Table 3.1). The enzymatic reaction was stopped by addition of 700 µL of CTAB solution (2.5 % CTAB in 0.5 N NaOH (w/v)). Then, the mixture was incubated at room temperature for an additional 20 minutes to allow development of sufficiently high turbidity. Subsequently, the incubation mixture was transferred to acryl cuvettes. The turbidity, quantified as optical density (OD), was measured at a wavelength of 580 nm using a Cary 100 UV-Vis spectrometer (Varian, Darmstadt, Germany) or at a wavelength of 578 nm using a LP700 spectrophotometer (Dr. Bruno Lange GmbH, Berlin, Germany). Samples without inhibitor (minimal signal) and without, both, inhibitor and enzyme (maximal signal), were measured as references.

3.2.3.3 96-well microtiter plate assay

The turbidimetric assay described by Di Ferrante[22] was modified to allow a quantification in 96-well microtiter plates as described by Hofinger.[23] Incubation mixtures contained the following compounds: 31 µL of incubation buffer, 8 µL of BSA solution (0.2 mg/mL in water), 13 µL of HA (2 mg/mL in water), and 13 µL of H_2O. For the investigation of test compounds 3 µL of inhibitor solution (inhibitor dissolved in 100 % DMSO) were added. Subsequently, 10 µL of enzyme solution were added, and the samples were immediately incubated at 37 °C for 30 minutes (cf. Table 3.1). The enzymatic reaction was stopped by addition of 200 µL of alkaline CTAB solution (2.5 % CTAB in 0.5 N NaOH (w/v)). Afterwards, the plates were incubated at room temperature for 20 min to provide sufficient turbidity. The turbidity, quantified as optical density (OD), was measured at λ = 580 nm (absorbance mode) using a Tecan Genios Pro microtiter plate reader (Tecan Deutschland GmbH, Crailsheim, Germany) with an XFluor Genios Pro software (version 4.55). The plates were shaken in the reader for 10 seconds. The OD was measured after 2 seconds of settling time by 10 flashes at the center of each well. Samples without inhibitor (minimal signal) and without, both, inhibitor and enzyme (maximal signal), were measured as references.

3.2.3.4 Compound solubility

Poor solubility of the putative inhibitors may lead to false-positive results in the turbidimetric assay. The solubility was determined prior to the investigation of hyaluronidase inhibition, taking the concentration range from 0.5 µM to 1 mM into consideration. To assess the solubility of the test compounds, a sample containing 920 µL citrate phosphate buffer (pH 5.0), 120 µL BSA solution (0.2 mg/mL) and 40 µL of a solution of the respective test compound (compound dissolved in 100 % DMSO) was measured at a wavelength of 580 nm using a Cary 100 UV-Vis spectrometer (Varian, Darmstadt, Germany). A cuvette filled with 920 µL of buffer, 120 µL of BSA solution, and 40 µL of DMSO served as reference. Precipitation of the compounds at the investigated concentrations (≤ 1 mM) with alkaline CTAB solution (2.5 % CTAB in 0.5 N NaOH (w/v)) was not observed and can be excluded.

3.2.3.5 Calculation of enzyme inhibition and IC_{50} values

The effect of the test compounds on the enzymatic activity was calculated according to Equation 3.2.

$$relative\ activity\ (\%) = \frac{(E_{max} - E_{sample})}{(E_{max} - E_{min})} \cdot 100 \qquad \text{Equation 3.2}$$

E_{max}: mean extinction of the incubation mixture without inhibitor (without enzyme)

E_{sample}: mean extinction of the incubation mixture containing inhibitor (containing enzyme)

E_{min}: mean extinction of the incubation mixture without inhibitor (containing enzyme)

The highest final concentration of the test compounds in the different assay formats was adjusted to 1 mM. Regularly, a dilution series covering a concentration range from 0.5 µM to 1 mM was used for the determination of IC_{50} values using cuvettes and 96-well assay format. The activities were plotted against the logarithm of the inhibitor concentration, and IC_{50} ± SEM values were calculated by curve fitting of the experimental data with Sigma Plot 11.0 (SPSS Inc., Chicago, USA) from at least two independent experiments performed in duplicate.

3.2.3.6 Determination of turbidity in the incubation mixture the presence of plasma proteins

To determine the turbidity in the incubation mixture of the turbidimetric assay, four different samples were prepared as follows (Table 3.2). The absorbance was measured at a wavelength of 600 nm.

Table 3.2 Composition of incubation mixtures for the investigation of high turbidity in positive control.

sample		**sample**	
1a (centrifuged)[a]	**2b (non-centrifuged)**	**2a (centrifuged)[a]**	**2b (non-centrifuged)**
120 µL McIlvaine's buffer (pH 5.0)		120 µL McIlvaine's buffer (pH 5.0)	
50 µL H_2O		50 µL H_2O	
35 µL human plasma		35 µL human plasma	
30 µL HA (2 mg/mL)		30 µL H_2O (instead of HA)	
25 µL DMSO (instead of inhibitor solution)		25 µL DMSO (instead of inhibitor solution)	

[a] sample 1a and 2a were centrifuged for 5 min at 10, 000 U/min, rt.

3.3 Inhibitory activity of selected non-steroidal anti-inflammatory drugs

3.3.1 Salicylates

Because of the numerous reports on the inhibitory effect of salicylates, compounds derived from salicylic acid were selected. The structures of the analyzed compounds are shown in Figure 3.1.

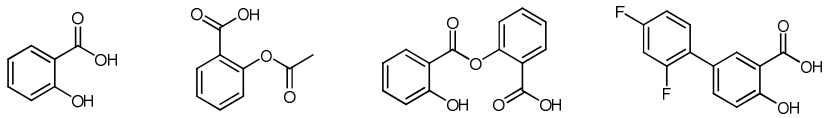


Figure 3.1 Structures of investigated salicylic acid derivatives (**1**-**4**).

The hyaluronidase inhibitory activities and calculated $logD_{5.0}$ values of compounds **1**-**4** are summarized in Table 3.3.

Table 3.3 Inhibitory activity on hyaluronidases and $logD_{5.0}$ values of salicylic acid derivatives **1**-**4**.

compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[b]
1	inactive	inactive	-0.7
2	inactive	inactive	-0.1
3	40 % at 1 mM	inactive	0.7
4	195 ± 35	30 % at 1 mM	0.9

[a] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of salicylic acid (**1**), acetylsalicylic acid (**2**), salsalat (**3**), and diflunisal (**4**) is depicted as concentration-response curve in Figure 3.2.

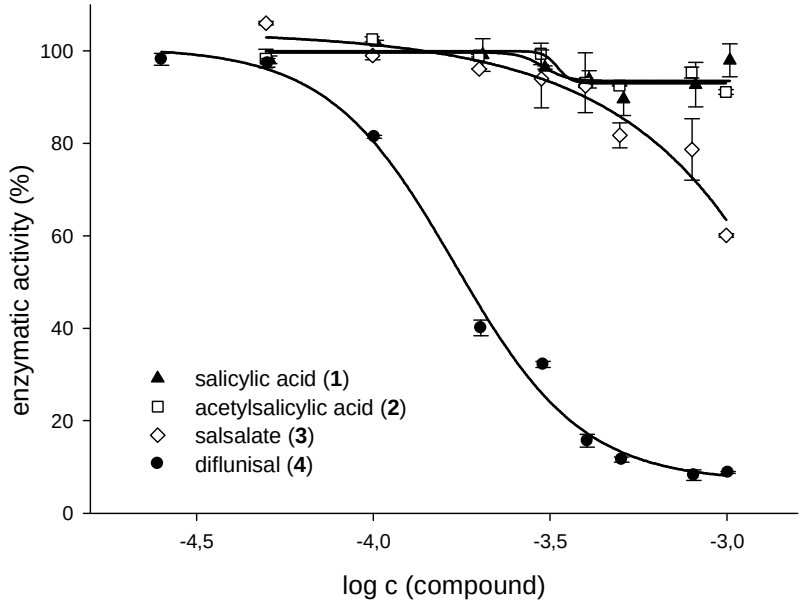


Figure 3.2 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of salicylic acid (**1**), acetylsalicylic acid (**2**), salsalat (**3**) and diflunisal (**4**).

Except for diflunisal (**4**), the investigated substances were inactive or showed only weak inhibition of the investigated enzymes (turbidimetric assay, pH 5.0). As a result, salicylic acid and acetylsalicylic acid do neither inhibit the bacterial hyaluronidase from *Streptococcus agalactiae* strain 4755 nor the bovine testicular hyaluronidase (BTH), when tested in the turbidimetric assay. This is in contrast to the results published by Guerra, who reported

inhibitory activity for sodium salicylate against bacterial and testicular hyaluronidase in the spreading test.[11]

3.3.2 Acetic acid derivatives

Five derivatives of phenyl- and heteroarylacetic acid were selected to investigate inhibitory activity against both, *Sag*Hyal$_{4755}$ and BTH. The structures of the analyzed compounds are shown in Figure 3.3.

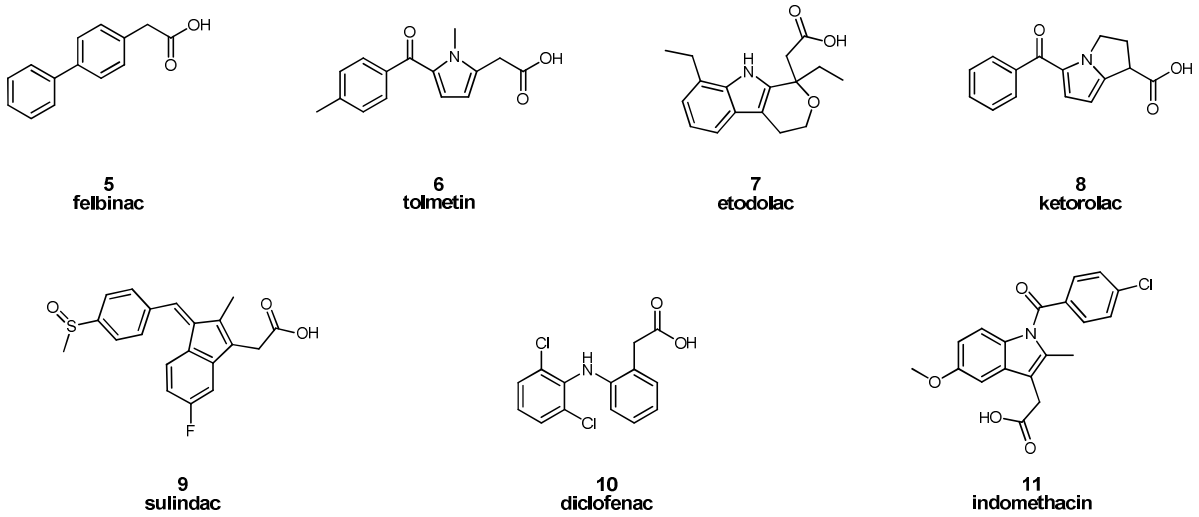


Figure 3.3 Structures of investigated phenyl- and heteroarylacetic acid derivatives.

The hyaluronidase inhibitory activities and calculated $logD_{5.0}$ values of compounds **5**-**9** are summarized in Table 3.4.

Table 3.4 Inhibitory activity on hyaluronidases and $logD_{5.0}$ values of phenyl- and heteroarylacetic acid derivatives **5**-**9**.

compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[d]
5	30 % at 500 µM	inactive	2.5
6	40 % at 1 mM	inactive	1.8
7	inactive	inactive	2.7
8	25 % at 1 mM	inactive	1.9
9	442 ± 50	inactive	1.7
10	161 ± 11[b]	> 1000[b]	3.7
11	350[c]	540[c]	3.2

[a] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] as determined by Spickenreither[5]; [c] as determined by Salmen[8]; [d] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of felbinac (**5**), tolmetin (**6**), etodolac (**7**), ketorolac (**8**) and sulindac (**9**) is depicted as concentration-response curve in Figure 3.4.

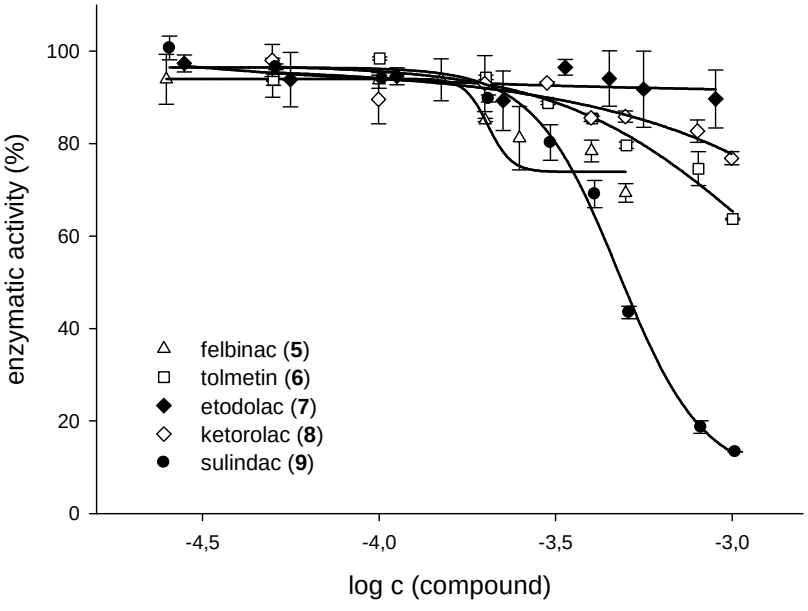


Figure 3.4 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of felbinac (**5**), tolmetin (**6**), etodolac (**7**), ketorolac (**8**) and sulindac (**9**).

Diclofenac (**10**) and indomethacin (**11**) had been published before and were used as reference compounds throughout this thesis.[5,8] The investigated compounds are more lipophilic than the selected salicylates (**1**-**4**). Sulindac (**8**) showed weak inhibition on *Sag*Hyal$_{4755}$, but was inactive BTH (turbidimetric assay, pH 5.0). In conclusion, the selected acetic acid derivatives showed only minor or no inhibition on the investigated bacterial and bovine hyaluronidase.

3.3.3 Propionic acid derivatives

Seven drugs bearing a 2-arylpropionic acid motif were investigated with respect to inhibition of *Sag*Hyal$_{4755}$ and BTH. The structures of the selected propionic acid derivatives are shown in Figure 3.5.

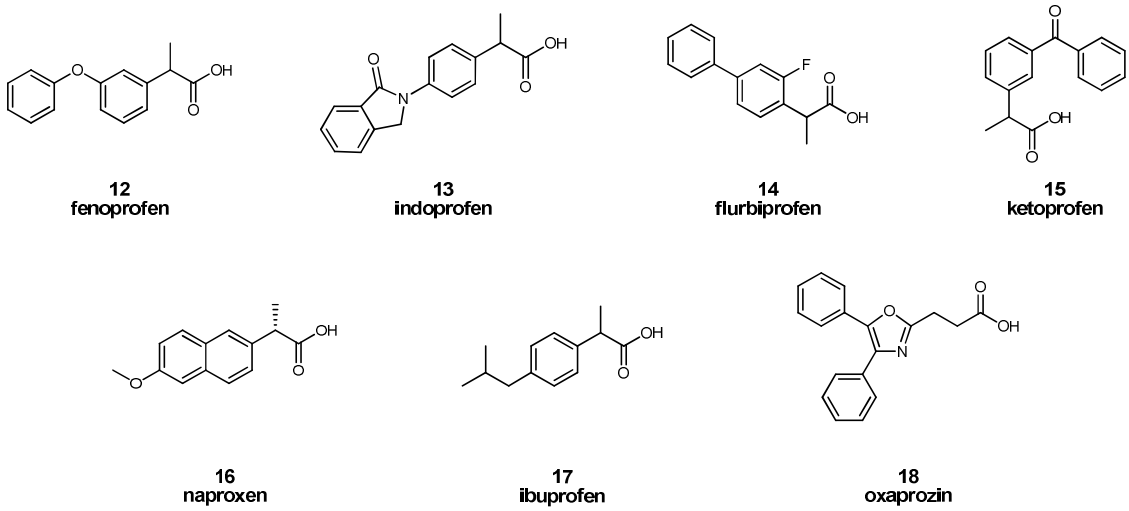


Figure 3.5 Structures of investigated 2-arylpropionic acid derivatives (**12**-**18**).

The hyaluronidase inhibitory activities and calculated log$D_{5.0}$ values of compounds **12**-**18** are summarized in Table 3.5.

Table 3.5 Inhibitory activity on hyaluronidases and $logD_{5.0}$ values of 2-arylpropionic acid derivatives **12**-**18**.

compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[b]
12	80 % at 1 mM	inactive	2.9
13	30 % at 1 mM	inactive	2.2
14	335 ± 131	inactive	2.8
15	70 % at 1 mM	inactive	2.1
16	794 ± 313	inactive	2.5
17	396 ± 69	inactive	2.8
18	40 % at 1 mM	inactive	2.3

[a] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of fenoprofen (**12**), indoprofen (**13**), flurbiprofen (**14**), ketoprofen (**15**), naproxen (**16**), ibuprofen (**17**) and oxaprozin (**18**) is depicted as concentration-response curves in Figure 3.6.

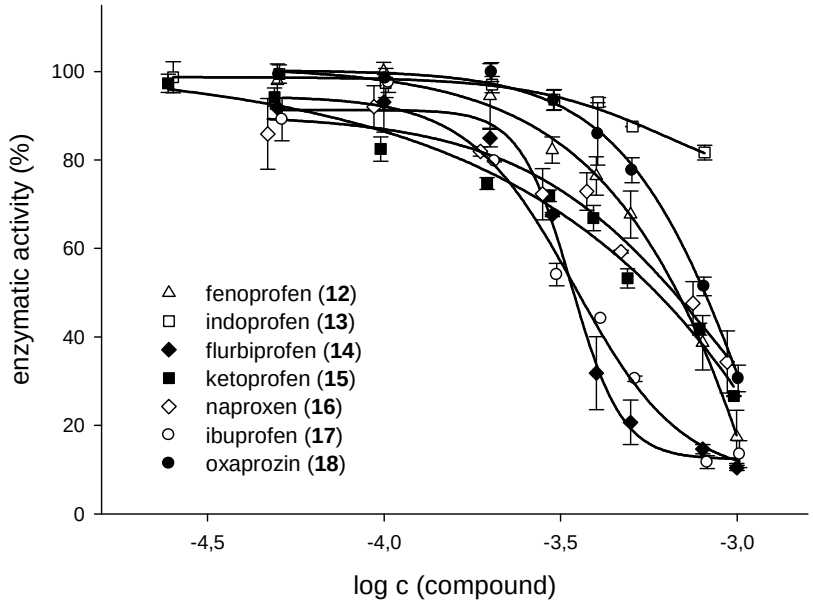


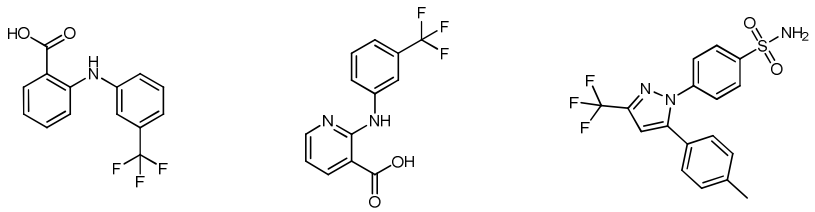
Figure 3.6 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of fenoprofen (**12**), flurbiprofen (**14**), naproxen (**16**), ibuprofen (**17**) and oxaprozin (**18**).

Fenoprofen (**12**) was reported as a hyaluronidase inhibitor before.[24] All 2-arylpropionic acid derivatives showed only weak inhibition of *Sag*Hyal$_{4755}$ and no inhibition of BTH. With $logD_{5.0}$

values of 2.8, flurbiprofen (**14**, IC_{50} = 335 µM) and ibuprofen (**17**, IC_{50} = 396 µM) were the two most active compounds investigated in this series.

3.3.4 *N*-Anthranilic acids and celecoxib

Flufenamic acid (**19**) and niflumic acid (**20**) were selected as representatives of *N*-anthranilic acid derivatives. Moreover, celecoxib (**21**) was tested due to the reported hyaluronidase inhibition of coxibs by Suleyman, et al.[19] All compounds were investigated for their inhibitory effect on the bacterial hyaluronidase *Sag*Hyal$_{4755}$ and the bovine testis hyaluronidase. The structures of the compounds are displayed in Figure 3.7.



19 flufenamic acid | **20** niflumic acid | **21** celecoxib

Figure 3.7 Structures of investigated anthranilic acid derivatives (**19**, **20**) and celecoxib (**21**).

The hyaluronidase inhibitory activities and calculated log$D_{5.0}$ values of compounds **19**-**21** are summarized in Table 3.6.

Table 3.6 Inhibitory activity on hyaluronidases and log$D_{5.0}$ values of *N*-anthranilic acid derivatives and celecoxib **19**-**21**.

compound	***Sag*Hyal$_{4755}$ IC_{50} (µM)[a]**	**BTH IC_{50} (µM)[a]**	**log$D_{5.0}$[b]**
19	151 ± 31	inactive	3.9
20	206 ± 24	10 % at 1 mM	2.5
21	inactive	inactive	2.6

[a] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of flufenamic acid (**19**), niflumic acid (**20**), and oxaprozin (**21**) is depicted as concentration-response curves in Figure 3.8.

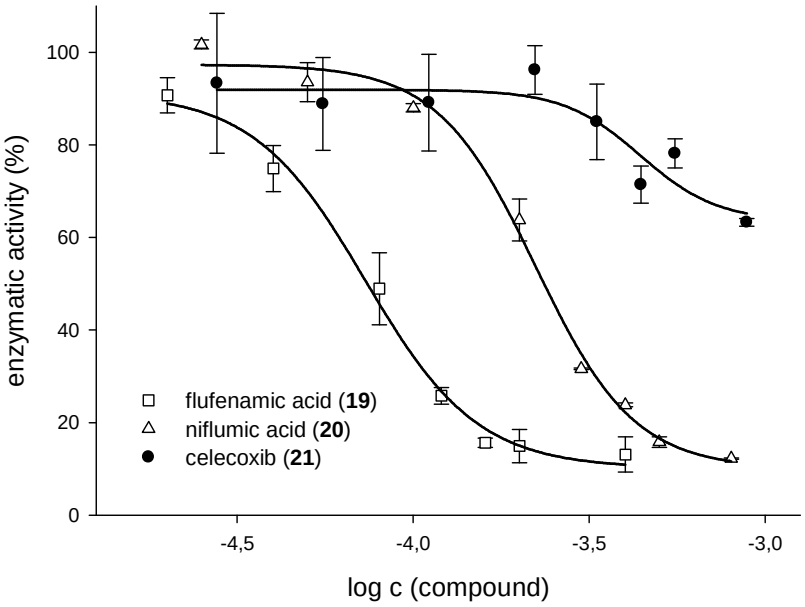
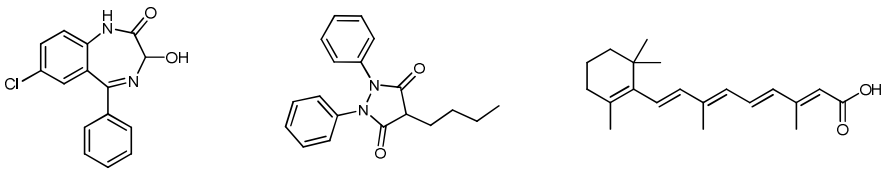


Figure 3.8 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of flufenamic acid (**19**), niflumic acid (**20**) and oxaprozin (**21**).

The purported inhibitory effect of celecoxib by Suleyman et al. was not confirmed for the bacterial hyaluronidase *Sag*Hyal$_{4755}$ and the bovine testis hyaluronidase. Unfortunately, Suleyman et al., did not state which hyaluronidase was used in their testing procedures.[19] The *N*-anthranilic acid derivatives showed IC_{50} values between 151 µM and 206 µM on *Sag*Hyal$_{4755}$, but only very weak or no inhibition on BTH. Compared to the aforementioned substances, flufenamic acid (**19**) and niflumic acid (**20**) are the most lipophilic with $logD_{5.0}$ values of 3.9 and 2.5, respectively.

3.3.5 Miscellaneous compounds

To investigate additional small molecules, compounds **22**-**24** (Figure 3.9) were tested for hyaluronidase inhibitory activity.



22
oxazepam

23
phenylbutazon

24
retinoic acid

Figure 3.9 Structures of investigated compounds **22**-**24**.

The hyaluronidase inhibitory activities and calculated $logD_{5.0}$ values of compounds **22**-**24** are summarized in Table 3.7.

Table 3.7 Inhibitory activity on hyaluronidases and $logD_{5.0}$ values of coxibs and benzodiazepine **22**-**24.**

compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[b]
22	70 % at 1 mM	inactive	2.2
23	650	inactive	2.9
24	15 % at 100 µM	inactive	5.8

[a] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of oxazepam (**22**) and phenylbutazone (**23**) is depicted as concentration-response curves in Figure 3.10.

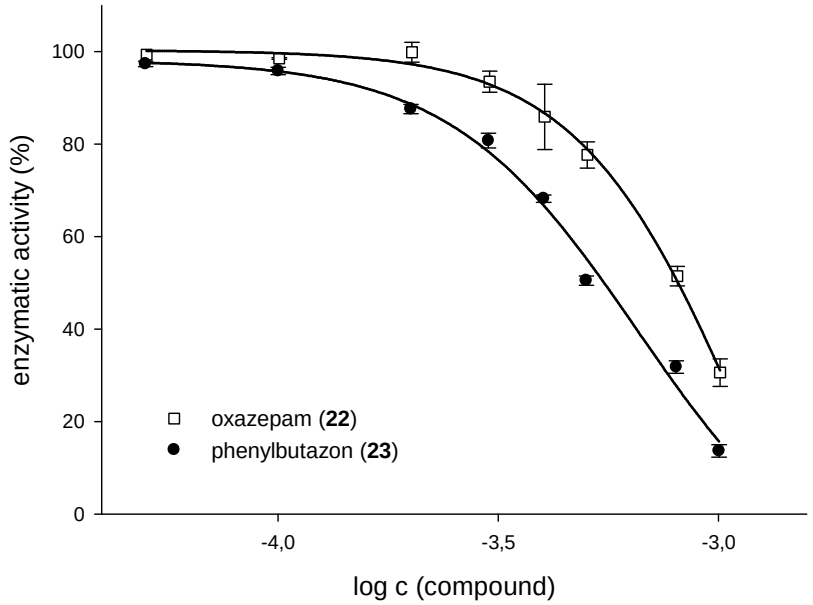


Figure 3.10 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of oxazepam (**22**) and phenylbutazone (**23**).

Phenylbutazone (**23**), which also belongs to the group of NSAIDs, was reported as an inhibitor previously.[4] The other two compounds **22** and **24** show weak inhibition of *Sag*Hyal$_{4755}$ and no inhibition of BTH.

3.4 Inhibitory activity of diflunisal in the presence of plasma proteins

Plasma protein binding is a very important aspect in the development of new drugs because it affects the bioavailability of drugs. Human serum albumin (HSA) is the most abundant multifunctional non-glycosylated, negatively charged protein in blood plasma (over 50 % of total plasma protein content). It is a globular protein (M_r = 66 kDa, concentration 0.52-0.75 mM ≈ 34-50 mg/mL), has multiple hydrophobic binding sites, and is capable of binding several endogenous ligands and drugs.[25-27] Diflunisal (**4**) is reported to bind to plasma proteins > 98 %.[10,28]

In the turbidimetric assay a standard bovine serum albumin (BSA) concentration of 0.2 mg/mL is applied. Therefore, possible plasma protein binding of the tested compounds is considered negligible. To determine whether the inhibitory activity of diflunisal against *Sag*Hyal$_{4755}$ is diminished by serum albumin binding, the BSA concentration was raised to 10 mg/mL. Besides diflunisal (**4**), the reference compounds vitamin C palmitate (Vcpal), diclofenac, (**10**) and indomethacin (**11**) were tested, too.

The turbidimetric assay was carried out in cuvettes f (270 µL per cuvette). The amount of BSA solution was 35 µL (cf. section 3.2.3). Inhibitory activity of the compounds was tested at one concentration. The final assay concentration of diflunisal (**4**), diclofenac (**10**), and indomethacin (**11**) was set to 1 mM, Vcpal was tested at a final concentration of 50 µM due to its high potency (IC_{50} = 4 µM[5]). Samples without inhibitor (minimal signal response) and without both inhibitor and enzyme (maximal signal response) were also measured as positive and negative controls under assay conditions.

The percent inhibition of all tested compounds in the presence of both, 0.2 mg/mL and 10 mg/mL, BSA is summarized in Table 3.8.

Table 3.8 Percentual inhibition of diflunisal, Vcpal, diclofenac and indomethacin of *Sag*Hyal$_{4755}$ in the presence of different BSA concentrations.

compound	**final assay concentration (mM)**	**percent inhibition of *Sag*Hyal$_{4755}$[a]**	
		c(BSA) = 0.2 mg/mL[b]	**c(BSA) = 10 mg/mL[b]**
diflunisal (**4**)	1	93 ± 4	88 ± 4
Vcpal	0.05	95 ± 1	6 ± 2
diclofenac (**10**)	1	93 ± 6	90 ± 4
indomethacin (**11**)	1	93 ± 4	89 ± 1

[a] percent inhibition was determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] mean values ± SEM (N = 2, experiments performed in duplicates).

When the BSA concentration was raised from 0.2 mg/mL to 10 mg/mL, in case of Vcpal, a significant drop of potency from 95 % to 6 % inhibition was observed. This can be explained by almost complete binding of vitamin C palmitate to bovine serum albumin. Studies by Spickenreither revealed that about five molecules of Vcpal can be bound per BSA molecule.[5]

For the other three compounds, diflunisal (**4**), diclofenac (**10**), and indomethacin (**11**), the inhibitory activity of *Sag*Hyal$_{4755}$ was not as much affected (3 %-5 %). This observation is contrary to reports in literature on the plasma protein binding of diflunisal (> 98 %[10,28]). Possibly, a BSA concentration of 10 mg/mL is too low for complete serum albumin. In addition to serum albumin, many other proteins (e.g. globulins) are present in plasma. Hence, the compounds **4**, **10** and **11** may bind to other proteins.

The inhibitory activity of diflunisal (**4**), Vcpal, diclofenac (**10**), and indomethacin (**11**) was also determined in the presence of human plasma and fetal calf serum (FCS, Biochrom AG, Berlin, Germany) in order to investigate inactivation due to protein binding. The turbidimetric assay was performed as described above (cf. section 3.2.3), the amount of plasma applied in the assay was 35 µL.

The percent inhibition of the compounds in the presence of BSA (0.2 mg/mL), fetal calf serum (FCS), and human plasma is summarized in Table 3.9.

Table 3.9 Percentual inhibition of diflunisal, Vcpal, diclofenac and indomethacin of *Sag*Hyal$_{4755}$ in the presence of BSA (0.2 mg/mL), FCS and human plasma.

compound	**final concentration (mM)**	**percent-inhibition of *Sag*Hyal$_{4755}$[a]**		
		c(BSA) = 0.2 mg/mL	**fetal calf serum (FCS)[b]**	**human plasma[c]**
diflunisal (**4**)	1	93 ± 4	81 ± 18	could not be determined
Vcpal	0.05	95 ± 1	0	
diclofenac (**10**)	1	93 ± 6	92 ± 6	
indomethacin (**11**)	1	93 ± 4	86 ± 7	

[a] Percentual inhibition was determined at pH 5.0 in the turbidimetric assay (cuvettes); mean values ± SEM (N = 2, experiments performed in duplicate); [b] protein concentration: 34.6 ± 1 mg/mL FCS (as determined in the Bradford assay);[29] [c] albumin concentration in the blood serum of an average adult human is 31.8-45 mg/mL.[30]

In case of Vcpal, the exchange of BSA (0.2 mg/mL) with fetal calf serum (FCS) in the turbidimetric assay led to an exceptional loss of inhibitory activity. Therefore, it can be concluded that Vcpal binds to plasma proteins completely. Compared to the assay conditions in the absence of FCS and human plasma, the inhibition of *Sag*Hyal$_{4755}$ by diflunisal (**4**), diclofenac (**10**), and indomethacin (**11**) was reduced by 1-12 %. With regard to the inhibitory activity in the turbidimetric assay, the lowest protein binding in FCS can be observed for diclofenac (**10**) and indomethacin (**11**).

When human plasma was added instead of BSA (0.2 mg/mL), in the turbidimetric assay, the inhibitory activity of the compounds could not be determined because the turbidity of the sample without inhibitor (minimal signal response – no turbidity) was as high as the turbidity of the sample without both inhibitor and enzyme (maximal signal response – high turbidity). To exclude the possibility, that the utilized human plasma was too old, the assay was repeated under the same conditions using mouse plasma and a freshly prepared human plasma. However, the turbidity in the negative control was again too high to determine the percent inhibition on *Sag*Hyal$_{4755}$.

To solve this problem several possibilities were taken into consideration. It is conceivable that the hyaluronan, which is added in the turbidimetric assay, forms an insoluble complex with some components in the plasma to cause the high turbidity. Several reports have been given about the precipitation of high molecular weight hyaluronan after the addition of acidified horse serum or serum from other sources.[31-36] Therefore, the absorbance of 4

different incubation mixtures (**1a/b**, **2a/b**; cf. Table 3.2 in Section 3.2.3.6) was measured at a wavelength of 600 nm. The different incubation mixtures were prepared both, with and without hyaluronan. Both, the centrifuged samples (**1a** and **2a**) as well as the non-centrifuged samples (**1b** and **2b**) did not show any turbidity. All absorbance values were very low (around 0.1). Therefore, an insoluble complex between hyaluronan and plasma components can be excluded.

Turbidity might result from very high amounts of hyaluronan in the plasma, so that during the incubation period (30 min, 37 °C) complete degradation of hyaluronan by the streptococcal hyaluronidase *Sag*Hyal$_{4755}$ does not occur. A hyaluronan Assay Kit[37] (HA-ELISA, K-1200, Echelon Biosciences Inc., Salt Lake City, UT, USA) was used to determine the amount of hyaluronan in 5 different plasma and FCS samples as described by the manufacturer.[38] Hyaluronectin (HN) is an HA-binding glycoprotein found in the brain. It has been used to make ELISA-like assays for hyaluronan.[38] The principle of this assay is that hyaluronectin binds to HA, which is adsorbed onto a plastic microtest plate. The binding of HN to the HA-coated plate decreases when a solution containing HA (solution to be assayed) is added. The amount of bound HN is measured by ELISA using purified specific anti-HN antibodies conjugated with alkaline phosphatase.[39,40]

The plasma from wild type mice (mouse plasma 1 and 2) and the plasma from Hyal-2 KO-mice were obtained from Prof. Dr. B. Flamion (University of Namur, Belgium; preparation of plasma is not described in this thesis, for detailed information cf. Hamberger[41]). The plasma samples were used in the HA assay without further dilution. The results are summarized in Table 3.10.

Table 3.10 Amount of hyaluronan in 5 different plasma and FCS samples as determined in the HA assay.

plasma	**amount of hyaluronan (ng/mL)[a]**
mouse plasma 1	2590
mouse plasma 2	241
Hyal-2 KO-mouse plasma	7877
human plasma[b]	456
fetal calf serum (FCS)	8028

[a] determined in the hyaluronan enzyme-linked assay-kit (N = 1, performed in triplicate); [b] from female test person.

The level of hyaluronan in mouse plasma 1, the KO-mouse plasma, and the fetal calf serum was very high between 2, 590-8, 028 ng/mL, as expected. The amount of hyaluronan in the second mouse plasma (2) was about ten-fold lower compared to mouse plasma 1, which is

normal, because hyaluronan levels can vary depending on the species and age.[42] Also the plasma of the female test person, , showed the expected concentration values of hyaluronan for human plasma of adults.[43] The amount of hyaluronan in fetal serum was much higher compared to the adult serum. However, in case of FCS the percent inhibition of *Sag*Hyal$_{4755}$ could be determined. In case of the human plasma it was not possible due to high turbidity in the reference sample that should not show any turbidity at all. These results proved that the high turbidity in the minimal response signal sample is not caused by high levels of hyaluronan or an insoluble complex between hyaluronan and components of the plasma.

Possibly, the turbidimetric assay is not suited for serum-shift assays to estimate plasma protein binding of the tested compounds and its effect on the inhibitory activity on *Sag*Hyal$_{4755}$. Therefore, the assay system was changed to the colorimetric Morgan-Elson assay. The incubation mixtures were prepared as described in Section 3.2.2. The final assay concentration of diflunisal (**4**) and diclofenac (**10**) was set to 1 mM, Vcpal was tested in a final concentration of 50 µM. Samples without inhibitor (maximal signal response, maximal coloration) and without both inhibitor and enzyme (minimal signal response, minimal coloration) were, also, measured as positive and negative controls under assay conditions. The yellow colored compound indomethacin (**11**) was not tested in the Morgan-Elson assay because it undergoes a side reaction, which provides a colored product, leading to false-negative results.[4] The percent inhibition of the tested compounds in the presence of BSA (0.2 mg/mL), fetal calf serum (FCS), and human plasma is summarized in Table 3.11.

Table 3.11 Percentual inhibition of diflunisal, Vcpal and diclofenac of *Sag*Hyal$_{4755}$ in the presence of BSA (0.2 mg/mL), FCS and human plasma.

compound	**final concentration (mM)**	**percent inhibition of *Sag*Hyal$_{4755}$[a]**		
		c(BSA) = 0.2 mg/mL	**fetal calf serum (FCS)[b]**	**human plasma[c]**
diflunisal (**4**)	1	90 ± 6	84 ± 1	could not be determined
Vcpal	0.05	99 ± 1	0	
diclofenac (**10**)	1	88 ± 6	87 ± 2	

[a] Percent inhibition was determined at pH 5.0 in the Morgan-Elson assay (cuvettes); mean values ± SEM (N = 2, experiments performed in duplicates); [b] protein concentration of FCS: 34.6 ± 1 mg/mL (as determined in the Bradford assay);[29] [c] the albumin concentration in the blood serum of an average adult human is 31.8-45 mg/mL.[30]

As expected, all tested compounds show very good inhibition in the final assay concentration (cf. Table 3.11) between 88-99 % in the presence of BSA (0.2 mg/mL). Compared to the percent inhibition in the presence of BSA (0.2 mg/mL) determined in the turbidimetric assay, the three tested compounds show comparable results. Inhibitory activity was slightly diminished in case of diflunisal (6 %) and diclofenac (1 %), but completely lost in case of

vitamin C palmitate (Vcpal) due to its high plasma protein binding. However, inhibition could still not be determined in the presence of human plasma. The samples with minimal and maximal response signals show the same coloration and, therefore, the same absorbance values (λ = 586 nm). The problem as to why inhibitory activity cannot be measured in the presence of human plasma has yet to be solved.

The presence of an endogenous inhibitor of hyaluronidase, which was characterized as a thermolabile, magnesium dependent glycoprotein, in human serum and tissues was reported in the middle of the last century (1946-1955).[44-47] In 2000, Mio et al. identified the serum inhibitor as a member of the inter-α-inhibitor family.[48] The results presented in this section might indicate the presence of a substance in the plasma that inhibits the bacterial hyaluronidase yielding high turbidity of the positive controls.

3.5 Peak plasma concentrations of commercial drugs

The pharmacokinetic parameter c_{max} (peak plasma concentration) is the maximum concentration (in µg/mL) of a drug after a single dose. The peak plasma concentrations of the investigated non-steroidal anti-inflammatory drugs were taken into consideration.

The highest final concentration (f. c.) in the incubation mixture in the turbidimetric assay was 1 mM, if not otherwise indicated, due to poor solubility of the drug in aqueous buffer. The concentration in µg/mL for each drug was calculated and compared with the literature values for the peak plasma concentration c_{max} in human plasma (Table 3.12). Moreover, in this context, the inhibitory activities of the investigated NSAIDs on the bacterial hyaluronidase *Sag*Hyal$_{4755}$ and the bovine testicular hyaluronidase BTH were discussed.

Table 3.12 Peak plasma concentrations and concentration in incubation mixture and inhibitory activity of *Sag*Hyal$_{4755}$ of NSAIDs.

drugs in clinical use	peak plasma concentration		final assay concentration of 1 mM corresponding to µg/mL	*Sag*Hyal$_{4755}$ IC_{50} (µM)[g]	BTH IC_{50} (µM)[g]
	dose (mg)[a]	c_{max} (µg/mL)			
salicylic acid (**1**)		150-300[49]	138.1	inactive	inactive
acetylsalicylic acid (**2**)	1000-2000	24 ± 4[10]	180.1	inactive	inactive
diflunisal (**4**)	250	41 ± 11[50]	250.2	195 ± 35	30 % at 1 mM
felbinac (**5**)	3000[b]	0.034 ± 0.029[51]	106.1 (500 µM)	inactive	inactive
tolmetin (**6**)	400	40[52]	279.3	40 % at 1 mM	inactive
etodolac (**7**)	200 / 600	14 ± 4 / 37 ± 9[53]	287.4	inactive	inactive
ketorolac (**8**)	10-30[c]	0.8-3.0[10]	255.3	25 % at 1 mM	inactive
sulindac (**9**)	200	4.7[54]	356.4	442 ± 50	inactive
diclofenac (**10**)	50 / 100[d]	0.42-2.0[10]	296.2	161 ± 11[5]	> 1000[5]
indomethacin (**11**)	50[e]	2.4[10]	357.8	350[8]	540[8]
fenoprofen (**12**)	600	50[55]	242.3	80 % at 1 mM	inactive
indoprofen (**13**)	100	8.3 ± 1.6[56]	281.3	30 % at 1 mM	inactive
flurbiprofen (**14**)	100	12[57]	244.3	335 ± 131	inactive
ketoprofen (**15**)	4 x 50[f]	2.4 ± 1[58]	254.3	70 % at 1 mM	inactive
naproxen (**16**)	10-30[c]	55 ± 14[10]	230.3	794 ± 313	inactive
ibuprofen (**17**)	800	61 ± 5.5[10]	206.3	396 ± 69	inactive
oxaprozin (**18**)	1200	80 ± 6[59]	293.2	40 % at 1 mM	inactive
flufenamic acid (**19**)	200	6-20[60]	112.5 (400 µM)	151 ± 31	inactive
niflumic acid (**20**)	740	0.180[61]	282.2	206 ± 24	10 % at 1 mM
celecoxib (**21**)	500	0.705 ± 0.286[10]	381.4	inactive	inactive
oxazepam (**22**)	1200	450[62]	286.7	70 % at 1 mM	inactive
phenylbutazone (**23**)	2 x 100	20[63]	307.4	650[4]	inactive

[a] Following an oral dose, if not otherwise indicated; [b] following a topical application; [c] range of c_{max} from different studies following a single 30 mg IM or 10 mg-oral dose in healthy adults; [d] following a single 50 mg enteric-coated tablet (EC) or 100 mg-sustained-release tablet (SR) given to healthy adults; [e] effective at concentrations of 0.3-3 µg/mL; [f] following 4 x 50 mg ketoprofen capsules in fasted and fed state; [g] mean values ± SEM (N = 2, experiments performed in duplicate), highest concentration of the test compounds in the assay was 1 mM; IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes).

For all but two drugs (salicylic acid and oxazepam), the final concentration in the assay is higher compared to the peak plasma concentration (c_{max}) value from literature. The peak

plasma concentration of felbinac after a topical application of 3 g is 34 ng/mL, which is approximately 3000-fold lower compared to the highest final concentration in the incubation mixture (f. c. = 500 µM corresponding to 106.1 µg/mL). The smallest difference between peak plasma concentration and highest assay concentration was found for ibuprofen (f. c. = 1 mM corresponding to 206 µg/mL; c_{max} = 61 µg/mL) and oxaprozin (f. c. = 1 mM corresponding to 293 µg/mL; c_{max} = 80 µg/mL) for which the highest assay concentration is about 3-4-fold higher compared to the peak plasma concentration c_{max}.

Compared to the peak plasma concentrations after a single dose, very high concentrations between 112-381 µg/mL were used *in vitro* in the turbidimetric assay for inhibition of *Sag*Hyal$_{4755}$ and BTH. Except for diflunisal (**4**), diclofenac (**10**), and flufenamic acid (**19**), which show IC_{50} values below 200 µM on *Sag*Hyal$_{4755}$, all of the other compounds showed weak or no inhibition of both enzymes *in vitro*. These results and previous investigations in our workgroup allow the conclusion that the bacterial hyaluronidase is more sensitive compared to the mammalian hyaluronidases. Moreover, the hyaluronidases from prokaryotes (*Sag*Hyal$_{4755}$) and mammals (BTH, human hyaluronidases) must be clearly distinguished, as these enzymes exhibit different catalytic mechanisms to degrade their substrate hyaluronan. BTH is orthologous to the human hyaluronidase PH-20. Hence, the investigation on BTH can be regarded as an approach to evaluate potential inhibitors of PH-20.

Taken together, the concentrations that are applied *in vitro* in the turbidimetric assay are relatively high (dilution series from 1 mM to 10 µM) and, except for salicylic acid and oxazepam, at least 3-4-fold higher compared to the peak plasma concentration *in vivo*. However, since in case of the bovine testis hyaluronidase the compounds showed very weak or no inhibition, it is expected that none of the compounds will show any effect *in vivo* on human hyaluronidases. The non-steroidal anti-inflammatory drugs are very potent inhibitors of the COX-1 and COX-2 enzymes, but cannot be used as hyaluronidase inhibitors.

3.6 Summary and conclusion

Non-steroidal anti-inflammatory drugs, phenylbutazone and retinoic acid were investigated under standardized conditions in the presence of *Sag*Hyal$_{4755}$ and BTH. Compounds bearing characteristic structural elements, i.e. lipophilic moieties combined with an acidic functional residue, were preferentially chosen. However, the high degree of lipophilicity of previously identified active inhibitors of *Sag*Hyal$_{4755}$ usually resulted in very high plasma protein binding

and a high $logD_{5.0}$ value as well as surfactant properties.[5] Among commercially available drugs, diflunisal (**4**; IC_{50} = 195 µM) and flufenamic acid (**19**; IC_{50} = 108 µM) showed the best inhibition of the bacterial hyaluronidase *Sag*$Hyal_{4755}$ under the present conditions. Diflunisal (**4**) additionally showed weak inhibition of the bovine testicular hyaluronidase (BTH). Moreover, out of the investigated compounds that showed IC_{50} values around 200 µM (**4**, **10**, **19**, **20**), diflunisal (**4**) had the lowest calculated $logD_{5.0}$ value (calculated $logD_{5.0}$ = 0.9).
In other words, under the current conditions, only inhibitors of *Sag*$Hyal_{4755}$ were identified. Only the compounds diflunisal (**4**, 30 % at 1 mM) and niflumic acid (**20**, 10 % at 1 mM) showed weak inhibition of BTH which, also, suggests inactivity of these compounds at the related human PH-20 hyaluronidase.

Diflunisal (**4**, IC_{50} = 195 µM), was identified as a putative lead compound for the design and synthesis of chemically related inhibitors. Interestingly, diflunisal is devoid of a long lipophilic carbon chain. With respect to the calculated $logD_{5.0}$ ($logD_{5.0}$ = 0.9), this might also represent a privileged structural element to overcome high plasma protein binding and thus might provide access to a new class of potent inhibitors of *Sag*$Hyal_{4755}$.

Several attempts were undertaken to determine the plasma protein binding of diflunisal (**4**) and the three reference compounds vitamin C palmitate (Vcpal), diclofenac (**10**), and indomethacin (**11**) using the both the colorimetric and turbidimetric assay. The inhibitory activity of these compounds on the bacterial hyaluronidase *Sag*$Hyal_{4755}$ was determined in presence of an enhanced BSA concentration (10 mg/mL), fetal calf serum (FCS) and human plasma. In case of Vcpal, a significant drop of inhibition was observed in both assays, possible due to very high plasma protein binding. The inhibitory effect of diclofenac (**10**), diflunisal (**4**) and indomethacin (**11**) was only slightly diminished in the presence of BSA (10 mg/mL) and FCS in both investigated assay systems. The human plasma caused a very high turbidity in the positive control, which could indicate the presence of an inhibitor in the human plasma that has been reported for a long time. However, this is a problem that has yet to be dealt with.

The peak plasma concentration c_{max} of the tested NSAIDs were compared with the highest final assay concentration corresponding to µg/mL. From the *in vitro* inhibitory activity on the bovine testis hyaluronidase, it was possible to draw conclusions for the related human hyaluronidase PH-20. The NSAIDs were tested in relatively high concentrations (1 mM to 10 µM dilution series) but no or only weak inhibition of BTH could be observed. Therefore, it is possible to conclude that none of the investigated drugs will the human hyaluronidase *in vivo*.

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4 Diflunisal analogs as inhibitors of bacterial hyaluronidase

4.1 Introduction

The inhibition of hyaluronidase by salicylates was reported in the years 1946 to 1950.[1-4] Aiming at drug-like molecules, a set of several commercially available drugs, mainly NSAIDs, purported to have hyaluronidase inhibiting activities, was investigated for inhibition of bacterial hyaluronidase from *S. agalactiae* (*Sag*Hyal$_{4755}$) and bovine testis hyaluronidase (BTH; cf. chapter 3). The biarylic salicyclic acid analog diflunisal[5] (Figure 4.1) was identified as an inhibitor with an IC_{50} value in the micromolar range on *Sag*Hyal$_{4755}$ (IC_{50} = 195 µM).

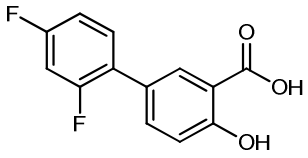


Figure 4.1 Chemical structure of diflunisal.

Over the past decade, potent inhibitors of hyaluronidase with a preference for the bacterial enzymes were discovered in our laboratory.[6-11] Characteristics of the most active inhibitors are acidic functional groups and a high degree of lipophilicity. The structural features of these compounds are reminiscent of the amphiphilic repetitive motives of hyaluronan. In general, the potency of the inhibitors correlates with the lipophilicity. Unfortunately, the drug-like properties of highly lipophilic substances are strongly affected due to high plasma protein binding.

Aiming at inhibitors with improved drug-like properties, the focus was set on a series of compounds that are structurally related to diflunisal (Figure 4.2). For the synthesis of the diflunisal derivatives, the Suzuki-Miyaura cross-coupling reaction of aryl halides with organoboron compounds was applied.

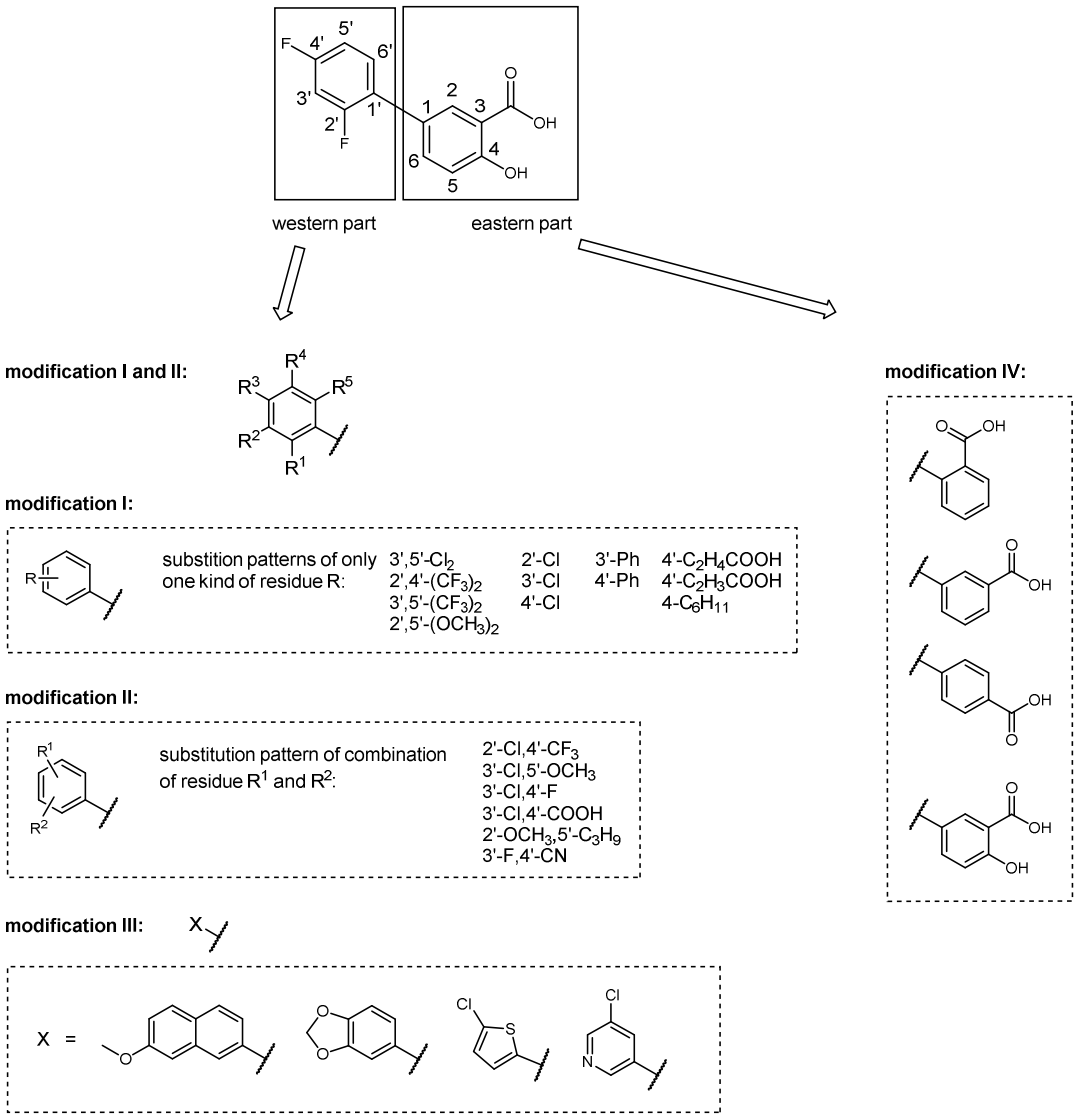


Figure 4.2 Structural modifications of diflunisal

The diflunisal core-structure was modified in the “eastern part” (variations in positions 2-4; Figure 4.2) and the “western part” (variations in positions 2’-6’; Figure 4.2) of the molecule. For the selection of the moieties to be introduced into the diflunisal scaffold, based on relevant experience from our laboratory, the contribution of the substructures to the physicochemical and drug-like properties of the hyaluronidase inhibitors as well as the commercial availability of educts were taken into consideration.

The two fluorine substituents of diflunisal were exchanged by several residues (cf. modification I in Figure 4.2), such as chlorine, trifluoromethyl, cyclohexyl, propanoic and acrylic acid and methoxy groups. On the one hand, the 2’,4’-F_2 substitution pattern of diflunisal was maintained, on the other hand it was changed to investigate the influence of different substituents at other positions (3’,5’; 2’,5’). Monosubstitution with chlorine (2’-Cl;

4'-Cl) or trifluoromethyl (3'-CF_3) were performed. Moreover, a bioisosteric exchange of chlorine by trifluoromethyl groups was performed (3',5'-Cl_2; 3',5'-$(CF_3)_2$).

Compared to the diflunisal molecule, compounds bearing chlorine or trifluoromethyl groups are more lipophilic in terms of the $logD_{5.0}$ value but suffer from limited solubility. Hence, to retain good solubility, an additional negatively charged carboxylic group was introduced into the western part. Both, a propanoic acid residue (4'-C_2H_4COOH) and an acrylic acid residue (4'-C_2H_3COOH) were introduced into the molecule. To create molecules that are sterically more demanding, a cyclohexane ring (4'-C_6H_{11}) or an additional phenyl ring (3'-Ph and 4'-Ph) were introduced into the western part. Additional structural modifications of the western part (cf. modification II in Figure 4.2) include various different substituents (3'-Cl, 5'-OMe; 2'-OMe, 5'-C_3H_9; 3'-Cl,4'-F and 3'-Cl, 4'-CN) were combined. Other structural modifications of the western part (cf. modification III in Figure 4.2) were performed by an exchange of the substituted phenyl ring with heterocycles, such as 2-methoxynaphthalene, benzo[*d*][1,3]dioxole, 2-chlorothiophene and 3-chloropyridine.

The eastern part of the diflunisal molecule contains a salicylic acid moiety. To vary the position of the carboxylic group (position 3 in diflunisal) and the hydroxyl group in position 4, the salicylic acid was replaced by 2-, 3- and 4-benzoic acid (cf. modification IV in Figure 4.2).

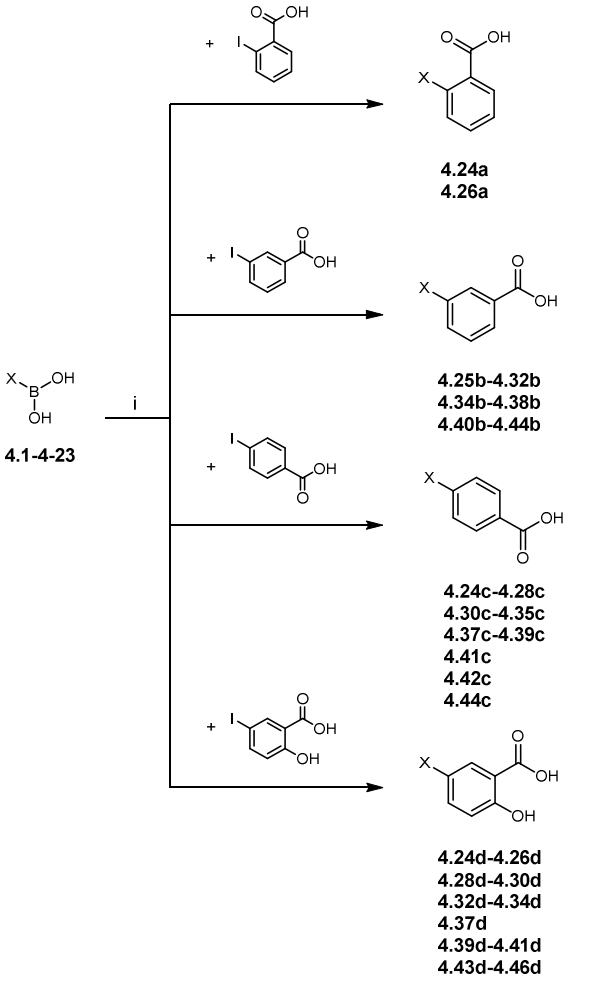
In the present chapter, the synthesis of selected diflunisal derivatives and the correlated inhibitory activity towards the bacterial hyaluronidase are described.

4.2 Chemistry

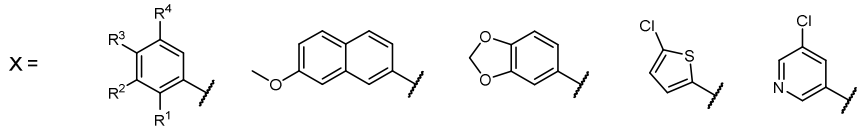
Carbon-carbon coupling reactions based on transition-metal catalysts have evolved as powerful tools in organic synthesis. Among them, palladium-catalyzed cross-coupling reactions are most prominent.[12] The palladium-mediated cross-coupling of organic electrophiles, such as aryl halides with organoboron compounds in the presence of an inorganic base is known as the Suzuki-Miyaura reaction and has become one of the most powerful and convenient synthetic tools for the preparation of biarylic compounds.[13-16]

The synthesis of the diflunisal analogs were carried out utilizing iodobenzoic acid or iodosalicylic acid as building blocks for the eastern part and organoboronic acids for the

western part of the molecules (cf. Figure 4.2). The Suzuki-Miyaura cross-coupling was carried out as outlined in Scheme 4.1.



	R^1	R^2	R^3	R^4
4.1, 4.24a-d	Cl	H	Cl	H
4.2, 4.25b-d	H	H	Ph	H
4.3, 4.26a-d	H	H	Cl	H
4.4, 4.27b/c	CF_3	H	CF_3	H
4.5, 4.28b-d	Cl	H	CF_3	H
4.6, 4.29b/d	Cl	H	H	H
4.7, 4.30b-d	H	CF_3	H	CF_3
4.8, 4.31b/c	H	Cl	H	H
4.9, 4.32b-d	H	Cl	H	OCH_3
4.10, 4.33c/d	H	Cl	H	Cl
4.11, 4.34b-d	H	Ph	H	H
4.12, 4.35b/c	H	Cl	F	H
4.13, 4.36b	H	Cl	COOH	H
4.14, 4.37b-d	OCH_3	H	H	C_3H_9
4.15, 4.38b/c	H	F	CN	H
4.16, 4.39c/d	H	H	$C_3H_5O_2$	H
4.17, 4.40b/d	H	H	$C_3H_3O_2$	H
4.18, 4.41b-d	OCH_3	H	H	OCH_3
4.19, 4.42b/c	H	H	C_6H_{11}	H



Scheme 4.1 Synthesis of the 2-, 3- and 4-carboxylic acid analogs. Reagents and conditions: $Pd(dppf)Cl_2 \bullet CH_2Cl_2$, K_2CO_3 (2 M), toluene, ethanol (1:1:1, v/v/v), microwave 140 °C, 20 min.

The biaryl compounds were synthesized in one step, starting from commercially available aryl boronic acids and aryl iodides according to the procedure described by Chalker et al.[17] Microwave irradiation (140 °C, 20 min) in toluene and ethanol in the presence of potassium carbonate (2 M) was used for rapid coupling to afford compounds **4.24**-**4.46**.

The mechanism of the Suzuki-Miyaura reaction is described as a general catalytic cycle, which involves oxidative addition of the aryl halide, transmetalation of the boronic acid and

reductive elimination of the biaryl.[18] These intermediates have been proven by isolation and spectroscopic analyses.[19,20] Oxidative addition of the aryl iodide to the palladium(0) complex affords a sTable *trans*-σ-palladium(II) complex.[21] Reductive elimination of organic partners from the palladium(II) complex reproduces the palladium(0) complex. Water and base were found necessary to activate the boronic acid.[18,22]

The target compounds **4.24**-**4.46** were separated from starting materials and isolated by flash chromatography in accepTable yields.

4.3 Pharmacological results and discussion

4.3.1 General conditions

All synthesized diflunisal derivatives were investigated for inhibition of the bacterial hyaluronan lyase *Sag*Hyal$_{4755}$ and the bovine testicular enzyme BTH (Neopermease®) at pH 5.0 in a turbidimetric assay based on the method of Di Ferrante[23] as described in chapter 1. In the following, inhibitory activities are expressed as IC_{50} values unless IC_{50} values could not be determined, due to poor solubility in aqueous buffer. In such cases the percent inhibition of the enzyme at the highest possible concentration of the compound is given.

4.3.2 Inhibitory activities of biphenyl-2-carboxylic acid derivatives

The IC_{50} values determined for the analogs of biphenyl-2-carboxylic acids are summarized in Table 4.1.

Table 4.1 Inhibitory activity[a] and calculated $logD_{5.0}$ values[b] of biphenyl-2-carboxylic acid derivatives **4.24a** and **4.26a**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[b]
4.24a	443 ± 38	inactive	2.5
4.26a	264 ± 81	inactive	2.4

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **4.24a** and **4.26a** is depicted as concentration-response curves in Figure 4.3.

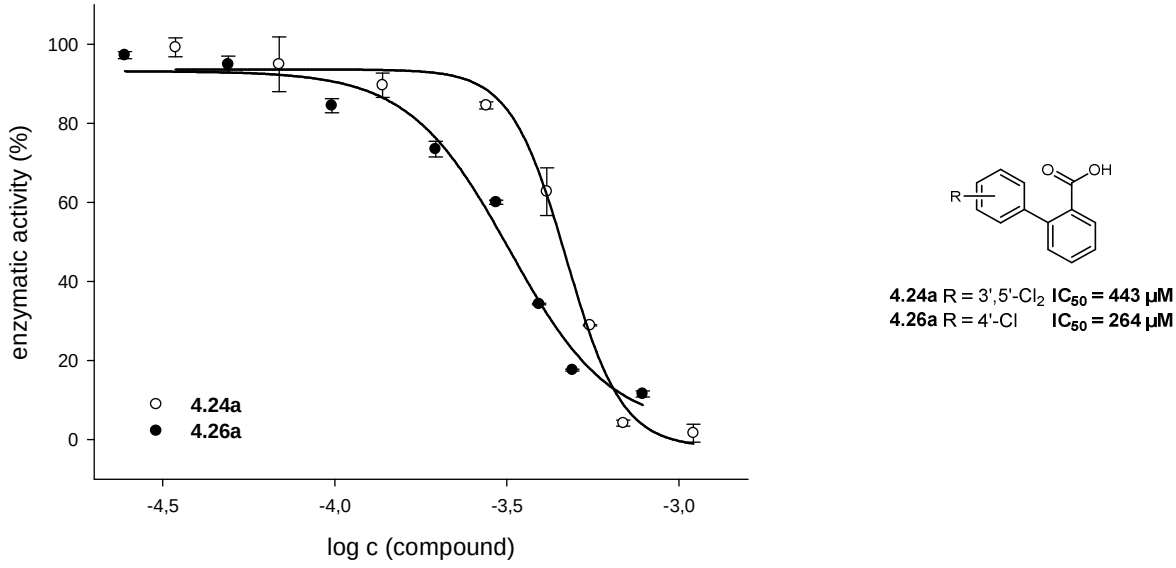


Figure 4.3 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **4.24a** and **4.26a**.

Both compounds showed only weak inhibition of *Sag*Hyal$_{4755}$ and no inhibition of BTH. Compared to diflunisal (IC_{50} = 195 µM), the inhibitory activity of these particular compounds was weaker. Therefore, the substitution pattern of compounds bearing the biphenyl-2-carboxylic acid motif was not further investigated.

4.3.3 Inhibitory activities of biphenyl-3-carboxylic acid derivatives

IC_{50}-values determined for the analogs of biphenyl-3-carboxylic acids are summarized in Table 4.2.

Table 4.2 Inhibitory activity and calculated $logD_{5.0}$ values[b] of biphenyl-3-carboxylic acid derivatives **4.24b**-**4.32b**, **4.34b**-**4.38b** and **4.40b**-**4.44b**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[c]
4.24b	121 ± 33	inactive	3.2
4.25b	inactive	inactive	3.7
4.26b	279 ± 100	25 % at 500 µM	3.0
4.27b	94 ± 1	inactive	2.5
4.28b	133 ± 33	inactive	2.2
4.29b	248 ± 185	inactive	2.5
4.30b	inactive	inactive	3.0
4.31b	inactive	inactive	2.9
4.32b	inactive	inactive	4.5
4.34b	20 % at 100 µM[b]	inactive	3.9
4.35b	40 % at 200 µM[b]	inactive	2.8
4.36b	inactive	inactive	-0.4
4.37b	inactive	inactive	4.0
4.38b	inactive	inactive	2.1
4.40b	20 % at 100 µM[b]	inactive	0.7
4.41b	inactive	inactive	1.5
4.42b	inactive	inactive	4.9
4.43b	inactive	inactive	2.5
4.44b	20 % at 100 µM[b]	inactive	2.3

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] % inhibition of *Sag*Hyal$_{4755}$ at indicated inhibitor concentration; [c] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in presence of **4.24b** and **4.26b**-**4.29b** is depicted as concentration-response curves in Figure 4.4.

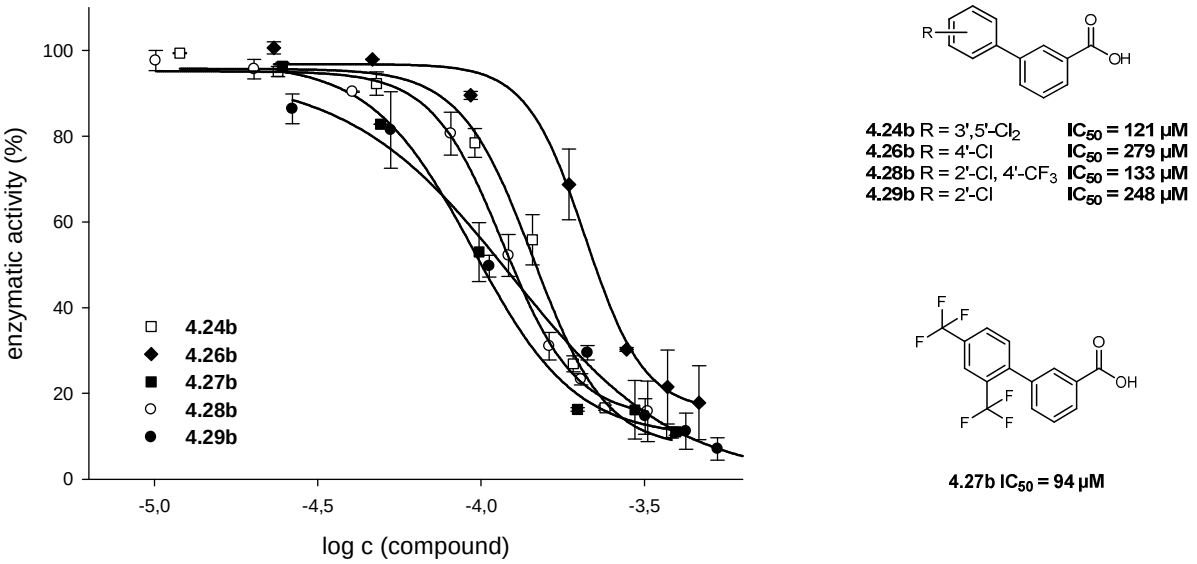


Figure 4.4 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **4.24b**-**4.29b**.

As a result, three compounds (**4.24b**, **4.28b** and **4.27b**) analyzed in this section showed higher inhibitory potency on *Sag*Hyal$_{4755}$ than diflunisal. Notably, compound **4.27b** bears two trifluoromethyl groups instead of the fluorine substituents in positions 2' and 4' of the diflunisal molecule. Additionally, the hydroxyl group in position 4 in the eastern part is missing. With an IC_{50} value of 94 µM compound **4.27b** was 2-fold more potent than diflunisal. Shifting the arrangement of the trifluoromethyl groups from 2', 4' to 3', 5' revealed both poorer solubility (50 µM compared to 400 µM) and complete loss of inhibitory activity. Substances **4.24b** (IC_{50} = 121 µM), **4.26b** (IC_{50} = 279 µM), **4.28b** (IC_{50} = 133 µM) and **4.29b** (IC_{50} = 248 µM) showed moderate, compounds **4.34b**, **4.35b**, **4.40b** and **4.44b** very weak inhibition of *Sag*Hyal$_{4755}$.

It is remarkable that compounds **4.24b** and **4.26b** were superior to compounds **4.24a**, **4.26a** as a consequence of the different position of the carboxylic acid group (position 2, cf. Figure 4.3) in the eastern part of the molecules.

The dicarboxylic acid **4.36b**, which is less lipophilic ($logD_{5.0}$ = -0.4) than compounds bearing one carboxylic group, was found to be inactive on *Sag*Hyal$_{4755}$. The calculated $logD_{5.0}$ value of the dichloro-substituted compound **4.24b** ($logD_{5.0}$ = 3.2) is higher than that of the corresponding bis(trifluoromethyl)-substituted analog **4.27b** ($logD_{5.0}$ = 2.5). However, the IC_{50} value of **4.27b** is slightly lower than the IC_{50} value of **4.24b**. Both substances are superior to diflunisal. The combination of the structural elements of **4.24b** and **4.27b** in compound **4.28b** revealed a calculated $logD_{5.0}$ and an IC_{50} value in the same range ($logD_{5.0}$ = 3.3;

IC_{50} = 133 µM). Mono-substitution with chlorine in position 2' (**4.26b**) and 3' (**4.29b**) resulted in about the same inhibitory effect on *Sag*$Hyal_{4755}$ with IC_{50} values of 279 µM and 248 µM, respectively, whereas the 4'-chloro-substituted analog **4.31b** was inactive.

Substances **4.25b**, **4.32b**, **4.34b**, **4.37b** and **4.42b** bear lipophilicity-increasing moieties in the western part resulting in $logD_{5.0}$ values ranging from 3.8-4.9. These compounds were inactive on *Sag*$Hyal_{4755}$ except for **4.34b**, which showed weak inhibition. The introduction of other substituents, such as 2-carboxyvinyl (**4.40b**), cyano (**4.38b**), methoxy (**4.41b**) and *tert*-butyl or replacing the substituted phenyl by 7-methoxy-2-naphthyl- (**4.43b**) or a benzo[*d*][1,3]dioxol-5-yl (**4.44b**) residues did not increase inhibitory activity.

The mammalian enzyme BTH was only weakly inhibited by **4.26b** (25 % at 500 µM) and not inhibited by all the other compounds included in this section. This is in accordance with previous reports for diflunisal, which showed only very weak inhibition of BTH (30 % at 1 mM, cf. chapter 3).

4.3.4 Inhibitory activities of biphenyl-4-carboxylic acid derivatives

IC_{50} values determined for the analogs of biphenyl-4-carboxylic acids are summarized in Table 4.3.

Table 4.3 Inhibitory activity[a] and calculated $logD_{5.0}$ values[b] of biphenyl-4-carboxylic acid derivatives **4.24c**-**4.28c**, **4.30c**-**4.35c**, **4.37c**-**4.39c**, **4.41c**, **4.42c** and **4.44c**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[c]
4.24c	inactive	inactive	3.1
4.25c	20 % at 100 µM[b]	inactive	3.9
4.26c	10 % at 50 µM[b]	inactive	3.0
4.27c	50 % at 300 µM[b]	inactive	2.5
4.28c	20 % at 100 µM[b]	inactive	2.2
4.30c	30 % at 200 µM[b]	inactive	3.0
4.31c	inactive	inactive	2.9
4.32c	25 % at 200 µM[b]	inactive	2.5
4.33c	55 ± 6	inactive	3.4
4.34c	31 ± 2	>400 µM	3.9
4.35c	inactive	inactive	2.8
4.37c	inactive	inactive	4.0
4.38c	20 % at 200 µM[b]	inactive	2.0
4.39c	inactive	inactive	0.8
4.41c	inactive	inactive	1.5
4.42c	inactive	inactive	4.9
4.44c	15 % at 100 µM[b]	inactive	2.3

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] % inhibition of *Sag*Hyal$_{4755}$ and BTH, respectively, at indicated inhibitor concentration; [c] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in presence of **4.33c** and **4.34c** is depicted as concentration-response curves in Figure 4.5.

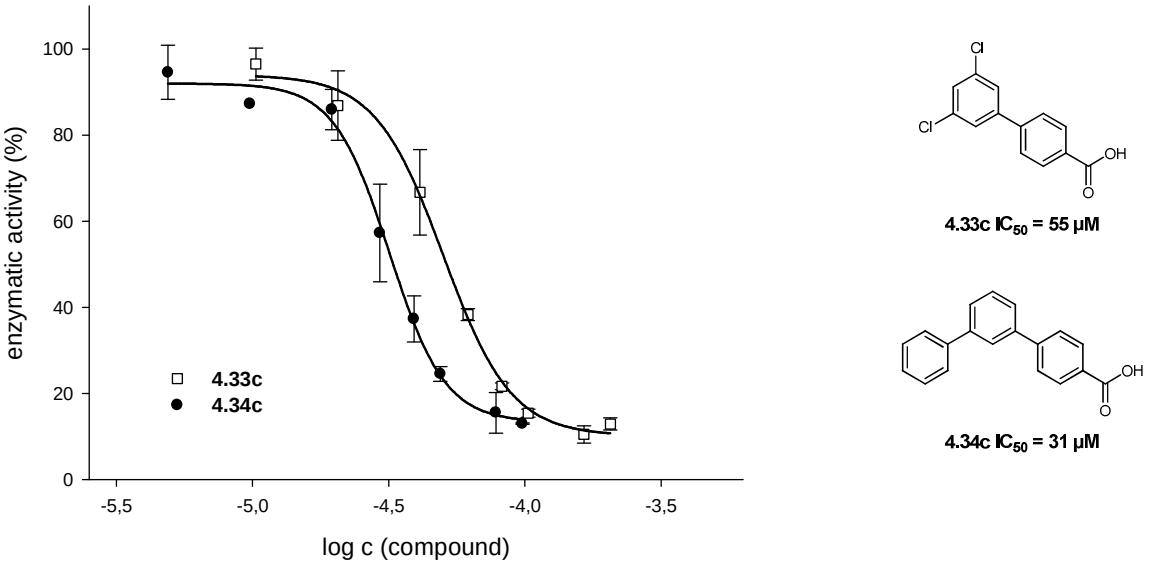


Figure 4.5 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **4.33c** and **4.34c**.

Compounds **4.33c** (IC_{50} = 55 µM) and **4.34c** (IC_{50} = 31 µM) were the most potent inhibitors of *Sag*Hyal$_{4755}$ among the synthesized diflunisal analogs. Compared to diflunisal, **4.33c** and **4.34c** possess more lipophilic moieties in the western part of the molecule (cf. Figure 4.5), resulting in calculated $logD_{5.0}$ values between 3.4 and 3.9. Compound **4.34c** possesses an additional phenyl ring in position 3' of the western part, which makes the molecule more sterically demanding. Compound **4.25c**, which only differs from **4.34c** in the position of the phenyl ring, shows only 20 % inhibition at 100 µM. Compared to the chlorine residues, the additional phenyl ring in **4.34c** is bulkier, which might be beneficial for the inhibition of *Sag*Hyal$_{4755}$.

Whereas compounds **4.24a** and **4.24b** showed weak inhibition of 400 µM and moderate inhibition (IC_{50} = 121 µM) on *Sag*Hyal$_{4755}$, the respective compound **4.24c** was inactive. The same applies for compounds **4.26a** and **4.26b**, which both showed weak inhibition with IC_{50} values of 321 µM and 280 µM, respectively, whereas compound **4.26c** only revealed 10 % inhibition at 50 µM due to poor solubility. Compound **4.27b** (IC_{50} = 94 µM) was more potent than **4.27c**, which showed 50 % inhibition at 300 µM; an IC_{50} value could not be determined, due to poor solubility. For compound **4.28c** (20 % inhibition at 100 µM) an IC_{50} value could not be determined, whereas compound **4.28b** (IC_{50} = 133 µM) was a moderate inhibitor. By contrast, compound **4.34c** revealed an IC_{50} value of 31 µM, whereas the corresponding isomer **4.34b** compound was inactive. Compounds **4.25b**, **4.30b**, **4.32b** and **4.38b** were inactive, whereas the isomers **4.25c**, **4.30c**, **4.32c** and **4.38c** showed weak inhibition (IC_{50} value not determined due to poor solubility). The reverse was true for the isomers **4.35b**

(40 % at 200 µM) and **4.35c** (inactive). Compounds **4.31b/c**, **4.37b/c** and **4.41b/c** were both inactive and poorly soluble.

Changing the arrangement of the trifluoromethyl groups from 2',4'- (**4.27c**) to 3',5'-position (**4.30c**) had only minor influence on the inhibition of *Sag*Hyal$_{4755}$. The combination of a chlorine and a trifluoromethyl group (**4.28c**) was not superior to attaching only one kind of substituent in the western part (cf. **4.24c**, **4.27c**, **4.30c**). Mono-chlorinated compounds (**4.26c**, **4.31c**) were inactive, and the introduction of different other substitution patterns in the western part of the molecule (cf. **4.32c**, **4.35c**, **4.37c**, **4.38c**, **4.39c**, **4.41**, **4.42c**, **4.44c**) did not lead to increased inhibition of *Sag*Hyal$_{4755}$.

Interestingly, compound **4.34c** showed weak inhibition of > 400 µM of the bovine testicular hyaluronidase, whereas all other compounds were inactive on this mammalian enzyme.

4.3.5 Inhibitory activities of 4-hydroxybiphenyl-3-carboxylic acid analogs

IC_{50} values determined for the analogs of biphenyl-3-carboxylic acids are summarized in Table 4.4.

Table 4.4 Inhibitory activity[a] and calculated $logD_{5.0}$ values[b] of biphenyl-4-carboxylic acid analogs **4.24d**-**4.26d**, **4.28d**-**4.30d**, **4.32d**-**4.34d**, **4.37d**, **4.39d**-**4.41d** and **4.43d**-**4.46d**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[c]
4.24d	68 ± 12	25 % at 1 mM	1.9
4.25d	33 ± 6	inactive	2.9
4.26d	148 ± 48	40 % at 1 mM	1.7
4.28d	50 ± 18	10 % at 1 mM	1.0
4.29d	154 ± 33	50 % at 1 mM	1.3
4.30d	48 ± 8	inactive	1.8
4.32d	171 ± 33	inactive	1.3
4.33d	inactive	inactive	2.2
4.34d	inactive	inactive	2.6
4.37d	80 % at 100 µM[b]	inactive	2.8
4.39d	50 % at 100 µM[b]	inactive	-0.5
4.40d	60 % at 100 µM[b]	inactive	-2.0
4.41d	40 % at 100 µM[b]	inactive	0.3
4.43d	76 ± 36	inactive	1.3
4.44d	40 % at 100 µM[b]	inactive	1.0
4.45d	90 ± 14	10 % at 200 µM[b]	1.5
4.46d	181 ± 23	40 % at 1 mM	0.3

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] % inhibition of *Sag*Hyal$_{4755}$ and BTH, respectively, at indicated inhibitor concentration; [c] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in presence of **4.24d**, **4.25d**, **4.30d** and **4.43d** is depicted as concentration-response curves in Figure 4.6.

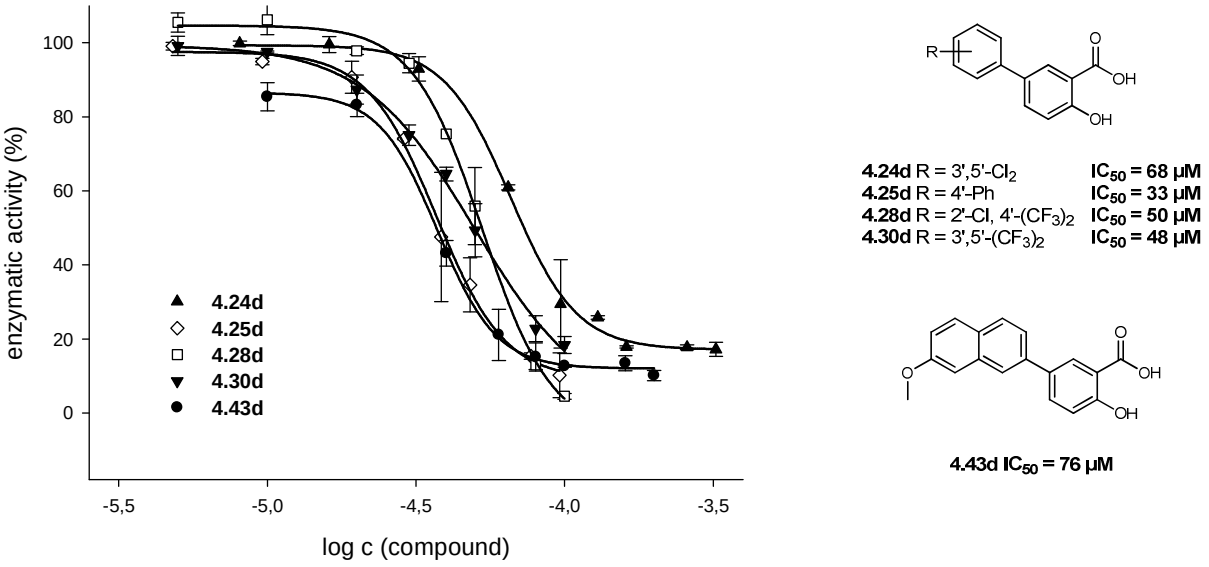


Figure 4.6 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **4.24d**, **4.25d**, **4.28d**, **4.30d** and **4.43d**.

In this group of compounds the most potent hyaluronate lyase inhibitors related to diflunisal were identified. The structures were designed as salicylic acid derivatives. 4-hydroxy-1,1':4',1''-terphenyl-3-carboxylic acid (**4.25d**, IC_{50} = 33 µM), bearing a phenyl moiety in position 4' in the western part, was 6-fold more potent than diflunisal and next to **4.34c** one of the most active inhibitors of *Sag*Hyal$_{4755}$ among the diflunisal analogs. By contrast, compounds **4.25b** and **4.25c** (20 % inhibition at 100 µM) were inactive or very weakly active, respectively. Five additional compounds among those listed in Table 4.4 showed IC_{50} values below 100 µM (**4.24d**, **4.28d**, **4.30d**, **4.43d**), whereas the corresponding analogs included in sections 4.3.3 and 0 revealed weak or no inhibition of the bacterial hyaluronidase.

Compared to diflunisal, in the four compounds **4.24a-d**, the fluorine atoms in positions 2' and 4' were replaced by chlorine atoms. Compound **4.24d** with an IC_{50} value of 68 µM is a more active inhibitor than the respective **4.24a-c** compounds, which showed weak (**4.24a**) or moderate (**4.24b**) inhibition or proved to be inactive (**4.24c**) on *Sag*Hyal$_{4755}$. Taken together, the compounds **4.24b** and **4.24d**, which bear the carboxylic group in position 3, were superior to their structural isomer **4.24c** and the compound with the salicylic acid motif (**4.24d**) was the most potent inhibitor.

Among the four mono-chlorinated compounds **4.26a-d**, **4.26d** was most potent, though at a lower level compared to the dichlorinated analog. Likewise, the combination of a chlorine and a trifluoromethyl substituent in positions 2'and 4', respectively, was most effective with the salicylic acid scaffold as demonstrated for compound **4.28d** (IC_{50} = 50 µM) (cf. **4.28b/c** in

sections 4.3.3 and 4.3.4). The same holds for compounds **4.30b** and **4.30c** in comparison with **4.30d**: whereas **4.30b** is inactive and **4.30c** was too poorly soluble to determine an IC_{50} value, **4.30d** was the most potent inhibitor of *Sag*Hyal$_{4755}$ with an IC_{50} value of 48 µM. Likewise, the salicylic acid moiety (**4.32d**) gave higher inhibitory activity when comparing compounds **4.32b**-**d**. By contrast, compound **4.34d** did not show any inhibition of *Sag*Hyal$_{4755}$, whereas substance **4.34c** (IC_{50} = 31 µM) was the most potent inhibitor among all synthesized diflunisal analogs. Compounds **4.29b** and **4.29d** showed moderate inhibition with IC_{50} values of 248 µM and 154 µM. The solubility of **4.40b** and **4.40d** was too low (100 µM) to determine IC_{50} values. In contrast to **4.43b** (inactive), compound **4.43d** (IC_{50} = 76 µM) showed moderate inhibition of *Sag*Hyal$_{4755}$, again suggesting that the hydroxyl group contributes to the inhibition of the enzyme.

Substances **4.26d** (IC_{50} = 148 µM) and **4.29d** (IC_{50} = 154 µM) are mono-chlorinated in position 4' or 2', respectively. Their inhibitory activity on *Sag*Hyal$_{4755}$ was lower than that of the dichlorinated analog**4.24d** (IC_{50} = 68 µM). Hyaluronidase inhibitory activity was lost, when the halogen atoms were attached to positions 3' and 5'. All the other structural variations (cf. **4.32d**, **4.37d**, **4.39d**, **4.41d** and **4.44d**) resulted in inactive compounds. In case of the salicylic acid derivatives, the replacement of the halogenated phenyl ring gave a hyaluronidase inhibitor with a 7-methoxy-2-naphthyl residue in the western part of the molecule (**4.43d**: IC_{50} = 76 µM). Compounds **4.45d** and **4.46d** bear heterocyclic residues (5-chlorothiophen-2yl- and 5-chloropyridin-3-yl residues, respectively) in the western part. With regard to the $logD_{5.0}$ value, the more lipophilic substance, thiophene **4.45d** ($logD_{5.0}$ = 1.5) showed higher inhibitory activity (IC_{50} = 90 µM) compared to **4.46d** ($logD_{5.0}$ = 0.3, IC_{50} = 181 µM).

Compounds **4.26d**, **4.28d**, **4.29d**, **4.30d**, **4.45d** and **4.46d** showed weak inhibition of the mammalian enzyme from bovine testis (BTH). All other compounds included in this section were inactive.

4.4 Inhibitory activities of selected compounds on *Spn*Hyl

The compounds described in this chapter were designed as inhibitors of the hyaluronate lyase from *S. agalactiae* (*Sag*Hyal$_{4755}$). To compare the results from *Sag*Hyal$_{4755}$ with data from an additional bacterial enzyme, an ensemble of eight inhibitors was selected for investigations on hyaluronidase from *S. pneumonia* (*Spn*Hyl). Under identical assay conditions (including the pH value) these substances showed only weak inhibition or were inactive (**4.25d**) on *Spn*Hyl (Table 4.5).

Table 4.5 Inhibitory activity[a,b] of selected biphenyl-carboxylic acid derivatives **4.27b**, **4.33c**, **4.34c**, **4.24d**, **4.25d**, **4.28d**, **4.30d**, **4.43d**.

Compound	***Sag*Hyal$_{4755}$ IC_{50} (µM)[a]**	***Spn*Hyl IC_{50} (µM)[a,b]**
4.27b	94 ± 1	9 % at 200 µM[b,c]
4.33c	55 ± 6	10 % at 200 µM[b,c]
4.34c	31 ± 2	70 % at 500 µM[c]
4.24d	68 ± 12	80 %
4.25d	33 ± 6	inactive
4.28d	50 ± 18	397 ± 69
4.30d	48 ± 8	50 % at 500 µM[c]
4.43d	76 ± 36	18 % at 200 µM[b,c]

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] IC_{50} values determined at pH 5.0 in the turbidimetric assay (96-well plate); [c] % inhibition of *Spn*Hyl at indicated inhibitor concentration.

For compound **4.28d** an IC_{50} value of 397 µM was determined on *Spn*Hyl, which was 8-fold higher than for inhibition of *Sag*Hyal$_{4755}$. Moreover, **4.25d** was the only substance that was inactive on *Spn*Hyl, suggesting preference for the enzyme *Sag*Hyal$_{4755}$.

4.5 Inhibitory activities of vitamin C palmitate in the presence of palladium catalyst

Metal ions have early been described to possess inhibitory activities of hyaluronidases. Meyer reported on the inhibitory activities of Fe^{3+}, Fe^{2+}, Cu^{2+} and Zn^{2+} for the testicular hyaluronidase in 1951.[24] Cd^{2+} and Zn^{2+} are also supposed to inhibit the bacterial hyaluronidase from *Streptococcus dysgalactiase* and *Streptococcus zooepidemicus*.[25] Moreover, the existence of a highly potent endogenous inhibitors, characterized as a thermolabile, magnesium dependent glycoprotein, present in human serum and tissues was reported.[26-28]

For the synthesis of the compounds described and analyzed in this chapter, the palladium catalyst [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium complex with dichloromethane ($Pd(dppf)Cl_2 \bullet CH_2Cl_2$, cf. Figure 4.7) was used.

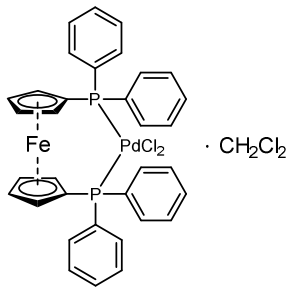


Figure 4.7 Structure of [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium complex with dichloromethane ($Pd(dppf)Cl_2 \bullet CH_2Cl_2$).

After completion of the reaction, the catalyst was removed from the reaction mixture and the crude products were purified by flash column chromatography. Due to this work-up procedure, traces of catalyst should not be present in the inhibitor solutions that were used in the turbidimetric assay for the determination of hyaluronidase inhibition. However, since several metal ions and their salts have been reported for hyaluronidase inhibition, a potential contribution of $Pd(dppf)Cl_2 \bullet CH_2Cl_2$ to false-negative results was taken into consideration. Therefore, the reference compound vitamin C palmitate (Vcpal; Figure 4.8), which is known as a potent inhibitor of the bacterial hyaluronidase *Sag*$Hyal_{4755}$, was tested in the turbidimetric assay in the presence of different concentrations (1.2-4.9 mol percent) of $Pd(dppf)Cl_2 \bullet CH_2Cl_2$.

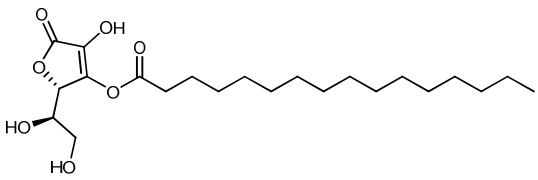


Figure 4.8 Structure of vitamin C palmitate (Vcpal).

The results are summarized in Table 4.6.

Table 4.6 Inhibitory activities of vitamin C palmitate (Vcpal) in the presence of different concentrations of the palladium catalyst $Pd(dppf)Cl_2•CH_2Cl_2$.

Compound	***Sag*Hyal$_{4755}$ IC$_{50}$ (µM)[a]**			
	Control, without $Pd(dppf)Cl_2•CH_2Cl_2$	1.2 mol%[b] $Pd(dppf)Cl_2•CH_2Cl_2$	2.7 mol%[b] $Pd(dppf)Cl_2•CH_2Cl_2$	4.9 mol%[b] $Pd(dppf)Cl_2•CH_2Cl_2$
Vcpal	4.1 ± 0.1[a]	4.7 ± 1	5.9 ± 3	6.6 ± 3

[a] As determined by Spickenreither[9]; [b] with regard to a 1.35 mM stock solution of Vcpal, final assay concentration of stock solution is 50 µM (dilution series: 50 µM to 2.5 µM).

With regard to the IC_{50} values, no relevant differences became obvious for the inhibition of *Sag*Hyal$_{4755}$ by Vcpal in the absence or presence of palladium catalyst. Compared to the IC_{50} value of Vcpal (IC_{50} = 4.1 µM) determined by Spickenreither[9], the determined IC_{50} values for Vcpal in the presence of $Pd(dppf)Cl_2•CH_2Cl_2$ were in the range from 4.7 µM to 6.6 µM, corresponding to less than a 2-fold decrease in inhibitory potency. If $Pd(dppf)Cl_2•CH_2Cl_2$ had an inhibitory effect on *Sag*Hyal$_{4755}$, the inhibitory activity of Vcpal should be increased, resulting in IC_{50} values lower than 4.1 µM. As a result, traces of palladium catalyst, if present in the incubation mixture, do not affect the inhibitory effect of the synthesized compounds.

4.6 Summary and conclusion

Aiming at inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$, the scaffold of the NSAID diflunisal was selected as a lead compound for structural modifications. This approach, starting from an approved drug, was expected to harbor the potential of improving the drug-like properties compared to previously identified, highly lipophilic hyaluronidase inhibitors, in particular regarding $logD_{5.0}$ values and plasma protein binding. According to a Suzuki-Miyaura cross-coupling procedure, a library of biarylic benzoic and salicylic acid analogs was

synthesized. These compounds revealed in part markedly lower lipophilicity than the reference compound (diflunisal) and IC_{50} values in the two-digit micromolar range.

The diflunisal core structure was changed in the eastern part by varying the position of the carboxylic acid at the biphenyl scaffold. In a first attempt, the carboxylic acid was introduced in the molecule in position 2. This did not result in better inhibitory activity of *Sag*Hyal$_{4755}$ compared to diflunisal although the lipophilicity was increased by chlorine residues.

When the carboxylic acid was shifted to position 3, three compounds exhibit lower IC_{50} values on the bacterial hyaluronidase (**4.24b**, **4.27b**, **4.28b**) compared to the lead compound. Such molecules incorporated either chlorine residues (cf. **4.24b**) or trifluoromethyl groups (cf. **4.27b**) or the combination of both (cf. **4.28b**). Hence, it can be concluded, that for the inhibition of *Sag*Hyal$_{4755}$ position 3 is more favorable compared to position 2 for the carboxylic acid moiety (cf. **4.24a** and **4.24b**). By introducing the carboxylic acid in position 4 at the biphenyl scaffold, the inhibitory effect was further enhanced in case of two compounds (**4.33c** and **4.34c**) which showed about 4-fold lower IC_{50} values than diflunisal. The lipophilicity of those compounds was increased by the 3',5'-dichloro substitution pattern and an additional phenyl ring, respectively. Although compound **4.34c** with an IC_{50} value of 31 µM was the most active inhibitor out of the whole series, retaining the salicylic acid moiety in the eastern part also led to more potent inhibitors of *Sag*Hyal$_{4755}$ compared to diflunisal. Again, the introduction of chlorine substituents (**4.24d**), trifluoromethyl groups (**4.30d**), the combination of both (**4.28d**) and an additional phenyl ring (**4.25d**) were most effective to achieve inhibitors with IC_{50} values in the two-digit micromolar range. Moreover, the incorporation of a 2 methoxy-2-naphthyl (**4.43d**) residue, replacing the substituted phenyl ring in diflunisal, led to an IC_{50} value of 76 µM.

To sum up, the best results with regard to the IC_{50} values were achieved when the carboxylic acid was incorporated in position 4 and when the salicylic acid moiety was utilized as eastern part. Moreover, the two most potent inhibitors from the diflunisal series were compounds in which the western part was substituted with an additional phenyl ring (**4.34c** in position 3', **4.25d** in position 4'). Regarding lipophilicity, these two compounds had the highest $logD_{5.0}$ values (**4.34c** $logD_{5.0}$ = 3.9; **4.25d** $logD_{5.0}$ = 2.9) of all substances of the respective series. The $logD_{5.0}$ value of **4.25d** is lower compared to the value of **4.34c** because of the additional hydroxyl group in position 4. As the two compounds were equipotent in terms of the IC_{50} value, the hydroxyl group appears to have no positive influence on the inhibition. However, by not diminishing the inhibitory effect, the OH group had no negative influence either. This suggests that it is favorable to leave the hydroxyl group incorporated in the molecule,

because it increases the solubility of the respective compound in aqueous buffer and decreases the lipophilicity, which is important with respect to possible *in vivo* studies.

So far, only inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ were identified. The second bacterial hyaluronidase *Spn*Hyl was only weakly inhibited by the biphenyl carboxylic acids. Compound **4.25d** was inactive on *Spn*Hyl and preferred *Sag*Hyal$_{4755}$ (IC_{50} = 33 µM). The mammalian hyaluronidase BTH was not affected by the small molecule inhibitors, except for compound **4.34c** (IC_{50} > 400 µM). This also suggests inactivity of these compounds at the human ortholog of BTH, the human PH-20 hyaluronidase.

The inhibitory activity of vitamin C palmitate on *Sag*Hyal$_{4755}$ was investigated in the turbidimetric assay in the presence of palladium catalyst to exclude a possible effect of traces of catalyst from the Suzuki-Miyaura cross-coupling reaction in the incubation mixture. No difference in inhibitory activity of Vcpal on *Sag*Hyal$_{4755}$ was observed.

In summary, the structural modifications of an approved drug was suiTable to obtain novel inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ that showed significantly higher inhibitory potency than the reference compounds diflunisal.

4.7 Experimental section

4.7.1 General conditions

Chemicals and solvents were purchased from Acros Organics (Geel, Belgium), Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (Munich, Germany) and used without further purification. Deuterated solvents for NMR spectroscopy were from Deutero GmbH (Kastellaun, Germany). All solvents were of analytical grade of distilled prior to use. If moisture-free conditions were required, reactions were performed in dried glassware under inert atmosphere (argon or nitrogen). DMF (H_2O < 0.01 %) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) and stored over 3 Å molecular sieves. Millipore water was used throughout for the preparation of buffers and HPLC eluents.

Nuclear Magnetic Resonance spectra were recorded with Avance 300 (^{1}H: 300 MHz, ^{13}C: 75.5 MHz), Avance 400 (^{1}H: 400 MHz, ^{13}C: 100.6 MHz) and Avance III 600 Kryo

spectrometer (^{1}H: 600 MHz, ^{13}C: 150.9 MHz) from Bruker BioSpin GmbH (Rheinstetten, Germany). Chemical shifts are given in δ (ppm) relative to external standards. Coupling constants (*J*) are reported in Hz. Multiplicities are specified with the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (mutiplet), s br (broad singlet), as well as combinations thereof. The Avance 400 and Avance 600 Kryo NMR spectrometer were used to measure 2D-NMR techniques (HSQC, HMBC, COSY) to assign ^{1}H and ^{13}C chemical shifts.

Flash chromatography was performed in glass columns on silica gel (Merck silica gel 60, particle size 0.040-0.063 mm). Automated flash chromatography was performed on a Varian IntelliFlash 310 using pre-packed Varian Superflash columns (Varian, Darmstadt, Germany). Reactions were routinely monitored by thin layer chromatography (TLC) on aluminum plates coated with silica gel (Merck silica gel 60 F_{254}, thickness 0.2 mm). The spots were visualized with UV light at 254 nM and/or a solution of bromocresol green (0.04 %) in ethanol (carboxylic acids). Microwave assisted reactions were performed on an Initiator 2.0 synthesizer (Biotage, Uppsala, Sweden). Lyophilisation was done on a Christ alpha 2-4 LD equipped with a vacuubrand RZ 6 rotary vane vacuum pump. Melting points (mp) were measured on a BÜCHI 530 electrically heated copper block apparatus (Büchi AG, Flawil, Switzerland) using open capillaries and are uncorrected. Compounds were dried under reduced pressure (0.1-1 Torr) at room temperature.

Analytical RP-HPLC was performed with a Eurospher-100 C18 column (250 x 4 mm, 5 µm, Knauer, Berlin, Germany) on a Merck Hitachi system consisting of a L-6200-A pump, an AS-2000A auto sampler, a L-4000A UV-VIS detector and a F-1050 fluorescence spectrophotometer. UV-detection was done at 220 nm. Mixtures of acetonitrile (A) and 0.05 % aq. TFA (B) were used as mobile phase. Helium degassing was used throughout. Compound purities were calculated as percentage peak area of the analyzed compound by UV detection at 220 nm. The applied gradient was: 0 to 30 min (A/B): 20/80 to 80/20 (v/v). Purity of the compounds tested for hyaluronidase inhibition was > 95 %. Preparative HPLC was performed with a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps, a K-2001 detector and a RP-column (Nucleodur-100 C_{18}, 250 x 21 mm, Macherey-Nagel, Düren, Germany) at a flow rate of 16 mL/min. Mixtures of acetonitrile and 0.1 % aq TFA were used as mobile phase. Acetonitrile was removed from the eluates under reduced pressure prior to lyophilisation.

Mass spectra (MS) were recorded on a Finnigan MAT 95 (EI-MS 70 eV), Finnigan SSQ 710A (CI-MS (NH_3)) or a Finnigan ThermoQuest TSQ 7000 (ES-MS). For high resolution mass

spectrometry an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an ESI source or a Finnigan MAT 95 with a liquid secondary ionization system (LSI-MS) was used. The peak intensity in percent relative to the strongest signals is indicated in parenthesis.

4.7.2 Chemistry

4.7.2.1 Preparation of compounds 4.24-4.46

General procedure[17]

The pertinent biphenyl carboxylic acid (1eq) and the pertinent boronic acid (1eq) were dissolved in a 1:1:1 mixture of toluene, ethanol and 2M potassium carbonate solution (v/v/v). $Pd(dppf)Cl_2 \bullet CH_2Cl_2$ (5 mol%) were added. Subsequently, the reaction mixture was stirred under microwave irradiation at 120 °C for 10 min. After cooling to room temperature the reaction mixture was taken up in water and washed with ethyl acetate. The resulting water phase was acidified to pH 2 with 0.5 N HCl. The precipitated crude product was filtered off, dried *in vacuo* and purified with flash chromatography (DCM/MeOH 100/0-80/20 v/v).

2',4'-Dichlorobiphenyl-2-carboxylic acid[29] (4.24a)

The title compound was prepared from 2-iodobenzoic acid (248 mg, 1 mmol) and 2,4-dichlorophenylboronic acid (**4.1**, 191 mg, 1 mmol) according to the general procedure. Flash chromatography yielded a white solid (40 mg, 15 %); mp 160 °C. RP-HPLC (220 nm): 95.0 % (t_R = 25.8 min, k = 10.1). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.26 (m, 1H, Ph-***H***-5'), 7.31 (d, *J* 8.2 Hz, 1H, Ph-***H***-6'), 7.46 (m, 1H, Ph-***H***-3'), 7.54 (m, 1H, Ph-***H***-4), 7.61-7.69 (m, 2H, Ph-***H***-5,6), 7.96 (m, 1H, Ph-***H***-3), 12.73 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 126.9, 128.1, 128.3, 129.9, 130.8, 130.9, 131.6, 131.8, 132.3, 132.8, 138.4, 139.6, 167.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 231.0 ([$M^{+\bullet}$], 100). HRMS (EI-MS) *m/z* calculated for 265.9901 [$M^{+\bullet}$], found 265.9902 [$M^{+\bullet}$]. $C_{13}H_8Cl_2O_2$ (265.99).

2',4'-Dichlorobiphenyl-3-carboxylic acid[29] (4.24b)

The title compound was prepared from 3-iodobenzoic acid (248 mg, 1 mmol) and 2,4-dichlorophenylboronic acid (**4.1**, 191 mg, 1 mmol) according to the general procedure. Flash chromatography yielded a white solid (97.7 mg, 37 %); mp 194 °C. RP-HPLC (220 nm): 89.4 % (t_R = 25.6 min, k = 10.0). ^{1}H-NMR (300 MHz, [D_6]DMSO) : δ (ppm) 7.49 (d, *J* 8.3 Hz, 1H, Ph-***H***), 7.54 (m, 1H, Ph-***H***), 7.62 (t, *J* 7.7 Hz, 1H, Ph-***H***), 7.69 (m, 1H, Ph-***H***), 7.77 (d, *J* 1.9 Hz, 1H, Ph-***H***), 7.96 (t, *J* 1.5 Hz, 1H, Ph-***H***), 8.00 (m, 1H, Ph-***H***), 13.15 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 127.7 (Ph-***C***), 128.8 (Ph-***C***), 129.2 (Ph-***C***), 129.8 (Ph-***C***), 130.8 (Ph-***C***), 132.2 (Ph-***C***), 132.6 (Ph-***C***), 133.2 (Ph-***C***), 133.5 (Ph-***C***),

137.7 (Ph-***C***), 166.9 (***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 310.9 ([*M*+HCOO$^-$], 100). HRMS (ESI) *m/z* [*M*+H]$^+$ calculated for $C_{13}H_9Cl_2O_2^+$: 264.9829, found 264.9845. $C_{13}H_8Cl_2O_2$ (265.99).

2',4'-Dichlorobiphenyl-4-carboxylic acid[29] (4.24c)

The title compound was prepared from 4-iodobenzoic acid (248 mg, 1 mmol) and 2,4-dichlorophenylboronic acid (**4.1**, 191 mg, 1 mmol) according to the general procedure. Flash chromatography yielded a white solid (110 mg, 41 %); mp >210 °C. RP-HPLC (220 nm): 100 % (t_R = 26.7 min, *k* = 10.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.48 (d, *J* 8.3 Hz, 1H), 7.51-7.60 (m, 3H), 7.78 (d, *J* 2.0 Hz, 1H), 8.03 (d, *J* 8.4 Hz, 2H) 13.09 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 127.7, 129.2, 129.3, 129.4, 130.2, 132.1, 132.5, 133.4, 137.8, 141.7, 166.9. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 266.0 ([$M^{+\bullet}$], 100), 249.0 ([*M*-OH$^\bullet$], 35). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{13}H_8Cl_2O_2^{\bullet+}$: 265.9901, found 265.9898. $C_{13}H_8Cl_2O_2$ (265.99).

2',4'-Dichloro-4-hydroxybiphenyl-3-carboxylic acid[29] (4.24d)

The title compound was prepared from 5-iodosalicylic acid (264 mg, 1 mmol) and 2,4-dichlorophenylboronic acid (**4.1**, 191 mg, 1 mmol) according to the general procedure. Flash chromatography yielded a white solid (30 mg, 10 %); RP-HPLC (220 nm): 94.0 % (t_R = 26.6 min, *k* = 10.4). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.05 (d, *J* 8.6 Hz, 1H, Ph-***H***-5), 7.45 (d, *J* 8.3 Hz, 1H, Ph-***H***-6'), 7.50 (m, 1H, Ph-***H***-5'), 7.59 (m, 1H, Ph-***H***-6), 7.73 (d, *J* 2.0 Hz, 1H, Ph-***H***-3'), 7.82 (d, *J* 2.3 Hz, 1H, Ph-***H***-2). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.0 (C_{quat}, Ph-***C***-4), 117.2 (Ph-***C***-5), 127.8 (Ph-***C***-5'), 128.4 (C_{quat}, Ph-***C***-1), 129.3 (Ph-***C***-3'), 130.8 (Ph-***C***-2), 132.4, (C_{quat}, Ph-***C***-2'), 132.7 (Ph-***C***-6'), 132.8 (C_{quat}, Ph-***C***-4'), 136.4 (Ph-***C***-6), 137.6 (C_{quat}, Ph-***C***-1'), 160.8 (C_{quat}, Ph-***C***-3), 171.5 (***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 564.9 ([2*M*-H$^+$]$^-$, 100), 326.8 ([*M*+HCOO$^-$], 35), 280.8 ([*M*-H$^+$]$^-$, 20). HRMS (ESI) *m/z* [*M*+H]$^+$ calculated for $C_{13}H_9Cl_2O_3^+$: 280.9778, found 280.9792. $C_{13}H_8Cl_2O_3$ (281.99).

1,1':4',1''-Terphenyl-3-carboxylic acid[30] (4.25b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and biphenyl-4-boronic acid (**4.2**, 139 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (139 mg, 42 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 27.7 min, *k* = 10.9). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.39 (t, *J* 7.3 Hz, 1H), 7.49 (t, *J* 7.5 Hz, 2H), 7.62 (t, *J* 7.7 Hz, 1H), 7.73 (d, *J* 7.3 Hz, 2H), 7.80 (s, 4H), 7.92-8.02 (m, 2H), 8.25 (s, 1H), 13.12 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 126.5, 127.1, 127.2, 127.2, 127.5, 128.2, 128.9, 129.3, 130.9, 131.5, 138.1, 139.4, 139.5, 139.8,

167.1. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 274.2 ([$M^{+\bullet}$], 100). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{19}H_{14}O_2^{+\bullet}$: 274.0994, found 274.0989. $C_{19}H_{14}O_2$ (274.10).

1,1':4',1''-Terphenyl-4-carboxylic acid[31] (4.25c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and biphenyl-4-boronic acid (**4.2**, 139 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (80 mg, 42 %); mp > 210 °C RP-HPLC (220 nm): 96.0 % (t_R = 19.0 min, *k* = 7.1), ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.40 (t, *J* 7.3 Hz, 1H, Ph-***H***),7.50 (t, *J* 7.5 Hz, 2H, Ph-***H***),7.74 (d, *J* 7.2 Hz, 2H, Ph-***H***), 7.78-7.91 (m, 6H, Ph-***H***), 8.04 (d, *J* 8.4 Hz, 2H, Ph-***H***), 12.99 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 126.5 (2C, Ph-***C***), 127.2 (2C, Ph-***C***), 127.4 (Ph-***C***), 127.6 (Ph-***C***), 128.9 (2C, Ph-***C***), 129.9 (2C, Ph-***C***), 137.8 (Ph-***C***), 139.3 (Ph-***C***), 139.8 (Ph-***C***), 143.6 (Ph-***C***), 167.0 (***C***OOH). MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 274.1 ([$M^{+\bullet}$], 100). HRMS (EI-MS) *m/z* [*M*+H]$^+$ calculated for $C_{19}H_{15}O_2^+$: 274.0994, found 274.0987. $C_{19}H_{14}O_2$ (274.10).

4-Hydroxy-1,1':4',1''-terphenyl-3-carboxylic acid (4.25d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and biphenyl-4-boronic acid (**4.2**, 297 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (44 mg, 10 %); RP-HPLC (220 nm): 100 % (t_R = 28.5 min, *k* = 11.2). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.08 (d, *J* 8.6 Hz, 1H), 7.37 (t, *J* 7.3 Hz, 1H), 7.48 (t, *J* 7.5 Hz, 2H), 7.66-7.79 (m, 6H), 7.89 (m, 1H), 8.10 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 113.3, 117.8, 126.4, 126.6, 127.1, 127.4, 127.7, 128.9, 130.6, 133.7, 137.9, 138.7, 139.5, 160.6, 171.7. MS (ES-MS) *m/z* (rel. int. in %) 579.1 ([2*M*-H$^+$]$^-$, 25), 335.0 ([*M*+HCOO$^-$], 100), 288.9 ([*M*-H$^+$]$^-$, 90). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{19}H_{13}O_3^-$: 290.0947, found 289.0875. $C_{19}H_{14}O_3$ (290.09).

4'-Chlorobiphenyl-2-carboxylic acid[32] (4.26a)

The title compound was prepared from 2-iodobenzoic acid (174 mg, 0.7 mmol) and 4-chlorophenylboronic acid (**4.3**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (27 mg, 11 %); mp 142 °C (ref.:[32] 165.5-166 °C). RP-HPLC (220 nm): 100 % (t_R = 23.7 min, *k* = 9.2). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.35 (t, *J* 10.7 Hz, 3H), 7.43-7.53 (m, 3H), 7.58 (t, *J* 7.8 Hz, 1H), 7.77 (d, *J* 7.4 Hz, 1H), 12.85 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 127.6, 127.9, 129.3, 130.1, 130.3, 131.0, 131.8, 132.0, 139.7, 139.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 232.1 ([$M^{+\bullet}$], 100), 215.0 ([*M*-OH$^\bullet$], 60). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_2^-$: 232.0252, found 232.0254. $C_{13}H_9ClO_2$ (232.03).

4'-Chlorobiphenyl-3-carboxylic acid[33] (4.26b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 4-chlorophenylboronic acid (**4.3**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (28 mg, 18 %); mp 187-189 °C (ref.:[33] 248 °C). RP-HPLC (220 nm): 100 % (t_R = 25.9 min, k = 10.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.50-7.65 (m, 3H), 7.70-7.78 (m, 2H), 7.87-8.00 (m, 2H), 8.17 (t, J 1.7 Hz, 1H), 13.14 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 127.2, 128.5, 128.5, 128.9, 129.3, 131.0, 131.5, 132.7, 138.0, 139.1, 167.0. MS (ES-MS) *m/z* (rel. int. in %) 231.0223 ([M-H]$^-$). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_2^-$: 231.0218, found 231.0223. $C_{13}H_9ClO_2$ (232.03).

4'-Chlorobiphenyl-4-carboxylic acid[33] (4.26c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 4-chlorophenylboronic acid (**4.3**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (46 mg, 28 %); mp > 210 °C (ref.:[33] 290-291 °C). RP-HPLC (220 nm): 100 % (t_R = 25.1 min, k = 9.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.55 (d, J 8.5 Hz, 2H), 7.79 (m, 4H), 8.02 (d, J 8.3 Hz, 2H), 13.02 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 126.7, 128.7, 128.9, 129.8, 129.9, 133.1, 137.7, 142.8, 166.9. MS (ES-MS) *m/z* (rel. int. in %) 231.0222 ([*M*-H]$^-$). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_2^-$: 231.0218, found 231.0221. $C_{13}H_9ClO_2$ (232.03).

4'-Chloro-4-hydroxybiphenyl-3-carboxylic acid[34] (4.26d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 4-chlorophenylboronic acid (**4.3**, 235 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (75 mg, 20 %); mp 169 °C. RP-HPLC (220 nm): 100 % (t_R = 26.6 min, k = 10.4). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.06 (d, J 8.6 Hz, 1H), 7.48 (d, J 8.6 Hz, 2H), 7.65 (d, J 8.6 Hz, 2H), 7.83 (m, 1H), 8.03 (d, J 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.3, 117.8, 127.8, 128.8, 129.8, 131.8, 133.7, 137.7, 160.7, 171.6. MS (ES-MS) *m/z* (rel. int. in %) 495.0 ([2*M*-H$^+$]$^-$, 100), 361.0 ([*M*+CF$_3$COO$^-$], 20) 292.9 ([*M*+HCOO$^-$], 30), 246.9 ([*M*-H$^+$]$^-$, 15). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_3^-$: 248.0201, found 248.0206. $C_{13}H_9ClO_3$ (248.02).

2',4'-Bis(trifluoromethyl)biphenyl-3-carboxylic acid (4.27b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 2,4-bis(trifluoromethyl)phenylboronic acid (**4.4**, 180 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (80 mg, 34 %); mp 170 °C. RP-

HPLC (220 nm): 100 % (t_R = 27.2 min, *k* = 10.7). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.63 (d, *J* 4.6 Hz, 2H, Ph-***H***-), 7.72 (d, *J* 7.8 Hz, 1H, Ph-***H***-), 7.90 (s, 1H, Ph-***H***-2), 8.01-8.09 (m, 1H, Ph-***H***-4), 8.15 (d, *J* 8.9 Hz, 2H, Ph-***H***-) 13.20 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 122.4 (q, *J* 28.08 Hz, ***C***F$_3$), 123.2 (m, Ph-***C***-3‘), 124.2 (q, *J* 26.12 Hz, ***C***F$_3$), 128.0 (q, *J* 30.04 Hz, C$_{quat}$, Ph-***C***-CF$_3$), 129.9 (q, *J* 33.46 Hz, C$_{quat}$, Ph-***C***-CF$_3$), 128.6 (Ph-***C***-5), 129.1 (Ph-***C***-2), 129.3 (2C, Ph-***C***-4,5‘), 130.6 (C$_{quat}$, Ph-***C***-3), 132.9 (Ph-***C***-6), 133.6 (Ph-***C***-6‘), 138.0 (C$_{quat}$, Ph-***C***-1), 143.9 (C$_{quat}$, Ph-***C***-1‘), 166.8 (C$_{quat}$, ***C***OOH). MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 334.1 ([*M*$^{+\bullet}$], 100), 317.0 ([*M*-OH$^\bullet$], 50). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{15}H_7F_6O_2^-$: 333.0356, found 333.0353. $C_{15}H_8F_6O_2$ (334.04).

2’,4’-Bis(trifluoromethyl)biphenyl-4-carboxylic acid (4.27c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 2,4-bis(trifluoromethyl)phenylboronic acid (**4.4**, 180 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (150 mg, 64 %); mp 175-178 °C. RP-HPLC (220 nm): 100 % (t_R = 27.5 min, *k* = 10.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.50 (d, *J* 8.1 Hz, 2H), 7.71 (d, *J* 7.9 Hz, 2H), 8.04 (d, *J* 8.4 Hz, 1H), 8.16 (d, *J* 8.6 Hz, 2H) 13.15 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 122.0, 123.6, 125.5, 125.6, 128.1, 128.6, 129.4, 129.5 (2 interfering carbon signals), 129.8, 131.2, 133.8, 142.6, 144.4, 167.6. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 334.1 ([*M*$^{+\bullet}$], 100), 317.0 ([*M*-OH$^\bullet$], 75). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{15}H_7F_6O_2^-$: 333.0356, found 333.0360. $C_{15}H_8F_6O_2$ (334.04).

2’-Chloro-4’-(trifluoromethyl)biphenyl-3-carboxylic acid (4.28b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 2-chloro-4-(trifluoromethyl)phenylboronic acid (**4.5**, 157 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (98 mg, 57 %); mp 150 °C. RP-HPLC (220 nm): 100 % (t_R = 27.2 min, *k* = 10.7). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.65 (t, *J* 7.7 Hz, 1H), 7.70 (d, *J* 8.0 Hz, 1H), 7.72-7.76 (m, 1H), 7.82 (m, 1H), 7.98-8.07 (m, 3H), 13.19 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 124.3, 126.6, 128.8, 129.2, 129.6, 129.7, 130.0, 130.9, 132.2, 132.4, 133.4, 137.5, 142.9, 166.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 300.1 ([*M*$^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_7ClF_3O_2^-$: 299.0092, found 299.0086. $C_{14}H_8ClF_3O_2$ (300.02).

2’-Chloro-4’-(trifluoromethyl)biphenyl-4-carboxylic acid (4.28c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 2-chloro-4-(trifluoromethyl)phenylboronic acid (**4.5**, 157 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (73 mg, 43 %); mp 210 °C. RP-HPLC (220 nm): 100 % (t_R = 27.7 min, *k* = 10.9). ^{1}H-NMR (300 MHz, [D$_6$]DMSO):

δ (ppm) 7.61 (d, *J* 8.4 Hz, 2H), 7.69 (d, *J* 8.0 Hz, 1H), 7.83 (m, 1H), 7.99-8.10 (m, 3H), 13.13 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 124.3, 126.7, 129.2, 129.4, 129.7, 130.2, 130.6, 132.1, 132.3, 141.5, 142.9, 166.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 300.1 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_7ClF_3O_2^-$: 299.0092, found 299.0086. $C_{14}H_8ClF_3O_2$ (300.02).

2'-Chloro-4-hydroxy-4'-(trifluoromethyl)biphenyl-3-carboxylic acid (4.28d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 2-chloro-4-(trifluoromethyl)phenylboronic acid (**4.5**, 337 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (240 mg, 50 %); mp 185 °C. RP-HPLC (220 nm): 100 % (t_R = 28.1 min, *k* = 11.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.09 (d, *J* 8.7 Hz, 1H, Ph-***H***-5), 7.64-7.67 (m, 2H, Ph-***H***-6,6'), 7.77-7.80 (m, 1H, Ph-***H***-5'), 7.88 (d, *J* 2.4 Hz, 1H, Ph-***H***-2), 7.97 (d, *J* 1.2 Hz, 1H, Ph-***H***-3'), 11.52 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.0 (C$_{quat}$, Ph-***C***-3), 117.3 (Ph-***C***-5), 122.5 (***C***F$_3$), 124.4 (Ph-***C***-5'), 126.7 (Ph-***C***-3'), 128.1 (C$_{quat}$, Ph-***C***-1), 130.9 (Ph-***C***-2), 132.3 (Ph-***C***-6'), 132.4 (C$_{quat}$, Ph-***C***-4'), 136.3 (Ph-***C***-6), 142.7 (C$_{quat}$, Ph-***C***-1'), 161.1 (C$_{quat}$, Ph-***C***-4), 171.4 (***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 630.9 ([2*M*-H]$^-$, 100), 360.9 ([*M*+HCOO$^-$], 15), 314.9 ([*M*-H]$^-$, 25). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_7ClF_3O_3^-$: 315.0041, found 315.0047. $C_{14}H_8ClF_3O_3$ (316.01).

2'-Chlorobiphenyl-3-carboxylic acid[35] (4.29b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 2-chlorophenylboronic acid (**4.6**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (121 mg, 61 %); mp 196 °C (ref.:[35] 185-187 °C). RP-HPLC (220 nm): 100 % (t_R = 22.1 min, *k* = 8.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.38-7.48 (m, 3H), 7.55-7.65 (m, 2H), 7.69 (m, 1H), 7.93-8.03 (m, 2H), 13.12 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 127.6, 128.5, 128.6, 129.5, 129.8, 130.7, 131.1, 131.4, 133.5, 138.7, 138.8, 166.9. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 232.1 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*+H]$^+$ calculated for $C_{13}H_{10}ClO_2^+$: 232.0291, found 233.0091. $C_{13}H_9ClO_2$ (232.03).

2'-Chloro-4-hydroxybiphenyl-3-carboxylic acid (4.29d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 2-chlorophenylboronic acid (**4.6**, 235 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (273 mg, 64 %); mp 188 °C. RP-HPLC (220 nm): 100 % (t_R = 25.2 min, *k* = 9.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ(ppm) 7.05 (d, *J* 8.6 Hz, 1H), 7.33-7.46 (m, 3H), 7.51-7.64 (m, 2H), 7.83 (d, *J* 2.1 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 112.7, 117.0, 127.5, 129.1, 129.5, 129.8, 130.7, 131.2, 131.3, 136.4,

138.4, 160.5, 171.5. MS (ES-MS) *m/z* (rel. int. in %) 247.0175 ([*M*-H]$^-$). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_3^-$: 248.0201, found 248.0206. $C_{13}H_9ClO_3$ (248.02).

3',5'-Bis(trifluoromethyl)biphenyl-3-carboxylic acid[36] (4.30b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 3,5-bis(trifluoromethyl)phenylboronic acid (**4.7**, 180 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (35 mg, 15 %); mp 198 °C (ref.:[36] 205-207 °C). RP-HPLC (220 nm): 100 % (t_R = 27.9 min, *k* = 11.0). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 7.64 (t, *J* 7.8 Hz, 1H), 7.90-8.02 (m, 2H), 8.11 (d, *J* 7.8 Hz, 1H), 8.21 (s, 2H), 8.31 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 123.1, 126.7, 128.6, 129.4, 130.7, 131.2, 132.8, 133.3, 133.7, 139.6, 144.1, 169.3. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 334.0 ([*M*$^{+\bullet}$], 100), 317.0 (50), 289.1 (10), 269.0 (35), 220.1 (20), 201.0 (10), 170.1 (5), 149.1 (10), 133.6 (5), 125.1 (10), 109.8 (5), 75.1 (5), 51.1 (5). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{15}H_7F_6O_2^-$: 334.0389, found 334.0385. $C_{15}H_8F_6O_2$ (334.04).

3',5'-Bis(trifluoromethyl)biphenyl-4-carboxylic acid[37] (4.30c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 3,5-bis(trifluoromethyl)phenylboronic acid (**4.7**, 181 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (121 mg, 52 %); mp 204 °C. RP-HPLC (220 nm): 98 % (t_R = 27.0 min, *k* = 10.6). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 7.83 (d, *J* 8.5 Hz, 2H), 8.00 (s, 1H), 8.16 (d, *J* 8.6 Hz, 2H), 8.24 (s, 2H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 122.6, 123.1, 126.7, 128.6, 128.8, 131.8, 132.4, 133.3, 143.5, 144.0, 169.2. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 334.0 ([*M*$^{+\bullet}$], 100), 317.0 ([*M*-OH$^\bullet$], 60), 315.0 ([*M*-F$^\bullet$]$^+$, 15). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{15}H_7F_6O_2^{-\cdot}$: 334.0389, found 334.0386. $C_{15}H_8F_6O_2$ (334.04).

4-Hydroxy-3',5'-bis(trifluoromethyl)biphenyl-3-carboxylic acid (4.30d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 3,5-bis(trifluoromethyl)phenylboronic acid (**4.7**, 386 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (204 mg, 39 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 29.2 min, *k* = 11.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.65 (t, *J* 1.9 Hz, 1H), 7.80 (d, *J* 1.9 Hz, 2H), 7.87 (d, *J* 8.5 Hz, 2H), 8.02 (d, *J* 8.5 Hz, 2H), 13.08 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.6, 127.2, 127.5, 129.8, 130.5, 134.7, 141.2, 142.4, 166.7. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 266.0 ([M$^{+\bullet}$], 100), 249.0 ([*M*-CH$_3^\bullet$], 45). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_7Cl_2O_2^-$: 267.9834, found 267.9835. $C_{13}H_8Cl_2O_2$ (265.99).

3'-Chlorobiphenyl-3-carboxylic acid[35] (4.31b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 3-chlorophenylboronic acid (**4.8**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (90 mg, 55 %); mp 185 °C (ref.:[35] 178-180 °C). RP-HPLC (220 nm): 100 % (t_R = 25.6 min, *k* = 10.0). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.48 (m, 2H), 7.56-7.71 (m, 2H), 7.76 (t, *J* 1.7 Hz, 1H), 7.89-8.02 (m, 2H), 8.18 (t, *J* 1.6 Hz, 1H), 13.16 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.5, 126.5, 127.4, 127.6, 128.8, 129.3, 130.8, 131.2, 131.5, 133.8, 138.9, 141.3, 167.0. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 232.1 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_2^-$: 232.0252, found 232.0254. $C_{13}H_9ClO_2$ (232.03).

3'-Chlorobiphenyl-4-carboxylic acid[38] (4.31c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 3-chlorophenylboronic acid (**4.8**, 110 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (120 mg, 68 %); mp > 210 °C (ref.:[38] 249-250 °C). RP-HPLC (220 nm): 100 % (t_R = 24.5 min, *k* = 9.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.42-7.58 (m, 2H), 7.71 (d, *J* 7.2 Hz, 1H), 7.75-7.88 (m, 3H), 8.02 (d, *J* 8.2 Hz, 2H), 13.06 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.6, 126.6, 126.9, 128.0, 129.9, 130.1, 130.8, 133.8, 141.1, 142.6, 166.9. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 232.0 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_8ClO_2^-$: 232.0252, found 232.0247. $C_{13}H_9ClO_2$ (232.03).

3'-Chloro-5'-methoxybiphenyl-3-carboxylic acid (4.32b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 3-chloro-5-methyoxyphenylboronic acid (**4.9**, 131 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (70 mg, 28 %); mp 191-193 °C. RP-HPLC (220 nm): 100 % (t_R = 26.1 min, *k* = 10.2). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.07 (t, *J* 2.0 Hz, 1H), 7.16-7.22 (m, 1H), 7.31 (t, *J* 1.6 Hz, 1H), 7.60 (t, *J* 7.8 Hz, 1H), 7.96 (t, *J* 6.7 Hz, 2H), 8.16 (t, *J* 1.6 Hz, 1H), 13.15 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.7, 111.5, 113.4, 118.8, 127.4, 128.9, 129.3, 131.3, 131.4, 134.4, 138.9, 142.2, 160.5, 167.0. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 262.0 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_{10}ClO_3^-$: 262.0358, found 262.0356. $C_{14}H_{11}ClO_3$ (262.04).

3'-Chloro-5'-methoxybiphenyl-4-carboxylic acid (4.32c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 3-chloro-5-methyoxyphenylboronic acid (**4.9**, 131 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white, crystalline solid (40 mg, 22 %); mp 194-197 °C. RP-HPLC (220 nm): 100 % (t_R = 26.6 min, k = 10.4). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.06-7.11 (m, 1H), 7.21-7.26 (m, 1H), 7.36 (t, *J* 1.7 Hz, 1H), 7.83 (d, *J* 8.5 Hz, 2H), 8.01 (d, *J* 8.5 Hz, 2H), 13.06 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.7, 111.6, 113.7, 118.9, 127.0, 129.8, 130.2, 134.4, 141.8, 142.6, 160.5, 166.9. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 262.1 ([$M^{+\bullet}$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_{10}ClO_3^-$: 262.0358, found 262.0354. $C_{14}H_{11}ClO_3$ (262.04).

3'-Chloro-4-hydroxy-5'-methoxybiphenyl-3-carboxylic acid (4.32d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 3-chloro-5-methyoxyphenylboronic acid (**4.9**, 279 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (94 mg, 22 %); mp 206-207 °C. RP-HPLC (220 nm): 100 % (t_R = 26.8 min, k = 10.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.83 (s, 3H), 6.99 (t, *J* 2.0 Hz, 1H), 7.05 (d, *J* 8.6 Hz, 1H), 7.08-7.13 (m, 1H), 7.22 (t, *J* 1.6 Hz, 1H), 7.85 (m, 1H), 8.02 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.6, 110.8, 112.5, 113.3, 117.7, 118.2, 128.2, 129.6, 134.0, 134.3, 141.9, 160.5, 161.0, 171.6. MS (ES-MS) *m/z* (rel. int. in %) 555.0 ([2*M*-H$^+$]$^-$, 100), 390.9 ([*M*+CF_3COO^-], 20), 323.0 ([*M*+$HCOO^-$], 35), 276.9 ([*M*-H$^+$]$^-$, 20). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_{10}ClO_4^-$: 278.0307, found 278.0304. $C_{14}H_{11}ClO_4$ (278.03).

3',5'-Dichlorobiphenyl-4-carboxylic acid (4.33c)

The title compound was prepared from 4-iodobenzoic acid (129 mg, 0.52 mmol) and 3,5-dichlorophenylboronic acid (**4.10**, 100 mg, 0.52 mmol) according to the general procedure. Flash chromatography yielded a white solid (94 mg, 67 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 29.2 min, k = 11.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.66 (t, *J* 3.7 Hz, 1H, Ph-***H***-4'), 7.81 (d, *J* 1.8 Hz, 2H, Ph-***H***-2',6'), 7.88 (d, *J* 8.7 Hz, 2H, Ph-***H***-2,6), 8.02 (d, *J* 8.6 Hz, 2H, Ph-***H***-3,5), 13.08 (br s, 1H, COO***H***). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.6 (2C, Ph-***C***-2',6'), 127.2 (2C, Ph-***C***-2,6), 127.5 (Ph-***C***-4'), 129.8 (2C, Ph-***C***-3,5), 130.6 (C_{quat}, Ph-***C***-1'), 134.7 (2C, C_{quat}, Ph-***C***-3',5'), 141.1 (C_{quat}, Ph-***C***-1), 142.4 (C_{quat}, Ph-***C***-4), 166.7 (C_{quat}, ***C***OOH). MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 266.0 ([$M^{+\bullet}$], 100), 249.0 ([*M*-$CH_3^\bullet$], 45). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_7Cl_2O_2^-$: 267.9834, found 267.9835. $C_{13}H_8Cl_2O_2$ (265.99).

3',5'-Dichloro-4-hydroxybiphenyl-3-carboxylic acid[29] (4.33d)

The title compound was prepared from 5-iodosalicylic acid (347 mg, 1.3 mmol) and 3,5-dichlorophenylboronic acid (**4.10**, 251 mg, 1.3 mmol) according to the general procedure. Flash chromatography yielded a white solid (177 mg, 48 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 29.3 min, *k* = 11.6). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.06 (d, *J* 8.7 Hz, 1H), 7.55 (t, *J* 1.8 Hz, 1H), 7.68 (d, *J* 1.9 Hz, 2H), 7.90 (m, 1H), 8.06 (d, *J* 2.5 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.5, 117.9, 124.8, 126.3, 128.2, 128.5, 134.1, 134.6, 142.5, 161.3, 171.5. MS (ES-MS) *m/z* (rel. int. in %) 564.9 ([2*M*-H$^+$]$^-$, 100), 394.9 ([*M*+CF$_3$COO$^-$], 10), 326.9 ([*M*+HCOO$^-$], 20), 280.9 ([*M*-H$^+$]$^-$, 30). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_7Cl_2O_3^-$: 281.9812, found 281.9806. $C_{13}H_8Cl_2O_3$ (281.99).

1,1':3',1''-Terphenyl-3-carboxylic acid (4.34b)

The title compound was prepared from 3-iodobenzoic acid (173 mg, 0.7 mmol) and biphenyl-3-boronic acid (**4.11**, 138 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (137 mg, 71 %); mp 172-175 °C. RP-HPLC (220 nm): 100 % (t_R = 27.0 min, *k* = 10.6). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.39 (t, *J* 7.3 Hz, 1H), 7.49 (t, *J* 7.4 Hz, 2H), 7.54-7.73 (m, 5H), 7.77 (d, *J* 7.3 Hz, 2H), 7.92 (s, 1H), 8.00 (m, 2H), 8.26 (s, 1H), 13.14 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.1, 125.9, 126.2, 126.9, 127.4, 127.5, 128.3, 128.9, 129.2, 129.6, 131.3, 131.4, 139.89, 139.93, 140.4, 141.0, 167.2. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 274.3 ([*M*$^{+\bullet}$], 100), 257.2 ([*M*-OH$^\bullet$], 5). HRMS (EI-MS) *m/z* [*M*$^{+\bullet}$] calculated for $C_{19}H_{14}O_2^{+\bullet}$: 274.0993, found 274.0991. $C_{19}H_{14}O_2$ (274.10).

1,1':3',1''-Terphenyl-4-carboxylic acid[38,39] (4.34c)

The title compound was prepared from 4-iodobenzoic acid (173 mg, 0.7 mmol) and biphenyl-3-boronic acid (**4.11**, 138 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (37 mg, 19 %; mp ref.:[39] 221 °C). RP-HPLC (220 nm): 96 % (t_R = 27.5 min, *k* = 10.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.08 (d, *J* 8.6 Hz, 1H), 7.34-7.66 (m, 7H), 7.75 (d, *J* 7.3 Hz, 2H), 7.84 (s, 1H), 7.94 (m, 1H), 8.11 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, DMSO): δ (ppm) 113.4, 117.7, 124.5, 125.3, 125.4, 126.9, 127.5, 128.1, 128.8, 129.5, 131.1, 134.1, 139.7, 140.0, 140.9, 160.6, 171.7. HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{19}H_{13}O_2^-$: 273.0916, found 273.0915. $C_{19}H_{14}O_2$ (274.10).

4-Hydroxy-1,1':3',1''-terphenyl-3-carboxylic acid (4.34d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and biphenyl-3-boronic acid (**4.11**, 297 mg, 1.5 mmol) according to the general procedure. Flash

chromatography yielded a yellow solid (84 mg, 19 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 27.5 min, k = 10.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.40 (t, J 7.3 Hz, 1H), 7.50 (t, J 7.4 Hz, 2H), 7.59 (t, J 7.7 Hz, 1H), 7.67-7.75 (m, 2H), 7.78 (d, J 7.3 Hz, 2H), 7.91 (d, J 8.4 Hz, 2H), 7.97 (s, 1H), 8.05 (d, J 8.4 Hz, 2H), 13.01 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 125.3, 126.0, 126.6, 126.9 (2 interfering carbon signals), 127.0 (2 interfering carbon signals), 127.6, 128.9 (2 interfering carbon signals), 129.6, 129.7, 129.8 (2 interfering carbon signals), 139.6, 139.8, 141.0, 144.2, 167.1. HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{19}H_{13}O_3^-$: 289.0870, found 289.0871. $C_{19}H_{14}O_3$ (290.09).

3'-Chloro-4'-fluorobiphenyl-3-carboxylic acid (4.35b)

The title compound was prepared from 3-iodobenzoic acid (173 mg, 0.7 mmol) and 3-chloro-4-fluorophenylboronic acid (**4.12**, 122 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white, crystalline solid (57 mg, 32 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 25.9 min, k = 10.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.52 (t, J 9.0 Hz, 1H), 7.60 (t, J 7.7 Hz, 1H), 7.73 (m, 1H), 8.00-7.90 (m, 3H), 8.17 (s, 1H), 13.15 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 117.2, 117.5, 120.0, 120.2, 127.4, 127.5, 128.8, 129.3, 131.2, 131.5, 155.3, 158.6, 167.0. MS (ES-MS) *m/z* (rel. int. in %) 498.9 ([2*M*-H$^+$]$^-$, 30), 294.9 ([*M*+HCOO$^-$], 100), 248.9 ([*M*-H$^+$]$^-$, 5). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_7ClFO_2^-$: 250.0158, found 250.015. $C_{13}H_8ClFO_2$ (250.02).

3'-Chloro-4'-fluorobiphenyl-4-carboxylic acid[40] (4.35c)

The title compound was prepared from 4-iodobenzoic acid (173 mg, 0.7 mmol) and 3-chloro-4-fluorophenylboronic acid (**4.12**, 122 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a grey solid (122 mg, 69 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 26.3 min, k = 10.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.53 (t, J 9.0 Hz, 1H), 7.76 (m, 1H), 7.82 (d, J 8.5 Hz, 2H), 7.93-8.05 (m, 3H), 13.05 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 117.2, 117.5, 120.0, 126.9, 127.6, 128.9, 129.8, 130.0, 136.8, 141.7, 166.9. MS (ES-MS) *m/z* (rel. int. in %) 498.9 ([2*M*-H$^+$]$^-$, 5), 294.9 ([*M*+HCOO$^-$], 100), 248.9 ([*M*-H$^+$]$^-$, 5). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{13}H_7ClFO_2^-$: 250.0158, found 250.0158. $C_{13}H_8ClFO_2$ (250.02).

3'-Chlorobiphenyl-3,4'-dicarboxylic acid (4.36b)

The title compound was prepared from 3-iodobenzoic acid (173 mg, 0.7 mmol) and 4-borono-2-chlorobenzoic acid (**4.13**, 140 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (105 mg, 54 %); RP-HPLC (220 nm): 98 % (t_R = 19.3 min, k = 7.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.64 (t, J 7.8 Hz, 1H), 7.77 (m, 1H), 7.90 (m, 2H), 8.01 (m, 2H), 8.23 (s, 1H), 13.32 (br s, 1H). ^{13}C-NMR (75 MHz,

[D_6]DMSO): δ (ppm) 125.5, 127.5, 128.6, 129.3, 129.4, 130.1, 131.4, 131.57, 131.63, 132.6, 137.9, 143.2, 166.3, 166.9. MS (ES-MS) *m/z* (rel. int. in %) 596.9 ([2*M*+HCOO$^-$], 70), 551.1 ([2*M*-H], 30), 366.9 ([*M*+HCOO$^-$+HCOOH], 100), 320.9 ([*M*-H$^+$]$^-$, 65). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_8ClO_4^-$: 276.015, found 276.0147. $C_{14}H_9ClO_4$ (276.02).

5'-*tert*-Butyl-2'-methoxybiphenyl-3-carboxylic acid (4.37b)

The title compound was prepared from 3-iodobenzoic acid (89 mg, 0.36 mmol) and 5-*tert*-butyl-2-methoxyphenylboronic acid (**4.14**, 75 mg, 0.36 mmol) according to the general procedure. Flash chromatography yielded a brown solid (32 mg, 31 %); mp 118-128 °C. RP-HPLC (220 nm): 100 % (t_R = 26.7 min, *k* = 10.5). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.30 (s, 9H), 3.74 (s, 3H), 7.05 (d, *J* 8.7 Hz, 1H), 7.27 (d, *J* 2.5 Hz, 1H), 7.38 (m, 1H), 7.53 (t, *J* 7.7 Hz, 1H), 7.71 (d, *J* 7.9 Hz, 1H), 7.89 (d, *J* 7.8 Hz, 1H), 8.02 (s, 1H), 12.98 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 31.2, 33.7, 55.5, 111.4, 125.8, 127.1, 127.5, 128.1, 128.2, 129.9, 130.4, 133.7, 138.8, 142.9, 153.8, 167.3. MS (ES-MS) *m/z* (rel. int. in %) 283.1339 ([*M*-H$^+$]$^-$). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{19}O_3^-$: 284.1374, found 284.1373. $C_{18}H_{20}O_3$ (284.14).

5'-*tert*-Butyl-2'-methoxybiphenyl-4-carboxylic acid (4.37c)

The title compound was prepared from 4-iodobenzoic acid (89 mg, 0.36 mmol) and 5-*tert*-butyl-2-methoxyphenylboronic acid (**4.14**, 75 mg, 0.36 mmol) according to the general procedure. Flash chromatography yielded a brown solid (90 mg, 88 %); mp 146-157 °C. RP-HPLC (220 nm): 100 % (t_R = 27.2 min, *k* = 10.7). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.29 (s, 9H), 3.75 (s, 3H), 7.06 (d, *J* 8.7 Hz, 1H), 7.30 (d, *J* 2.5 Hz, 1H), 7.39 (m, 1H), 7.60 (d, *J* 8.3 Hz, 2H), 7.97 (d, *J* 8.3 Hz, 2H), 12.89 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 31.2, 33.7, 55.5, 111.4, 126.1, 127.1, 128.0, 128.8, 128.9, 129.4, 142.9, 143.0, 153.9, 167.1. MS (ES-MS) *m/z* (rel. int. in %) 567.2 ([2*M*-H$^+$]$^-$, 100), 382.9 ([*M*+HCOO$^-$], 80), 329.0 ([*M*-H$^+$]$^-$, 30). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{19}O_3^-$: 284.1413, found 284.1341. $C_{18}H_{20}O_3$ (284.14).

5'-*tert*-Butyl-4-hydroxy-2'-methoxybiphenyl-3-carboxylic acid (4.37d)

The title compound was prepared from 5-iodosalicylic acid (126 mg, 0.48 mmol) and 5-*tert*-butyl-2-methoxyphenylboronic acid (**4.14**, 100 mg, 0.48 mmol) according to the general procedure. Flash chromatography yielded a white solid (61 mg, 42 %); mp 140-150 °C. RP-HPLC (220 nm): 100 % (t_R = 27.2 min, *k* = 10.7). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.28 (s, 9H), 3.73 (s, 3H), 7.00 (m, 2H), 7.23 (d, *J* 2.5 Hz, 1H), 7.32 (m, 1H), 7.62 (m, 1H), 7.87 (d, *J* 2.3 Hz, 1H), 11.26 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 31.2 (3 interfering carbon signals), 33.7, 55.5, 111.3, 112.4, 116.7, 125.2, 126.8, 127.8, 129.4,

130.5, 136.6, 142.8, 153.8, 159.9, 171.8. MS (ES-MS) *m/z* (rel. int. in %) 599.2 ([2*M*-H$^+$]$^-$, 60), 345.0 ([*M*+HCOO$^-$], 100), 299.0 ([*M*-H$^+$]$^-$, 30). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{19}O_4^-$: 300.1363, found 299.1290. $C_{18}H_{20}O_4$ (300.14).

4'-Cyano-3'-fluorobiphenyl-3-carboxylic acid (4.38b)

The title compound was prepared from 3-iodobenzoic acid (173 mg, 0.7 mmol) and 4-cyano-3-fluorophenylboronic acid (**4.15**, 115 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a white solid (111 mg, 66 %); mp 185-190 °C. RP-HPLC (220 nm): 100 % (t_R = 20.4 min, *k* = 7.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.65 (t, *J* 7.8 Hz, 1H), 7.78 (m, 1H), 7.89-8.07 (m, 4H), 8.26 (s, 1H), 13.24 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 98.9, 113.9, 114.5, 114.8, 123.7, 127.8, 129.5, 131.5, 134.3, 137.4, 146.6, 161.1, 164.5, 166.8. MS (ES-MS) *m/z* (rel. int. in %) 480.9 ([2*M*-H$^+$]$^-$, 100), 285.8 ([*M*+HCOO$^-$], 30). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_7FNO_2^-$: 241.0499, found 241.0497. $C_{14}H_8FNO_2$ (241.05).

4'-Cyano-3'-fluorobiphenyl-4-carboxylic acid (4.38c)

The title compound was prepared from 4-iodobenzoic acid (173 mg, 0.7 mmol) and 4-cyano-3-fluorophenylboronic acid (**4.15**, 115 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a grey solid (54 mg, 32 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 20.7 min, *k* = 7.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.81 (m, 1H), 7.95 (m, 3H), 8.04 (t, *J* 7.6 Hz, 3H), 13.15 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 99.3, 113.9, 114.6, 114.9, 123.9, 127.5, 129.9, 131.2, 134.3, 140.9, 146.4, 161.1, 164.5. MS (ES-MS) *m/z* (rel. int. in %) 480.9 ([2*M*-H$^+$]$^-$, 20), 285.8 ([*M*+HCOO$^-$], 100). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_7FNO_2^-$: 241.0499, found 241.0492. $C_{14}H_8FNO_2$ (241.05).

4'-(2-Carboxyethyl)biphenyl-4-carboxylic acid (4.39c)

The title compound was prepared from 4-iodobenzoic acid (173 mg, 0.7 mmol) and 4-(2-carboxyethyl)phenylboronic acid (**4.16**, 136 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (181 mg, 95 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 16.7 min, *k* = 6.2). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.58 (t, *J* 7.5 Hz, 2H), 2.87 (t, *J* 7.5 Hz, 2H), 7.35 (d, *J* 8.2 Hz, 2H), 7.53-7.67 (m, 3H), 7.91 (m, 2H), 8.16 (s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 30.0, 35.1, 126.8 (2 interfering carbon signals), 127.2, 128.1, 129.1 (2 interfering carbon signals), 129.4, 131.1, 131.5, 137.1, 140.4, 140.8, 167.4, 173.9. MS (ES-MS) *m/z* (rel. int. in %) 331.0 ([*M*+HCOO$^-$], 100), 284.9 ([*M*-H$^+$]$^-$, 40). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{16}H_{13}O_4^-$: 270.0894, found 269.0822. $C_{16}H_{14}O_4$ (270.09).

4'-(2-Carboxyethyl)-4-hydroxybiphenyl-3-carboxylic acid (4.39d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 4-(2-carboxyethyl)phenylboronic acid (**4.16**, 291 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (230 mg, 52 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 17.7 min, *k* = 6.6). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.56 (t, *J* 7.6 Hz, 3H), 2.85 (t, *J* 7.5 Hz, 2H), 7.04 (d, *J* 8.6 Hz, 1H), 7.30 (d, *J* 8.2 Hz, 2H), 7.53 (d, *J* 8.2 Hz, 2H), 7.81 (m, 1H), 8.00 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 30.2, 35.5, 113.7, 118.2, 126.5 (2 interfering carbon signals), 128.1, 129.3 (2 interfering carbon signals), 131.6, 134.2, 137.1, 140.2, 160.7, 172.1, 174.3. MS (ES-MS) *m/z* (rel. int. in %) 571.0 ([2*M*-H$^+$]$^-$, 100), 330.9 ([*M*+HCOO$^-$], 60), 284.9 ([*M*-H$^+$]$^-$, 20). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{16}H_{13}O_5^-$: 286.0842, found 285.0769. $C_{16}H_{14}O_5$ (286.08).

(*E*)-4'-(2-carboxyvinyl)biphenyl-3-carboxylic acid (4.40b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and (*E*)-3-(4-boronophenyl)acrylic acid (**4.17**, 134 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a brown solid (180 mg, 92 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 17.6 min, *k* = 6.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 6.60 (d, *J* 16.0 Hz, 1H), 7.63 (m, 8.4 Hz, 2H), 7.79 (q, *J* 8.5 Hz, 4H), 7.97 (m, 2H), 8.22 (s, 1H). MS (ES-MS) *m/z* (rel. int. in %) 535.1 ([2*M*-H$^+$]$^-$, 100), 313.0 ([*M*+HCOO$^-$], 25). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{16}H_{11}O_4^-$: 268.0739, found 267.0666. $C_{16}H_{12}O_4$ (268.07).

(*E*)-4'-(2-carboxyvinyl)-4-hydroxybiphenyl-3-carboxylic acid (4.40d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and (*E*)-3-(4-boronophenyl)acrylic acid (**4.17**, 288 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a brown solid (380 mg, 86 %); mp > 210 °C. RP-HPLC (220 nm): 97 % (t_R = 18.7 min, *k* = 7.0). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 6.56 (d, *J* 16.0 Hz, 1H), 7.07 (d, *J* 8.7 Hz, 1H), 7.65 (m, 3H), 7.77 (d, *J* 8.4 Hz, 2H), 7.90 (m, 1H), 8.09 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 25.0, 66.9, 117.8, 118.9, 126.4 (2 interfering carbon signals), 127.9, 128.8 (2 interfering carbon signals), 130.2, 132.9, 133.8, 140.4, 143.3, 160.8, 167.5, 171.6. MS (ES-MS) *m/z* (rel. int. in %) 567.0 ([2*M*-H$^+$]$^-$, 90), 328.9 ([*M*+HCOO$^-$], 80), 282.9 ([*M*-H$^+$]$^-$, 100). $C_{16}H_{12}O_5$ (284.07).

2',5'-Dimethoxybiphenyl-3-carboxylic acid (4.41b)

The title compound was prepared from 3-iodobenzoic acid (174 mg, 0.7 mmol) and 2,5-dimethoxyphenylboronic acid (**4.18**, 127 mg, 0.7 mmol) according to the general

procedure. Flash chromatography yielded a brown solid (150 mg, 83 %); mp 137-144 °C. RP-HPLC (220 nm): 100 % (t_R = 20.6 min, k = 7.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.71 (s, 3H), 3.75 (s, 3H), 6.85-6.98 (m, 2H), 7.07 (d, *J* 8.9 Hz, 1H), 7.54 (t, *J* 7.7 Hz, 1H), 7.72 (d, *J* 7.9 Hz, 1H), 7.90 (d, *J* 7.8 Hz, 1H), 8.04 (s, 1H), 12.96 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.3, 55.9, 113.1, 115.7, 127.8, 128.5, 129.4, 129.8, 130.3, 133.6, 138.0, 150.0, 153.1, 167.5. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 258.2 ([$M^{+\bullet}$], 100), 243.2 ([M-$CH_3^{\bullet}$], 10). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{15}H_{14}O_4^{+\bullet}$: 258.0892, found 258.0892. $C_{15}H_{14}O_4$ (258.09).

2',5'-Dimethoxybiphenyl-4-carboxylic acid[41] (4.41c)

The title compound was prepared from 4-iodobenzoic acid (174 mg, 0.7 mmol) and 2,5-dimethoxyphenylboronic acid (**4.18**, 127 mg, 0.7 mmol) according to the general procedure. Flash chromatography yielded a grey solid (140 mg, 77 %); mp 174-181 °C. RP-HPLC (220 nm): 100 % (t_R = 20.8 min, k = 7.9). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.71 (s, 3H), 3.75 (s, 3H), 6.88-6.99 (m, 2H), 7.08 (d, *J* 8.9 Hz, 1H), 7.61 (d, *J* 8.4 Hz, 2H), 7.96 (d, *J* 8.4 Hz, 2H), 12.88 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.3, 55.9, 113.1, 114.1, 115.8, 128.9 (2 interfering carbon signals), 129.3 (2 interfering carbon signals), 142.3, 150.1, 153.1, 167.2. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 258.2 ([$M^{+\bullet}$], 100), 243.2 ([M-$CH_3^{\bullet}$], 10). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{15}H_{14}O_4^{+\bullet}$: 258.0892, found 258.0888. $C_{15}H_{14}O_4$ (258.09).

4-Hydroxy-2',5'-dimethoxybiphenyl-3-carboxylic acid (4.41d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 2,5-dimethoxyphenylboronic acid (**4.18**, 273 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a grey solid (320 mg, 78 %); mp 177-184 °C. RP-HPLC (220 nm): 100 % (t_R = 21.6 min, k = 8.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.70 (s, 3H), 3.74 (s, 3H), 6.81-6.93 (m, 2H), 7.01 (m, 2H), 7.64 (m, 1H), 7.89 (d, *J* 2.3 Hz, 1H), 11.30 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.3, 55.9, 112.5, 113.0, 115.5, 116.7, 128.7, 129.2, 130.5, 136.4, 150.0, 153.2, 160.0, 171.7. MS (ES-MS) *m/z* (rel. int. in %) 547.0 ([2M-H$^+$]$^-$, 60), 318.9 ([M+HCOO$^-$], 100), 272.9 ([M-H$^+$]$^-$, 40). HRMS (ESI) *m/z* [M-H$^+$]$^-$ calculated for $C_{15}H_{13}O_5^-$: 274.0845, found 273.0772. $C_{15}H_{14}O_5$ (274.08).

4'-Cyclohexylbiphenyl-3-carboxylic acid (4.42b)

The title compound was prepared from 3-iodobenzoic acid (84 mg, 0.35 mmol) and 4-cyclohexylphenylboronic acid (**4.19**, 71 mg, 0.35 mmol) according to the general procedure. Flash chromatography yielded a white solid (60 mg, 63 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 18.1 min, k = 6.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.37

(m, 6H), 1.77 (m, 5H), 7.34 (d, *J* 8.2 Hz, 2H), 7.54-7.64 (m, 3H), 7.89-7.94 (m, 2H), 8.15 (t, *J* 1.7 Hz, 1H), 13.02 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 25.9, 26.7 (2 interfering carbon signals), 34.3 (2 interfering carbon signals), 43.8, 127.1 (2 interfering carbon signals), 127.4, 127.8 (2 interfering carbon signals), 128.4, 129.8, 131.3, 131.7, 137.1, 140.8, 147.8, 167.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 280.2 ([$M^{+\bullet}$], 100). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{19}H_{20}O_2^{+\bullet}$: 280.1469, found 280.1463. $C_{19}H_{20}O_2$ (280.15).

4'-Cyclohexylbiphenyl-4-carboxylic acid[42] (4.42c)

The title compound was prepared from 4-iodobenzoic acid (84 mg, 0.35 mmol) and 4-cyclohexylphenylboronic acid (**4.19**, 71 mg, 0.35 mmol) according to the general procedure. Flash chromatography yielded a white solid (20 mg, 20 %; mp ref.:[42] 288 °C). RP-HPLC (220 nm): 100 % (t_R = 14.0 min, *k* = 5.0). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.37 (m, 7H), 1.77 (m, 6H), 7.35 (d, *J* 8.3 Hz, 2H), 7.65 (d, *J* 8.3 Hz, 2H), 7.77 (d, *J* 8.4 Hz, 2H), 8.00 (d, *J* 8.4 Hz, 2H), 12.92 (br s, 1H). MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 280.2 ([$M^{+\bullet}$], 100). HRMS (EI-MS) *m/z* [$M^{+\bullet}$] calculated for $C_{19}H_{20}O_2^{+\bullet}$: 280.1463, found 280.1469. $C_{19}H_{20}O_2$ (280.15).

3-(7-Methoxynaphthalen-2-yl)benzoic acid (4.43b)

The title compound was prepared from 3-iodobenzoic acid (183 mg, 0.74 mmol) and 7-methoxynaphthalen-2-ylboronic acid (**4.20**, 141 mg, 0.69 mmol) according to the general procedure. Flash chromatography yielded a grey solid (152 mg, 74 %); mp > 210 °C. ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.93 (s, 3H), 7.17 (m, 1H), 7.27 (d, *J* 2.5 Hz, 1H), 7.58 (t, *J* 7.8 Hz, 1H), 7.76 (m, 1H), 7.87 (t, *J* 8.9 Hz, 2H), 7.93-8.03 (m, 2H), 8.06 (s, 1H), 8.37 (s, 1H). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for 278.0943, found 277.0870. $C_{18}H_{14}O_3$ (278.09).

2-Hydroxy-5-(7-methoxynaphthalen-2-yl)benzoic acid (4.43d)

The title compound was prepared from 5-iodosalicylic acid (412 mg, 1.56 mmol) and 7-methoxynaphthalen-2-ylboronic acid (**4.20**, 304 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a white solid (277 mg, 60 %); mp > 210 °C. RP-HPLC (220 nm): 98 % (t_R = 26.4 min, *k* = 10.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 3.89 (s, 3H), 7.09 (d, *J* 8.6 Hz, 1H), 7.19 (m, 1H), 7.34 (d, *J* 2.5 Hz, 1H), 7.76 (m, 1H), 7.93 (m, 3H), 8.11 (d, *J* 1.5 Hz, 1H), 8.16 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 55.1, 117.8, 118.9, 124.3, 125.0, 127.4, 128.7, 129.5, 130.2, 131.1, 133.2, 133.9, 146.6, 147.6, 147.9, 157.2, 160.3, 171.7. MS (ES-MS) *m/z* (rel. int. in %) 587.1 ([2*M*-H$^+$]$^-$, 35), 339.0 ([*M*+HCOO$^-$], 100) 293.0 ([*M*-H$^+$]$^-$, 55). HRMS (EI-MS) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{13}O_4^-$: 294.0895, found 293.0823. $C_{18}H_{14}O_4$ (294.09).

3-(Benzo[*d*][1,3]dioxol-5-yl)benzoic acid[43] (4.44b)

The title compound was prepared from 3-iodobenzoic acid (177 mg, 0.71 mmol) and benzo[*d*][1,3]-5-ylboronic acid (**4.21**, 121 mg, 0.73 mmol) according to the general procedure. Flash chromatography yielded a brown solid (167 mg, 97 %); mp > 210 °C (ref.:[43] 227-228 °C). RP-HPLC (220 nm): 98 % (t_R = 20.8 min, k = 7.9). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 6.08 (s, 2H), 7.02 (d, *J* 8.1 Hz, 1H), 7.18 (m, 1H), 7.28 (d, *J* 1.8 Hz, 1H), 7.55 (t, *J* 7.7 Hz, 1H), 7.82-7.92 (m, 2H), 8.10 (s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 101.2, 107.1, 108.7, 120.4, 127.7, 129.1, 130.8, 131.3, 133.4, 140.1, 147.1, 148.0, 167.1. HRMS (EI-MS) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_9O_4^-$: 242.0578, found 241.0505. $C_{14}H_{10}O_4$ (242.06).

4-(Benzo[*d*][1,3]dioxol-5-yl)benzoic acid[44] (4.44c)

The title compound was prepared from 4-iodobenzoic acid (179 mg, 0.72 mmol) and benzo[*d*][1,3]-5-ylboronic acid (**4.21**, 122 mg, 0.73 mmol) according to the general procedure. Flash chromatography yielded a grey solid (127 mg, 73 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 21.2 min, k = 8.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 6.09 (s, 2H), 7.03 (d, *J* 8.1 Hz, 1H), 7.24 (m, 1H), 7.34 (d, *J* 1.8 Hz, 1H), 7.74 (d, *J* 8.4 Hz, 2H), 7.97 (d, *J* 8.4 Hz, 2H), 12.89 (br s, 1H). ^{13}C-NMR (75 MHz, DMSO): δ (ppm) 101.2, 107.1, 108.7, 120.7, 126.4 (2 interfering carbon signals), 129.0, 129.8 (2 interfering carbon signals), 133.1, 147.5, 148.0, 167.0. HRMS (EI-MS) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_9O_4^-$: 242.0580, found 241.0507. $C_{14}H_{10}O_4$ (242.06).

5-(Benzo[*d*][1,3]dioxol-5-yl)-2-hydroxybenzoic acid[45] (4.44d)

The title compound was prepared from 5-iodosalicylic acid (402 mg, 1.52 mmol) and benzo[*d*][1,3]-5-ylboronic acid (**4.21**, 256 mg, 1.54 mmol) according to the general procedure. Flash chromatography yielded a brown solid (112 mg, 28 %); mp > 210 °C (ref.:[45] 241-245 °C). RP-HPLC (220 nm): 100 % (t_R = 21.5 min, k = 8.2). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 6.05 (s, 2H), 7.04 (m, 3H), 7.20 (d, *J* 1.8 Hz, 1H), 7.76 (m, 1H), 7.94 (d, *J* 2.5 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 101.0, 106.7, 108.6, 113.1, 117.6, 119.6, 127.6, 131.1, 133.7, 142.9, 147.9, 160.1, 160.5, 171.7. MS (ES-MS) *m/z* (rel. int. in %) 515.0 ([2*M*-H$^+$]$^-$, 35), 302.9 ([*M*+HCOO$^-$], 100) 256.9 ([*M*-H$^+$]$^-$, 25). HRMS (EI-MS) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{14}H_9O_5^-$: 258.0533, found 257.0460. $C_{14}H_{10}O_5$ (258.05).

5-(5-Chloropyridin-3-yl)-2-hydroxybenzoic acid (4.45d)

The title compound was prepared from 5-iodosalicylic acid (396 mg, 1.5 mmol) and 5-chloropyridin-3-ylboronic acid (**4.22**, 244 mg, 1.5 mmol) according to the general procedure. Flash chromatography yielded a yellow solid (160 mg, 42 %); mp > 210 °C. RP-HPLC (220 nm): 98 % (t_R = 25.8 min, *k* = 10.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.02 (d, *J* 8.7 Hz, 1H), 7.12 (d, *J* 3.9 Hz, 1H), 7.31 (d, *J* 3.9 Hz, 1H), 7.77 (m, 1H), 7.91 (d, *J* 2.4 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.5, 118.1, 122.7, 123.4, 126.4, 128.1, 132.3, 141.2, 160.7, 171.2. MS (ES-MS) *m/z* (rel. int. in %) 506.9 ([2*M*-H$^+$]$^-$, 100), 299.0 [*M*+HCOO$^-$], 90), 253.0 [*M*-H$^+$]$^-$, 40). HRMS (ESI) *m/z* [*M*-H$^+$]$^-$ calculated for $C_{11}H_6ClO_3S^-$: 253.9763, found 253.9762. $C_{11}H_7ClO_3S$ (253.98).

5-(5-Chlorothiophen-2-yl)-2-hydroxybenzoic acid (4.46d)

The title compound was prepared from 5-iodosalicylic acid (378 mg, 1.52 mmol) and 5-chlorotiophen-2-ylboronic acid (**4.23**, 220 mg, 1.54 mmol) according to the general procedure. Flash chromatography yielded a grey solid (190 mg, 51 %); mp > 210 °C. RP-HPLC (220 nm): 97 % (t_R = 18.1 min, *k* = 6.7). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 7.09 (d, *J* 8.1 Hz, 1H), 7.94 (d, *J* 7.6 Hz, 1H), 8.18 (d, *J* 36.9 Hz, 2H), 8.59 (s, 1H), 8.83 (s, 1H), 11.44 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 113.6, 118.0, 126.4, 128.8, 131.4, 133.2, 134.2, 136.0, 145.5, 146.3, 161.3, 171.4. MS (ES-MS) *m/z* (rel. int. in %) 496.9 ([2*M*-H$^+$]$^-$, 100), 293.9 [*M*+HCOO$^-$], 95), 247.9 [*M*-H$^+$]$^-$, 50). HRMS (ESI) *m/z* [*M*-H]$^-$ calculated for $C_{12}H_8ClNO_3^-$: 249.0152, found 249.0146. $C_{12}H_8ClNO_3$ (249.02).

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5 Indole-2-carboxylic acids and 2-(6,7-dichloro-1*H*-indol-2-yl)-1,3,4-oxadiazoles as inhibitors of bacterial hyaluronidases

5.1 Introduction

In 1975, the anti-inflammatory drug indomethacin was reported as a hyaluronidase inhibitor for the first time.[1] Investigations by Spickenreither on indomethacin and indole derivatives, such as *N*-substituted indole-3-butanoic acid, and *N*-acylated and *N*-alkylated indole-3-alkanoic acid derivatives, showed that these compounds are inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ and the mammalian enzymes BTH, Hyal-1 and PH-20.[2] Structurally related compounds of indomethacin designed in our work group by M. Spickenreither[2] led to the discovery of substances that are among the most potent inhibitors of bacterial and mammalian hyaluronidases known so far. For example *N*-alkylated and 2-phenyl-substituted indoles have been identified as potent inhibitors of streptococcal hyaluronidases with IC_{50} values in the lower micromolar range (Figure 5.1).

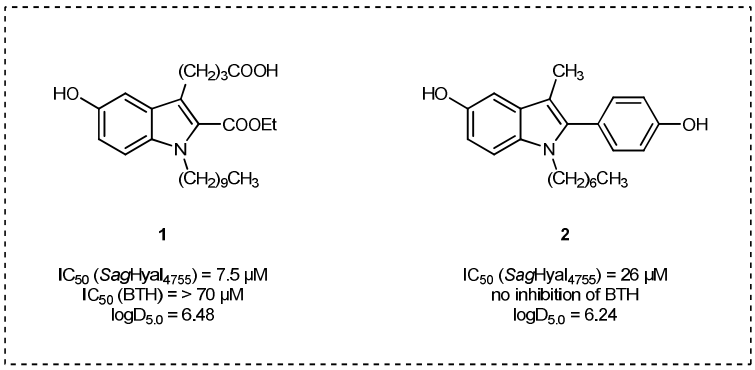


Figure 5.1 Structures, inhibitory activity of *Sag*Hyal$_{4755}$ and BTH and calculated log$D_{5.0}$ of *N*-alkylated indole **1** and 2-phenylindole **2**.

The two compounds 4-(1-decyl-2-(ethoxycarbonyl)-5-hydroxy-1*H*-indol-3-yl)butanoic acid (**1**) and 1-heptyl-2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**2**) showed inhibition of *Sag*Hyal$_{4755}$ with IC_{50} values of 7.5 µM and 26 µM, respectively.[2,3] Moreover, X-ray analysis confirmed that hydroxylated 2-phenylindoles bind to the active site of another streptococcal hyaluronidase, *Spn*Hyl.[3-5]

Searching for suiTable inhibitors for *in vivo* application, *N*-alkylated indoles proved to be inappropriate due to high log$D_{5.0}$ values as a consequence of long lipophilic alkyl moieties and extremely high plasma-protein binding (cf. Figure 5.1). Furthermore, compounds possessing a negatively charged carboxyl group can have surfactant properties. Moreover, substances with a 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol scaffold are known for anti-estrogenic activity at low nanomolar concentrations.[6-8]

In search for novel substitution patterns on the indole, 6,7-dichloro-1*H*-indole derivatives were investigated for inhibitory activity on *Sag*Hyal$_{4755}$ and BTH. In addition, compounds derived from the β-carboline alkaloid bauerine C and originally developed as potent and selective kinase inhibitors, were tested. These substances were kindly provided by Prof. Dr. F. Bracher (Department of Pharmacy, LMU Munich).[9-11] Among the investigated small molecules (data not shown; for detailed information cf. Textor[5]) compounds **3** and **4** (Figure 5.2) were identified as moderate inhibitors of *Sag*Hyal$_{4755}$. However, inhibitors of BTH were not identified among the 6,7-dichloro-1*H*-indoles.

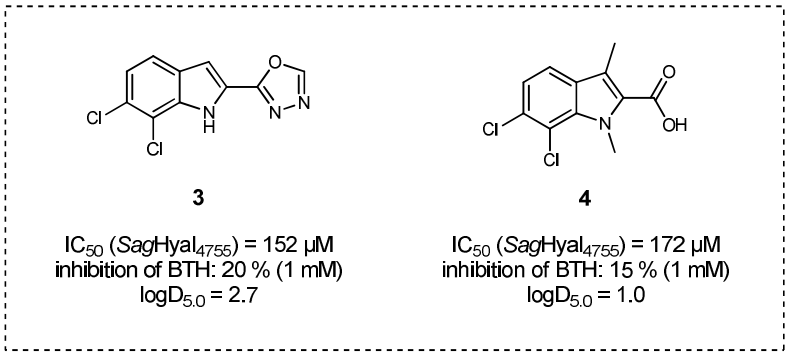


Figure 5.2 Structures, inhibitory activities of *Sag*Hyal$_{4755}$ and BTH and calculated log$D_{5.0}$ values of 6,7-dichloro-1*H*-indole derivatives **3** and **4**.

Compound **3** bears an oxadiazole ring in position 2 of the indole scaffold. Such moieties are frequently used as bioisosteric replacements for ester and amide groups.[12,13]

Inspired by compounds **3** and **4**, novel 2-phenylindole derivatives were explored in our workgroup. Compound **5** (Figure 5.3) was found to be one of the most potent inhibitor of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ known so far with an IC_{50} value of 6 µM. Moreover, an IC_{50} value of 93 µM was determined on the streptococcal hyaluronidase *Spn*Hyl.

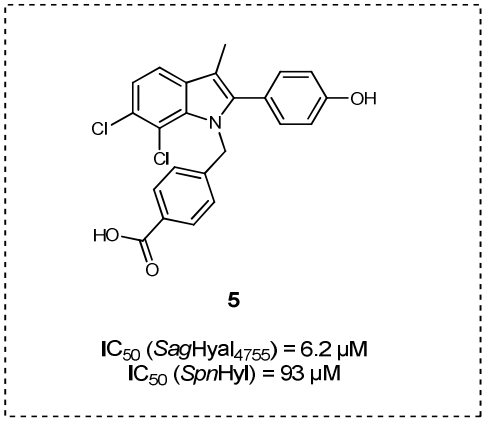


Figure 5.3 Structure and inhibitory activity of *Sag*Hyal$_{4755}$ and BTH of 4-[(6,7-dichloro-2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-1-yl)methyl]benzoic acid (**5**).

It becomes obvious from compounds **3**-**5** that long lipophilic alkyl moieties, which are characteristic of previously identified inhibitors of streptococcal hyaluronidases, are not an essential structural feature. Moreover, antiestrogenic activities and cytostatic effects on hormone sensitive breast cancer cells were not observed for the indole-type hyaluronidase inhibitor **3**.[5] Therefore, in the present project, the 6,7-dichloro-1*H*-indole motif was selected as a core structures for the design of small molecules, aiming at more potent hyaluronidase inhibitors with improved drug-like properties. The structural modifications of compounds **3** and **4** are outlined in Figure 5.4.

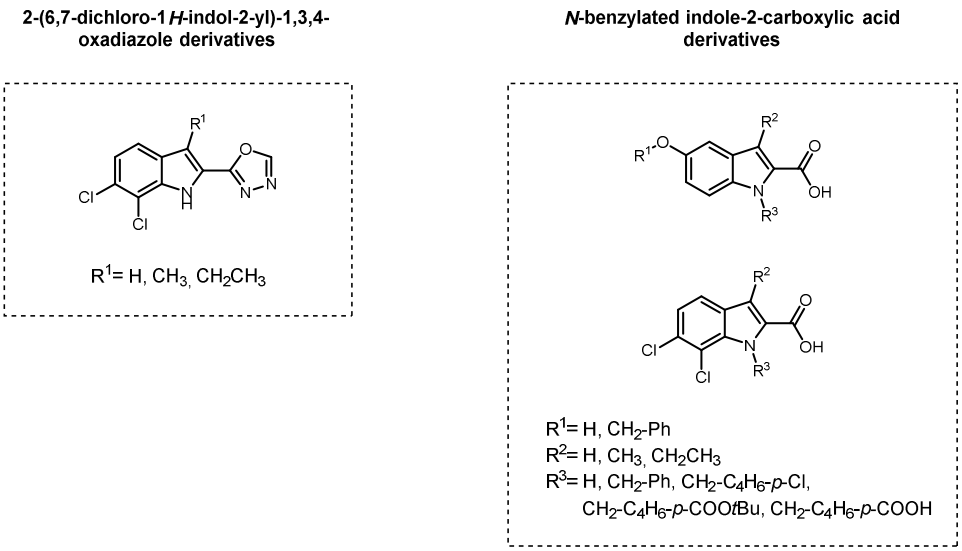


Figure 5.4 Structural modifications of **3** and **4** for the development of inhibitors of *Sag*Hyal$_{4755}$.

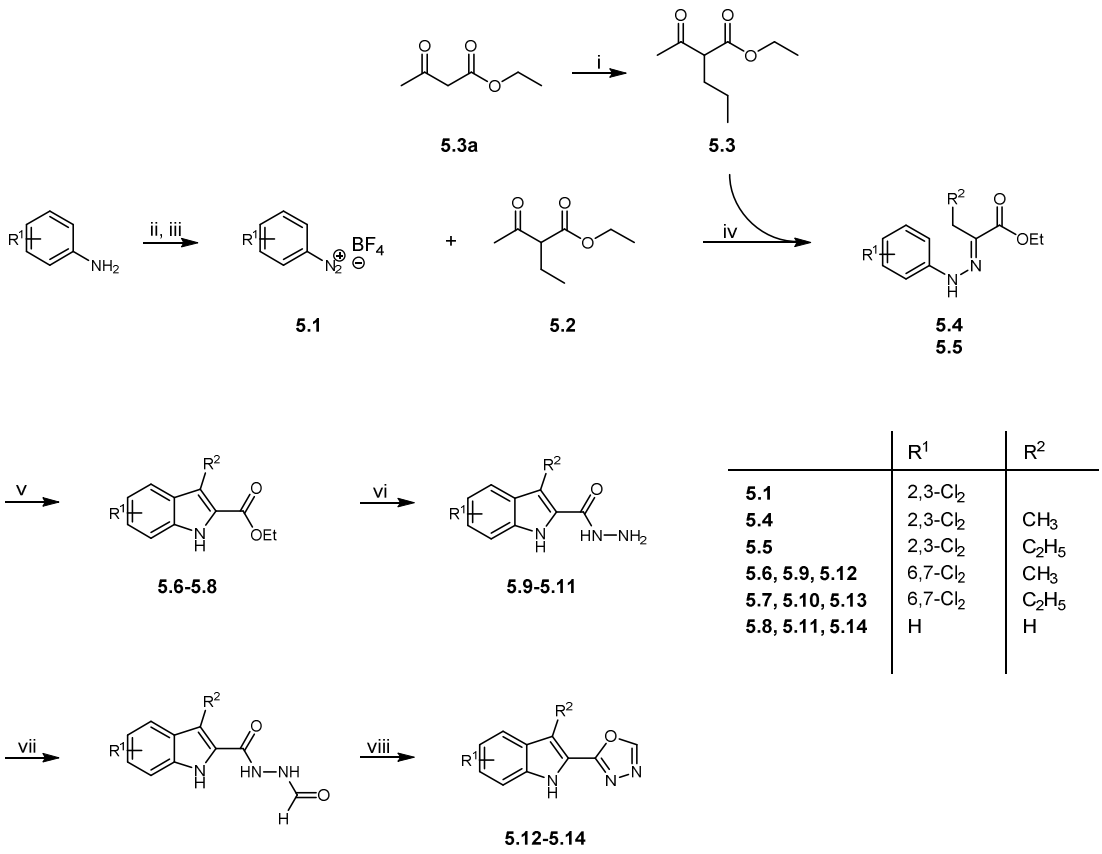
Both, the 2-(6,7-dichloro-1*H*-indole-2-yl)-1,3,4-oxadiazole and 6,7-dichloro-1*H*-indole-2-carboxylic acid structures were varied by introducing a methyl and ethyl group, respectively, in position 3 (cf. R^1 in Figure 5.3). The 6,7-dichloro substitution pattern will be exchanged by

an O-benzyl and hydroxyl group, respectively, in position 5 (cf. Figure 5.3). Aiming at drug-like inhibitors, based on the experience with previously identified inhibitors of hyaluronidase, the physicochemical properties were taken into consideration. The introduction of a benzyl or a chlorobenzyl moiety at the indole-*N* of the 1*H*-indole-2-carboxylic acid derivatives results in higher lipophilicity of the molecule, which is known to contribute to higher potency of inhibitors of the bacterial hyaluronidases. Moreover, by introducing an additional negatively charged carboxylic group at the benzyl moiety, the solubility of the compounds should be increased.

5.2 Chemistry

5.2.1 Synthesis of 2-(6,7-dichloro-1*H*-indol-2-yl)-1,3,4-oxadiazoles

The synthesis of the 2-(6,7-dichloro-1*H*-indole-2-yl)-1,3,4-oxadiazole compounds was carried out as outlined in Scheme 5.1.



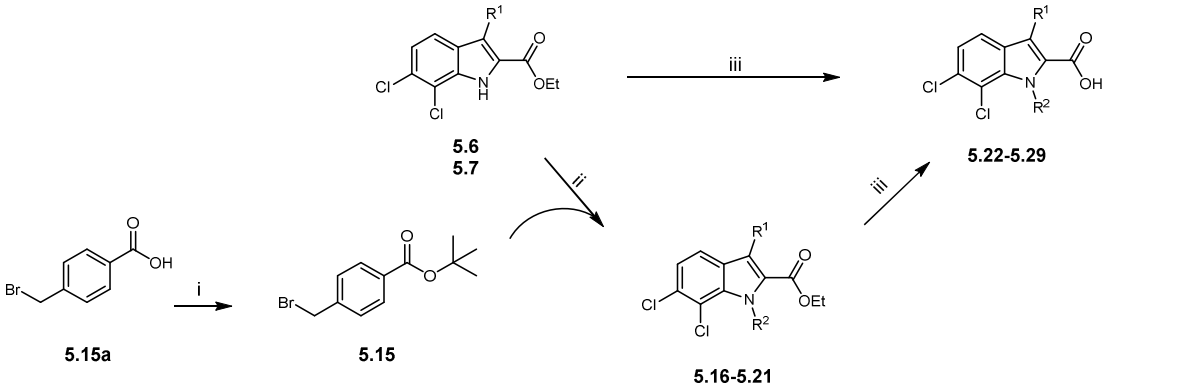
	R^1	R^2
5.1	2,3-Cl_2	
5.4	2,3-Cl_2	CH_3
5.5	2,3-Cl_2	C_2H_5
5.6, 5.9, 5.12	6,7-Cl_2	CH_3
5.7, 5.10, 5.13	6,7-Cl_2	C_2H_5
5.8, 5.11, 5.14	H	H

Scheme 5.1 Synthesis of the 2-(indol-2-yl)-1,3,4-oxadiazole derivatives **5.12-5.14**. Reagents and conditions: (i) $NaNO_2$, HCl (conc.)/H_2O, 0 °C; (ii) $NaBF_4$, rt, 20 min; (iii) NaH, THF, C_3H_7I, reflux, 6 h; (iv) NaH, THF; (v) HCl (conc.), reflux 36 h; (vi) $H_4N_2 \cdot H_2O$, reflux 72 h; (vii) HCOOH; (viii) $POCl_3$.

The indole scaffold was prepared using the Japp-Klingemann variant of the Fischer indole synthesis.[14-17] First, 2,3-dichlorophenyldiazonium tetrafluoroborate was prepared from 2,3-dichloroaniline.[18] Subsequently, the reaction of the diazonium salt with the respective oxobutanoate was carried out to give the hydrazono compounds **5.4** and **5.5**. These, in turn were refluxed with concentrated hydrochloric acid to achieve the indole ring closure at high temperature.[2,19] To obtain the 6,7-dichloro-1*H*-indole-2-carbohydrazides, ethyl 1*H*-indole-2-carboxylates were refluxed for 3 days with hydrazine hydrate. Finally, oxadiazole ring closure was accomplished by treating the indole-2-carbohydrazides with formic acid and phosphoryl chloride.[20-22] After removal of side-products by flash chromatography, compounds **5.12**-**5.14** were obtained in moderate yields.

5.2.2 Synthesis of 1*H*-indole-2-carboxylic acid compounds

The *N*-benzylated 6,7-dichloro-1*H*-indole-2-carboxlic acid derivatives were synthesized according to the scheme outlined in Scheme 5.2.



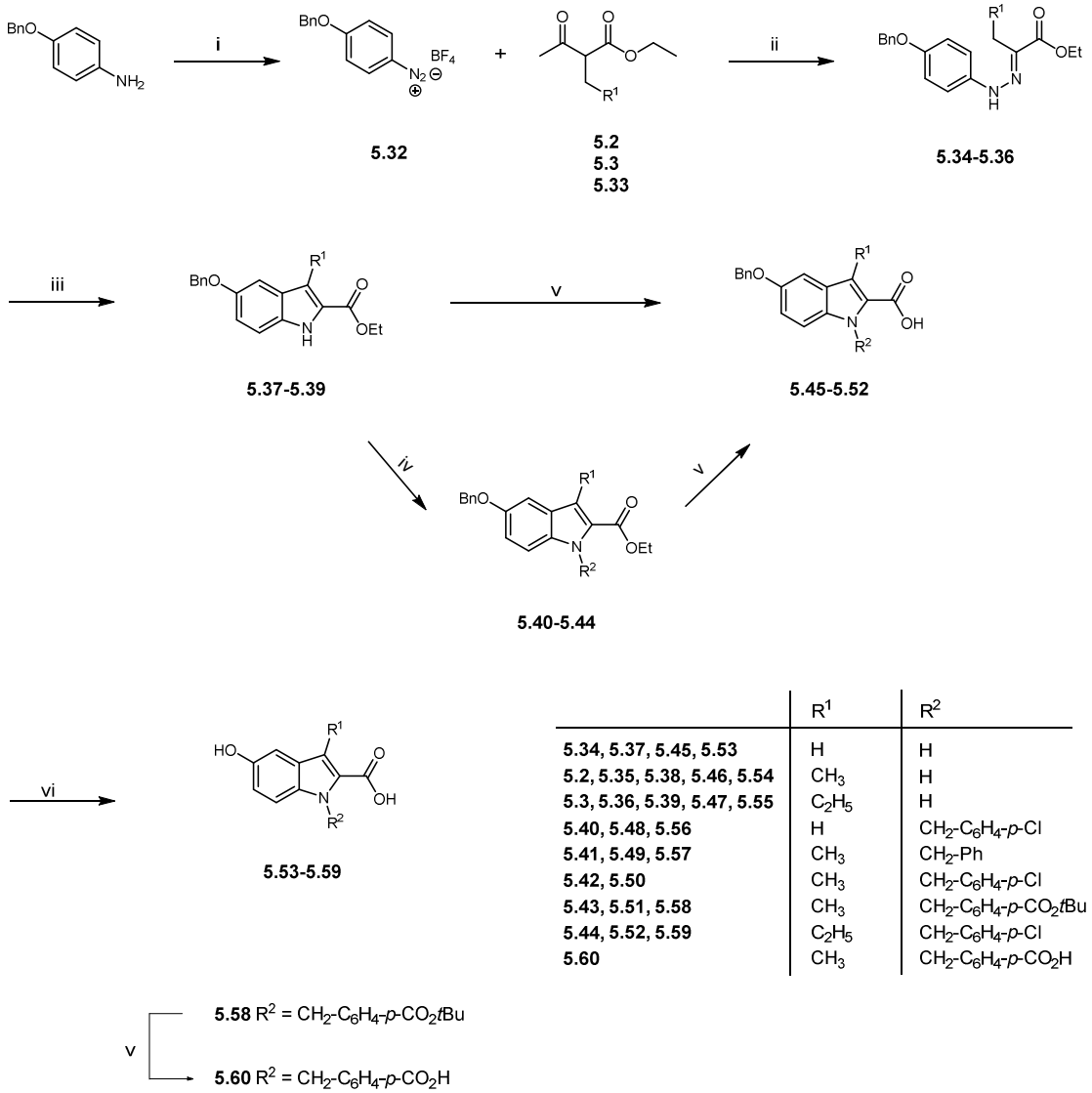
	R^1	R^2
5.6, 5.22	CH_3	H
5.7, 5.23	C_2H_5	H
5.16, 5.24	CH_3	CH_2-Ph
5.17, 5.25	CH_3	CH_2-C_6H_4-*p*-Cl
5.18, 5.26	CH_3	CH_2-C_6H_4-*p*-CO_2*t*Bu
5.19, 5.27	C_2H_5	CH_2-Ph
5.20, 5.28	C_2H_5	CH_2-C_6H_4-*p*-Cl
5.21, 5.29	C_2H_5	CH_2-C_6H_4-*p*-CO_2*t*Bu
5.30	CH_3	CH_2-C_6H_4-*p*-CO_2H
5.31	C_2H_5	CH_2-C_6H_4-*p*-CO_2H

iii → **5.26**, **5.29** R^2 = CH_2-C_6H_4-*p*-CO_2*t*Bu
iii → **5.30**, **5.31** R^2 = CH_2-C_6H_4-*p*-CO_2H

Scheme 5.2 Synthesis of the 6,7-dichloro-1*H*-indole derivatives **5.22**-**5.31**. Reagents and conditions: (i) TBTA, BF_3Et_2O, cyclohexane/THF, rt, 17 h; (ii) NaH, DMF, R^2-Br, 0 °C; (iii) LiOH, THF/EtOH/H_2O = 3:0.2:1 (v/v/v).

The building blocks **5.6** and **5.7** were prepared as described in Section 5.2.1. *N*-benzylation of the indole scaffold was carried out by treating the indole-2-carboxylate with sodium hydride in dimethylformamide and benzylation with the corresponding benzyl bromide.[23] Saponification of the ester moieties in position 2 (**5.6**, **5.7**, **5.16**-**5.21**) and in position 4 of the *N*-benzyl residue (**5.26**, **5.29**) with lithium hydroxide yielded the target molecules (**5.22**-**5.31**).[5] The crude products were purified by RP-HPLC.

The synthesis of the *N*-benzylated 5-benzyloxy- and 5-hydroxy-1*H*-indole-2-carboxylic acid derivatives is outlined in Scheme 5.3.



	R^1	R^2
5.34, 5.37, 5.45, 5.53	H	H
5.2, 5.35, 5.38, 5.46, 5.54	CH_3	H
5.3, 5.36, 5.39, 5.47, 5.55	C_2H_5	H
5.40, 5.48, 5.56	H	CH_2-C_6H_4-*p*-Cl
5.41, 5.49, 5.57	CH_3	CH_2-Ph
5.42, 5.50	CH_3	CH_2-C_6H_4-*p*-Cl
5.43, 5.51, 5.58	CH_3	CH_2-C_6H_4-*p*-CO_2*t*Bu
5.44, 5.52, 5.59	C_2H_5	CH_2-C_6H_4-*p*-Cl
5.60	CH_3	CH_2-C_6H_4-*p*-CO_2H

Scheme 5.3 Synthesis of the 5-benzyloxy- and 5-hydroxy-1*H*-indole-2-carboxylic acid derivatives **5.53**-**5.60**. Reagents and conditions: (i/ii) $NaNO_2$, HCl (conc.) / H_2O, 0 °C; (ii) $NaBF_4$, rt, 20 min; (iii) NaH, THF (iv) HCl (conc.), reflux, 36 h; (v) NaH, DMF, R^2-Br, 0 °C; (vi) LiOH, THF/EtOH/H_2O = 3:0.2:1 (v/v/v), RT, 72 h; (vii) 10 % Pd/C (cat.), H_2 (atm), THF, rt, overnight.

The synthesis of the indole scaffold, as well as the *N*-benzylation was performed as described above. Hydrogenolytic cleavage of the benzyl ether compounds **5.45**-**5.52** was achieved in the presence of palladium on activated charcoal.[2] Saponification of the ester moieties (**5.37**-**5.44** and **5.58**) was carried out as described above. RP-HPLC purification yielded the pure target compounds.

5.3 Pharmacological results and discussion

5.3.1 General conditions

All synthesized indole compounds were investigated for inhibition of the bacterial hyaluronate lyase *Sag*Hyal$_{4755}$ and the bovine testicular enzyme BTH (Neopermease®) in a turbidimetric assay based on the method of Di Ferrante as described in chapter 1.[24] If IC_{50} values could not be determined, due to poor solubility in aqueous buffer, the percent inhibition of the compound was given.

5.3.2 Inhibition of hyaluronidases by 2-(1*H*-indol-2-yl)-1,3,4-oxadiazoles

The IC_{50} values determined for the 2-(1*H*-indol-2-yl)-1,3,4-oxadiazoles (**5.12**-**5.14**) are summarized in Table 5.1.

Table 5.1 Inhibitory activity[a] on hyaluronidases and calculated $logD_{5.0}$ values[b] of 2-(1*H*-indol-2-yl)-1,3,4-oxadiazoles **5.12**-**5.14**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[c]
5.12	20 % at 100 µM[b]	inactive	2.7
5.13	30 % at 200 µM[b]	inactive	3.2
5.14	inactive	inactive	1.7

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] % inhibition of *Sag*Hyal$_{4755}$ at indicated inhibitor concentration; [c] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The structures of compounds **5.12**-**5.14** are similar to that of parent compound **3**: compounds **5.12** (20 % at 100 µM) and **5.13** (30 % at 200 µM) bear a methyl or an ethyl group, respectively, in position 3 of the indole scaffold. Nevertheless, compared to **3** (IC_{50} = 152 µM) these analogs are less potent hyaluronidase inhibitors. In compound **5.14** the 6,7-dichloro

substitution was omitted, resulting in reduced lipophilicity and a lower calculated $logD_{5.0}$ value ($logD_{5.0}$ = 1.7). However, the inhibitory effect on *Sag*Hyal$_{4755}$ was completely lost. Due to poor solubility, IC_{50} values of compounds **5.12** and **5.13** could not be determined.

None of the oxadiazole derivatives **5.12**-**5.14** revealed inhibition of BTH. In view of low activity and very poor solubility, the synthesis of compounds bearing an oxadiazole moiety was not further pursued.

5.3.3 Inhibition of hyaluronidases by 6,7-dichloro-1*H*-indole-2-carboxylic acid derivatives

The IC_{50} values determined for the 6,7-dichloro-1*H*-indole-2-carboxylic acid derivatives (**5.16**, **5.22**-**5.31**) are summarized in Table 5.2.

Table 5.2 Inhibitory activity[a] on hyaluronidases and calculated $logD_{5.0}$ values[b] of 6,7-dichloro-1*H*-indole-2-carboxylic acid derivatives **5.16** and **5.22**-**5.31**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[b]
5.16	inactive	inactive	6.2
5.22	30 % at 100 µM	inactive	1.4
5.23	143 ± 53	20 % at 1 mM	1.8
5.24	20 % at 100 µM	inactive	3.7
5.25	16 ± 1	inactive	4.2
5.26	19 ± 3	inactive	4.8
5.27	25 ± 5	inactive	4.1
5.28	61 ± 14	inactive	4.6
5.29	29 ± 15	inactive	5.1
5.30	72 ± 6	inactive	2.1
5.31	41 ± 3	inactive	2.5

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **5.25**-**5.27**, **5.29** and **5.31** is depicted as concentration-response curves in Figure 5.5.

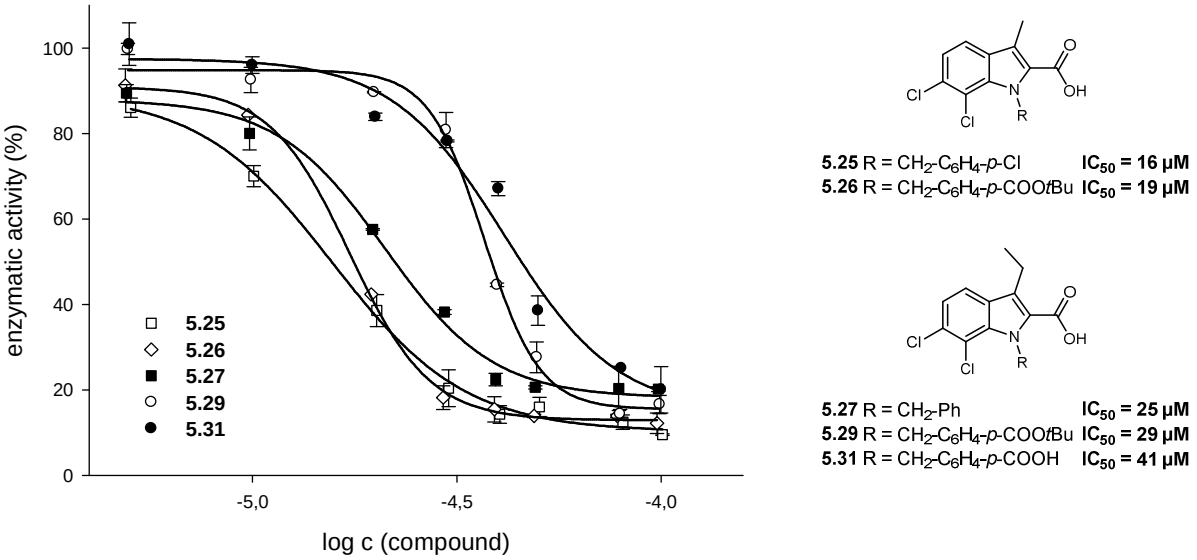


Figure 5.5 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **5.25**-**5.27**, **5.29** and **5.31**.

Whereas, the ethyl ester **5.16** was devoid of inhibitory effect, the corresponding carboxylic acid **5.24** showed weak inhibition of *Sag*Hyal$_{4755}$ (20 % inhibition at 100 µM) and better solubility in aqueous buffer. Interestingly, compound **5.27**, the 3-ethyl-substituted analog of **5.24**, revealed comparable solubility (100 µM) and higher inhibitory potency on *Sag*Hyal$_{4755}$ (IC_{50} = 25 µM).

The only difference between compounds **5.22** (30 % at 100 µM) and 6,7-dichloro-1,3-dimethyl-1*H*-indole-2-carboxylic acid (**4**, IC_{50} = 172 µM) is the methyl group at the indole nitrogen, but interestingly, the latter showed higher inhibitory activity on *Sag*Hyal$_{4755}$ and weak inhibition of BTH (15 % at 1 mM), whereas **5.22** was inactive at the mammalian enzyme. The homolog of **5.22**, substance **5.23**, bearing an ethyl instead of a methyl group in position 3, proved to be superior (IC_{50} = 143 µM) to **5.22** and **5.24**.

The indole-2-carboxylic acids **5.22** (30 % at 100 µM) and **5.23** (IC_{50} = 143 µM) were slightly more potent as inhibitors of *Sag*Hyal$_{4755}$, compared to the corresponding oxadiazoles **5.12** (20 % at 100 µM) and **5.13** (30 % at 200 µM). All other compounds included in this section (**5.25-5.31**) showed moderate to good inhibition of *Sag*Hyal$_{4755}$ in the two-digit micromolar range (IC_{50} = 16-72 µM). As expected, these compounds were not capable of inhibiting the mammalian enzyme BTH, except for compound **5.23** which showed very weak inhibition by 20 % at 1 mM. Indole **5.25** (IC_{50} = 16 µM), bearing another chloro substituent in position 4 of the benzyl moiety turned out to be the most potent hyaluronidase inhibitor among these compounds, confirming again the correlation: the higher the lipophilicity (**5.25**: calculated

$logD_{5.0}$ = 4.2) the higher the potency. However, differing from this statement, an ethyl group instead of a methyl group (**5.25**) in position 3 did not result in a further increase but in almost 4-fold decrease in inhibitory activity (**5.28**, IC_{50} = 61 µM).

Surprisingly, the *tert*-butyl ester protected compound **5.26** (IC_{50} = 19 µM) showed more than 3-fold higher inhibitory activity than the deprotected compound **5.30** (IC_{50} = 72 µM). The same tendency became obvious for the 3-ethyl-substituted analogs: the *tert*-butyl ester **5.29** (IC_{50} = 29 µM) and the corresponding deprotected carboxylic acid (**5.31,** IC_{50} = 41 µM). Thus, an additional carboxyl residue had minor influences on the IC_{50} values, whereas the bulky *tert*-butyl group increased lipophilicity and inhibitory activity.

In summary, among the 6,7-dichloro-1*H*-indole-2-carboxylic acid derivatives, the *N*-benzylated compounds showed the highest inhibitory potencies. The 2-carboxylic group should be unprotected to increase both, the effect on *Sag*$Hyal_{4755}$ and the solubility. In this group of compounds inhibitors of the streptococcal hyaluronidase *Sag*$Hyal_{4755}$ were found with IC_{50} values in the lower two-digit micromolar range. With regard to the lipophilicity, for all compounds (**5.22**-**5.31**: $logD_{5.0}$ = 1.4-5.1), except for **5.16** ($logD_{5.0}$ = 6.2), the calculated $logD_{5.0}$ values were below or at 5.1.

5.3.4 Inhibition of hyaluronidases by 5-benzyloxy- and 5-hydroxy-1*H*-indole-2-carboxylic acid derivatives

The IC_{50} values determined for the 5-benzyloxy- (**5.43**, **5.48**-**5.50** and **5.52**) and 5-hydroxy-1*H*-indole-2-carboxylic acid derivatives (**5.53**-**5.57**, **5.59** and **5.60**) are summarized in Table 5.3.

Table 5.3 Inhibitory activity[a] and calculated $logD_{5.0}$ values[b] of 5-(benzyloxy)- and 5-hydroxy-1*H*-indole-2-carboxylic acid derivatives **5.43**, **5.48**-**5.50**, **5.52**-**5.57**, **5.59** and **5.60**.

Compound	***Sag*Hyal$_{4755}$ IC_{50} (µM)[a]**	**BTH IC_{50} (µM)[a]**	**$logD_{5.0}$[b]**
5.43	inactive	inactive	5.1
5.48	20 ± 6	inactive	5.4
5.49	10 % at 20 µM	inactive	5.0
5.50	60 % at 200 µM	inactive	5.5
5.52	inactive	inactive	5.9
5.53	inactive	inactive	-0.3
5.54	inactive	inactive	-0.3
5.55	20 % at 1 mM	inactive	0.0
5.56	inactive	inactive	3.4
5.57	inactive	inactive	3.0
5.59	50 % at 1 mM	inactive	3.4
5.60	40 % at 1 mM	inactive	1.4

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **5.48** is depicted as concentration-response curve in Figure 5.6.

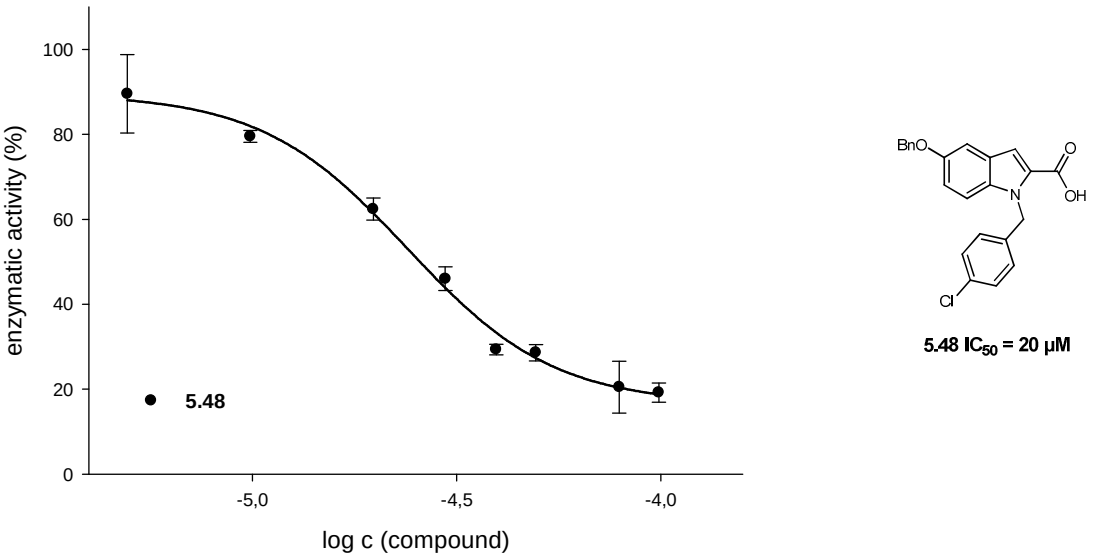


Figure 5.6 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **5.48**.

Most of the synthesized indole derivatives described in this section were inactive or showed only weak inhibition of the bacterial hyaluronidase *Sag*Hyal$_{4755}$. Compound **5.43** bears an ethyl ester group in position 2 and a *tert*-butyl ester in position 4 of the benzyl moiety. Hence, the solubility of this substance was very poor and no inhibitory activity of *Sag*Hyal$_{4755}$ could be detected. In compound **5.60** both carboxylic groups are unprotected, resulting in increased solubility. However, this compound showed only weak inhibition of the bacterial hyaluronidase by 40 % at a concentration of 1 mM. The corresponding 6,7-dichloro-substituted compound (cf. section 5.3.3, **5.30**: IC_{50} = 72 µM) was more potent and lipophilic (**5.30**: $logD_{5.0}$ = 2.1, **5.60**: $logD_{5.0}$ = 1.4).

The 5-benzyloxy-substituted indole **5.48** (IC_{50} = 20 µM, $logD_{5.0}$ = 5.4) was the most potent hyaluronidase inhibitor among this group of compounds. Compared to substances **5.50** (60 % at 200 µM) and **5.52** (inactive), **5.48** bears no residue in position 3. Interestingly, when the benzyloxy group of compound **5.48** was cleaved to give 1-(4-chlorobenzyl)-5-hydroxy-1H-indole-2-carboxylic acid (**5.52**), the inhibitory activity was completely lost. The 6,7-dichloro-substituted analogs **5.25** (IC_{50} = 16 µM) and **5.28** (IC_{50} = 61 µM), were superior to the compounds bearing the 5-benzyloxy-moiety (**5.48**, **5.50**, **5.52**), although the lipophilicity of the latter was higher than that of compounds **5.25** and **5.28** ($logD_{5.0}$ **5.25** = 4.2; $logD_{5.0}$ **5.28** = 4.6). The benzyloxy-substitution (**5.48**-**50** and **5.52**) increased the lipophilicity and reduced the solubility in aqueous buffer. This prevented the determination of IC_{50} values of compounds such as **5.49** ($logD_{5.0}$ = 5.0).

With regard to inhibition of *Sag*Hyal$_{4755}$, this series was less successful than the 6,7-dichloro-1*H*-indole-2-carboxylic acid series. Only one compound (**5.48**) with an IC_{50} value in the two-digit micromolar range (IC_{50} = 20 µM) was identified. None of these compounds showed inhibition of bovine testicular hyaluronidase.

5.4 Inhibitory activities of selected compounds on *Spn*Hyl

To compare the inhibition of *Sag*Hyal$_{4755}$ with data from an additional bacterial enzyme, an ensemble of 10 inhibitors was investigated on hyaluronidase from *S. pneumonia, Spn*Hyl. All compounds were tested in the 96-well plate format in duplicate at a final assay concentration of 200 µM. Under identical assay conditions (including the pH value) these substances were inactive or showed very weak inhibition of *Spn*Hyl (Table 5.4).

Table 5.4 Inhibitory activities[a] of compounds **5.23**, **5.25**-**5.31** and **5.53**-**5.55** on *Sag*Hyal$_{4755}$ and *Spn*Hyl.

Compound	***Sag*Hyal$_{4755}$ IC_{50} (µM)[a]**	**inhibitory activity of *Spn*Hyl (%) at a concentration of 200 µM**
5.23	143 ± 53	inactive
5.25	16 ± 1	40
5.26	19 ± 3	12
5.27	25 ± 5	10
5.28	61 ± 14	30
5.29	29 ± 15	inactive
5.30	72 ± 6	inactive
5.31	41 ± 3	inactive
5.53	inactive	inactive
5.54	inactive	inactive
5.55	inactive	12

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes).

Compound **5.55** showed very weak inhibition of *Spn*Hyl by 12 % at 200 µM and was the only compound that was more active on the hyaluronidase from *S. pneumonia* than on the enzyme from *S. agalactiae*.

5.5 Summary

Indole derivatives that are structurally related to 2-(6,7-dichloro-1*H*-indol-2-yl)-1,3,4-oxydiazole (**3**) and 6,7-dichloro-1,3-dimethyl-1*H*-indole-2-carboxylic acid (**4**; cf. Figure 5.2) were synthesized and investigated for inhibition of the bacterial hyaluronidases *Sag*Hyal$_{4755}$ and *Spn*Hyl. Out of this series, the most active inhibitors are characterized by 6,7-dichloro substitution, *N*-benzylation and a carboxylic group in position 2. Compound **5.25** (6,7-dichloro-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylic acid) showed the highest inhibition of *Sag*Hyal$_{4755}$ (IC_{50} = 16 µM).

Starting from the parent compounds (**3**) and (**4**), the hydrophobicity of the derivatives was increased by *N*-benzylation, resulting in increased inhibition of the bacterial hyaluronate lyase *Sag*Hyal$_{4755}$. Moreover, instead of 6,7-dichloro substitution a hydroxyl group and the corresponding O-benzyl-protected phenolic moiety were introduced in position 5 of the indole scaffold. Although the latter resulted in higher lipophilicity, except for one compound this strategy did not positively influence the potency of the inhibitors. The calculated $logD_{5.0}$ values of the most potent compounds from this section (**5.25**-**5.27**, **5.29**, **5.48**) possess higher lipophilicity with regard to the $logD_{5.0}$ value compared to the parent compounds (**1**, **2**). The comparison of the four compounds **5.12**, **5.13**, **5.22** and **5.23**, allows the conclusion that the presence of a carboxyl group in position 2 of the indole is better suited for inhibition of *Sag*Hyal$_{4755}$ than an oxadiazole ring.

The influence of a methyl or ethyl group in position 3 remained unclear: for some molecules the methyl group seems to be more suited, in other compounds the ethyl group leads to higher inhibitory activity on *Sag*Hyal$_{4755}$. Generally, *N*-benzylated indoles were more potent than *N*-unsubstituted analogs. An additional chloro substituent (cf. **5.25**, **5.48**) or a *tert*-butyl carboxylate in position 4 of the attached benzyl-moiety, increased the inhibition of *Sag*Hyal$_{4755}$. Interestingly, the conversion of the hydrophobic *tert*-butyl ester to the polar carboxylic group (**5.30**, **5.40**) led to a slight decrease in inhibitory activity.

Except for compound **5.16**, in which the ester in position 2 moiety is still present, all 1*H*-indole analogs bearing the 6,7-dichloro motif showed at least weak inhibition of *Sag*Hyal$_{4755}$, most probably due to the lipophilicity contribution of the chlorine atoms. Except for 5-(benzyloxy)-1-(4-chlorobenzyl)-1*H*-indole-2-carboxylic acid (**5.48**, IC_{50} = 20 µM), all 6,7-dichloro-1*H*-indole-2-carboxylic acid derivatives showed higher inhibitory activity on the bacterial hyaluronidase *Sag*Hyal$_{4755}$ than the 5-benzyloxy- and 5-hydroxy-substitueted

1*H*-indole analogs. Although lipophilicity is obviously one of the most important physicochemical properties, the 6,7-dichloro-1*H*-indoles had lower $logD_{5.0}$ values and were more potent than the 5-benzyloxy-substiuted derivatives.

The most pronounced increase in inhibition of the hyaluronate lyase resulted from introduction of hydrophobic residues at the indole moiety. Especially, when the benzyl moiety was chloro-substituted, IC_{50} values in the lower two-digit micromolar range were achieved (cf. **5.25** and **5.48**). A negatively charged carboxylic group was also found to increase the affinity of the inhibitors.

In summary, the combination of the 6,7-dichloro substitution pattern with *N*-benzylation and a negatively charged carboxylic acid in position 2 proved to be most efficient and is considered promising to pursue further development of inhibitors for the bacterial hyaluronidase. The synthesized compounds showed very weak inhibition or were devoid of inhibitory activity on the second bacterial hyaluronidase, *Spn*Hyl. Moreover, all hyaluronate lyase inhibitors of the indole series were inactive on the bovine testicular enzyme (BTH).

5.6 Experimental section

5.6.1 General conditions

Cf. section 4.7.1

5.6.2 Chemistry

5.6.2.1 Preparation of compounds 5.1, 5.32

General Procedure[2,18,25]

To a suspension of the pertinent aniline (1 eq) in water (0.34 mL/mmol) and concentrated HCl (0.23 mL/mmol) was added dropwise a solution of sodium nitrite in water at 0 °C. After stirring for 10 min a solution of sodium tetrafluoroborate (1 eq) in water (0.24 mL/mmol) was added and the cooling bath was removed. Stirring was continued for additional 20 min at room temperature. The resulting orange solid was isolated by filtration and washed three times with ice cold water, methanol and ether.

2,3-Dichlorobenzenediazonium tetrafluoroborate (5.1)

The title compound was prepared from 2,3-dichloroaniline (10 g, 61.7 mmol) according to the general procedure. After drying under reduced pressure the title compound was obtained as a pale orange solid. (11 g, 69%); mp 150-151 °C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.99 (t, *J* 8.4 Hz, 1H), 8.58 (m, 1H), 8.87 (m, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 118.8, 130.8, 133.4, 133.6, 134.6, 142.2. MS (ESI) *m/z* (rel. int. in %) 172.9 ([M^+], 100). HRMS (ESI): *m/z* [M^+] calculated for $C_6H_3Cl_2N_2^+$: 172.9666, found 172.9665. HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_6H_4Cl_2N_2^+$: 172.9666, found 172.9665. $C_6H_3Cl_2N_2^+$•BF_4^- (259.97).

4-(Benzyloxy)benzenediazonium tetrafluoroborate[18] (5.32)

The title compound was prepared from 4-benzyloxyaniline (15 g, 64 mmol) according to the general procedure. After drying under reduced pressure the title compound was obtained as a pale yellow solid (15.8 g, 89%); mp 152-153 °C (ref.[18]: 137 °C). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 5.42 (s, 2H,), 7.34-7.61 (m, 7H), 8.54-8.70 (m, 2H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 71.26, 103.5, 117.8 (2 interfering carbon signals), 128.2 (2 interfering carbon signals), 128.6 (2 interfering carbon signals), 134.8, 135.0 (2 interfering carbon signals), 167.7. HRMS (ESI): *m/z* [M^+] calculated for $C_{13}H_{11}N_2O^+$: 211.0866, found 211.0864. MS (ES-MS) *m/z* (rel. int. in %) 211.0 ([M^+], 100). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_{13}H_{12}N_2O^+$: 211.0866, found 211.0864. $C_{13}H_{11}NO_2^+$•BF_4^- (298.10).

5.6.2.2 Preparation of ethyl 2-acetylpentanoate (5.3)

General procedure[26-28]

To a suspension of NaH (60 % in mineral oil, 1 g) in dry THF (25 mL) was added ethyl acetoacetate (10.4 g, 80 mmol) dropwise. The mixture was stirred at room temperature for 30 min during which time the colorless suspension became a clear yellow solution. 1-Iodopropane (20.4 g, 120 mmol) was added, and the mixture was refluxed overnight. After cooling to room temperature, sat aq. NH_4Cl was added. The aqueous layer was separated and extracted with ethyl acetate three times. The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (petroleum ether/EtOAc 70/30 v/v) to give a yellowish liquid (5.62 g, 42 %). R_f = 0.89, ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 0.86 (t, *J* 7.3 Hz, 3H), 1.20 (t, *J* 7.1 Hz, 5H), 1.73 (q, *J* 1.0 Hz, 2H), 2.15 (s, 3H), 3.35 (t, *J* 7.4 Hz, 1H), 4.12 (q, *J* 5.0 Hz, 2H). ^{13}C-NMR (75 MHz, [D_1]$CHCl_3$): δ (ppm) 13.8, 14.1, 20.6, 28.7, 30.2, 59.7, 61.3, 169.9, 203.4. MS (CI-MS) (NH_3) *m/z* (rel. int. in %) 207.2 ([M+NH_4^++NH_3], 10), 190.1 ([M+NH_4^+], 100), 173.1 ([M+H^+]$^+$, 5). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_9H_{17}O_3^+$: 173.1199, found 173.1189. $C_9H_{16}O_3$ (172.22).

5.6.2.3 Preparation of compounds 5.4-5.5, 5.34-5.36

General procedure[2]

To a suspension of NaH (60 % suspension in mineral oil, 1.14 eq) in anhydrous THF was added dropwise a solution of the pertinent butanoate (**5.2**, **5.3**, **5.33**; 1.02 eq). The mixture was refluxed for 30 min. After cooling to -5 °C, **5.1** or **5.32** (1 eq) was added in portions, and the resulting dark red solution was stirred overnight at room temperature. The mixture was poured into water and extracted with ether three times. The combined organic layers were washed with water, dried over $MgSO_4$, filtered, and evaporated.

(*E*)-Ethyl 2-[2-(2,3-dichlorophenyl)hydrazono]butanoate (5.4)

The title compound was prepared from **5.1** (11.4 g, 44 mmol) and **5.2** (7.5 g, 45 mmol), according to the general procedure. The crude product, a dark oil, was used for the next step without further purification (13 g, 98 %). $C_{12}H_{14}Cl_2N_2O_2$ (288.04).

(*E*)-Ethyl 2-[2-(2,3-dichlorophenyl)hydrazono]pentanoate (5.5)

The title compound was prepared from **5.1** (11.4 g, 44 mmol) and **5.3** (8 g, 45 mmol), according to the general procedure. The crude product, a dark oil, was used for the next step without further purification (12 g, 86 %). $C_{13}H_{16}Cl_2N_2O_2$ (302.06).

(*E*)-Ethyl 2-{2-[4-(benzyloxy)phenyl]hydrazono}propanoate (5.34)

The title compound was prepared from **5.32** (13.7 g, 46 mmol) and **5.36** (5.37 g, 47 mmol), according to the general procedure. The crude product, a dark oil, was used for the next step without further purification (19.2 g, 62 %). $C_{18}H_{20}N_2O_3$ (312.15).

(*E*)-Ethyl 2-{2-[4-(benzyloxy)phenyl]hydrazono}butanoate (5.35)

The title compound was prepared from **5.32** (10 g, 47 mmol) and **5.2** (5.73 g, 47 mmol), according to the general procedure. The crude product, which was a dark oil, was used for the next step without further purification (13.7 g, 100 %). $C_{19}H_{22}N_2O_3$ (325.16).

(*E*)-Ethyl 2-{2-[4-(benzyloxy)phenyl]hydrazono}pentanoate (5.36)

The title compound was prepared from **5.32** (3.9 g, 13.1 mmol) and **5.3** (2.3 g, 13.4 mmol), according to the general procedure. The crude product, a dark oil, was used for the next step without further purification (5 g, 100 %). $C_{20}H_{24}N_2O_3$ (340.18).

5.6.2.4 Preparation of compounds 5.6, 5.7, 5.37-5.39

General procedure

A solution of the pertinent hydrazone (**5.4-5.5**, **5.34-5.36**) and concentrated HCl in anhydrous EtOH was refluxed for 3 days. After cooling the solution was diluted with water and extracted three times with $CHCl_3$. The combined organic layers were washed with water, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified with flash chromatography (PE/EtOAc 80/20 v/v) and recrystallized from petroleum ether.

Ethyl 6,7-dichloro-3-methyl-1*H*-indole-2-carboxylate (5.6)

The title compound was prepared from **5.4** (15.2 g, 53 mmol), according to the general procedure. The purified product was recrystallized from petroleum ether to yield yellow crystals (1.3 g, 12 %); mp 141-148 °C, R_f = 0.73, ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.36 (t, *J* 7.1 Hz, 3H, $COOCH_2C\boldsymbol{H_3}$), 2.50 (s, 3H, $C\boldsymbol{H_3}$), 4.35 (q, *J* 7.1 Hz, 2H, $COOC\boldsymbol{H_2}CH_3$), 7.25 (d, *J* 8.6 Hz, 1H, indole-***H***), 7.64 (d, *J* 8.6 Hz, 1H, indole-***H***), 11.77 (br s, 1H, N***H***). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 11.3, 13.8, 25.8, 61.1, 112.0, 115.2, 121.8, 128.8, 131.7, 134.0, 141.3, 162.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 271.1 ([$M^{+\bullet}$], 45), 225.0 ([*M*-EtOH], 100). HRMS (ESI): *m/z* [*M*+H^+]$^+$ calculated for $C_{12}H_{13}Cl_2NO_2^+$: 272.0240, found 272.0240. $C_{12}H_{11}Cl_2NO_2$ (271.02).

Ethyl 6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylate (5.7)

The title compound was prepared from **5.5** (11.4 g, 38 mmol), according to the general procedure. The purified product was recrystallized from petroleum ether to yield a white solid (2.2 g, 20 %); R_f = 0.69, mp 120-121 °C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.18 (t, *J* 7.5 Hz, 3H), 1.36 (t, *J* 7.1 Hz, 3H), 3.03 (q, *J* 7.4 Hz, 2H), 4.36 (q, *J* 7.1 Hz, 2H), 7.27 (d, *J* 8.6 Hz, 1H), 7.70 (d, *J* 8.6 Hz, 1H), 11.79 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 14.1, 15.4, 17.4, 60.4, 114.9, 120.1, 121.3, 125.0, 125.5, 127.0, 127.4, 134.0, 161.0. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 285.0 ([$M^{+\bullet}$], 70), 255.0 ([*M*-$C_2H_5^\bullet$], 100). HRMS (ESI): *m/z* [*M*+H^+]$^+$ calculated for $C_{13}H_{14}Cl_2NO_2^+$: 285.0398, found 285.0395. $C_{13}H_{13}Cl_2NO_2$ (285.03).

Ethyl 5-(benzyloxy)-1*H*-indole-2-carboxylate[29] (5.37)

The title compound was prepared from **5.34** (19.3 g, 62 mmol), according to the general procedure. The purified product was recrystallized from petroleum ether to yield a yellow solid (1.4 g, 8 %); mp 157-158 °C (ref.:[29] 161-163 °C) ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.33 (t, *J* 7.1 Hz, 3H, $COOCH_2C\boldsymbol{H}_3$), 4.32 (q, *J* 7.1 Hz, 2H, $COOC\boldsymbol{H}_2CH_3$), 5.09 (s, 2H, benzyl-$C\boldsymbol{H}_2$), 5.95-7.07 (m, 2H), 7.21 (d, *J* 2.4 Hz, 1H), 7.28-7.43 (m, 4H), 7.44-7.52 (m, 2H),

11.76 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 14.2, 60.2, 69.5, 103.4, 107.1, 113.4, 115.6, 125.9, 127.6 (2 interfering carbon signals), 128.3 (2 interfering carbon signals), 132.7, 137.3, 152.9, 161.1. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 295.2 ([$M^{+\bullet}$], 55), 204.1 ([*M*-$C_7H_7^{\bullet}$], 75), 204.1 ([*M*-$C_7H_7^{\bullet}$-EtOH], 50), 91.1 ([C_7H_7]$^+$, 100). $C_{18}H_{17}NO_3$ (295.33).

Ethyl 5-(benzyloxy)-3-methyl-1*H*-indole-2-carboxylate (5.38)

The title compound was prepared from **5.35** (13.66 g, 42 mmol), according to the general procedure. The purified product was recrystallized from petroleum ether to yield a white-yellow solid (4.8 g, 44 %); mp 143-145 °C. ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.35 (t, *J* 7.1 Hz, 3H), 2.50 (s, 3H), 4.32 (q, *J* 7.1 Hz, 2H), 5.12 (s, 2H), 5.99 (m, 1H), 7.20 (d, *J* 2.3 Hz, 1H), 7.26-7.45 (m, 4H), 7.49 (d, *J* 5.8 Hz, 2H), 11.34 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 9.8, 14.3, 59.9, 69.6, 101.9, 113.2, 115.8, 118.2, 123.5, 127.6 (2 interfering carbon signals), 127.7, 128.3, 131.6, 137.2, 152.4, 161.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 309.2 ([$M^{+\bullet}$], 45), 218.1 ([*M*-$C_7H_7^{\bullet}$], 100), 172.0 ([*M*-$C_7H_7^{\bullet}$-EtOH], 95), 91.1 ([C_7H_7]$^+$, 60). HRMS (ESI): *m/z* [*M*+H$^+$]$^+$ calculated for $C_{19}H_{20}NO_3^+$: 310.1438, found 310.1438. $C_{19}H_{19}NO_3$ (309.14).

Ethyl 5-(benzyloxy)-3-ethyl-1*H*-indole-2-carboxylate (5.39)

The title compound was prepared from **5.36** (5 g, 14.6 mmol), according to the general procedure. The purified product was recrystallized from petroleum ether to yield an orange solid (638 g, 14 %); mp 150-158 °C. ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.17 (t, *J* 7.4 Hz, 3H), 1.34 (t, *J* 7.1 Hz, 3H), 3.01 (q, *J* 7.4 Hz, 2H), 4.33 (q, *J* 7.1 Hz, 2H), 5.12 (s, 2H), 5.99 (m, 1H), 7.20 (d, *J* 2.3 Hz, 1H), 7.27-7.45 (m, 4H), 7.45-7.51 (m, 2H), 11.33 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 14.2, 15.4, 59.9, 69.6, 101.8, 113.3, 115.7, 122.7, 124.6, 125.7, 127.6, 127.6, 128.3, 131.7, 137.4, 152.4, 161.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 323.1 ([$M^{+\bullet}$], 35), 232.1 ([*M*-$C_7H_7^{\bullet}$], 90), 185.0 ([*M*-$C_7H_7^{\bullet}$-EtOH], 95), 91.1 ([C_7H_7]$^+$, 60). $C_{20}H_{21}NO_3$ (323.15).

5.6.2.5 Preparation of compounds 5.9-5.11

General procedure[20,30]

The pertinent indole ester (**5.6-5.8**, 1 eq) was dissolved in ethanol (5.2 mL/mmol). Hydrazine monohydrate (21 eq) was added, and the mixture was heated to reflux overnight. The reaction mixture was cooled to -15 °C to give a precipitate.

6,7-Dichloro-3-methyl-1*H*-indole-2-carbohydrazide (5.9)

The title compound was prepared from **5.6** (800 mg, 2.94 mmol), according to the general procedure. The precipitate was filtered off to give the product as white-yellow crystals (530 mg, 70 %); mp > 210 °C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 2.50 (s, 3H), 4.56 (s, 2H), 7.25 (d, *J* 8.5 Hz, 1H), 7.61 (d, *J* 8.5 Hz, 1H), 9.55 (br s, 1H), 11.35 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 9.5, 114.0, 115.4, 119.6, 120.8, 125.0, 127.96, 128.04, 133.1, 160.8. HRMS (ESI): *m/z* [*M*+H^+]$^+$ calculated for $C_{10}H_{10}Cl_2N_3O^+$: 258.0200, found 258.0197. $C_{10}H_9Cl_2N_3O$ (257.01).

6,7-Dichloro-3-ethyl-1*H*-indole-2-carbohydrazide (5.10)

The title compound was prepared from **5.6** (190 mg, 0.51 mmol), according to the general procedure. The precipitate was filtered off to give the product as orange solid (103 mg, 56 %); R_f = 0.95, ^{1}H-NMR (300 MHz, [D_4]MeOH): δ (ppm) 1.22 (t, *J* = 7.3 Hz, 3H), 3.06 (q, *J* 7.4 Hz, 2H), 5.23 (s, 2H), 5.80 (d, *J* 5.5 Hz, 2H), 7.07-7.21 (m, 4H), 7.53 (d, *J* 8.5 Hz, 1H). ^{13}C-NMR (75 MHz, [D_4]MeOH): δ (ppm) 15.0, 19.1, 115.7, 120.5, 122.9, 125.1, 125.8 (2 interfering carbon signals), 127.8, 129.4 (2 interfering carbon signals), 130.2, 134.3, 135.2, 141.6, 167.5. HRMS (ESI): *m/z* [*M*+H^+]$^+$ calculated for $C_{18}H_{18}Cl_2N_3O^+$: 362.0818, found 362.0817. $C_{18}H_{17}Cl_2N_3O$ (361.07).

1*H*-Indole-2-carbohydrazide[31] (5.11)

The title compound was prepared from **5.8** (1 g, 5.3 mmol), according to the general procedure. The precipitate was filtered off to give the product as white crystals (1.6 g, 85 %); mp > 210 °C (ref.[31]: 250-251 °C dec). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.07 (m, 1H), 7.15 (m, 1H), 7.26 (m, 1H), 7.46 (m, 1H), 7.66 (m, 1H), 11.89 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 101.7, 112.1, 119.6, 121.3, 123.0, 127.0, 130.4, 135.2, 161.1. HRMS (ESI): *m/z* [*M*+H^+]$^+$ calculated for $C_9H_{10}N_3O^+$: 175.0817, found 175.0817. $C_9H_9N_3O$ (175.07).

5.6.2.6 Preparation of compounds 5.12-5.14

General procedure[20,32]

The pertinent indole carbohydrazide (**5.9-5.11**, 1 eq) was dissolved in formic acid (9.3 mL/mmol) and heated to reflux for 3 h. After cooling to room temperature, the solvent was removed under pressure. The precipitate was dissolved in methanol and cooled with ice to give the product as a precipitate which was filtered off and dried under pressure. Subsequently, the precipitate was suspended in phosphoryl chloride (7.7 mL/ mmol) and heated to reflux for 20 min. The reaction mixture was put on ice to cool down, neutralized with 2.5 M NaOH and washed three times with ethyl acetate. The combined organic layers

were dried over $MgSO_4$, filtered, and the solvent evaporated. The crude product was purified by flash chromatography (PE/EtOAc 50/50 v/v).

2-(6,7-Dichloro-3-methyl-1*H*-indol-2-yl)-1,3,4-oxadiazole (5.12)

The title compound was prepared from **5.9** (250 mg, 0.97 mmol), according to the general procedure. Flash chromatography yielded a red solid (30 mg, 12 %); R_f = 0.66, RP-HPLC (220 nm): 76 % (t_R = 26.0 min, k = 10.2).^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 2.59 (s, 3H), 7.32 (d, *J* 8.5 Hz, 1H), 7.70 (d, *J* 8.5 Hz, 1H), 9.45 (br s, 1H), 12.36 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 9.5, 115.0, 115.9, 119.8, 119.9, 121.5, 125.7, 127.8, 134.7, 153.7, 158.4. MS (ES-MS) *m/z* (rel. int. in %) 268.0 ([*M*+H$^+$]$^+$, 100). HRMS (ESI): *m/z* [*M*+H$^+$]$^+$ calculated for $C_{11}H_8Cl_2N_3O^+$: 268.0039, found 268.0039. $C_{11}H_7Cl_2N_3O$ (267.00).

2-(6,7-Dichloro-3-ethyl-1*H*-indol-2-yl)-1,3,4-oxadiazole (5.13)

The title compound was prepared from **5.10** (130 mg, 0.55 mmol), according to the general procedure. Flash chromatography yielded a red solid (70 mg, 45 %); ^{1}H-NMR (300 MHz, [D_4]MeOH): δ (ppm) 1.30 (t, *J* 7.5 Hz, 3H), 3.16 (q, *J* 7.5 Hz, 2H), 7.23 (d, *J* 8.6 Hz, 1H), 7.61 (d, *J* 8.6 Hz, 1H), 8.08 (s, 1H), 9.06 (br s, 1H). MS (ES-MS) *m/z* (rel. int. in %) 595.0 ([2M+Na$^+$], 20), 285.0 ([*M*H$^+$]$^+$, 100). HRMS (ESI): *m/z* [*M*+H$^+$]$^+$ calculated for $C_{12}H_{10}Cl_2N_3O^+$: 282.0198, found 282.0197. $C_{12}H_9Cl_2N_3O$ (281.01).

2-(1*H*-Indol-2-yl)-1,3,4-oxadiazole (5.14)

The title compound was prepared from **5.11** (500 mg, 2.85 mmol), according to the general procedure. Flash chromatography yielded a red solid (500 mg, 95 %); mp 190 °C. RP-HPLC (220 nm): 97 % (t_R = 17.1 min, k = 5.3). ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 7.10 (t, *J* 7.5 Hz, 1H), 7.20-7.25 (m, 1H), 7.28 (d, *J* 7.0 Hz, 1H), 7.51 (s, 1H), 7.68 (d, *J* 7.4 Hz, 1H), 9.37 (br s, 1H), 12.30 (br s, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 104.9, 112.2, 120.3, 120.8, 121.4, 124.1, 127.1, 137.7, 153.8, 158.9. MS (ES-MS) *m/z* (rel. int. in %) 185.1 ([*M*+H$^+$]$^+$, 100). HRMS (ESI): *m/z* [*M*+H$^+$]$^+$ calculated for $C_{10}H_8N_3O^+$: 185.0662, found 185.065. $C_{10}H_7N_3O$ (185.06).

5.6.2.7 Preparation of *tert*-butyl 4-(bromomethyl)benzoate (5.15)

General procedure[19]

4-(Bromomethyl)benzoic acid (**5.15a**; 3.22 g, 15 mmol, 1 eq) was dissolved in anhydrous THF (1.33 mL/mmol). Under an atmosphere of nitrogen, *tert*-butyl 2,2,2-trichloroacetimidate (TBTA, 5.6 g, 30 mmol, 2 eq) was dissolved in anhydrous cyclohexane (0.33 mL/mmol) and

added dropwise. After adding boron trifluoride etherate (BF_3Et_2O; 340 mg, 2.4 mmol, 0.16 eq), the reaction mixture was stirred for 17 h at ambient temperature. To the crude product, solid $NaHCO_3$ was added and stirred for additional 30 min. Subsequently, the reaction mixture was filtered over celite, washed twice with cyclohexane, and evaporated under reduced pressure. The crude brown oil was purified by flash chromatography (PE/EtOAc 80/20 v/v) to yield white crystals (2.9 g, 72 %); mp 139-144 °C, R_f = 0.75, ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.54 (s, 9H), 4.75 (s, 2H), 7.57 (t, *J* 9.6 Hz, 2H), 7.90 (t, *J* 13.3 Hz, 2H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 27.7 (3 interfering carbon signals), 33.1, 80.3, 125.0 (2 interfering carbon signals), 128.8 (2 interfering carbon signals), 128.9, 147.7, 164.8. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 272.1 ([$M^{+\bullet}$], 5), 255.0 ([M–$CH_3^{\bullet}$]$^+$, 5), 215.0 ([M–$C_7H_7^{\bullet}$]$^+$, 45), 135.1 (100). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_{12}H_{16}BrO_2^+$: 271.0327, found 273.0302. $C_{12}H_{15}BrO_2$ (270.03).

5.6.2.8 Preparation of compounds 5.16-5.21, 5.40-5.44

General procedure[5]

With ice cooling, NaH (60 % suspension in mineral oil, 1.14 eq) was suspended in anhydrous DMF (1.5 mL/mmol) and stirred for 15 min under an atmosphere of nitrogen. Subsequently, the pertinent indole compound (**5.6**, **5.7**, **5.37**, **5.38** and **5.39**, 1.0 eq) in anhydrous DMF (2.25 mL/mmol) was added dropwise at 0 °C under an atmosphere of nitrogen. After stirring for 45 min at 0 °C, the corresponding benzyl halide (1.2 eq) in anhydrous DMF (4.5 mL/mmol) was slowly added. After removing the cooling bath, the mixture was stirred for additional 2.5 h at ambient temperature and then poured into ice water. The aqueous solution was extracted at least three times with EtOAc. The combined organic layers were washed with water and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by RP flash chromatography (MeCN/H_2O 20/80-95/5 v/v).

Ethyl 1-benzyl-6,7-dichloro-3-methyl-1*H*-indole-2-carboxylate (5.16)

The title compound was prepared from **5.6** (1 g, 3.7 mmol) and (bromomethyl)-benzene (805 mg, 1.25 mmol), according to the general procedure. Flash chromatography yielded a white solid (910 mg, 68 %); mp 110 °C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.24 (t, *J* 7.1 Hz, 3H), 2.51 (s, 3H), 4.28 (q, *J* 7.1 Hz, 2H), 5.14 (s, 2H), 5.82 (d, *J* 5.9 Hz, 2H), 7.13-7.31 (m, 3H), 7.36 (d, *J* 8.6 Hz, 1H), 7.75 (d, *J* 8.6 Hz, 1H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 10.3, 13.8, 48.4, 60.8, 114.5, 120.7, 120.8, 122.1, 125.1, 125.7, 127.7, 128.2, 128.4, 128.6, 129.7, 133.7, 139.5, 161.2. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 361.0 ([$M^{+\bullet}$], 10), 91.1 ([C_7H_7]$^+$, 100). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_{19}H_{18}Cl_2NO_2^+$: 362.0712, found 362.0710. $C_{19}H_{17}Cl_2NO_2$ (361.06).

Ethyl 6,7-dichloro-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylate (5.17)

The title compound was prepared from **5.6** (340 mg, 1.25 mmol) and 1-(bromomethyl)-4-chlorobenzene (321 mg, 1.56 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (140 mg, 28 %) which was used for the preparation of **5.25** without further purification.

Ethyl 1-[4-(*tert*-butoxycarbonyl)benzyl]-6,7-dichloro-3-methyl-1*H*-indole-2-carboxylate (5.18)

The title compound was prepared from **5.6** (430 mg, 1.6 mmol) and *tert*-butyl 4-(bromomethyl)benzoate (543 mg, 2 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (220 mg, 30 %) which was used for the preparation of **5.26** without further purification.

Ethyl 1-benzyl-6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylate (5.19)

The title compound was prepared from **5.7** (890 mg, 3.11 mmol) and (bromomethyl)benzene (665 mg, 3.89 mmol), according to the general procedure. RP flash chromatography yielded a white solid (240 mg, 21 %); R_f = 0.88, ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.19 (t, *J* 7.5 Hz, 3H), 1.25 (t, *J* 5.4 Hz, 3H), 3.02 (q, *J* 7.4 Hz, 2H), 4.29 (q, *J* 7.1 Hz, 2H), 5.14 (s, 2H), 5.82 (d, *J* 7.0 Hz, 2H), 7.15-7.30 (m, 3H), 7.38 (d, *J* 8.5 Hz, 1H), 7.80 (d, *J* 8.6 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 13.7, 15.4, 17.8, 48.5, 60.9, 120.5, 122.2, 125.1 (2 interfering carbon signals), 125.7, 127.0, 127.3, 127.4, 127.7, 128.4 (2 interfering carbon signals), 129.6, 133.7, 139.6, 161.1. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 375.1 ([$M^{+\bullet}$], 20), 284.0 ([M-$C_7H_7^{\bullet}$], 20), 91.1 ([C_7H_7]$^+$, 100). $C_{20}H_{19}Cl_2NO_2$ (375.08).

Ethyl 6,7-dichloro-1-(4-chlorobenzyl)-3-ethyl-1*H*-indole-2-carboxylate (5.20)

The title compound was prepared from **5.7** (1 g, 3.5 mmol) and 1-(bromomethyl)-4-chlorobenzene (864 mg, 4.2 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (120 mg, 8 %) which was used for the preparation of **5.28** without further purification.

Ethyl 1-[4-(*tert*-butoxycarbonyl)benzyl]-6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylate (5.21)

The title compound was prepared from **5.7** (430 mg, 1.58 mmol) and *tert*-butyl 4-(bromomethyl)benzoate (543 mg, 2 mmol), according to the general procedure. RP flash

chromatography yielded a white fluffy solid (240 mg, 33 %) which was used for the preparation of **5.29** without further purification.

Ethyl 5-(benzyloxy)-1-(4-chlorobenzyl)-1*H*-indole-2-carboxylate (5.40)

The title compound was prepared from **5.37** (470 mg, 1.6 mmol) and 1-(bromomethyl)-4-chlorobenzene (411 mg, 2 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (260 mg, 39 %), which was used for the preparation of **5.48** without further purification.

Ethyl 1-benzyl-5-(benzyloxy)-3-methyl-1*H*-indole-2-carboxylate (5.41)

The title compound was prepared from **5.38** (500 mg, 1.62 mmol) and 1-(bromomethyl)-4-chlorobenzene (350 mg, 2.02 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (415 mg, 65 %); mp 200 °C. ^{1}H-NMR (300 MHz, [D_6]DMSO): δ (ppm) 1.27 (t, *J* 7.1 Hz, 3H), 2.53 (s, 3H), 4.27 (q, *J* 7.1 Hz, 2H), 5.14 (s, 2H), 5.75 (s, 2H), 5.94 (m, 2H), 7.05 (m, 1H), 7.17-7.31 (m, 3H), 7.31-7.52 (m, 7H). ^{13}C-NMR (75 MHz, [D_6]DMSO): δ (ppm) 10.7, 14.0, 20.7, 59.7, 69.6, 102.2, 111.8, 117.2, 119.6, 124.4, 125.0 (2 interfering carbon signals), 125.8, 125.9, 127.6 (3 interfering carbon signals), 128.3 (4 interfering carbon signals), 133.5, 137.2, 138.8, 152.7, 161.5. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 399.2 ([$M^{+\bullet}$], 10), 308.2 ([M-$C_7H_7^{\bullet}$]$^+$, 25), 91.1 ([$C_7H_7^+$], 100). $C_{26}H_{25}NO_3$ (399.18).

Ethyl 5-(benzyloxy)-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylate (5.42)

The title compound was prepared from **5.38** (500 mg, 1.6 mmol) and 1-(bromomethyl)-4-chlorobenzene (410 mg, 2 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (120 mg, 17 %), which was used for the preparation of **5.50** without further purification.

Ethyl 5-(benzyloxy)-1-(4-(*tert*-butoxycarbonyl)benzyl)-3-methyl-1*H*-indole-2-carboxylate (5.43)

The title compound was prepared from **5.38** (500 mg, 1.6 mmol) and 1-(bromomethyl)-4-chlorobenzene (545 mg, 1.25 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (560 mg, 70 %); mp 110-120 °C. RP-HPLC (220 nm): 100 % (t_R = 34.9 min, *k* = 14.0). ^{1}H-NMR (300 MHz, [D_6]Acetone): δ (ppm) 1.33 (t, *J* 7.1 Hz, 3H), 1.55 (s, 9H), 2.59 (s, 3H), 4.31 (q, *J* 7.1 Hz, 2H), 5.17 (s, 2H), 5.89 (s, 2H), 7.03-7.13 (m, 3H), 7.27-7.45 (m, 5H), 7.52 (d, *J* 7.4 Hz, 2H), 7.85 (d, *J* 8.3 Hz, 2H). ^{13}C-NMR (75 MHz, [D_6]Acetone): δ (ppm) 11.2, 14.6, 28.3 (3 interfering carbon signals), 48.6, 61.1, 71.0, 81.2, 103.4, 112.6, 118.3, 121.3, 125.8, 127.0 (2 interfering carbon signals), 128.5 (2

interfering carbon signals), 128.6, 129.3 (2 interfering carbon signals), 130.3 (2 interfering carbon signals), 131.7, 135.0, 138.8, 145.2, 154.7, 163.2, 165.8. HRMS (ESI): *m/z* $[M+H^+]^+$ calculated for $C_{31}H_{34}NO_5^+$: 500.2437, found 500.2432. $C_{31}H_{33}NO_5$ (499.24).

Ethyl 5-(benzyloxy)-1-(4-chlorobenzyl)-3-ethyl-1*H*-indole-2-carboxylate (5.44)

The title compound was prepared from **5.39** (500 mg, 1.6 mmol) and 1-(bromomethyl)-4-chlorobenzene (390 mg, 1.9 mmol), according to the general procedure. RP flash chromatography yielded a white fluffy solid (230 mg, 34 %), which was used for the preparation of **5.52** without further purification.

5.6.2.9 Preparation of compounds 5.22-5.31, 5.45-5.52, 5.60

General procedure[5]

A suspension of the pertinent carboxylate (**5.16-5.21**, **5.40-5.44**, 1 eq) and LiOH (2.5 eq) in a 3:1:0.2 mixture of THF, H_2O and EtOH (v/v/v) was stirred at room temperature for 3 days. The reaction mixture was acidified with 0.5 N HCl to pH 2 to yield the product as precipitate.

6,7-Dichloro-3-methyl-1*H*-indole-2-carboxylic acid (5.22)

The title compound was prepared from **5.6** (70 mg, 0.26 mmol), according to the general procedure. Precipitation yielded a yellow solid (60 mg, 94 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 23.1 min, k = 8.9). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 2.56 (s, 3H, indole-C***H***$_3$-3), 7.18 (d, *J* 8.6 Hz, 1H, indole-***H***-5), 7.55 (d, *J* 8.6 Hz, 1H; indole-***H***-4). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 9.7 (indole-***C***H$_3$-3), 114.7 (C$_{quat}$, indole-***C***-7), 119.3 (C$_{quat}$, indole-***C***-9), 120.1 (indole-***C***-4), 121.0 (indole-***C***-5), 125.5 (C$_{quat}$, indole-***C***-2), 127.1 (C$_{quat}$, indole-***C***-6), 128.1 (C$_{quat}$, indole-***C***-8), 133.8 (C$_{quat}$, indole-***C***-3), 162.8 (***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 485.9 ([2*M*-H$^+$]$^-$, 100) 288.0 ([*M*+HCOO$^-$], 20). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{10}H_6Cl_2NO_2^-$: 242.9813, found 242.9811. $C_{10}H_7Cl_2NO_2$ (242.99).

6,7-Dichloro-3-ethyl-1*H*-indole-2-carboxylic acid (5.23)

The title compound was prepared from **5.7** (25 mg, 0.1 mmol), according to the general procedure. Precipitation yielded a yellow solid (25 mg, 97 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 23.9 min, k = 9.3). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 1.24 (t, *J* 7.5 Hz, 3H, indole-C***H***$_3$-3), 3.11 (q, *J* 7.5 Hz, 2H, indole-C***H***$_2$-3), 7.19 (d, *J* 8.6 Hz, 1H, indole-***H***-5), 7.58 (d, *J* 8.6 Hz, 1H, indole-***H***-4). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 15.9 (indole-***C***H$_3$-3), 18.9 (indole-***C***H$_2$-3), 115.6 (C$_{quat}$, indole-***C***-7), 120.9 (C$_{quat}$, indole-***C***-9), 122.6 (indole-***C***-4), 128.5 (indole-***C***-5), 128.7 (C$_{quat}$, indole-***C***-2), 129.4 (C$_{quat}$, indole-***C***-6), 134.1

(C_{quat}, indole-***C***-8), 135.8 (C_{quat}, indole-***C***-3), 164.6 (C_{quat}, ***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 514.9 ([2*M*-H$^+$]$^-$, 100) 302.0 ([*M*+HCOO$^-$], 35). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{11}H_8Cl_2NO_2^-$: 255.997, found 255.9968. $C_{11}H_9Cl_2NO_2$ (257.00).

1-Benzyl-6,7-dichloro-3-methyl-1*H*-indole-2-carboxylic acid (5.24)

The title compound was prepared from **5.16** (64 mg, 0.18 mmol), according to the general procedure. Precipitation yielded a white solid (40 mg, 60 %); mp 200 °C. RP-HPLC (220 nm): 100 % (t_R = 29.5 min, *k* = 11.6). R_f = 0.78, ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.53 (s, 3H), 5.22 (s, 2H), 5.81 (d, *J* 7.0 Hz, 2H), 7.14-7.29 (m, 3H), 7.36 (d, *J* 8.5 Hz, 1H), 7.75 (d, *J* 8.5 Hz, 1H), 13.46 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 9.9, 47.8, 113.8, 120.4, 120.6, 121.9, 125.1, 125.6, 128.4, 128.5, 128.7, 129.3, 133.6, 139.6, 162.5. MS (ES-MS) *m/z* (rel. int. in %) 332.0 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{17}H_{12}Cl_2NO_2^-$: 333.0283, found 333.0288. $C_{17}H_{13}Cl_2NO_2$ (333.03).

6,7-Dichloro-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylic acid (5.25)

The title compound was prepared from **5.17** (60 mg, 0.15 mmol), according to the general procedure. Precipitation yielded a white-yellow solid (50 mg, 91 %); mp > 210 °C. RP-HPLC (220 nm): 96 % (t_R = 30.6 min, *k* = 12.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.52 (s, 3H), 5.18 (s, 2H), 5.84 (d, *J* 8.4 Hz, 2H), 7.24-7.48 (m, 3H), 7.75 (d, *J* 8.5 Hz, 1H) 13.49 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.4, 47.8, 114.4, 120.5, 120.7, 122.0, 127.0 (2 interfering carbon signals), 128.4 (2 interfering carbon signals), 128.8, 129.4, 131.2, 133.4, 138.9, 162.9. MS (ES-MS) *m/z* (rel. int. in %) 365.0 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{17}H_{11}Cl_3NO_2^-$: 365.9893, found 365.9895. $C_{17}H_{12}Cl_3NO_2$ (365.99).

1-[4-(*tert*-Butoxycarbonyl)benzyl]-6,7-dichloro-3-methyl-1*H*-indole-2-carboxylic acid (5.26)

The title compound was prepared from **5.18** (70 mg, 0.16 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-30 min: MeCN/0.1% aq. TFA 60/40-95/5, t_R = 21. 9 min) and removal of the eluate by evaporation and lyophilisation afforded **5.25** as a white fluffy solid (20 mg, 29 %); mp 200 °C. RP-HPLC (220 nm): 100 % (t_R = 19.3 min, *k* = 7.3) ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.50 (s, 9H), 2.53 (s, 3H), 5.25 (s, 2H), 5.93 (d, *J* 8.4 Hz, 2H), 7.38 (d, *J* 8.5 Hz, 1H), 7.78 (m, 3H), 13.47 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.3, 27.6 (3 interfering carbon signals), 80.3, 113.7, 120.7, 125.2 (2 interfering carbon signals), 127.8, 128.5, 128.7, 128.8, 129.2 (2 interfering carbon signals), 129.4, 129.8, 133.6, 137.5, 162.9, 164.5. MS (ES-MS) *m/z* (rel. int. in %) 432.1 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{22}H_{20}Cl_2NO_4^-$: 432.0788, found 432.0788. $C_{22}H_{21}Cl_2NO_4$ (433.08).

1-Benzyl-6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylic acid (5.27)

The title compound was prepared from **5.19** (50 mg, 0.13 mmol), according to the general procedure. Precipitation yielded a white solid (36 mg, 80 %); mp 200 °C. RP-HPLC (220 nm): 83 % (t_R = 30.9 min, *k* = 12.2). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 1.24 (t, *J* 7.5 Hz, 3H), 3.03 (q, *J* 7.5 Hz, 2H), 5.16 (s, 2H), 5.85 (d, *J* 7.0 Hz, 2H), 7.13-7.30 (m, 3H), 7.36 (d, *J* 8.5 Hz, 1H), 7.77 (d, *J* 8.6 Hz, 2H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 15.5, 17.7, 105.2, 105.8, 114.6, 120.3, 122.0, 125.1 (2 interfering carbon signals), 125.6, 125.7, 127.9, 128.2, 128.4 (2 interfering carbon signals), 129.3, 139.7, 162.7. MS (ES-MS) *m/z* (rel. int. in %) 348.1 ([*M*+H$^+$], 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{14}Cl_2NO_2^-$: 345.0407, found 345.0417. $C_{18}H_{15}Cl_2NO_2$ (347.05).

6,7-Dichloro-1-(4-chlorobenzyl)-3-ethyl-1*H*-indole-2-carboxylic acid (5.28)

The title compound was prepared from **5.20** (100 mg, 0.351 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-25 min: MeCN/0.1% aq. TFA 28/72-95/5, t_R = 22.2 min) and removal of the eluate by evaporation and lyophilisation afforded **5.28** as a white fluffy solid (60 mg, 45 %); mp > 210 °C. RP-HPLC (220 nm): 96 % (t_R = 32.7 min, *k* = 13.0). ^{1}H-NMR (300 MHz, [D$_6$]Aceton): δ (ppm) 1.26 (t, *J* 7.5 Hz, 3H), 3.15 (q, *J* 7.5 Hz, 2H), 5.32 (s, 2H), 5.94 (t, *J* 5.6 Hz, 2H), 7.20-7.37 (m, 3H), 7.75 (d, *J* 8.6 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]Aceton): δ (ppm) 15.0, 19.0, 49.1, 121.0, 123.1, 128.3 (2 interfering carbon signals), 129.3 (2 interfering carbon signals), 129.4, 130.7, 133.8, 135.1, 140.4. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{13}Cl_3NO_2^-$: 381.9990, found 380.0020. $C_{18}H_{14}Cl_3NO_2$ (381.01).

1-[4-(*tert*-Butoxycarbonyl)benzyl]-6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylic acid (5.29)

The title compound was prepared from **5.21** (100 mg, 0.2 mmol), according to the general procedure. Precipitation yielded a white solid (80 mg, 89 %); mp 140 °C. RP-HPLC (220 nm): 96 % (t_R = 34.8 min, *k* = 13.9). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 1.25 (t, *J* 7.4 Hz, 3H), 1.53 (s, 9H), 3.08 (q, *J* 7.4 Hz, 2H), 5.29 (s, 2H), 5.87 (t, *J* 8.2 Hz, 2H), 7.20 (d, *J* 8.5 Hz, 1H), 7.56 (d, *J* 8.5 Hz, 1H), 7.75 (d, *J* 8.3 Hz, 2H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 15.6, 17.3, 27.6 (3 interfering carbon signals), 27.7, 68.3, 70.8, 80.3, 114.2, 119.4, 121.1, 125.4 (2 interfering carbon signals), 127.2, 128.6, 129.0 (2 interfering carbon signals), 129.5, 132.2, 143.2, 145.0, 164.5. MS (ES-MS) *m/z* (rel. int. in %) 445.1 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{23}H_{22}Cl_2NO_4^-$: 447.0964, found 447.095. $C_{23}H_{23}Cl_2NO_4$ (447.10).

1-(4-Carboxybenzyl)-6,7-dichloro-3-methyl-1*H*-indole-2-carboxylic acid (5.30)

The title compound was prepared from **5.26** (70 mg, 0.16 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-25 min: MeCN/0.1% aq. TFA 28/72-95/5, t_R = 10.1 min) and removal of the eluate by evaporation and lyophilisation afforded **5.30** as a white fluffy solid (60 mg, 45 %); mp 200 °C. RP-HPLC (220 nm): 100 % (t_R = 24.4 min, *k* = 9.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.55 (s, 3H, indole-C***H***$_3$-3), 5.26 (s, 2H, NC***H***$_2$), 5.94 (d, *J* 8.3 Hz, 2H, benzyl-***H***), 7.37 (d, *J* 8.6 Hz, 1H, indole-***H***-5), 7.78 (d, *J* 8.5 Hz, 1H, indole-***H***-4), 7.84 (d, *J* 8.3 Hz, 2H, benzyl-***H***). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.1 (indole-***C***H$_3$-3), 48.3 (N***C***H$_2$), 114.3 (C$_{quat}$, indole-***C***-7), 120.5 (indole-***C***-4), 120.7 (C$_{quat}$, indole-***C***-9), 122.0 (indole-***C***-5), 125.1 (benzyl-***C***-2,6), 128.2 (C$_{quat}$, indole-***C***-2), 128.7 (C$_{quat}$, indole-***C***-6), 129.2 (C$_{quat}$, benzyl-***C***-4), 129.4 (C$_{quat}$, indole-***C***-8), 129.5 (benzyl-***C***-3,5), 133.4 (C$_{quat}$, indole-***C***-3), 144.8 (C$_{quat}$, benzyl-***C***-1), 162.8 (C$_{quat}$, indole-***C***OOH), 165.8 (C$_{quat}$, benzyl-***C***OOH). MS (ES-MS) *m/z* (rel. int. in %) 375.0 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{12}Cl_2NO_4^-$: 377.0182, found 377.0185. $C_{18}H_{13}Cl_2NO_4$ (377.02).

1-(4-Carboxybenzyl)-6,7-dichloro-3-ethyl-1*H*-indole-2-carboxylic acid (5.31)

The title compound was prepared from **5.29** (70 mg, 0.16 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-20 min: MeCN/0.1% aq. TFA 55/45-90/10, t_R = 11.8 min) and removal of the eluate by evaporation and lyophilisation afforded **5.31** as a white fluffy solid (40 mg, 64 %); RP-HPLC (220 nm): 99 % (t_R = 19.4 min, *k* = 7.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.19 (t, *J* 7.4 Hz, 3H), 3.05 (q, *J* 7.4 Hz, 2H), 5.24 (s, 2H), 5.94 (d, *J* 8.3 Hz, 2H), 7.37 (d, *J* 8.5 Hz, 1H), 7.81 (m, 3H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 15.4, 17.9, 48.3, 114.5, 120.5, 122.1, 125.2 (2 interfering carbon signals), 125.8, 127.77, 127.82, 129.1, 129.4, 129.5 (2 interfering carbon signals), 133.5, 145.0, 162.8, 165.8. MS (ES-MS) *m/z* (rel. int. in %) 435.0 ([*M*+HCOO$^-$], 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{19}H_{14}Cl_2NO_4^-$: 391.0338, found 391.0335. $C_{19}H_{15}Cl_2NO_4$ (391.04).

5-(Benzyloxy)-1*H*-indole-2-carboxylic acid[29,33] (5.45)

The title compound was prepared from **5.37** (80 mg, 0.27 mmol), according to the general procedure. Precipitation yielded a white solid (40 mg, 55 %; mp ref.:[33] 190 °C). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 5.08 (s, 2H), 7.00 (m, 1H), 7.06 (d, *J* 0.8 Hz, 1H), 7.16 (d, *J* 2.2 Hz, 1H), 7.34 (m, 4H), 7.43-7.49 (m, 2H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 71.7, 105.0, 114.1, 118.1, 128.7 (3 interfering carbon signals), 128.8 (2 interfering carbon signals), 128.9, 129.5, 134.6, 139.2, 154.9. HRMS (ESI): *m/z* [*M*+H$^+$]$^+$ calculated for $C_{16}H_{14}NO_3^+$: 265.0810, found 265.0812. $C_{16}H_{13}NO_3$ (267.09).

5-(Benzyloxy)-3-methyl-1*H*-indole-2-carboxylic acid (5.46)

The title compound was prepared from **5.38** (100 mg, 0.32 mmol), according to the general procedure. Precipitation yielded a white solid (100 mg, 100 %); ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 2.53 (s, 3H), 5.08 (s, 2H), 7.00 (m, 1H), 7.12 (d, *J* 2.2 Hz, 1H), 7.25-7.41 (m, 4H), 7.43-7.50 (m, 2H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 10.1, 71.8, 103.4, 114.0, 118.3, 120.1, 125.6, 128.7 (2 interfering carbon signals), 128.8, 129.5 (2 interfering carbon signals), 129.8, 133.6, 139.2, 154.5, 165.7. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{17}H_{14}NO_3^-$: 280.0972, found 280.0972. $C_{17}H_{16}NO_3$ (281.11).

5-(Benzyloxy)-3-ethyl-1*H*-indole-2-carboxylic acid (5.47)

The title compound was prepared from **5.39** (80 mg, 0.25 mmol), according to the general procedure. Precipitation yielded a white solid (40 mg, 54 %); ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 1.21 (t, *J* 5.1 Hz, 3H), 3.07 (q, *J* 7.5 Hz, 2H), 5.10 (s, 2H), 7.00 (m, 1H), 7.13 (d, *J* 2.3 Hz, 1H), 7.25-7.41 (m, 4H), 7.43-7.50 (m, 2H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 15.9, 18.9, 71.9, 103.5, 114.1, 118.2, 127.0, 128.75 (2 interfering carbon signals), 128.78, 128.80, 129.5 (2 interfering carbon signals), 130.6, 133.6, 139.2, 154.4, 165.5. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{16}NO_3^-$: 294.1128, found 294.1128. $C_{18}H_{17}NO_3$ (295.12).

5-(Benzyloxy)-1-(4-chlorobenzyl)-1*H*-indole-2-carboxylic acid (5.48)

The title compound was prepared from **5.40** (50 mg, 0.12 mmol), according to the general procedure. Precipitation yielded a white solid (35 mg, 75 %); RP-HPLC (220 nm): 93 % (t_R = 29.2 min, *k* = 11.5). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 5.08 (s, 2H), 5.82 (s, 2H), 5.96-7.05 (m, 3H), 7.19-7.25 (m, 3H), 7.28 (d, *J* 5.2 Hz, 2H), 7.30-7.40 (m, 3H), 7.45 (m, 2H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 45.8, 62.0, 69.5, 103.6, 125.3, 127.48, 127.53, 127.88, 127.91, 128.1, 128.3, 128.7, 131.1, 137.5, 152.7. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{23}H_{17}ClNO_3^-$: 390.0902, found 390.0902. $C_{23}H_{18}ClNO_3$ (391.10).

1-Benzyl-5-(benzyloxy)-3-methyl-1*H*-indole-2-carboxylic acid (5.49)

The title compound was prepared from **5.41** (100 mg, 0.25 mmol), according to the general procedure. Precipitation yielded a white solid (77 mg, 83 %); RP-HPLC (220 nm): 95 % (t_R = 19.3 min, *k* = 7.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.52 (s, 3H), 5.13 (s, 2H), 5.78 (s, 2H), 5.92-5.99 (m, 2H), 7.02 (m, 1H), 7.13-7.28 (m, 4H), 7.29-7.52 (m, 6H), 12.98 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.7, 47.1, 69.6, 102.2, 111.8, 115.6, 119.3, 125.1, 125.0 (2 interfering carbon signals), 125.7, 127.0, 127.6 (2 interfering carbon signals), 138.27 (2 interfering carbon signals), 128.29 (2 interfering carbon signals), 133.3, 137.2,

138.9, 152.7, 163.5. MS (ES-MS) *m/z* (rel. int. in %) 372.2 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{24}H_{20}NO_3^-$: 370.1449, found 370.1455. $C_{24}H_{21}NO_3$ (371.15).

5-(Benzyloxy)-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylic acid (5.50)

The title compound was prepared from **5.42** (120 mg, 0.28 mmol), according to the general procedure. Precipitation yielded a white solid (100 mg, 88 %); ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 2.52 (s, 3H), 5.10 (s, 2H), 5.76 (s, 2H), 5.89-7.00 (m, 3H), 7.11-7.23 (m, 4H), 7.25-7.41 (m, 3H), 7.46 (d, *J* 7.2 Hz, 2H). ^{13}C-NMR (151 MHz, [D$_4$]MeOH): δ (ppm) 10.6, 49.6, 72.0, 104.0, 112.2, 115.3, 128.66 (2 interfering carbon signals), 128.73, 129.2 (2 interfering carbon signals), 129.3 (2 interfering carbon signals), 129.4 (2 interfering carbon signals), 133.5, 133.9, 134.4, 139.3, 139.9, 154.6, 172.4. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{24}H_{19}ClNO_3^-$: 404.1039, found 404.1043. $C_{24}H_{20}ClNO_3$ (405.11).

5-(Benzyloxy)-1-(4-chlorobenzyl)-3-ethyl-1*H*-indole-2-carboxylic acid (5.52)

The title compound was prepared from **5.44** (230 mg, 0.51 mmol), according to the general procedure. Precipitation yielded a white-yellow solid (200 mg, 94 %); mp 135-145 °C. RP-HPLC (220 nm): 85 % (t_R = 31.2 min, *k* = 12.4). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.14 (t, *J* 7.0 Hz, 3H), 3.07 (q, *J* 7.0 Hz, 2H), 5.09 (s, 2H), 5.86 (s, 2H), 5.86 (m, 1H), 7.00-7.17 (m, 3H), 7.18-7.43 (m, 6H), 7.47 (d, *J* 5.8 Hz, 2H). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{25}H_{21}ClNO_3^-$: 418.1199, found 418.1198. $C_{25}H_{22}ClNO_3$ (419.13).

5.6.2.10 Preparation of compounds 5.53-5.59

General procedure[2]

The pertinent 5-benzyloxyindole derivative (**5.45-5.52**, 1 eq) was dissolved in a 1:1 mixture of MeOH and THF (v/v) and a catalytic amount of palladium on activated charcoal (10 % Pd) was added. A slow stream of hydrogen was then bubbled through the suspension overnight. Insoluble material was filtered off, and the solvent was evaporated to yield the target compound.

5-Hydroxy-1*H*-indole-2-carboxylic acid[34] (5.53)

The title compound was prepared from **5.45** (40 mg, 0.23 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-30 min: MeCN/0.1% aq. TFA 28/72-95/5, t_R = 7.9 min) and removal of the eluate by evaporation and lyophilisation afforded **5.53** as a light brown fluffy solid (30 mg, 74 %); mp > 210 °C (ref.:[34] 246 °C, decomposed). RP-HPLC (220 nm): 83 %

(t_R = 8.18 min, *k* = 2.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 5.78 (m, 1H), 5.88 (s, 2H), 7.23 (d, *J* 8.8 Hz, 1H), 8.88 (s, 1H), 11.45 (s, 1H), 12.73 (br s, 1H). MS (ES-MS) *m/z* (rel. int. in %) 175.0 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_9H_6NO_3^-$: 177.0385, found 175.0384. $C_9H_7NO_3$ (177.04).

5-Hydroxy-3-methyl-1*H*-indole-2-carboxylic acid (5.54)

The title compound was prepared from **5.46** (100 mg, 0.52 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-30 min: MeCN/0.1% aq. TFA 28/72-95/5, t_R = 8.7 min) and removal of the eluate by evaporation and lyophilisation afforded **5.54** as a light violet fluffy solid (50 mg, 50 %); mp 210 °C. RP-HPLC (220 nm): 94 % (t_R = 10.3 min, *k* = 3.4). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.42 (s, 3H), 5.78 (m, 1H), 5.84 (d, *J* 2.3 Hz, 1H), 7.18 (d, *J* 8.7 Hz, 1H), 8.85 (s, 1H), 11.06 (s, 1H), 12.70 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 9.7, 102.6, 112.7, 115.9, 115.4, 124.1, 128.3, 130.7, 150.6, 163.4. MS (ES-MS) *m/z* (rel. int. in %) 190.1 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{10}H_8NO_3^-$: 191.0542, found 191.0541. $C_{10}H_9NO_3$ (191.06).

3-Ethyl-5-hydroxy-1*H*-indole-2-carboxylic acid (5.55)

The title compound was prepared from **5.47** (40 mg, 0.2 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-30 min: MeCN/0.1% aq. TFA 28/72-95/5, t_R = 11.4 min) and removal of the eluate by evaporation and lyophilisation afforded **5.55** as a light brown fluffy solid (20 mg, 49 %); mp 208-209 °C. RP-HPLC (220 nm): 100 % (t_R = 19.4 min, *k* = 7.3). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.15 (t, *J* 7.4 Hz, 3H), 2.95 (q, *J* 7.4 Hz, 2H), 5.77 (m, 1H), 5.87 (d, *J* 2.2 Hz, 1H), 7.19 (d, *J* 8.8 Hz, 1H), 8.84 (s, 1H), 11.06 (s, 1H), 12.70 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 15.4, 17.4, 102.5, 112.9, 115.8, 123.2, 123.4, 127.3, 130.8, 150.5, 163.3. MS (ES-MS) *m/z* (rel. int. in %) 204.1 ([*M*-H$^+$]$^-$, 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{11}H_{10}NO_3^-$: 205.0698, found 205.0697. $C_{11}H_{11}NO_3$ (205.07).

1-(4-Chlorobenzyl)-5-hydroxy-1*H*-indole-2-carboxylic acid[35] (5.56)

The title compound was prepared from **5.48** (35 mg, 0.1 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-20 min: MeCN/0.1% aq. TFA 40/60-75/25, t_R = 12.4 min) and removal of the eluate by evaporation and lyophilisation afforded **5.56** as a white fluffy solid (30 mg, 99 %); RP-HPLC (220 nm): 90.5 % (t_R = 17.93 min, *k* = 6.7). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 5.73 (s, 2H), 5.81 (m, 1H), 5.94 (m, 3H), 7.12-7.34 (m, 4H), 9.00 (s, 1H),

12.89 (br s, 1H). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{16}H_{11}ClNO_3^-$: 300.0430, found 300.0430. $C_{16}H_{12}ClNO_3$ (301.05).

1-Benzyl-5-hydroxy-3-methyl-1*H*-indole-2-carboxylic acid (5.57)

The title compound was prepared from **5.49** (37 mg, 0.1 mmol), according to the general procedure. Precipitation yielded the product as a brown solid (23 mg, 82 %); RP-HPLC (220 nm): 100 % (t_R = 17.5 min, *k* = 5.5). ^{1}H-NMR (300 MHz, [D$_1$]CHCl$_3$): δ (ppm) 2.55 (s, 3H), 5.71 (s, 2H), 5.92 (m, 3H), 7.04 (d, *J* 2.3 Hz, 1H), 7.07-7.19 (m, 4H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.7, 103.1, 111.5, 115.3, 118.6, 124.8, 125.1 (benzyl-***C***-2,6), 125.7, 127.4, 128.2 (benzyl-***C***-3,5), 132.8, 139.1, 139.2, 151.2, 163.5. MS (ES-MS) *m/z* (rel. int. in %) 561.2 ([2*M*-H$^+$]$^-$, 20) 325.1 ([*M*+HCOO$^-$], 100). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{17}H_{14}NO_3^-$: 281.1012, found 281.1011. $C_{17}H_{15}NO_3$ (281.11).

5-(Benzyloxy)-1-(carboxybenzyl)-3-methyl-1*H*-indole-2-carboxylic acid (5.58)

The title compound was prepared from **5.43** (200 mg, 0.4 mmol), according to the general procedure. Precipitation yielded a white solid (85 mg, 20 %); ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.53 (s, 3H), 5.14 (s, 2H), 5.85 (s, 2H), 7.04 (m, 3H), 7.24-7.44 (m, 5H), 7.49 (d, *J* 5.8 Hz, 2H), 7.74-7.86 (m, 2H), 12.92 (br s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.7, 51.7, 69.6, 102.4, 111.8, 119.5, 125.2, 125.0 (2 interfering carbon signals), 127.0, 127.6 (2 interfering carbon signals), 128.3 (2 interfering carbon signals), 129.3, 129.4, 133.4, 137.3, 144.2, 152.9, 163.5, 167.0. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{25}H_{20}NO_5^-$: 414.1343, found 414.1341. $C_{25}H_{21}NO_5$ (415.14).

1-Benzyl-3-ethyl-5-hydroxy-1*H*-indole-2-carboxylic acid (5.59)

The title compound was prepared from **5.52** (50 mg, 0.12 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-15 min: MeCN/0.1% aq. TFA 46/54-73/27, t_R = 11.5 min) and removal of the eluate by evaporation and lyophilisation afforded **5.59** as a white fluffy solid (20 mg, 56 %); RP-HPLC (220 nm): 97.9 % (t_R = 19.27 min, *k* = 7.3). ^{1}H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 1.24 (t, *J* 7.5 Hz, 3H), 3.08 (q, *J* 7.5 Hz, 2H), 5.74 (s, 2H), 5.85 (m, 1H), 5.90-5.99 (m, 2H), 7.01 (d, *J* 2.3 Hz, 1H), 7.08-7.27 (m, 4H). ^{13}C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 15.06, 19.7, 104.5, 112.6, 117.4, 125.7, 127.3 (2 interfering carbon signals), 127.9, 128.3, 128.5, 129.4 (2 interfering carbon signals), 135.5, 140.7, 152.4, 165.7. HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{16}NO_3^-$: 194.1143, found 294.1142. $C_{18}H_{17}NO_3$ (295.12).

1-(4-Carboxybenzyl)-5-hydroxy-3-methyl-1*H*-indole-2-carboxylic acid (5.60)

The title compound was prepared from **5.51** (50 mg, 0.12 mmol), according to the general procedure. The product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-20 min: MeCN/0.1% aq. TFA 46/54-95/5, t_R = 13.1 min) and removal of the eluate by evaporation and lyophilisation afforded **5.60** as a white fluffy solid (36 mg, 92 %); mp > 210 °C. RP-HPLC (220 nm): 100 % (t_R = 14.3 min, k = 5.1). ^{1}H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 2.48 (s, 3H, indole-C***H***$_3$-3), 5.80 (s, 2H, benzyl-C***H***$_2$), 5.83 (m, 1H, indole-***H***-6), 5.95 (d, *J* 2.2 Hz, 1H, indole-***H***-4), 7.04 (d, *J* 8.4 Hz, 2H, benzyl-***H***-2,6), 7.28 (d, *J* 8.9 Hz, 1H, indole-***H***-7), 7.82 (d, *J* 8.3 Hz, 2H, benzyl-***H***-3,5), 9.02 (s, 1H, O***H***), 12.88 (br s, 2H, COO***H***). ^{13}C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 10.69 (indole-***C***H$_3$), 45.9 (benzyl-***C***H$_2$), 103.2 (indole-***C***-4), 111.4 (indole-***C***-7), 115.4 (indole-***C***-6), 118.8 (C$_{quat}$, indole-***C***-2), 124.9 (C$_{quat}$, indole-***C***-3), 125.1 (benzyl-***C***-2,6), 127.5 (C$_{quat}$, indole-***C***-9), 129.3 (C$_{quat}$, benzyl-***C***-4), 129.4 (benzyl-***C***-3,5), 132.8 (C$_{quat}$, indole-***C***-8), 144.3 (C$_{quat}$, benzyl-***C***-1), 151.4 (C$_{quat}$, indole-***C***-5), 163.5 (indole-***C***OOH), 167.0 (benzyl-***C***OOH). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{18}H_{14}NO_5^-$: 324.0885, found 324.0885. $C_{18}H_{15}NO_5$ (325.10).

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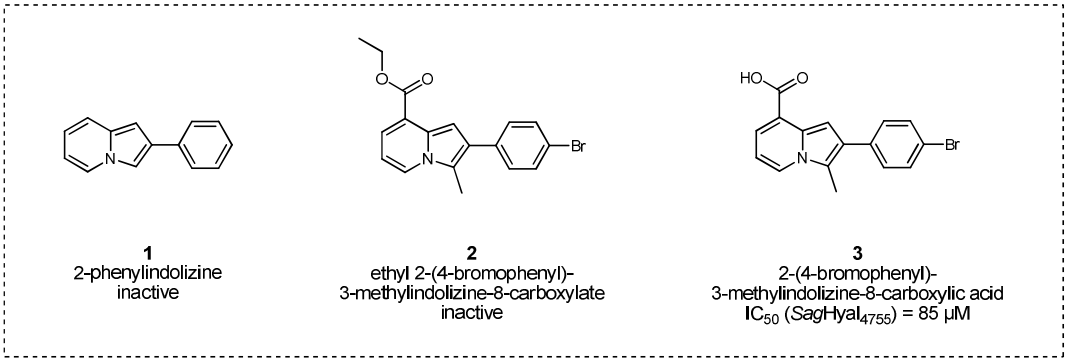
6 2-Phenylindolizines as hyaluronidase inhibitors

6.1 Introduction

Indoles and indolizines are constitutional isomers. Whereas the concept of isosterism[1] refers to the similarity of molecules or ions, which have the same number of atoms and valence electrons, bioisosteres are often more alike regarding biological rather than in physical or chemical properties. This means that compounds bearing isosteric molecular features do not necessarily represent bioisosteres. Therefore, the biological effects and biochemical mimicry are more important than the similarity of their physicochemical properties. In medicinal chemistry the concept of bioisosterism represents a rational approach to the design and discovery of new lead compounds.[2-4]

Previously, 32 inhibitors of *Sag*Hyal$_{4755}$ from our laboratory were selected for the computer-assisted evaluation by means of COSMO*sim* (cf. Textor,[5] Appendix II). The COSMO*sim* program enables an σ-profile-based drug similarity search for the discovery of new bioisosteres, for instance, as potential drug candidates. This approach to the quantification of drug similarity is based on the conductor-like screening model for realistic solvation (COSMO-RS) and considers the conductor surface polarization charge densities σ. The σ-profiles for surface interactions of molecules in liquid states were found to most likely carry a lot of information required for the estimation of desolvation and binding processes.[6] The COSMO*sim* calculations, based on the aforementioned compound library, proposed indolizines as novel, putative bioisosteres possessing inhibitory activity on the target enzyme, the streptococcal hyaluronidase *Sag*Hyal$_{4755}$. In collaboration with Origenis GmbH (Martinsried, Germany) virtual screening was performed, taking into consideration synthetic accessibility, drug-like properties and innovational character.[5] Figure 6.1 outlines the previously synthesized 2-phenylindolizines.



1
2-phenylindolizine
inactive

2
ethyl 2-(4-bromophenyl)-
3-methylindolizine-8-carboxylate
inactive

3
2-(4-bromophenyl)-
3-methylindolizine-8-carboxylic acid
IC_{50} (*Sag*Hyal$_{4755}$) = 85 µM

Figure 6.1 Structures and inhibitory activities of *Sag*Hyal$_{4755}$ of previously reported 2-phenylindolizine derivatives **1**-**3**.

The “minimal pharmacophore” 2-phenylindolizine (**1**) and ethyl 2-(4-bromophenyl)-3-methylindolizine-8-carboxylate (**2**) were inactive, whereas the corresponding carboxylic acid (**3**) showed inhibitory activity on *Sag*Hyal$_{4755}$ with an IC_{50} values in the two-digit micromolar range (IC_{50} = 85 µM). A negatively charged residue, such as the carboxylic acid moiety, most likely plays an important role for inhibitory activity.[5]

Indolizine comprises two condensed rings (5- and 6-membered) and a bridging nitrogen atom. It was discovered by Angeli in 1890[7] and first synthesized from α-picoline and acetic acid anhydride by Scholtz in 1912.[8-10] The generally accepted numbering of indolizines compared to indoles is outlined in Figure 6.2.

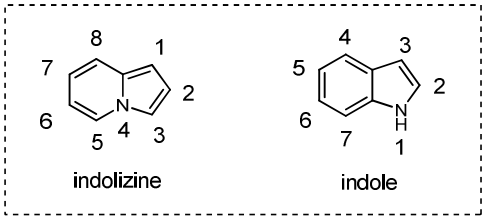


Figure 6.2 Molecular structure of indolizine, indole (including numbering system).

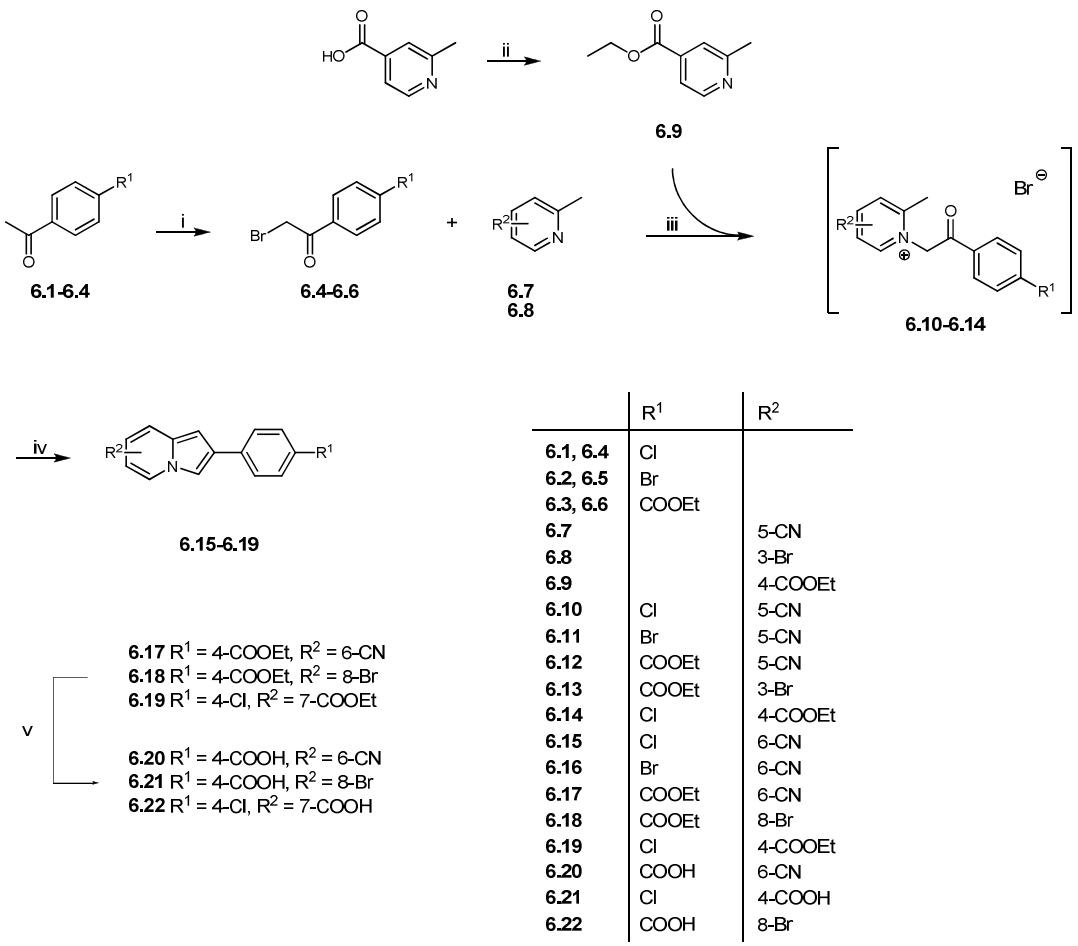
The indolizine ring system is a structural motif frequently found in natural products and has been used as a scaffold in pharmaceuticals. Synthetic indolizines have found wide-spread application in biological and pharmaceutical research.[11,12] As the substituted indolizines display a broad spectrum of potential pharmacological activities, they are associated with a wide range of biological activities, including anti-inflammatory,[13] hypoglycemic,[14,15] anti-acetylcholine,[16] anti-bacterial,[17] anti-cancer[18] activities, and estrogen receptor binding[19] and are reported as histamine H_3 receptor antagonists[20] and 15-lipoxygenase inhibitors.[21]

The current investigation considers 2-phenylindolizine derivatives with and without carboxylic groups. The synthesis and the structure-activity relationships of a small collection of 2-phenylindolizineschemically derived from compounds **1**-**3** is described in this chapter.

6.2 Chemistry

The synthesis of indolizine derivatives has been extensively investigated and many synthetic strategies for producing indolizine derivatives have been described in literature.[10,22] The

preparation of the 2-phenylindolizine derivatives **6.15**-**6.22** was accomplished as outlined in Scheme 6.1.

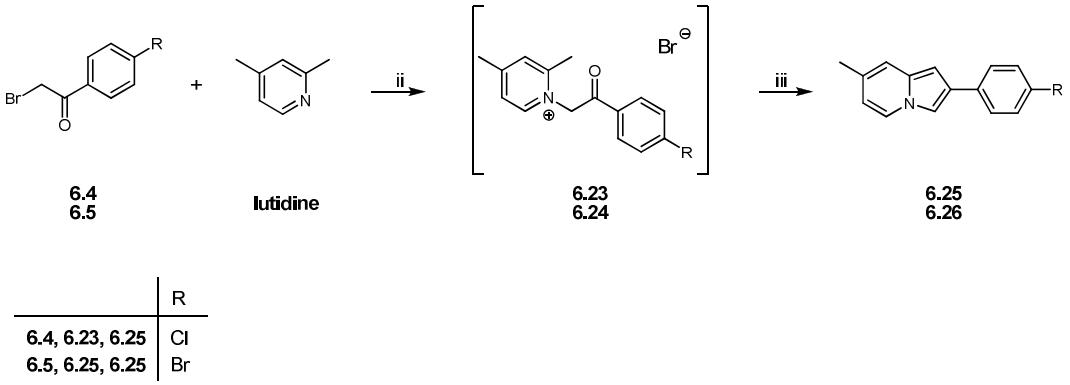


	R^1	R^2
6.1, 6.4	Cl	
6.2, 6.5	Br	
6.3, 6.6	COOEt	
6.7		5-CN
6.8		3-Br
6.9		4-COOEt
6.10	Cl	5-CN
6.11	Br	5-CN
6.12	COOEt	5-CN
6.13	COOEt	3-Br
6.14	Cl	4-COOEt
6.15	Cl	6-CN
6.16	Br	6-CN
6.17	COOEt	6-CN
6.18	COOEt	8-Br
6.19	Cl	4-COOEt
6.20	COOH	6-CN
6.21	Cl	4-COOH
6.22	COOH	8-Br

Scheme 6.1 Synthesis of substituted 2-phenylindolizine derivatives **6.15**-**6.22**. Reagents and conditions: (i) Et_2O, dioxane, Br_2, 0°C; (ii) ethanol, H_2SO_4, reflux, 12 h; (iii) acetone, microwave irradiation (130 °C, 4 min); (iv) K_2CO_3, H_2O, microwave irradiation (150 °C, 4 min); (v) LiOH, THF/EtOH/H_2O = 3:0.2:1 (v/v/v), RT, 72 h.

The synthesis of the 2-phenylindolizine derivatives was performed according to the Tschitschibabin indolizine synthesis in which a quarternary pyridinium halide, resulting from the reaction of a 2-alkyl pyridine and an α-halo carbonyl compound, undergoes an intramolecular condensation to the indolizine product.[23] Bromination of the pertinent 4-substituted acetophenones (**6.1**-**6.3**) afforded the corresponding α-bromoketones **6.4**-**6.6**. In a two-step reaction the respective 2-methylpyridine **6.7**-**6.9** was alkylated with the substituted 2-bromo-1-phenylethanones **6.4**-**6.6**, followed by ring closure in the presence of a base (potassium carbonate). The synthesis of **6.15**-**6.19** was carried out as a microwave assisted reaction.[5] Subsequently, the ester groups of compounds **6.17** and **6.19** were hydrolyzed with lithium hydroxide to yield the target compounds **6.20**-**6.22**.

2,4-Dimethylpyridine (lutidine) is insoluble in acetone. Therefore, a different synthetic route according to Dann, et al.[24], was applied. The synthesis of the 2-(4-bromophenyl)- and 2-(4-chlorophenyl)-7-methylindolizine is outlined in Scheme 6.2.



6.4
6.5

lutidine

6.23
6.24

6.25
6.26

	R
6.4, 6.23, 6.25	Cl
6.5, 6.25, 6.25	Br

Scheme 6.2 Synthesis of the substituted 2-phenylindolizine derivatives **6.25** and **6.26**. Reagents and conditions: 125-135 °C; (ii) $NaHCO_3$, H_2O, reflux.

The respective 4-substituted 2-bromo-1-phenylethanone (**6.4**, **6.5**) and 2,4-dimethylpyridine (lutidine) were melted at 125-135 °C until the mass solidified. The quaternary pyridinium halide was suspended in water and heated to reflux in the presence of a base (sodium hydrogen carbonate) to afford the 4-substituted 7-methyl-2-phenylindolizine compounds **6.25** and **6.26**.

Because of its electron-rich character, the indolizine core structure was reported to be prone to oxidation, therefore, electron withdrawing groups on the indolizine nucleus are supposed to make the compounds much less susceptible to oxidation by air.[25] For the synthesized 2-phenylindolizine derivatives **6.15**, **6.16**, **6.19**-**6.22**, **6.25** and **6.26** autoxidation or decomposition was not observed.

6.3 Pharmacological results and discussion

6.3.1 General conditions

All synthesized 2-phenylindolizine derivatives were investigated for inhibition of the bacterial hyaluronate lyase *Sag*$Hyal_{4755}$ and the bovine testicular enzyme BTH (Neopermease®) in a turbidimetric assay based on the method of Di Ferrante[26] as described in chapter 1. In case

that IC_{50} values could not be determined, due to poor solubility in aqueous buffer, the effect was expressed as percent inhibition of the compound at the highest concentration achieved in the assay.

6.3.2 Inhibitory activity of 2-phenylindolizine compounds

The IC_{50} values determined for the 2-phenylindolizine compounds **6.15**, **6.16**, **6.18**, **6.20**-**6.22**, **6.24**, **6.25** are summarized in Table 6.1.

Table 6.1 Inhibitory activity[a] and calculated $logD_{5.0}$ values[c] of 2-phenylindolizine derivatives **6.15**, **6.16**, **6.18**, **6.20**-**6.22**, **6.24**, **6.25**.

Compound	*Sag*Hyal$_{4755}$ IC_{50} (µM)[a]	BTH IC_{50} (µM)[a]	$logD_{5.0}$[c]
6.15	inactive	inactive	4.5
6.16	inactive	inactive	4.7
6.18	inactive	inactive	5.8
6.20	56 ± 1	inactive	2.1
6.21	30 % at 50 µM[b]	inactive	2.0
6.22	inactive	inactive	3.5
6.25	inactive	inactive	5.6
6.26	inactive	inactive	5.8

[a] Mean values ± SEM (N = 2, experiments performed in duplicate), IC_{50} values determined at pH 5.0 in the turbidimetric assay (cuvettes); [b] % inhibition of *Sag*Hyal$_{4755}$ at indicated concentration; [c] calculated with ACD-Labs (Advanced Chemistry Development Inc., Toronto, Canada) product version 12.0.

The concentration dependent enzymatic activity of *Sag*Hyal$_{4755}$ in presence of **6.15**, **6.20** and **6.22** is depicted in Figure 6.3.

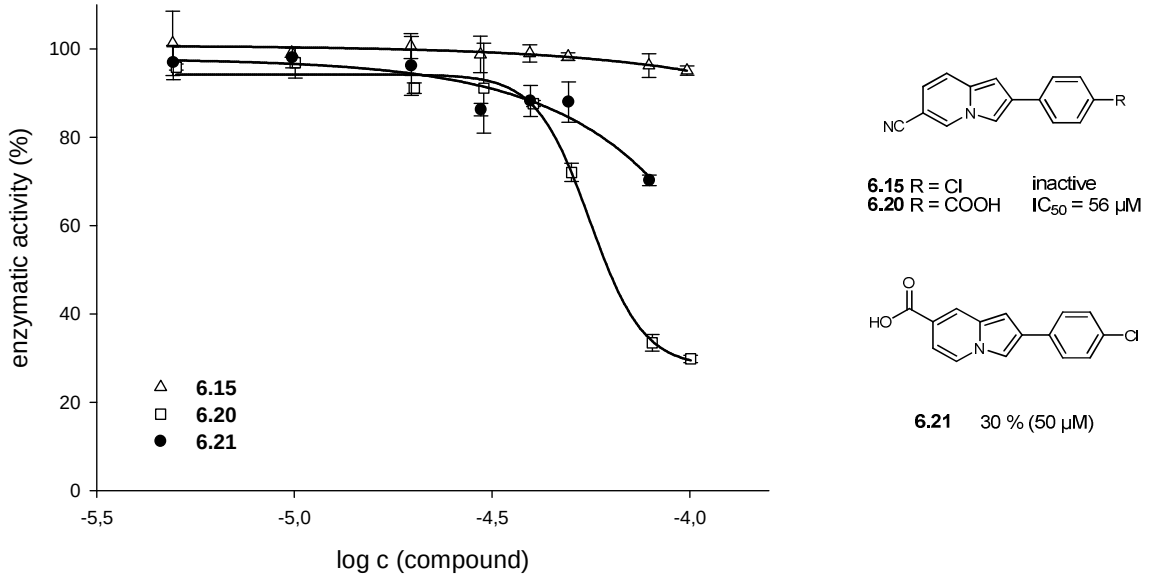


Figure 6.3 Enzymatic activity of *Sag*Hyal$_{4755}$ in the presence of **6.15**, **6.20** and **6.21**.

The majority of the synthesized 2-phenylindolizine derivatives described in this chapter were inactive on the bacterial hyaluronidase *Sag*Hyal$_{4755}$. Inhibitory activity was detected for compound **6.20**, which showed an IC_{50} value of 56 µM. In case of compound **6.21**, it was impossible to determine an IC_{50} value due to poor solubility in aqueous buffer. However, this compound showed 30 % inhibition at a concentration of 50 µM. It needs to be kept in mind that, in general; indolizines are very poorly soluble in aqueous buffer. Despite the negatively charged carboxyl group, compound **6.22**, which also bears a bromine residue (solubility ca. 50 µM) shows no inhibitory activity of *Sag*Hyal$_{4755}$. Regardless of that, the presence of a negatively charged group is important for inhibition of the hyaluronate lyase: all compounds devoid of a carboxylic acid were inactive.

Interestingly, with regard to the $logD_{5.0}$ value, the two most active compounds (**6.20**, **6.21**) were the ones offering the lowest lipophilicity (**6.20** $logD_{5.0}$ = 2.1, **6.21** $logD_{5.0}$ = 2.0), whereas those with higher $logD_{5.0}$ values around 4.5-5.8 (**6.15**, **6.16**, **6.18**, **6.24**, **6.25**), were devoid of inhibitory activity on *Sag*Hyal$_{4755}$. This is in contrast to the general observation that lipophilicity determines inhibitory potency of hyaluronidase inhibitors. Most probably, in case of the more lipophilic indolizine derivatives, poor solubility is the decisive factor. The mammalian enzyme BTH was not inhibited by molecules analyzed in this section.

6.4 Summary

The calculations of the *σ*-profile based drug similarity measure for the detection of new bioisosteric drugs, COSMO*sim*, suggested an indolizine skeleton as core structure of bioisosteric inhibitors of the bacterial hyaluronidase from *Streptococcus agalactiae* *Sag*Hyal$_{4755}$. A small library of eight compounds was designed, synthesized, and tested for inhibition of the bacterial hyaluronidase from *Streptococcus agalactiae* *Sag*Hyal$_{4755}$ and the mammalian hyaluronidase from bovine testis (BTH). Most of the tested compounds in this series were found to be inactive on the *Sag*Hyal$_{4755}$. 2-(4-chlorophenyl)-indolizine-7-carboxylic acid (**6.20**; IC_{50} = 56 µM) was the only compound that showed an IC_{50} value in the two-digit micromolar range. The inhibitory activity of compound **6.20** was increased compared to 2-(4-bromophenyl)-3-methylindolizine-8-carboxylic acid **3** (IC_{50} = 85 µM; cf. Figure 6.1) and was selective for the bacterial hyaluronidase *Sag*Hyal$_{4755}$ since it was inactive on BTH.

Although, the hydrophobicity of the substituents appeared to be key to high affinity of the inhibitors, it becomes clear that the structure-activity relationships are different for the investigated 2-phenylindolizine derivatives. The presence of a negatively charged carboxyl group, which also contributes to better solubility in aqueous buffer, seems to be more important than lipophilic residues. The successful combination of the carboxyl group together with the cyano residue in compound **6.20** (IC_{50} = 56 µM) should be kept in mind for the future development of hyaluronidase inhibitors. The indolizine scaffold should not yet be considered inappropriate, because a wide variety of substitution patterns has not been investigated.

6.5 Experimental section

6.5.1 General conditions

Cf. section 4.7.1

6.5.2 Chemistry

6.5.2.1 Preparation of compounds 6.4-6.6

General procedure[5]

To a solution of the pertinent 4-substituted phenylethanone (1 eq) in dioxane (3.3 mL/mmol) and dichloromethane (1.6 mL/mmol), bromine (1 eq) was slowly added under vigorous stirring. The solution was cooled with ice to maintain the temperature below 20 °C. Subsequently, the reaction mixture was stirred at ambient temperature for 1 hour. After washing with water (2 x 200 mL) the aqueous solution was extracted with EtOAc (4 x 250 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified by recrystallization from ethanol.

2-Bromo-1-(4-chlorophenyl)ethanone[27] 6.4

The title compound was prepared from 1-(4-chlorophenyl)ethanone (4.64 g, 30 mmol), according to the general procedure. Recrystallization yielded a white solid (3.9 g, 56 %); mp 98 °C (lit.[27] mp 270-272 °C), R_f = 0.68, ^{1}H-NMR (400 MHz, [D_1]$CHCl_3$): δ (ppm) 4.40 (s, 2H), 6.48 (m, 2H), 6.93 (m, 2H). ^{13}C-NMR (101 MHz, [D_1]$CHCl_3$): δ (ppm) 30.3, 129.3 (2 interfering carbon signals), 130.4 (2 interfering carbon signals), 132.3, 140.6, 190.2. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 231.9 ([$M^{+\bullet}$], 5), 139.0 ([M-$^{\bullet}CH_2Br$]$^+$, 100). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_8H_7BrClO^+$: 234.9344, found 234.9345. C_8H_6BrClO (231.93).

2-Bromo-1-(4-bromophenyl)ethanone[28] 6.5

The title compound was prepared from 1-(4-bromophenyl)ethanone (6 g, 30 mmol), according to the general procedure. Recrystallization yielded a white solid (5.3 g, 56 %); mp 113 °C, R_f = 0.68, ^{1}H-NMR (400 MHz, [D_1]$CHCl_3$): δ (ppm) 4.40 (s, 2H), 6.64 (m, 2H), 6.85 (m, 2H). ^{13}C-NMR (101 MHz, [D_1]$CHCl_3$): δ (ppm) 30.3, 129.3, 130.4 (2 interfering carbon signals), 132.3 (2 interfering carbon signals), 132.7, 190.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 276.9 ([$M^{+\bullet}$], 5), 183.0 ([M-$^{\bullet}CH_2Br$]$^+$, 80). HRMS (ESI): *m/z* [M+H^+]$^+$ calculated for $C_8H_7Br_2O^+$: 278.8840, found 278.8841.$C_8H_6Br_2O$ (275.88). $C_8H_6Br_2O$ (275.88).

Ethyl 4-(2-bromoacetyl)benzoate[29] 6.6

The title compound was prepared from ethyl-4-acetylbenzoate (4 g, 20.8 mmol), according to the general procedure. Recrystallization yielded white crystals (3 g, 53 %); mp 79 °C (lit.[29] mp 73-75 °C), R_f = 0.49 ^{1}H-NMR (400 MHz, [D_1]$CHCl_3$) δ (ppm) 1.42 (t, *J* 6.1 Hz, 3H), 4.42 (q, *J* 6.1 Hz, 2H), 4.47 (s, 2H), 8.01-8.07 (m, 2H), 8.12-8.19 (m, 2H). ^{13}C-NMR (75 MHz, [D_6]DMSO) δ (ppm) 14.0, 61.1, 65.6, 126.8 (2 interfering carbon signals), 129.3 (2 interfering carbon signals), 130.7, 136.9, 164.9, 198.9. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 270.0 ([$M^{+\bullet}$], 5), 226.0 ([M-$^\bullet$EtO]$^+$, 5), 176.1 ([M-$^\bullet CH_2Br$], 100). $C_{11}H_{11}BrO_3$ (269.99).

6.5.2.2 Preparation of ethyl 2-methylisonicotinoate[30] 6.8

A mixture of 2-methylpyridine-4-carboxylic acid (1 eq, 200 mg, 1.45 mmol), ethanol (2 mL/mmol) and H_2SO_4 (0.05 mL/mmol) was refluxed for 12 h in a round-bottomed flask. After evaporation of the solvent under reduced pressure, about 20 mL of Na_2CO_3 solution (1 M) were added into the mixture. The combined phases were extracted with ether. After evaporation of the solvent, the product was obtained as a yellowish liquid (0.1 g, 42 %). R_f = 0.2. ^{1}H-NMR (400 MHz, [D_6]DMSO): δ (ppm) 1.39 (t, *J* 7.1 Hz, 3H), 2.60 (s, 3H), 4.40 (q, *J* 7.1 Hz, 2H), 7.72 (d, *J* 5.2 Hz, 1H), 7.80 (s, 1H), 8.57 (d, *J* 5.2 Hz, 1H). ^{13}C-NMR (101 MHz, [D_6]DMSO): δ (ppm) 14.50, 23.89, 63.03, 121.56, 124.13, 140.30, 150.41, 160.69, 166.23. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 165.12 ([$M^{+\bullet}$], 70), 137.09 ([M-C_2H_4], 80), 120.07 ([M-OEt]$^\bullet$, 100). $C_9H_{11}NO_2$ (165.08).

6.5.2.3 Preparation of compounds 6.15-6.19

General procedure[24]

The pertinent 2-methylpyridine (1 eq) and the 4-substituted α-bromo-acetophenone (1 eq) were melted under stirring in a round-bottom flask in a pre-heated heating block. After 5-10 min the liquefied material became brown-orange solid. After cooling to room temperature the solid was pulverized and heated to reflux in petroleum ether to dissolve and remove residual starting material. The product was insoluble in petroleum ether and the supernatant was decanted. This procedure was repeated 5-10 times. The solid was filtered off and taken up in H_2O (10.7 mL/mmol starting material), $NaHCO_3$ was added in surplus, and the mixture was heated to reflux for a few minutes. The product precipitated under foaming of the reaction mixture. After cooling to room temperature the product was filtered off, washed with water, and dried *in vacuo*.

2-(4-Chlorophenyl)indolizine-6-carbonitrile 6.15

The title compound was prepared from **6.4** (810 mg, 3.47 mmol) and **6.7** (410 mg, 3.47 mmol), according to the general procedure to yield the product as a red-brown solid (300 mg, 34 %); mp 192-195 °C, R_f = 0.68, RP-HPLC (220 nm): 87 % (t_R = 19.1 min, k = 7.2). ^{1}H-NMR (400 MHz, [D$_6$]DMSO): δ (ppm) 6.87 (m, 1H), 6.01 (s, 1H), 6.47 (d, J 8.5 Hz, 2H), 6.55 (d, J 9.3 Hz, 1H), 6.77 (d, J 8.5 Hz, 2H), 8.11 (s, 1H), 9.03 (s, 1H). ^{13}C-NMR (101 MHz, [D$_6$]DMSO): δ (ppm) 94.9, 99.2, 112.0, 116.5, 116.9, 119.4, 126.6 (2 interfering carbon signals), 128.8 (2 interfering carbon signals), 129.6, 131.7, 132.0, 132.5, 133.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 252.04 ([M$^{+\bullet}$], 100), 216.11 ([M-HCl]$^+$, 25). HRMS (EI-MS): *m/z* [*M*H$^{+\bullet}$] calculated for $C_{15}H_9ClN_2$: 252.0454, found 252.0456. $C_{15}H_9ClN_2$ (252.05).

2-(4-Bromophenyl)indolizine-6-carbonitrile[24] 6.16

The title compound was prepared from **6.5** (150 mg, 0.55 mmol) and **6.7** (65 mg, 0.55 mmol), according to the general procedure to yield the product as a yellow solid (70 mg, 43 %); RP-HPLC (220 nm): 95.3 % (t_R = 19.1 min, k = 7.2). ^{1}H-NMR (300 MHz, [D$_6$]DMSO) δ (ppm) 6.88 (m, 1H), 6.02 (s, 1H), 6.55 (d, J 9.3 Hz, 1H), 6.58-6.66 (m, 2H), 6.66-6.75 (m, 2H), 8.13 (d, J 1.3 Hz, 1H), 9.04 (m, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO) δ (ppm) 94.9, 99.2, 112.0, 116.5, 116.9, 119.5, 120.2, 126.9 (2 interfering carbon signals), 129.6, 131.8 (2 interfering carbon signals), 132.0, 132.9, 133.5. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 296.00 ([M$^{+\bullet}$], 100), 216.11 ([M-HBr]$^+$, 80). HRMS (EI-MS) *m/z* calculated for 295.9949 [M$^{+\bullet}$], found 295.9952 [M$^{+\bullet}$]. $C_{15}H_9BrN_2$ (295.99).

Ethyl 4-(6-cyanoindolizin-2-yl)benzoate 6.17

The title compound was prepared from **6.6** (500 mg, 1.8 mmol) and **6.7** (215 mg, 1.8 mmol), according to the general procedure to yield the product as a yellow solid (344 mg, 66 %); RP-HPLC (220 nm): 94.7 % (t_R = 22.4 min, k = 8.6). ^{1}H-NMR (300 MHz, [D$_6$]DMSO) δ (ppm) 1.34 (t, J 6.1 Hz, 3H), 4.32 (q, J 6.1 Hz, 2H), 6.89 (m), 6.09 (s, 1H), 6.58 (d, J 9.3 Hz, 1H), 6.84-6.93 (m, 2H), 6.96-8.04 (m, 2H), 8.21 (d, J 1.3 Hz, 1H), 9.06 (d, J 1.1 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO) δ (ppm) 14.1, 60.6, 95.2, 99.5, 112.8, 116.7, 116.8, 119.7, 125.9 (2 interfering carbon signals), 128.2, 129.5, 129.8 (2 interfering carbon signals), 132.1, 133.6, 138.3, 165.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 290.1 ([M$^{+\bullet}$], 100), 262.2 ([M-C_2H_4]$^+$, 50), 245.1 ([M-EtO$^\bullet$]$^+$, 50), 216.1 ([M-COOEt$^\bullet$]$^+$, 50). HRMS (EI-MS) *m/z* calculated for $C_{18}H_{14}N_2O_2$: 290.1055 [M$^{+\bullet}$], found 290.1058 [M$^{+\bullet}$]. $C_{18}H_{14}N_2O_2$ (290.11).

Ethyl 4-(8-bromoindolizin-2-yl)benzoate 6.18

The title compound was prepared from **6.6** (500 mg, 1.8 mmol) and **6.8** (310 mg, 1.8 mmol), according to the general procedure to yield the product as a yellow solid (380 mg, 62 %); RP-HPLC (220 nm): 93.6 % (t_R = 22.6 min, *k* = 8.7). ^{1}H-NMR (300 MHz, [D$_6$]DMSO) δ (ppm) 1.34 (t, *J* 6.1 Hz, 3H), 4.33 (q, *J* 6.1 Hz, 2H), 6.54 (t, *J* 6.0 Hz, 1H), 6.94 (d, *J* 0.7 Hz, 1H), 6.08 (d, *J* 6.0 Hz, 1H), 6.85-6.95 (m, 2H), 6.95-8.03 (m, 2H), 8.27 (d, *J* 1.8 Hz, 1H), 8.31 (d, *J* 6.9 Hz, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO) δ (ppm) 14.1, 60.5, 98.0, 110.8, 111.7, 113.1, 120.7, 125.5, 125.6 (2 interfering carbon signals), 126.2, 126.8, 129.7 (2 interfering carbon signals), 131.8, 165.4. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 343.0 ([M$^{+\bullet}$], 100), 316.9 ([M-C_2H_4]$^+$, 60). HRMS (EI-MS) *m/z* calculated for 343.0208 [M$^{+\bullet}$], found 343.0212 [M$^{+\bullet}$]. $C_{17}H_{14}BrNO_2$ (343.02).

Ethyl 2-(4-chlorophenyl)indolizine-7-carboxylate 6.19

The title compound was prepared from **6.4** (350 mg, 1.5 mmol) and **6.9** (250 mg, 1.5 mmol), according to the general procedure to yield the product as a yellow solid (51 mg, 13 %); RP-HPLC (220 nm): 92.8 % (t_R = 22.8 min, *k* = 8.8). ^{1}H-NMR (300 MHz, [D$_6$]DMSO) δ (ppm) 6.90 (m, 1H), 6.09 (s, 1H), 6.58 (d, *J* 9.3 Hz, 1H), 6.88 (d, *J* 8.3 Hz, 2H), 6.99 (d, *J* 8.3 Hz, 2H), 8.21 (s, 1H), 9.07 (s, 1H), 12.93 (s, 1H). ^{13}C-NMR (75 MHz, [D$_6$]DMSO) δ (ppm) 95.1, 99.5, 112.7, 116.6, 116.8, 119.6, 125.8 (2 interfering carbon signals), 129.1, 129.7, 130.0 (2 interfering carbon signals), 132.1, 133.6, 136.9, 166.0. HRMS (EI-MS) *m/z* calculated for $C_{16}H_9N_2O_2^-$: 261.0672 [M-H$^+$]$^-$, found 261.0671 [M-H$^+$]$^-$. $C_{16}H_{10}N_2O_2$ (262.07).

6.5.2.4 Preparation of compounds 6.20-6.22

General procedure[5]

A suspension of the pertinent ethyl carboxylate (1 eq) and LiOH (2.5 eq) in a 3:1:0.2 mixture of THF, H_2O and EtOH (v/v/v) was stirred at room temperature for 3 days. The reaction mixture was adjusted to pH2 by addition of 0.5 M HCl to yield the product as precipitate.

4-(6-Cyanoindolizin-2-yl)benzoic acid 6.20

The title compound was prepared from **6.17** (344 mg, 1.18 mmol), according to the general procedure. The crude product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-15 min: MeCN/0.1% aq. TFA 30/70-80/20, t_R = 12.7 min) and removal of the eluate by evaporation and lyophilisation afforded **6.20** as a white fluffy solid (100 mg, 32 %); RP-HPLC (220 nm): 97.8 % (t_R = 22.2 min, *k* = 8.5). ^{1}H-NMR (300 MHz, [D$_6$]DMSO) δ (ppm) 6.90 (m, 1H), 6.09 (s, 1H), 6.58 (d, *J* 9.3 Hz, 1H), 6.88 (d, *J* 8.3 Hz, 2H), 6.99 (d, *J* 8.3 Hz, 2H), 8.21 (s, 1H), 9.07 (s, 1H), 12.93 (br s, 1H). ^{13}C-NMR (75 MHz,

[D_6]DMSO) δ (ppm) 95.1, 99.5, 112.7, 116.6, 116.8, 119.6, 125.8 (2 interfering carbon signals), 129.1, 129.7, 130.0 (2 interfering carbon signals), 132.1, 133.6, 136.9, 166.0. HRMS (EI-MS) *m/z* calculated for $C_{16}H_9N_2O_2^-$: 261.0672 [M-H$^+$]$^-$, found 261.0671 [M-H$^+$]$^-$. $C_{16}H_{10}N_2O_2$ (262.07).

4-(8-Bromoindolizin-2-yl)benzoic acid 6.21

The title compound was prepared from **6.18** (280 mg, 0.81 mmol), according to the general procedure. The crude product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-15 min: MeCN/0.1% aq. TFA 30/70-80/20, t_R = 11.7 min) and removal of the eluate by evaporation and lyophilisation afforded **6.21** as a white fluffy solid (100 mg, 39 %); RP-HPLC (220 nm): 98.3 % (t_R = 18.9 min, *k* = 7.1). ^{1}H-NMR (400 MHz, [D_6]DMSO) δ (ppm) 6.53 (t, *J* 6.0 Hz, 1H, indozline-***H***-6), 6.93 (d, *J* 0.8 Hz, 1H, indozline-***H***-1), 6.08 (d, *J* 6.6 Hz, 1H, indozline-***H***-7), 6.88 (d, *J* 8.6 Hz, 2H, phenyl-***H***-2,6), 6.97 (d, *J* 8.5 Hz, 2H, phenyl-***H***-3,5), 8.25 (d, *J* 1.8 Hz, 1H, indozline-***H***-3), 8.31 (d, *J* 6.9 Hz, 1H, indozline-***H***-5). ^{13}C-NMR (101 MHz, [D_6]DMSO) δ (ppm) 98.2 (indolizine-***C***-1), 110.8 (indolizine-***C***-6), 111.7 (indolizine-***C***-8), 113.0 (indolizine-***C***-3), 120.7 (indolizine-***C***-7), 125.4 (indolizine-***C***-4), 125.5 (phenyl-***C***-2,6), 126.4 (indolizine-***C***-2), 128.7 (phenyl-***C***-4), 129.9 (phenyl-***C***-3,5), 131.9 (indolizine-***C***-9), 138.5 (phenyl-***C***-1),166.0 (***C***OOH). HRMS (ESI): *m/z* [*M*-H$^+$]$^-$ calculated for $C_{15}H_9BrNO_2^-$: 313.9819, found 315.9800. $C_{15}H_{10}BrNO_2$ (314.99).

2-(4-Chlorophenyl)indolizine-7-carboxylic acid 6.22

The title compound was prepared from **6.19** (51 mg, 0.17 mmol), according to the general procedure. The crude product was purified with preparative HPLC (column: Nucleodur 250 x 21 mm; gradient: 0-15 min: MeCN/0.1% aq. TFA 30/70-80/20, t_R = 14.2 min) and removal of the eluate by evaporation and lyophilisation afforded **6.22** as a white fluffy solid (30 mg, 65 %); RP-HPLC (220 nm): 93.8 % (t_R = 22.6 min, *k* = 8.7). v^{1}H-NMR (600 MHz, [D_6]DMSO) δ (ppm) 6.95 (m, 1H, indolizine-***H***-6), 6.13 (s, 1H, indolizine-***H***-1), 6.43-6.51 (m, 2H, phenyl-***H***-3,5), 7-70-6.79 (m, 2H, phenyl-***H***-2,6), 8.09 (s, 1H, indolizine-***H***-8), 8.18 (d, *J* 1.1 Hz, 1H, indolizine-***H***-3), 8.24 (d, *J* 6.3 Hz, 1H, indolizine-***H***-5), 12.80 (br s, 1H, COO***H***). ^{13}C-NMR (150 MHz, [D_6]DMSO) δ (ppm) 101.4 (indolizine-***C***-1), 109.6 (indolizine-***C***-6), 113.1 (indolizine-***C***-3), 119.6 (C_{quat}, indolizine-***C***-7), 122.3 (indolizine-***C***-8), 125.4 (indolizine-***C***-5), 126.6 (phenyl-***C***-2,6), 128.8 (C_{quat}, indolizine-***C***-2), 129.0 (phenyl-***C***-3,5), 131.5 (C_{quat}, phenyl-***C***-1'), 131.9 (C_{quat}, indolizine-***C***-9), 133.3 (C_{quat}, phenyl-***C***-4), 166.8 (C_{quat}, ***C***OOH). HRMS (EI-MS) *m/z* calculated for $C_{15}H_9ClNO_2^-$: 270.0331 [M-H$^+$]$^-$, found 270.0326 [M-H$^+$]$^-$. $C_{15}H_{10}ClNO_2$ (271.04).

6.5.2.5 Preparation of compounds 6.25, 6.26

General procedure[5,24,31]

A mixture of 2-methylpyridine (1 eq) and the pertinent 4-substituted α-bromo-acetophenone (1 eq) was dissolved in 2 mL of anhydrous acetone. After microwave irradiation (130 °C, 4 min), the quaternary pyridinium halide was filtered off. The precipitate was re-dissolved in 2 mL of hot H_2O with the aid of sonification. Subsequently, K_2CO_3 (1 eq) was added to the solution. After microwave irradiation (150 °C, 4 min) the remaining precipitate was dissolved in DCM. The organic layer was washed with H_2O and dried over $MgSO_4$. After evaporation of the solvent, the product was purified as indicated.

2-(4-Chlorophenyl)-7-methylindolizine[32] 6.25

The title compound was prepared from **6.4** (933 mg, 4 mmol), according to the general procedure. Evaporation of the solvent yielded a silver colored, shiny solid as the pure product (310 mg, 32 %); mp >210 °C, R_f = 0.64, RP-HPLC (220 nm): 94.5 % (t_R = 18.9 min, k = 7.1). ^{1}H-NMR (400 MHz, [D_6]DMSO) δ (ppm) 2.22 (s, 3H, C***H***$_3$), 6.39 (m, 1H, indolizine-***H***-6), 6.60 (s, 1H, indolizine-***H***-1), 6.14 (s, 1H, indolizine-***H***-8), 6.41-6.43 (m, 2H, phenyl-***H***-3,5), 6.69-6.71 (m, 2H, phenyl-***H***-2,6), 6.88 (d, *J* 1.4 Hz, 1H, indolizine-***H***-3), 8.11 (d, *J* 6.1 Hz, 1H, indolizine-***H***-5). ^{13}C-NMR (101 MHz, [D_6]DMSO) δ (ppm) 20.5 (***C***H$_3$), 94.6 (indolizine-***C***-1), 109.2 (indolizine-***C***-3), 113.2 (indolizine-***C***-6), 116.4 (indolizine-***C***-8), 125.2 (C_{quat}, indolizine-***C***-5), 126.7 (C_{quat}. Indolizine-***C***-2), 126.0 (phenyl-***C***-2,6), 126.3 (indolizine-***C***-7), 128.6 (phenyl-***C***-3,5), 130.5 (C_{quat}, phenyl-***C***-1), 133.2 (C_{quat}, indolizine-***C***-9), 133.9 (C_{quat}, phenyl-***C***-4). MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 241.1 ([$M^{+\bullet}$], 100), 240.1 ([M-H^+], 35). HRMS (EI-MS): *m/z* [*M*$H^{+\bullet}$] calculated for $C_{15}H_{12}ClN$: 241.0658, found 241.0658. $C_{15}H_{12}ClN$ (241.07).

2-(4-Bromophenyl)-7-methylindolizine[33] 6.26

The title compound was prepared from **6.5** (1.3 g, 4.67 mmol), according to the general procedure. Evaporation of the solvent yielded a silver colored, shiny solid as the pure product (1.15 mg, 86 %; ref.:[33] mp 214-215 °C); RP-HPLC (220 nm): 95.1 % (t_R = 18.9 min, k = 7.1). ^{1}H-NMR (400 MHz, [D_6]DMSO) δ (ppm) 2.22 (s, 3H), 6.40 (m, 1H), 6.61 (s, 1H), 6.14 (s, 1H), 6.50-6.59 (m, 2H), 6.59-6.70 (m, 2H), 6.89 (d, *J* 1.4 Hz, 1H), 8.12 (d, *J* 6.0 Hz, 1H). ^{13}C-NMR (101 MHz, [D_6]DMSO) δ (ppm) 20.5, 94.5, 109.2, 113.2, 116.4, 125.2, 126.7, 126.3, 126.4 (2 interfering carbon signals), 128.6, 131.5 (2 interfering carbon signals), 133.5, 134.3. MS (EI-MS, 70 eV) *m/z* (rel. int. in %) 285.07 ([$M^{+\bullet}$], 100), 204.14 ([M-HBr]$^+$, 40). HRMS (EI-MS) *m/z* calculated for 285.0153 [$M^{+\bullet}$], found 285.0149 [$M^{+\bullet}$]. $C_{15}H_{12}BrN$ (285.02).

6.6 References

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7 Characterization of snake venoms regarding hyaluronidase activity

7.1 Introduction

Snakes are widely distributed all over the world, particularly present in high species diversity in the tropics. Currently, 2,700 species of snakes are recognized, divided into 420 genera and 18 families, of which 20 % are venomous.[1] Members of the four families *Elapidae* (cobras, coral snakes and their relatives), *Viperidae* (vipers, adders and pit vipers), *Colubridae* (colubrid snakes) and *Atractaspididae* (western rat snakes and mole vipers) represent major venomous snakes.[2]

Venoms of the puff adder *Bitis arietans* and the cobra *Naja siamensis* were investigated in this thesis with regard to their hyaluronidase activity. The puff adder *Bitis arietans* (Figure 7.1) can be identified because of its rough-scaled appearance and the alternating pattern of dark and light chevron-shaped markings. It is distributed in the savannah and grasslands of Morocco and western Arabia as well as throughout Africa, except for the Sahara and rain forest regions. Cobras of the genus *Naja* (Figure 7.1) are found over much of southern Asia and Africa. All cobras have an expandable hood, and usually the ventral surface of the neck is brightly marked.[3] Both, the Indian Cobras (*Naja naja*) and Siamese cobra (*Naja siamensis*) have a spectacle-like marking which is shown in Figure 7.1.

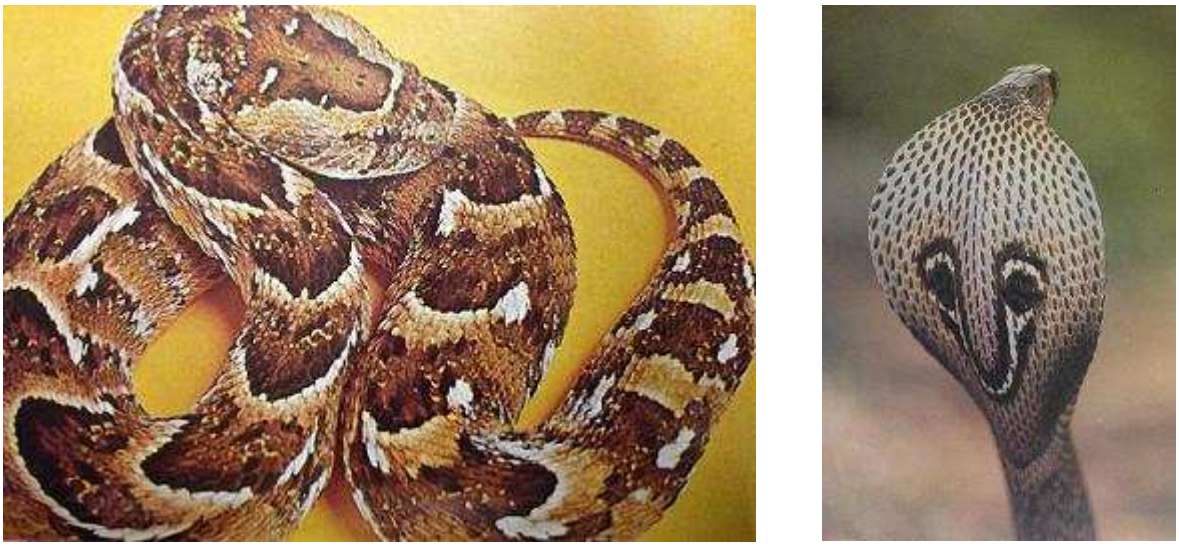

Figure 7.1 Puff adder *Bitis arietans* (left)[4] and Indian cobra *Naja naja* with its hood spread(right)[1].

Snake venoms are a complex and highly concentrated mixture of proteins and polypeptides, which possess enzymatic and a number of other biological activities and toxic properties that induce diverse pathological and pathophysiological effects.[5] The venom composition is highly variable among snake species, populations, and even among individual snakes.

Polypeptides without enzymatic activity are toxins with specific activity towards nerve structures and cell membranes or peptides with inhibitory activities towards enzymes, synergistic effects, and other nontoxic venom components.[6] The enzymes present in snake venom are mostly hydrolases and amino acid oxidases with digestive function, for instance proteinases, exo- and endopeptidases, phosphodiesterases, and phospholipases. Snakebites cause a wide variety of symptoms, including impairment of blood coagulation, bleeding, massive tissue destruction, and necrosis, cardiovascular irregularities and shock, and muscular paralysis, each of which can cause death.[6-9]

Hyaluronidase which is present in all snake venoms has a “spreading action”, thereby facilitating the distribution of the other venom components throughout the tissues of the prey by hydrolyzing the dermal barrier hyaluronic acid and destroying the structural integrity.[10] Therefore, the extent of the hyaluronidase activity can modulate the degree of venom toxicity.[11] As early as 1936, Duran-Reynals described the extraordinarily powerful spreading action of snake venom from rattlesnakes and cobras.[12] Moreover, it was found that the spreading power differs considerably in various species and the spreading action is independent of the toxic effect of the snake venoms.[13] However, in contrast to mammalian and microbial hyaluronidases, the enzyme from snake venoms has been least studied in animal venoms because it was claimed non-toxic without any significant properties. In the treatment of snakebites, the inhibition of the snake venom hyaluronidases represents a therapeutic target, since they will not only minimize local tissue damage, but will also retard the distribution of lethal toxins.[14-16]

Previously, snake venoms from *Bitis gabonica rhinoceros, Bitis nasicornis, Bitis arietans, Naja kaouthia, Naja siamensis, Naja melanoleuca* and *Naja mossambica* were characterized in our laboratory with regard to hyaluronidase activity.[17] Moreover, compounds from different substance classes, such as flavonoids and chalcone analogs, ascorbic acid and indole derivatives[18] as well as glucurono-6,3-lactones, were tested in the turbidimetric assay for inhibition of the snake venom hyaluronidases. In this chapter, the characterization of two different snake venoms from *Bitis arietans* and *Naja siamensis* regarding hyaluronidase activity and the investigation of possible hyaluronidase inhibitors are described.

7.2 Materials and methods

The freeze-dried snake venoms from *Bitis arietans* and *Naja siamensis* were a gift from Dr. Marc Kunze.

7.2.1 Determination of protein content

The protein content of the snake venoms was determined by the Bradford method.[19] Human serum albumin (Behringwerke, Marburg, Germany) was used for calibration at concentrations of 50-600 µg/mL. The determination was carried out in a miniaturized version of the Bio-Rad protein assay. Different dilutions of the snake venoms from *Bitis arietans* and *Naja siamensis* (c = 400 µg/mL and 600 µg/mL) were made. 25 µL of the calibration standards and the sample dilutions were transferred to an acryl cuvette (in duplicate). 1.25 mL of the 1:5 diluted Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Munich, Germany) was added. After 5 min of incubation, absorbance at λ = 595 nm was measured using a Cary 100 UV-Vis spectrometer (Varian, Darmstadt, Germany). All dilutions were within the calibrated concentration range and therefore used for the calculation of the protein content.

7.2.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins present in snake venoms from *Bitis arietans* and *Naja siamensis* were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the protocol of Laemmli.[20] Molecular masses were assigned with the help of PeqGold Protein-Marker I (PeqLab, Erlangen, Germany), which contains: β-galactosidase from *E. coli* (molecular weight: 116.0 kDa), BSA from bovine blood (molecular weight: 66.2 kDa), ovalbumin from chicken eggs (molecular weight: 45.0 kDa), lactate dehydrogenase from pork muscle (molecular weight: 35.0 kDa), 98I from *E. coli* (molecular weight: 25.0 kDa), β-lactoglobulin from cow's milk (molecular weight: 18.4 kDa) and lysozyme from chicken eggs (molecular weight: 14.4 kDa).[21] The buffers for polyacrylamide gels and gel electrophoresis were prepared as follows:

buffer A:	1.5 M tris/HCl (pH 8.8), 0.4 % SDS (m/v)
buffer B:	0.5 M tris/HCl (pH 6.8), 0.4 % SDS (m/v)
running buffer:	0.025 M tris/HCl (pH 8.3), 0.2 M glycine, 0.1 % SDS (m/v)
sample buffer:	0.075 M tris/HCl (pH 6.8), 0.5 M glycine, 0.3 % SDS (m/v), 4.5 % glycerol (v/v), 0.05 % bromophenol blue (m/v), 1 % mercaptoethanol (v/v)

12 % separation gel mixtures contained 2.2 mL of water, 2 mL of buffer A and 3.2 mL acrylamide/bisacrylamide solution 30 % (Sigma-Aldrich, Munich, Germany). Polymerization was initiated by adding 3.5 µL *N,N,N',N'*-tetramethylethylenediamine (TEMED, Serva, Heidelberg, Germany) and 35 µL of a 10 % (m/v) solution of ammonium peroxodisulfate (APS) (Serva, Heidelberg, Germany) in water. The mixture was filled into gel chambers (10 cm x 10 cm x 0.8 cm) and overlaid with saturated isobutyl alcohol. Before casting the stacking gel, the isobutyl alcohol was discarded. 5 % stacking gels contained 3.25 mL of water, 1.25 mL of buffer B and 0.5 mL of acrylamide/bisacrylamide solution 30 %. 3.75 µL TEMED and 50 µL of APS (10 % solution in water) were added to start polymerization.

Electrophoresis was performed with a PerfectBlue gel electrophoresis system (Twin S, Peqlab, Erlangen, Germany) at 150 V for approximately 2 hours. The electrode chambers were filled with running buffer. Snake venom samples (c = 50 mg/mL) were mixed with a one-third volume of sample buffer and heated for 5 min at 100 °C. The proteins were stained with a 0.1 % (m/v) solution of Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) in 50 % (v/v) methanol (Merck, Darmstadt, Germany) and 10 % (v/v) acetic acid (Merck, Darmstadt, Germany). Destaining of the gel was carried out in an aqueous solution of 7 % (v/v) acetic acid and 10 % (v/v) methanol. The gels were analyzed with a Bio-Rad gel detection system (GS-710 Imaging Densitometer) using Quantity One quantification software, version 4.0.3 (Bio-Rad, Munich, Germany).

7.2.3 Zymography

Zymography with HA substrate gels is an SDS-polyacrylamide gel electrophoresis to visualize enzymatic activity and determine the molecular weight of the hyaluronidase. It was first described by Cherr et al.[22] and essentially carried out according to Hamberger.[23] SDS polyacrylamide gels were prepared as described in section 7.2.2 with additional 67 µg/mL hyaluronan (5 mg/mL stock solution) in the separation gel. Samples for zymography were mixed with sample buffer (200 mM tris/HCl pH 6.8, 20 % (v/v) glycerol, 10 % (m/v) SDS,

0.05 % (m/v) bromophenol blue) in ratio of 1:1 and directly applied to the gel. After electrophoresis at 150 V for approximately 2 hours, SDS was replaced by triton X-100 (Carl Roth, Karlsruhe, Germany). The SDS was removed by shaking the gels in a 2.5 % (v/v) solution of triton X-100 for 30 min at room temperature. Subsequently, the gel was washed with water for 5 min and with incubation buffer for additional 30 min. The McIlvaine's incubation buffer was prepared as described in section 3.2.2 and adjusted to pH 5.0. After incubation at 37 °C for 24 h, the gel was stained with a 0.5 % (m/v) solution of alcian blue 8 GX (Sigma, St. Louis, MO, USA) in acetic acid for 1 h. The gel was unstained in 7 % acetic acid until light bands (regions of hyaluronan digest) became visible on the blue background. The proteins were counterstained with Coomassie Brilliant Blue G-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid. After a final unstaining step in an aqueous solution of 7 % (v/v) acetic acid and 10 % (v/v) methanol, the gels were analyzed with a Bio-Rad GS-710 gel scanner as described in section 7.2.2.

7.2.4 Colorimetric hyaluronidase activity assay (Morgan-Elson assay)

7.2.4.1 General procedures

The required reagents and solutions were prepared as described in section 3.2.2.1 McIlvaine's buffer was adjusted to the required pH value (pH 5.0).

7.2.4.2 Determination of hyaluronidase activity

The enzymatic activity of hyaluronidases can be quantified according to the definition of the International Union of Biochemistry by defining 1 unit (U) as the amount of enzyme that catalyzes the liberation of 1 µmol *N*-acetyl-D-glucosamine at the reducing ends of sugars per min under specified conditions. For this purpose, references of known *N*-acetyl-D-glucosamine (GlcNAc) concentration were used to create a linear calibration graph. The absorbance resulting from the formation of the red colored product measured at a wavelength of 586 nm was plotted against a dilution series of GlcNAc. The GlcNAc concentrations typically covered a range from 0.1 mM to 2 mM, incubation time was set to 75 minutes. The incubation mixture containing 200 µL buffer, 50 µL BSA solution (0.2 mg/mL in water), 50 µL of the respective GlcNAc dilution (replacing HA), 82 µL H_2O, and 50 µL BSA (replacing the enzyme solution) was treated as described previously. Additionally, a blank probe with 50 µL HA solution (instead of GlcNAc) was added. Snake venom solutions were prepared with and without protease inhibitor. For the quantification of hyaluronidase activity

50 µL of the respective snake venom solution (c = 5 mg/mL) was incubated with 50 µL HA (5 mg/mL in water). Enzymatic activity was calculated according to Equation 7.1.

$$1\ \mathrm{U} = \frac{1\ \mu\mathrm{mol\ GlcNAc}}{\mathrm{min}} \qquad \text{Equation 7.1}$$

U: enzymatic activity (U)

GlcNAc: *N*-acetyl-D-glucosamine at the reducing ends of sugars

According to this definition, a hyaluronidase activity of 0.1 mU (0.1 nmol GlcNAc/min) is equivalent to approximately 1 international unit (IU), 1 (relative) turbidity reducing unit ((r)TRU), 1 national formulatory unit (NFU), 1.5 Benger units and 3.3 viscosity units, respectively.[24]

7.2.5 Turbidimetric hyaluronidase activity assay

The turbidimetric assay was performed in 96-well titer plates as described in detail in chapter 3 (Section 3.5.2.1). Identical assay conditions (including the pH value) were applied. The lowest concentration of both snake venom solutions that led to 100 % turbidity was used in the turbidimetric assay. As determined by M. Kunze, in case of *Bitis arietans* and *Naja siamensis*, this concentration was c = 50 mg/mL.[17] 10 µL of the snake venom solution was added to the incubation mixture.

7.2.6 Plasma protein binding

An HPLC-based method, based upon work published by Valko et al.[25-28], for the determination of the lipophilicity of selected compounds was performed by Origenis GmbH, Martinsried, Germany. The substances were characterized with three different methods. A C_{18}-column was used for the determination of the chromatographic hydrophobicity index (CHI, logD = 7.4), a human serum albumin (HSA) column for the determination of protein binding and an immobilized artificial membrane column (IAM) for the determination of membrane affinity. UV/VIS was used for detection (Dr. M. Thormann, personal communication 2013).

7.3 Results and discussion

7.3.1 Protein content and determination of molecular mass

The protein content of the snake venom samples was determined in three independent experiments each performed in duplicate. The results are shown in Table 7.1.

Table 7.1 Protein content (Bradford assay) of the snake venoms from *Bitis arietans* and *Naja siamensis*.

Snake species	**amount of protein (µg/mL)[a]**	**amount of protein in %**
Bitis arietans	248.6 ± 36	62 (52)[b]
Naja siamensis	218.2 ± 25	55 (35)[b]

[a] Mean values ± SEM (N = 2, experiments performed in duplicate); [b] values in parentheses reported by M. Kunze[17].

The amounts of protein found in the venoms of *Bitis arietans* and *Naja siamensis* were 249 µg/mL and 218 µg/mL, respectively, corresponding to 62 % and 55 %, which is about 10-20 % lower than the values M. Kunze (Table 7.1). However, the results agreed in the fact that the venom from *Bitis arietans* contains more protein than the venom from *Naja siamensis*.

The separation of the complex mixture of proteins and polypeptides in the venoms from both snakes was performed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Figure 7.2). 500 µg of the freeze dried, unpurified snake venom was applied onto the gel.

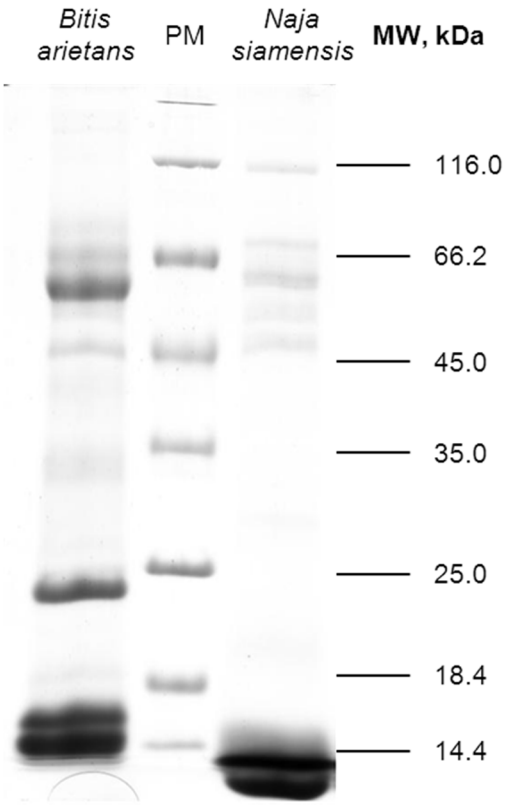

Figure 7.2 SDS-PAGE analysis of snake venoms from *Bitis arietans* and *Naja siamensis.*

The separation of the snake venom from *Bitis arietans* revealed two prominent protein bands at approximately 60 kDa and 23 kDa. In the area between 45 to 70 kDa several separated protein bands can be found in the lanes of both snake venoms. Additionally, in both lanes two very strong and broad protein bands can be observed in the area between 10 to 16 kDa of small protein fragments. Since the hyaluronidases present in the snake venoms are expected to possess a molecular mass > 33 kDa, this area can be neglected.

7.3.2 Hyaluronidase activity

Hyaluronidases from snake venoms are neutral pH endo-β-*N*-acetyl-D-hexosaminidases characterized as having molecular weights ranging from approximately 33 kDa to ~110 kDa.[29] For the determination and quantification of hyaluronidase activity in snake venoms, two different methods were in the present study: zymography and the colorimetric Morgan-Elson assay. After gel electrophoresis incubation at pH 5.0 and staining of the gel, light bands on dark background indicated hyaluronan degradation, i. e. enzymatic activity (Figure 7.3).

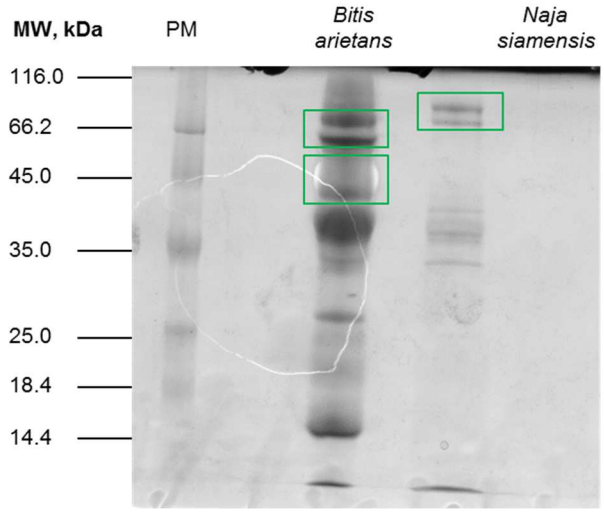

Figure 7.3 Determination of hyaluronidase activity by zymography (incubation at pH 5.0) followed by alcian blue staining and Coomassie counterstaining. Light bands on dark background indicate enzymatic activity.

Hyaluronidase activity was found in both snake venoms. In the venom from *Bitis arietans* two protein bands (66 kDa and 45 kDa) showed hyaluronidase activity. The presence of hyaluronidase isoforms in snake venoms, for example in *Naja naja,* was reported by Girish.[30,31] However, in the venom from *Naja siamensis*, no isoform of hyaluronidase was detected, only the protein band at approximately 65 kDa showed hyaluronidase activity. Kunze determined slightly higher molecular weights for the hyaluronidases in *Bitis arietans* (60 kDa and 70 kDa) and *Naja siamensis* (59 kDa). The reason why smaller molecular weights of the hyaluronidases were observed in this experiment may be that the snake venom, which was dissolved in water, was frozen and thawed for several times.[17] The molecular weight of hyaluronidases from different sources, such as human plasma or urine or animal venoms (snakes, scorpions, lizards, spiders and caterpillars), are between 32.5 kDa and 70.4 kDa[31-40]. This is in good agreement with the determined molecular weight of the investigated snake venoms from *Bitis arietans and Naja siamensis*.

The hyaluronidase activity of the two venoms from *Bitis arietans* and *Naja siamensis* was determined using the colorimetric Morgan-Elson assay at pH 5.0. The specific activity (mU/mg) takes the protein content of the freeze dried venoms into consideration (c (freeze dried venom) = 5 mg/mL). The enzymatic (mU/mL) and specific activities (mU/mg) are summarized in Table 7.2.

Table 7.2 Hyaluronidase enzymatic and specific activity (Morgan-Elson assay) of the snake venoms from *Bitis arietans* and *Naja siamensis*.

Snake species	pH of activity determination	enzymatic activity (mU/mL)[a]	specific activity (mU/mg)[a]
Bitis arietans	5.0	12.5 ± 1.4 (8.4)[b]	2.8 ± 0.7 (1.6)[b]
Naja siamensis	5.0	4.4 ± 2.0 (6.2)[b]	1.4 ± 0.8 (1.8)[b]

[a] Mean values ± SEM (N = 2, experiments performed in duplicate); [b] as determined by M. Kunze[17].

The hyaluronidase activity of the investigated venom sample from *Bitis arietans* (12.5 mU/mL) was almost 3-fold higher than the enzymatic activity of the venom from *Naja siamensis* (4.4 mU/mL). However, with regard to the protein content, the specific activity of the venom from *Bitis arietans* (2.8 mU/mg) was only 2-fold higher than the specific activity of the venom from *Naja siamensis* (1.4 mU/mg). The results determined by M. Kunze also revealed higher enzymatic activity in the venom from *Bitis arietans* (8.4 mU/mL) than in the venom from *Naja siamensis* (6.2 mU/mL), but the values were more similar. Moreover, with regard to the protein amount in the freeze dried snake venoms, the specific activity of hyaluronidase in the venom from *Naja siamensis* (1.8 mU/mg) was higher compared to the venom from *Bitis arietans* (1.6 mU/mg).[17]

Compared to the venoms from *Bitis arietans* and *Naja siamensis,* characterized by M. Kunze, the Bradford and Morgan-Elson assay showed slightly different results. It is important to characterize each venom sample regarding the amount of protein and hyaluronidase enzymatic and specific activity with respect to the investigation of potential hyaluronidase inhibitors.

7.3.3 Influence of pH on enzymatic activity

Animal and bacterial hyaluronidases exhibit their maximum enzymatic activity at acidic to neutral pH values (pH 4-7). According to a summary by Kemparaju, snake venom hyaluronidases exhibit their maximum activities between pH 4 and 7.[16] Previous studies by Hoechstetter showed that the pH optima of the bovine testicular hyaluronidase (BTH) is assay dependent (turbidimetric assay: pH 6; colorimetric assay: pH 4).[41] Therefore, it was of interest to investigate the pH profiles of the snake venom hyaluronidases.

In previous investigations by M. Kunze, the pH optima of several snake venom hyaluronidases were determined. The pH profiles of *Bitis arietans* and *Naja siamensis* are shown in Figure 7.4.

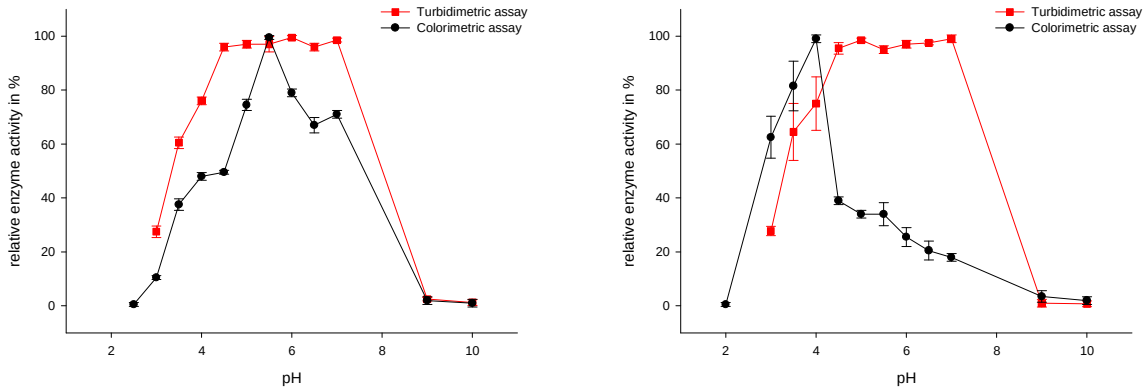

Figure 7.4 pH profiles of *Bitis arietans* (left) and *Naja siamensis* (right) as determined in the turbidimetric assay (red squares) and colorimetric assay (black circles) by M. Kunze.[17]

The resulting pH profiles of the snake venom hyaluronidases were similar to the profiles of BTH. The pH maximum was shifted towards more acidic pH values in the colorimetric Morgan-Elson assay. In the colorimetric assay, the curve of the pH profiles of the hyaluronidases in the venoms from the two snakes *Bitis arietans* and *Naja siamensis* showed sharp pH maxima (*Bitis arietans*: pH 5.5; *Naja siamensis*: pH 4). In the turbidimetric assay, however, both snake venom hyaluronidases showed broad areas of high activity between pH 4.5 and 7.5.[17]

The pH activity profiles of the hyaluronidases in the snake venoms from *Bitis arietans* and *Naja siamensis* (Figure 7.5) were determined in a pH range from 2 to 9 using both the colorimetric and turbidimetric assay. Identical parameters were chosen for both assay types. Only, the hyaluronan concentration in the turbidimetric assay differed by a factor of 2.5 to guarantee linearity in the turbidimetric assay.[42]

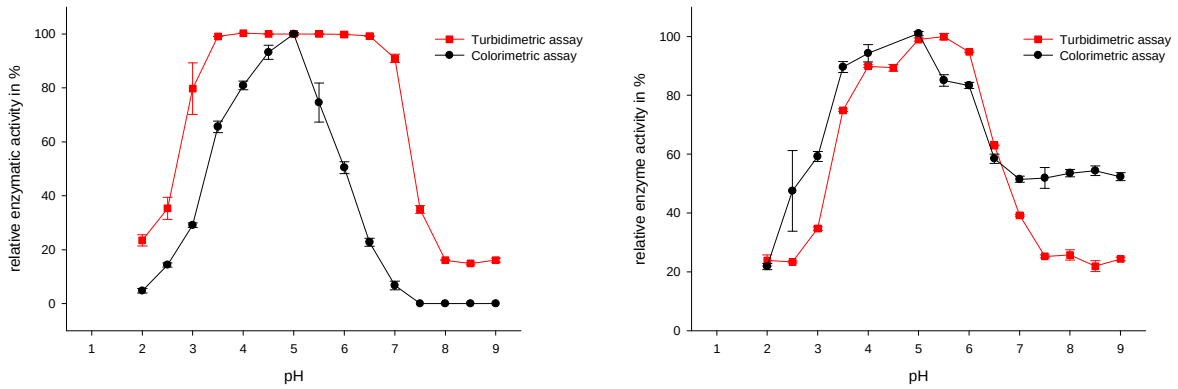

Figure 7.5 Enzymatic activity of the hyaluronidases from *Bitis arietans* (left) and *Naja siamensis* (right) as determined in the turbidimetric (red squares) and colorimetric assay (black circles). Maximum values set as 100 %.

In the colorimetric assay, the maximum activity of the hyaluronidase from *Bitis arietans* was detected at pH 5. As already described by Kunze[17], in the turbidimetric assay, the hyaluronidase revealed high activity between pH 3.5 and 6.5. The hyaluronidase in the venom from *Naja siamensis* showed its maximum activity at pH 5 in the colorimetric assay and at pH 5.5 in the turbidimetric assay. However, there was no sharp maximum in the curve of the pH profile when the Morgan-Elson assay was applied.

7.3.4 Inhibitory activities of selected compounds on snake venom hyaluronidases

The degradation of hyaluronic acid in the extracellular matrix of local tissues is presumed to be the key event in the enzyme mediated spreading process during snake envenomation.[30] Inhibition of the snake venom hyaluronidases appears to be a promising target in the treatment of snakebites. It would widen the time gap between the bite and the anti-venom administration by limiting easy diffusion of venom components, and it would, also, reduce the anti-venom load to achieve affective neutralization and hence the side effects of the therapy.[15] Sodium cromoglycate and sodium auro-thiomalate were reported to reduce the local tissue damage and to delay the time of death of mice injected with *Naja kaouthia* and *Calloselasma rhodaostoma* venoms.[43] *In vitro* testing of sodium cromoglycate in our laboratory confirmed the inhibition of several snake venom hyaluronidases only at very high concentrations (36 % inhibition at 10 mM).[17] To prevent local tissue damage and increase survival by retarding the diffusion of systemic toxins, inhibitors of the local acting venom hyaluronidases could be a useful tool in the treatment of snakebites.[16]

An selection of 4 compounds (Figure 7.6) was tested for inhibition of the hyaluronidases in the raw venoms from *Bitis arietans* and *Naja siamensis* (c (raw snake venom) = 50 mg/mL; according to M. Kunze[17]) in the turbidimetric assay (96-well plate format).

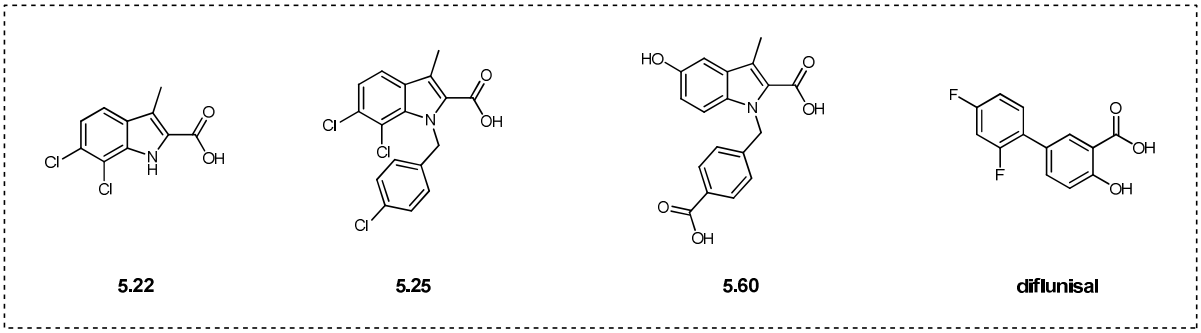

Figure 7.6 Structures of compounds **5.21**, **5.25**, **5.60** and diflunisal that were selected for investigation of plasma protein binding.

Each compound was tested in duplicate at a final assay concentration between 1 mM and 100 µM (depending on solubility of the compound in aqueous buffer). The results are summarized in Table 7.3.

Table 7.3 Inhibitory activity of selected compounds **5.22**, **5.25**, **5.60** and diflunisal.

Compound	**inhibitory activity (%)[a]**	
	Bitis arietans[a]	*Naja siamensis*[a]
5.22	inactive[b]	8 % 500 µM
5.25	inactive[c]	inactive[b]
5.60	inactive[d]	20 % 1 mM
diflunisal	inactive[d]	28 % 1 mM

[a] Inhibitory activity determined at pH 5.0 in the turbidimetric assay (96-well plate); [b] final assay concentration: 500 µM; [c] final assay concentration: 100 µM; [d] final assay concentration: 1 mM.

None of the tested substances showed inhibitory activity on the tested snake venom hyaluronidase from *Bitis arietans*. Whereas, compound **5.25** was inactive, weak inhibition was detected for compound **5.22** (8 % at 500 µM) and **5.60** (20 % at 1 mM) and diflunisal (28 % at 1 mM) on the hyaluronidase from *Naja siamensis*. However, it should be considered that without further purification the raw snake venom was applied in the turbidimetric assay. The poor inhibitory activity may be caused by high binding of the investigated compounds to

the proteins present in the raw snake venom. Therefore, the plasma protein binding of the tested compounds was determined.

7.3.5 Plasma protein binding of selected compounds

Human serum albumin (HSA) is the most prevalent protein in blood plasma. It has multiple hydrophobic binding sites and binds a diverse set of drugs, especially negatively charged hydrophobic compounds.[44] Therefore, lipophilicity is an important parameter in the process of designing new drugs.[45] Lipophilicity correlates positively with the logarithm of the *n*-octanol-water-partition coefficient logP.[46-49] Reversed-phase high performance liquid chromatography (HPLC) has been recognized as a method for the determination of lipophilicity, and it is based upon the direct correlation between logP (logD for ionized compounds) and the retention factor (log*k*').[50-52] Stationary phases for HPLC which imitate the phospholipid bilayers of physiological membranes by covalently linking phospholipids to silica particles, immobilized artificial membranes (IAM), were developed for the fast and accurate prediction of drug-membrane interactions.[45,53] The conversion of retention times into the chromatographic hydrophobicity index (CHI) with a set of standards, which refers to IAM chromatography (CHI_{IAM}), was proposed by Valko et al.[25] The relation between IAM binding data (CHI_{IAM}) of known drug molecules and HSA binding, both obtained by chromatography, showed that compounds with a certain lipophilicity bind to both membrane and HSA. The CHI_{IAM} values, which are more suiTable for interlaboratory comparison and high throughput screening, are a measure of compound interaction with phospholipids.[26,27] The HSA binding values were derived from the gradient retention times that were converted to the logarithm of the eliquibrium constants log*K* HSA using data from a calibration set of molecules.

The percentual protein bound to plasma values (% PPB; e. g. from literature) can be converted into the linear free energy related log*K* values (logarithm of apparent affinity constant) using Equation 7.2.

$$\log K = \log\left[\frac{\%\,PPB}{101 - \%\,PPB}\right] \qquad \text{Equation 7.2}$$

The value of 101 was taken arbitrarily to be able to calculate a log*K* for compounds that bind 100 % to HSA.[27] With the assumption that a binary complex is formed between the ligand

and HSA in the blood, and an excess of albumin compared to the concentration of the drug, these log*K* values can be converted to the logk_A affinity constant (Equation 7.3).[44]

$$\log k_A = \log \frac{[f_b]}{1-[f_b]} - \log[\mathrm{HSA}] \qquad \text{Equation 7.3}$$

k_A: equivalent binding affinity to HSA under the assumption that binding occurs exclusively to HSA

f_b: fraction bound to plasma proteins

c (HSA) = 0.6 mM

The plasma protein binding of three selected compounds, **5.22**, **5.25** and **5.60** (cf. Figure 7.6), was determined. The HSA binding affinities and measured chromatographic lipophilicity values of the tested compounds are summarized in Table 7.4.

Table 7.4 HSA binding affinities and measured chromatographic lipophilicity values of compounds **5.22**, **5.25** and **5.60**, respectively, each calculated in 2 independent experiments.

Cpd.	% PPB[a]	logK_a (HSA)	log$k$ (HSA)	confid.[b] HSA	CHI[c]	log$D$	confid.[b] log$D$	CHI IAM[d]	confid.[b] IAM
5.22	99.37	5.38	2.19	2	65.51	2.00	2	33.81	2
	99.29	5.33	2.14	2				32.25	1
5.25				0	82.28	2.56	2	53.50	1
	99.61	5.59	2.41	1				51.72	2
5.60	99.40	5.40	2.22	2	2.85	-2.96	2	2.88	2
	99.02	5.26	2.08	2				2.07	1

[a] Calculated percentual plasma protein binding; HPLC conditions: solvent A (5 % (v/v) isopropanol in 50 mM ammonium acetate buffer; pH 7.4)/isopropanol (100 %): 0 min: 100/0, 3.5 min: 0/30, 4.5 min: 0/30, 4.6 min: 100/0, 6 min: 100/0; [b] confidence: 2 = without any doubt, 1 = likely, 0 = unlikely; [c] a C_{18} column (40 °C) was used with an ammonium acetate buffer [50 mM; pH 7.4] + 5 % (v/v) MeCN as solvent (solvent B) under the following gradient conditions: solvent B/MeCN: 0 min: 100/0, 3 min: 0/100, 4 min: 0/100, 4.2 min: 100/0, 6 min: 100/0; [d] IAM column at 40 °C with the following gradient: solvent B/MeCN: 0 min: 100/0, 5 min: 0/100, 5.5 min: 0/100, 6.5 min: 100/0, 8.2 min: 100/0.

In general, the investigated compounds **5.22**, **5.25** and **5.60** show high plasma protein binding (> 99 %). However, with regard to the lipophilicity and membrane binding, the three substances differ considerably, showing CHI values between 2.85 and 82.28 and CHI IAM values between 2.07 and 53.50. Compound **5.60** bears two carboxylic residues, which cause the low CHI and CHI IAM values compared to the other two compounds.

To conclude, due to the high protein binding of the tested compounds, the free (non-protein-bound) compound concentration is too low to observe inhibitory activity on the snake venom hyaluronidases from *Bitis arietans* and *Naja siamensis*. The inhibition cannot be determined when applying the raw freeze-dried snake venom solutions. In order to find out whether the compounds show inhibitory effects on the snake venom hyaluronidases, the hyaluronidases need to be isolated from the venom and tested again.

7.4 Summary and conclusion

With regard to hyaluronidase activity, the venoms from two different snake species, *Bitis arietans* and *Naja siamensis*, were characterized. The Bradford assay, which was used to determine the total protein content of the lyophilized raw snake venom, revealed that the venoms contained between 55 % and 62 % protein. In accordance with previous results from our laboratories, the viper venom from *Bitis arietans* contained more protein than the cobra venom from *Naja siamensis*.[17]

Electrophoretic and zymographic experiments provided information about the molecular weight and activity of the snake venom hyaluronidases. The investigated hyaluronidases had a molecular weight between 45 and 65 kDa, which was in the same range as the molecular weights of hyaluronidases in venoms from other snakes, spiders, or scorpions (33-70 kDa). Additionally, in the venom from *Bitis arietans* hyaluronidase isoforms were found. Generally, the hyaluronidases have their maximum activity in the pH range between pH 4 and 7, as verified in both assays. However, in the colorimetric assay, both hyaluronidases show highest activity at only one pH value (pH 5.0).

The four compounds **5.22**, **5.25**, **5.60,** and diflunisal were tested in the turbidimetric assay for inhibition of the hyaluronidases in the venoms from *Bitis arietans* and *Naja siamensis*. Unfortunately, no inhibitory activity was detected for the hyaluronidase from *Bitis arietans*. Compounds **5.22**, **5.60** and diflunisal showed poor inhibition of the hyaluronidase from *Naja siamensis*. The poor results may be caused by high protein binding of the tested compounds, since the unpurified raw snake venoms were used in the turbidimetric assay. The plasma protein binding of the investigated compounds, determined with the help of an HPLC based method (cooperation with Origenis GmbH, Martinsried, Germany), was >99 %. Due to the high protein binding of the investigated compounds it remains unclear whether these substances are lacking inhibitory activity on snake venom hyaluronidases or the free fraction

of inhibitor is too low to produce a relevant effect. Nevertheless, for the treatment of a snake bite it is important that the hyaluronidase in the raw snake venom is inhibited. Therefore, inhibitors of snake venom hyaluronidases with considerably lower protein binding are needed.

7.5 References

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8 Summary

Hyaluronidases are enzymes that predominantly degrade hyaluronan, a major constituent of the extracellular matrix. They can be ubiquitously found throughout the animal kingdom and have been isolated from a large number of different organs and animal venoms. Additionally, hyaluronidases are produced by bacteria and fungi. In case of pathogenic bacteria and animal venoms, the hyaluronidases act as spreading factors. They cause local tissue damage to facilitate the diffusion of pathogens and toxins, respectively. Inhibitors of the hyaluronidases are required as pharmacological tools to further investigate their physiological and pathophysiological roles of these enzymes.

Previously, we identified hyaluronidase inhibitors among lipophilic vitamin C, indole, and benzoxazole derivatives. Due to the lack of drug-like properties and high plasma protein binding, such substances are considered inappropriate for *in vivo* studies. The goal of this thesis was the design, synthesis, identification, and pharmacological characterization of novel low-molecular weight inhibitors of the bacterial hyaluronidase *Sag*Hyal$_{4755}$ with improved drug-like properties.

In a first attempt, several commercially available drugs, especially non-steroidal anti-inflammatory drugs (NSAIDs), were investigated for inhibitory activity on the hyaluronate lyase *Sag*Hyal$_{4755}$ and the bovine testis hyaluronidase (BTH). All tested substances were inactive or only weakly active on BTH, suggesting inactivity of these compounds at the corresponding human enzyme, the PH-20 hyaluronidase. In contrast to the mammalian enzyme, the bacterial hyaluronidase was inhibited by several compounds. The salicylate diflunisal (IC_{50} (*Sag*Hyal$_{4755}$) = 195 µM) was identified as a putative lead compound for the design and synthesis of chemically related inhibitors of *Sag*Hyal$_{4755}$ with improved drug-like properties.

The scaffold of diflunisal was modified by introduction of a variously substituted aryl rings via Suzuki-Miyaura coupling led to potent inhibitors, for example 1,1':3',1''-terphenyl-4-carboxylic acid and 4-hydroxy-1,1':4',1''-terphenyl-3-carboxylic acid which revealed IC_{50} values of about 30 µM on *Sag*Hyal$_{4755}$ corresponding to a 6-fold increased potency compared to the lead diflunisal. However, inhibitors for the second bacterial hyaluronidase, *Spn*Hyl, were not found among the diflunisal analogs, indicating differences between the binding pockets of these related enzymes.

In a second synthetic approach, analogs of 6,7-dichloro-1*H*-indoles and 5-hydroxy-1*H*-indoles were synthesized. Whereas the investigated compounds showed only weak or no inhibition on both the bovine testicular hyaluronidase (BTH) and the bacterial hyaluronidase

*Spn*Hyl, IC_{50} values in the two-digit micromolar range were determined on *Sag*$Hyal_{4755}$. 6,7-Dichloro-1-(4-chlorobenzyl)-3-methyl-1*H*-indole-2-carboxylic acid was the most potent inhibitor from the series (IC_{50} = 16 µM).

In general, the potency of both, diflunisal- and indole-type hyaluronidase inhibitors, is strongly dependent on the presence of a negatively charged carboxylic group and chloro substituents as well as the overall lipophilicity of the compounds. The combination of these structural features resulted in inhibitory activity on *Sag*$Hyal_{4755}$ in the lower two-digit micromolar range.

In search of bioisosteric replacements of the indole scaffold, a small series of eight 2-phenylindolizine derivatives, with and without carboxylic acid moieties, was synthesized. Except for 4-(6-cyanoindolizin-2-yl)benzoic acid (IC_{50} = 56 µM), these compounds showed very weak or no inhibition of the streptococcal hyaluronidase *Sag*$Hyal_{4755}$. All synthesized compounds were devoid of inhibitory activity on BTH.

A subproject of this thesis focused upon the characterization of venoms from two different snake species, *Bitis arietans* (viper) and *Naja siamensis* (cobra), with regard to their hyaluronidase activity. Both venoms contain hyaluronidases with a molecular weight between 45 kDa and 65 kDa, which showed their maximum activity at acidic pH values (pH 4-7). The investigation of four selected compounds for inhibition of the two snake venom hyaluronidases revealed very weak or no inhibition. Since the pure reptile hyaluronidases were not available for more detailed investigations, lack of activity at these reptile enzymes cannot be ruled out. However, the high protein content in the raw venoms has to be taken into account as well. This assumption is supported by the high plasma protein binding of the tested compounds, as determined by an HPLC based method.

Taken together, substances derived from three different scaffolds were identified as two-digit micromolar inhibitors of the hyaluronate lyase *Sag*$Hyal_{4755}$, but not of the bacterial hyaluronidase *Spn*Hyl, the bovine testis hyaluronidase (BTH), and the snake venom hyaluronidases.

9 Appendix

9.1 Abbreviations

abs	absolute
Anal.	Analysis
aq	aqueous
Ar	aromatic
atm	standard atmosphere
AU	absorption units
Bn	Benzyl
BSA	bovine serum albumin
BTH	bovine testicular hyaluronidase
c	concentration
calcd.	calculated
cat.	catalytic amounts
CD44	cluster of differentiation 44
conc.	concentrated
COSY	correlated spectroscopy
cpd	compound
C_{quat}	quarternary carbon atom
CTAB	cetrimonium bromide, cetyltrimethylammonium bromide
CV	column volume
d	day(s) or doublet
δ	chemical shift
Da	Dalton
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DMAB	*p*-(dimethylamino)benzaldehyde
DMF	*N,N*-dimethylformamide

DMSO	dimethylsulfoxide
DMSO-d_6	per-deuterated DMSO
dppf	diphenylphosphino-ferrocene
DSCG	disodium cromoglycate
EC	enzyme commission number
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EI	electron impact ionization
ELISA	enzyme-linked immunosorbent assay
eq	equivalent(s)
ESI	electrospray ionization
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
f. c.	final concentration
FCS	fetal calf serum
GAG	glycosaminoglycan
GBS	Group B streptococcus
GlcNAc	*N*-acetyl-D-glucosamine
GlcUA	D-glucuronic acid
h	hour(s)
H_2O	water
H_2SO_4	sulfuric acid
HA	hyaluronic acid, hyaluronan
HAS	hyaluronan synthase
HCl	hydrochloric acid
HCOOH	formic acid
HMBC	heteronuclear multiple bond correlation

HN	hyaluronectin
H_2N_4	hydrazine hydrate
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
HSA	human serum albumine
HSQC	heteronuclear single quantum correlation
Hyal-1	(human) hyaluronidase 1
IC_{50}	concentration of an inhibitor required to give 50 % inhibition of enzymatic activity
IU	international units
J	coupling constant
LiOH	lithium hydroxide
m	mili or multiplet
M	molar
M	mass
M_r	molecular mass
Me	methyl
MeCN	acetonitrile
MeOH	methanol
min	minute(s)
mp	melting point
MS	mass spectral or mass spectrum
MTP	microtiter plate
mw	molecular weight
m/*z*	mass-to-charge ratio
N	number of experiments
$NaBF_4$	sodium tetrafluoroborate
Na_2CO_3	sodium carbonate
NaH	sodium hydride

$NaNO_2$	sodium nitrite
NMR	nuclear magnetic response
NP	normal phase
NSAID	non-steroidal anti-inflammatory drug
OAc	acetate
OD	optical density
O-sulfated	oxygen sulfated
PAD	proton acceptance and donation
PAGE	polyacrylamide gel electrophoresis
Pd/C	palladium on activated charcoal
PE	petroleum ether
pH	negative logarithm of the hydrogen ion concentration
Ph	phenyl
pK_a	negative logarithm of the acid dissociation constant (K_a)
$POCl_3$	phosphoryl chloride
PPB	plasma protein binding
ppm	part per million
q	quartet
RHAMM	receptor for hyaluronate-mediated motility
RP	reversed phase
rpm	revolutions per minute
rt	room temperature
s	singulet
Sag$Hyal_{4755}$	hyaluronate lyase from *Streptococcus agalactiae* strain 4755
sat.	saturated
SDS	sodium dodecylsulfate
SEM	standard error of the mean
SPAM1	spam adhesion molecule 1 (also termed PH-20)

*Spn*Hyl	hyaluronate lyase from *Streptococcus pneumoniae*
t	triplet
t_0	dead time
t_R	retention time
TBTA	*tert*-butyl-2,2,2-trichloroacetimidate
TFA	trifluoroacetic acid
THF	tetrahydrofurane
TIC	total ion count
TLC	thin layer chromatography
tris	tris(hydroxymethyl)aminomethane
(r)TRU	(relative) turbidity reducing unit
U	unit(s)
UV	ultraviolet
v/v	volume concentration in %
Vcpal	Vitamin C palmitate
w/v	mass concentration in %
X-ray	X-radiation, form of electromagnetic radiation

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