New Inhibitors of bacterial hyaluronidase - Synthesis and structure-activity relationships

Dissertation

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Für Sylvia

Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt.

Albert Einstein

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Introduction

1.1 Hyaluronic acid

1.1.1 Structure and physicochemical properties

Hyaluronic acid (hyaluronan, HA), a main component of the extracellular matrix, was first found in the vitreous humor of bovine eyes by Meyer and Palmer in the year 1934[2]. This versatile and fascinating macromolecule belongs to the family of glycosaminoglycanes (GAG), which are linear polysaccharides of high molecular weight composed of aminosugars (*N*-acetylglucosamine or *N*-acetylgalactosamine) and uronic acids (glucuronic acid or iduronic acid), along with chondroitin-, keratan- and dermatane sulfate, heparin and heparan sulfate.

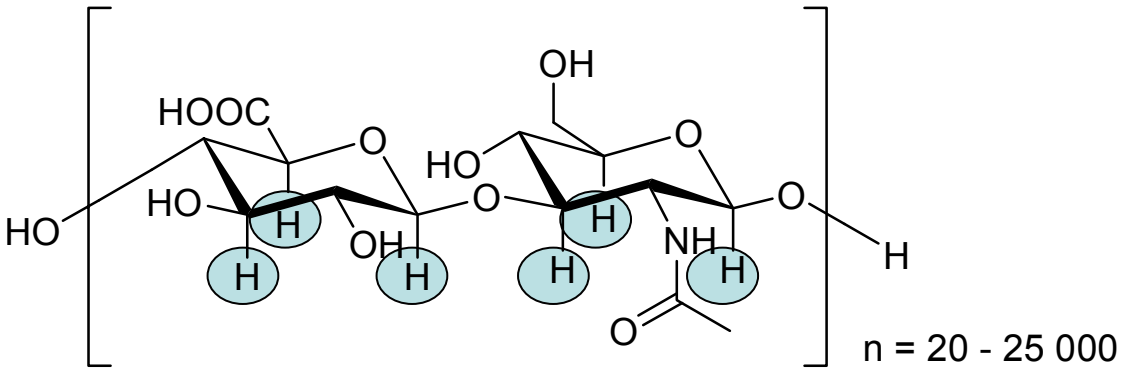


Fig. 1.1: Chemical structure of hyaluronic acid. H (blue colored): axial hydrogens that contribute to the hydrophobic face

The chemical structure of hyaluronan is the simplest of all GAGs consisting of straight-lined repeating disaccharide units of [→4)-β-D-glucuronic acid(1→3)-β-D-*N*-acetylglucosamine(1→]$_n$, where n can be up to 25000 dependent on the

tissue source (Fig. 1.1). When considering the energetically state the HA structure is very stable since the bulky groups (the hydroxyl groups, the carboxylate moiety, the *N*-acetyl residue and the adjacent sugars) are situated in sterically favorable equatorial positions while all of the small hydrogen atoms occupy the axial positions. In contrast to the other glycosaminoglycanes, hyaluronan is not covalently bound to a protein core and is not sulfated. Lately, the term hyaluronan, suggested by Balasz *et al*.[3], has substituted the terms hyaluronic acid and hyaluronate reflecting the fact that it exists *in vivo* as a polyanion due to the mostly charged carboxyl groups of the glucuronic acid residues (pK_a = 3-4, depending on ionic conditions[4]).

For decades, it was assumed that hyaluronan chains were expanded random coils in physiological solutions. However, nuclear magnetic resonance studies performed by Scott *et al*.[5] suggested an ordered structure of hyaluronan in solution. This conformation is characterized by a gently undulating, tape-like, twofold helix which is formed as a result of 180 °C rotations between alternating disaccharide units and is stabilized via internal hydrogen bonds and interactions with the solvent[6,7]. The striking feature of this secondary structure of hyaluronan is an extensive hydrophobic patch (represented by the axial hydrogen atoms) of about 8 CH-groups of 3 carbohydrate units. Thus, hyaluronan is amphiphilic because it includes properties of highly hydrophilic material simultaneously with hydrophobic patches, which is characteristic of lipids.

In addition, the application of rotary shadowing-electron microscopy revealed that HA self-aggregates into strands of a honeycomb meshwork in an aqueous solution whereby the thickness of the strands increased with HA concentration[8]. According to ^{13}C-NMR studies[9], this aggregation is stabilized both by hydrophobic interactions between the hydrophobic patches and by hydrogen bonds between acetamido and carboxylate groups of neighboring HA chains arranged antiparallel to each other. Since such hydrophobic and hydrophilic “bonds” can be formed on both sides of the hyaluronan polymer higher order aggregates can assemble causing strands of increased thickness in a HA meshwork. Recently, NMR spectroscopy investigations[10] demonstrated that the tertiary structures (aggregation of antiparallel HA chains) are specifically and reversibly disaggregated by mild physicochemical methods (raising temperature or pH). Further on, it was highlighted that the supramolecular organization of HA is on the edge of

stability indicating that reversible formation and breakdown of tertiary structures have a major influence on controlling biological properties.
These properties lead to highly viscous solutions, *i.e.* by binding water, the volume of HA increases by about 1000-fold compared to the non-hydrated state[11]. In the hydrated state, the diffusion of *e.g.* proteins and electrolytes is considerably facilitated. Generally, all molecules can pass through this network but with different velocity depending on their hydrodynamic volumes. Therefore, the hyaluronan network acts as a diffusion barrier *in vivo* and regulates the transport of other substances through the intercellular space[12].
The organized structure of HA is supposed to possess a substantial influence on the binding of HA to receptors and to the active site of hyaluronidases.

1.1.2 Occurrence and physiological importance

All vertebrates as well as some Streptococci strains produce hyaluronan. As a major component of the extracellular matrix, HA is found in the vitreous body of human eye (0.1-0.4 mg/g wet weight), in synovial joint fluid (3-4 mg/ml), in umbilical cord (~4 mg/ml), in rooster comb (up tp 7.5 mg/ml), in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/ml) or in the pathological matrix that occludes the artery in coronary restenosis. The largest amount of hyaluronan (7-8 g per average adult human, about 50% of the total in the body) resides in the skin tissue where it is present in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue). Furthermore, hyaluronic acid serves as a structural element in the matrix, *e.g.* in hyaline cartilages (1 mg/g wet weight) where it retains aggrecan molecules in the matrix through specific protein-hyaluronan interactions[4].
Lower concentrations of HA are found in the matrix of other connective tissues such as those surrounding smooth muscle cells in the aorta.
Hyaluronan as an essential structural element in the matrix plays an important role for tissue architecture by immobilizing specific proteins (aggrecan, versican, neurocan, brevican, CD44 etc.) in desired locations within the body. Moreover, hyaluronan is implicated in many biological processes including fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, growth and metastasis of tumor cells and whenever rapid tissue

turnover and repair are occuring[11-13]. The function of HA may be partly regulated dependent on its chain length, *e.g.* angiogenesis is presumably induced by small HA oligosaccharides, whereas high molecular weight HA exerts inhibitory effects[14].

HA interacts with a variety of receptors and hyaluronan binding proteins (hyaladherins) on the surface of cells[12,15]. The great number of hyaladherins known so far can be grouped into (i) the structural hyaluronan-binding proteins of the extracellular matrix, such as link protein and the aggregating proteoglycanes, (ii) cell surface hyaluronan receptors and (iii) intracellular hyaluronan binding proteins. The most studied hyaluronan receptor to date is CD44 (lymphocyte homing receptor), which is responsible for a wide variety of cellular functions, *e.g.* receptor mediated internalization/degradation of hyaluronan, cell migration and cell proliferation. Several other cell membrane-localized receptors have been identified including the RHAMM (receptor for hyaluronan which mediates motility), ICAM-1 (intercellular adhesion molecule-1), the LEC receptor (Liver Endothelial Cell clearance receptor)[12,16] and LYVE-1 (Lymphatic endothelial hyaluronan receptor)[17]. While most interactions between hyaluronan and hyaladherins are non-covalent the SHAP(Serum-derived Hyaluronan-Associated Protein)-complex represents the only case with covalent hyaluronan-protein crosslinking. The formation of this SHAP-hyaluronan complex plays an important role in the construction and maintenance of certain hyaluronan-rich extracellular matrices[18].

1.2 Hyaluronidases

1.2.1 History and occurrence

Hyaluronidase is a general term initially introduced by *Karl Meyer* in 1940 to describe enzymes that are able to break down primarily hyaluronan. Hyaluronidase activity was first identified in an extract of mammalian testes and other tissues as a ‘spreading factor’ that facilitated diffusion of antiviral vaccines, dyes and toxins injected subcutaneously[19]. A second line of independent investigation led to the first isolation of hyaluronan by Meyer and Palmer[2]. After the iden-

tification of a HA degrading enzyme in bacteria[20], it could soon be shown that the mammalian spreading factor was also an enzyme degrading hyaluronan[21].
In the following years similar hyaluronidase-like enzymes were identified from a large number of tissues and organisms, *e.g.* skin, liver, kidney, spleen, testes, uterus, placenta, from the venoms of snakes, lizards, fish, bees, wasps, scorpions, spiders, from body liquids (tear liquid, blood, sperm) as well as from some bacteria, fungi and invertebrate animals (leech, crustacean). The hyaluronidases from different sources vary in their molecular weight, substrate specificity, pH optima and catalytic mechanism[1,13,22,23]. Although widely distributed in nature, hyaluronidases are not well characterized and are a group of neglected enzymes owing to their difficult purification and lack of scientific interest over a large period of time. However, in recent years there is growing interest in the possible role of hyaluronan and hyaluronidase in numerous biological processes.

1.2.2 Classification of hyaluronidases

K. Meyer established the first classification scheme for hyaluronidases in 1971. Based on detailed biochemical analysis of the enzymes and their reaction products, the hyaluronidases are subdivided into three main families (Fig. 1.2)[1].

The first groups of hyaluronidases are the hyaluronate 4-glycanohydrolases (EC 3.2.1.35) degrading hyaluronan by cleavage of the β-1,4-glycosidic bond to the tetrasaccharide as the main product. Apart from the preferred substrate HA, these enzymes depolymerize also chondroitin, chondroitin-4- and chondroitin-6-sulfate and, to a small extent, dermatan sulfate. As a special characteristic, this class of enzymes reveals both hydrolytic and transglycosidase activity[24,25]. The best-known enzymes are the testicular, the lysosomal and the bee venom hyaluronidase.

The second type is represented by hyaluronidases occurring in the salivary glands of leeches and hookworms. These hyaluronate 3-glycanohydrolases (EC 3.2.1.36) hydrolyze the β-1,3-glycosidic bond of HA yielding sugar fragments bearing glucuronic acid at the reducing end. The main product of this reaction is a tetrasaccharide, too.

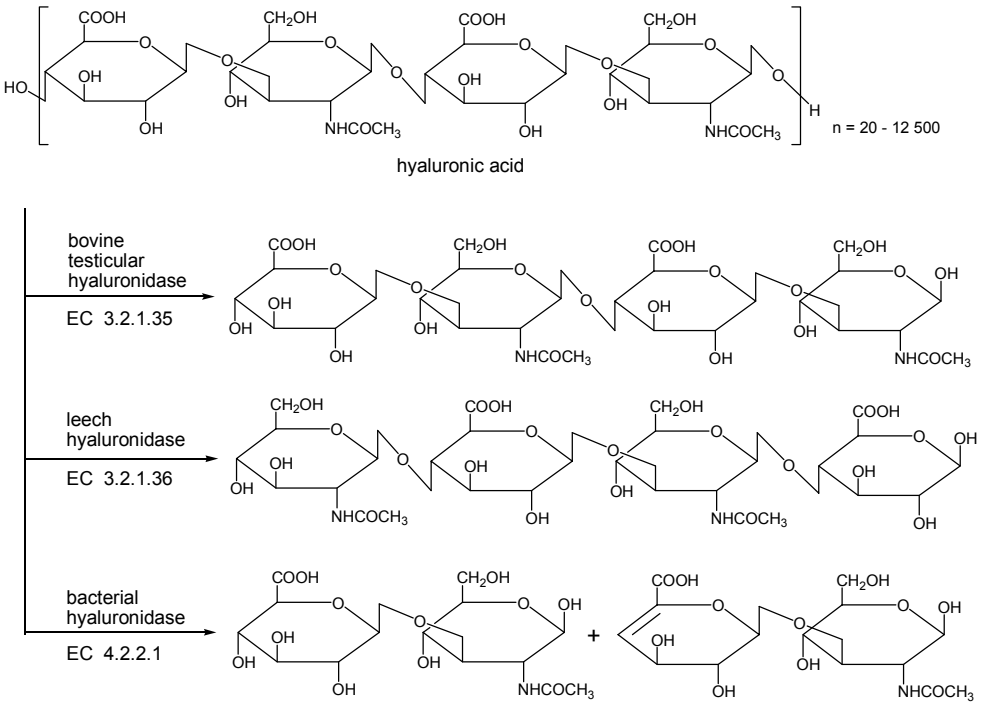


Fig. 1.2: Classification scheme of hyaluronidases according to Meyer[1]

The last group, the microbial hyaluronidases or hyaluronate lyases (EC 4.2.2.1 or EC 4.2.99.1), act *via* a β-elimination reaction resulting in the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-*D*-glucose as main product[22,26]. The hyaluronate lyases, isolated from various microorganisms as *e.g.* strains of *Clostridium*, *Micrococcus*, *Streptococcus* or *Streptomyces*, differ in substrate specificity[27,28].

Based on molecular genetic analysis the hyaluronidases can be divided alternatively in two main families – the hyaluronidases from eukaryotes and from prokaryotes – according to amino acid sequence homology[13,29].

1.2.3 Hyaluronidases from eukaryotes

1.2.3.1 Mammalian hyaluronidases

As a consequence of the knowledge gained by the “human genome project” in the last years, six hyaluronidase-like sequences in the human genome were identified with about 40% amino acid sequence identity. Three genes (*HYAL1*, *HYAL2* and *HYAL3*) coding for Hyal-1, Hyal-2 and Hyal-3 are located tightly

clustered on human chromosome 3p21.3. *HYAL4*, *HYALP1* (a pseudogene) and *PH20* (*SPAM 1*), coding for Hyal-4 and PH-20, are found on chromosome 7q31.3. Among these enzymes, Hyal-2, Hyal-4 and PH-20 are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) linker[29,30].

The activity of the PH-20 protein (also known as SPAM 1 (sperm adhesion molecule 1)) was first found by Gmachl *et al*.[31] due to the significant similarity to bee venom hyaluronidase which was the first cloned eukaryotic hyaluronidase[32]. The human testicular hyaluronidase is required for sperm penetration through the cumulus cell layer that surrounds the oocyte and for fertilization[33]. It is a multifunctional protein with a separate domain that binds to the zona pellucida[34]. The PH-20 occurs in two forms, a GPI-anchored one attached on the surface of mammalian sperm and a soluble one[35,36].

Hyal-1 and Hyal-2 constitute the major hyaluronidases of somatic tissue. Thus, they are present in most tissues and body fluids. However, Hyal-2 is not existent in the adult brain[37]. Both proteins are localized in lysosomes and additionally, Hyal-2 can be anchored to the plasma membrane by a GPI-linker. Hyal-1 produces small oligosaccharides with the tetrasaccharide as major product whereas Hyal-2 degrades high molecular weight HA to intermediate size fragments of about 20 kDa (approximately 50 disaccharide units)[30,38]. Therefore, they are believed to act in succession on depolymerizing HA: at the cell surface GPI-anchored Hyal-2 generates HA fragments of approx. 20 kDa, which are absorbed by the cell and digested to tetrasaccharides by the lysosomal Hyal-1[29,39]. Hyal-1 is also found in human urine[40] and mammalian plasma[41].

Mucopolysaccharidosis IX, a newly described lysosomal disorder, stems from mutations in the gene *HYAL1* [42,43]. Moreover, Hyal-1 and Hyal-2 seem to be involved in tumor formation. Hyal-1 is a candidate tumor suppressor gene that is inactivated in many tobacco-related lung tumors[44,45] and was found to promote tumor cell cycling[46]. Hyal-2 might share an oncogenic and a tumor suppressor gene function. On the one hand, an overexpression of Hyal-2 is reported accelerating tumor formation of murine astrocytoma cells[47] and on the other hand, Hyal-2 seems to speed up apoptosis[48]. Additionally, a distinct influence of the size of HA degradation products on proliferation and, to a lesser extent, on migration has been discussed[14], often referred to as angiogenic switch. In the be-

ginning of tumor growth, high molecular weight HA is necessary to provide the flow of nutrients at the primary site and subsequently, intermediate HA fragments generated by Hyal-2 induce angiogenesis[49,50].

Although Hyal-3 is widely expressed, *e.g*. in testes and bone marrow, no activity was measured using the available hyaluronidase assays[30]. Interestingly, Hyal-4 seems to be a chondroitinase which would be the first one found in vertebrate tissue. In contrast to Hyal-1 and PH-20 which are cleaving both HA and chondroitin sulfate, albeit at a slower rate, Hyal-4 has an absolute substrate specificity for chondroitin and chondroitin sulfate[30]. With the exception of PH-20, which is displaying activity at neutral pH, all known mammalian hyaluronidases are active at acidic pH.

1.2.3.2 Bovine testicular hyaluronidase (BTH)

As well-known representative of the mammalian hyaluronidases, the bovine testicular hyaluronidase (BTH) was adopted as a spreading factor in several medical fields for a long time, *e.g.* orthopaedia, surgery, opthalmology, dermatology or internal medicine[51,52]. BTH acts as endo-glycanohydrolase (EC 3.2.1.35) by cleaving the β-1,4-glycosidic bond of hyaluronan. The structurally related GAGs chondroitin, chondroitin-4- and -6-sulfate are accepted as substrate, too.

As confirmed by ion-spray mass spectrometry the enzymatic hydrolysis of HA by BTH generates tetrasaccharide and saturated disaccharide fragments[24]. A specific characteristic of BTH is that it exerts hydrolase activity as well as transglycosylase activity. According to Cramer *et al*.[25], transglycosylation reactions occur as long as HA oligosaccharides with 6 to 12 monomer units are available resulting in saturated HA oligosaccharides with *N*-acetylglucosamine at the reducing end. This is in contrast to the aforementioned formation of smaller fragments. The transglycosylase activity of BTH is dependent on the pH value and the salt content of the incubation buffer. Saitoh *et al*.[53] showed that the optimal pH value for transglycosylation reaction is at pH 7 whereas hydrolysis appears to be optimal at pH values below 5. The presence of NaCl negatively affects the transglycosylase activity. Nearly complete inhibition was found at NaCl concentrations above 0.5 M.[53].

Different pH optima of hyaluronidase activity are reported in the literature depending on the mostly heterogeneous enzyme composition of the BTH preparations, the source of substrate, the applied hyaluronidase assay[54] and the incubation conditions[1,54-56].

The bee venom hyaluronidase (BVH), a member of the hyaluronate 4-glycanohydrolases like BTH, was found to share 30% sequence identity with the mammalian PH-20. Compared to the human and the bovine hyaluronidases, in BVH a C-terminal domain of about 120-150 amino acid residues is lacking, but the active site residues are conserved[32,57]. The recent structural elucidation of a BVH-HA tetrasaccharide complex provides insights into the mode of substrate binding and the catalytic mechanism[57,58].

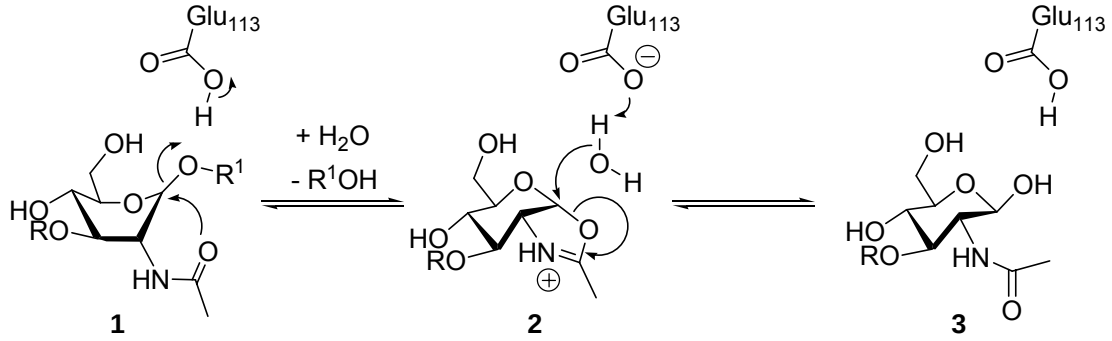


Fig. 1.3: Double-displacement substrate assisted mechanism of bee venom hyaluronidase. The saccharide in subsite[59] -1* (**1**) binds in boat conformation, and catalysis is proposed to occur via a formation of a covalent oxazolinium ion intermediate **2** to the product **3**. Adapted from Markovic-Housley and Schirmer[58].

Generally, glycosidases act *via* a double or single nucleophilic displacement mechanism which results in either retention or inversion of the configuration of the anomeric carbon atom[58,60]. In the case of BVH, an acid-base catalytic mechanism is suggested in which Glu113 serves as the proton donor and the *N*-acetyl group of the substrate is the nucleophile as illustrated in Fig. 1.3. It is assumed that the hydrolysis of BTH operates in this double-displacement substrate assisted mechanism as described for BVH. The acid-base catalyst Glu113 of BVH corresponds to Glu149 of BTH as a sequence alignment of BTH and BVH clearly showed[61].

* By convention, the sugar residue subsites are labelled from –n to +n, with –n at the non-reducing end and +n at the reducing end of the substrate. Cleavage occurs between the -1 and +1 subsites.

1.2.4 Hyaluronidases from prokaryotes

The bacterial enzymes capable of breaking down hyaluronan were reviewed by Suzuki[27] and Hynes and Walton[28]. The amino acid sequences of a variety of hyaluronidases from prokaryotes are known[62]. Among the bacterial hyaluronidases, the hyaluronate lyases from *Streptococcus pneumoniae* and from *S. agalactiae* (group B streptococcus, GBS) are the best characterized ones[26,63,64]. Both enzymes are cleaving the β-1,4-glycosidic bond of hyaluronan between D-glucuronic acid and *N*-acetyl-D-glucosamine in a β-elimination reaction resulting in the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-D-glucose[1,22,26,65]. Due to the elucidation of the crystal structures of hyaluronate lyase from *S. pneumoniae* and from *S. agalactiae* strain 3502 ($hylB_{3502}$) by X-ray analysis in the recent years more detailed information on the catalytic sites is available[63,64,66-68]. Considering the active site of *S. agalactiae* hylaruonate lyase there are two important parts. The three catalytic residues His479, Tyr488 and Asn429 are responsible for the substrate degradation and the aromatic patch comprising Trp371, Trp372 and Phe423 selects the cleavage sites on the HA chain and anchors these sites exactly in the degradation position within the cleft. In addition, the highly positively charged enzymatic cleft facilitates the binding of the negatively charged polymeric substrate chain[66].

The mechanism of hyaluronan binding and degradation, termed proton acceptance and donation (PAD), was proposed based on crystal structure of the native enzyme as well as the structure of the enzyme in complex with the disaccharide product of degradation[66] and in complex with hyaluronate hexasaccharide[69]. Additionally, the PAD mechanism is supported by a homology model of the enzyme in complex with the hyaluronate tetrasaccharide, site-directed mutagenesis studies[26] and the comparison with the related hyaluronate lyase from *S. pneumoniae* (hylSpn)[66,67,70]. In the first step, the negatively charged hyaluronan binds to the positively charged enzymatic cleft. Three disaccharide units can be accommodated in the cleft (see Fig. 1.4, only two, HA1 and HA2, of the three units are drawn). In the second step, the aromatic patch of the active site stabilizes and precisely positions HA1 and HA2 in a way that is optimal for degradation. In the next step, the glucuronic acid of HA1 is deprotonated at

C5 by His479 (Fig.1.4). Simultaneously, Tyr488, located at the bottom of the cleft and interacting with the O4 oxygen atom of the glycosidic linkage provides a proton to this glycosidic oxygen. The glycosidic bond is cleaved by 1,2-elimination forming the unsaturated final product (fourth step).

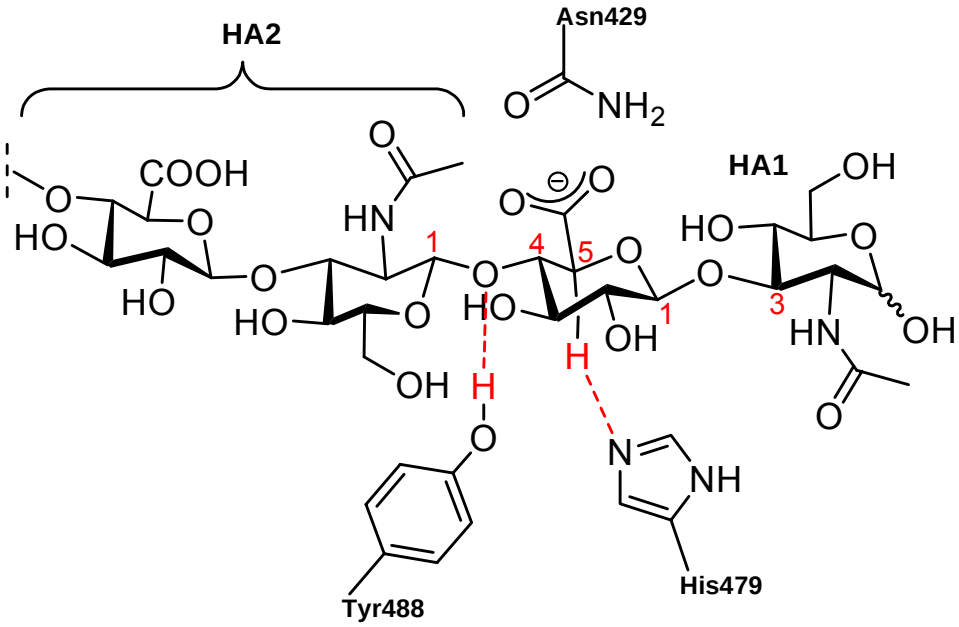


Fig. 1.4: Mechanism of hyaluronan degradation by *S. agalactiae* hyaluronate lyase strain 3502 according to Li and Jedrzejas [66]. Schematic representation of hyaluronic acid with HA1 and HA2 as disaccharide units and the position of the side chains of Tyr488, His 479 and Asn429 relative to the substrate.

In the final step, the catalytic residues are regenerated: His479 is deprotonated and Tyr488 is protonated by a water molecule.

Supported by elucidation of 3D structures of *S. pneumoniae* and *S. agalactiae* strain 3502 hyaluronidases in complex with hyaluronan fragments of various lengths, the PAD mechanism has been supposed to result in a processive mode of degradation of the polymeric substrate[69,71]. Furthermore, chondroitin and chondroitin sulfate were exposed to hyaluronate lyases. Hyaluronate lyase of GBS was found to cleave chondroitin and chondroitin-6-sulfate but not chondroitin-4-sulfate[72,73]. This profile of action is in accordance with the X-ray structure of hylSpn in complex with chondroitin and chondroitin sulfate disaccharides[74].

The three catalytic residues, Asn429, His479 and Tyr488 in hyaluronate lyase of *S. agalactiae* strain 3502, are conserved among all known bacterial hyaluronidases, but the aromatic patch residues, including Trp371, Trp372 and Phe423, are only partly conserved[66]. The difference in the aromatic patch com-

position may explain the different enzymatic activities of the hyaluronate lyases. The bacterial enzymes containing the complete aromatic patch like $hylB_{3502}$ and hylSpn act in a processive way and produce the disaccharide only whereas bacterial enzymes possessing Trp371 and Trp372 but not Phe423 operate in a nonprocessive degradation pattern and differ in their enzymatic products, *e.g*. *Streptomyces hyalurolyticus* hyaluronate lyase produces a mixture of tetra- and hexasaccharides and no disaccharides[75,76]. By contrast, Kuhn *et al*.[77] found that $hylB_{4755}$ degrades HA *via* a nonprocessive way since at all stages of digestion a mixture of oligosaccharides of different size were present.

Bacterial hyaluronate lyases are considered as virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronan[78,79]. Human infection by *S. agalactiae* is one of the major causes of meningitis and septicaemia and many other serious diseases leading to death in neonates[28,80,81]. To study the role of hyaluronan and hyaluronidases in bacterial infection, the design and development of hyaluronate lyase inhibitors become more and more important.

In this study, hyaluronidases from bovine testis and from *S. agalactiae* strain 4755 ($hylB_{4755}$) were used to determine the inhibitory potency of the synthesized putative inhibitors. $HylB_{4755}$ shows sequence identities of 98% with the homologous enzyme of *S. agalactiae* strain 3502[82] and of 53% with *S. pneumoniae* hyaluronidase[83].

1.3 Medical applications of hyaluronan and hyaluronidases

HA has found applications in various medical and pharmaceutical areas owing to its high water-binding capacity and the viscoelasticity of its solutions. In the late 1950s, hyaluronan was probably applied for the first time to humans, in fact as vitreous humor supplement/replacement during eye surgery, an application which has proved therapeutically useful up to now (*e.g*. in cataract surgery)[84]. Since HA retains moisture it is used in some cosmetics to keep skin young and

fresh-looking[85]. Sodium hyaluronate and a covalently cross-linked form of hyaluronan are successfully applied for the treatment of osteoarthritis[86]. Anabolic effects of HA on degraded bovine articular cartilages suppress their degeneration[87]. Moreover, HA normalizes the properties of synovial fluids[88] and produces an analgesic effect[89,90]. A rapid increase of hyaluronan levels can occur in many clinical situations, e.g. shock incidents, septicaemia and in burn patients[91].

The therapeutical benefit of hyaluronidases is based on the cleavage of hyaluronan in tissues resulting in increased membrane permeability, a reduced viscosity and a facilitated diffusion of injected fluids. These phenomena are referred to as spreading effect of hyaluronidases. The ability to promote penetration and spread are used to accelerate and increase absorption of injected drugs, *e.g*. antibiotics, to promote resorption of excess fluids, to improve the effectiveness of local anaesthesia and to diminish pain due to subcutaneous or intramuscular injection of fluids[13,52]. For many years, hyaluronidases, especially BTH preparations, are widely used in many fields like orthopaedia, surgery, ophthalmology, internal medicine, oncology, dermatology and gynaecology[52,92-94]. Because of the BSE risk the supply of BTH preparations was stopped. As a consequence, a number of cases of iatrogenic strabismus have been observed after cataract surgeries[95]. With respect to this shortage, pharmaceutical preparations with bacterial[54] or ovine[96] hyaluronidase are suggested as replacement of BTH. Sperm hyaluronidase is involved as a key player in successful fertilization in most mammalians, including humans[97,98].

Hyaluronidase has been investigated as an additive to chemotherapeutic drugs for augmentation of the anticancer effect[56,99-101]. There is evidence that hyaluronidase may have intrinsic anticancer effects and can suppress tumor progression. Furthermore, Zahalka *et al*.[102] showed in an animal model of T cell lymphoma that hyaluronidase blocks lymph node invasion by tumor cells. However, the findings are rather inconsistent. The specific function of Hyal1 and Hyal2 in different tumors is still contradictory and also the angiogenic effect of HA fragments must be regarded.

Selective and potent hyaluronidase inhibitors are not available so far. On one hand such compounds could be of potential therapeutical value as drugs. On the other hand hyaluronidase inhibitors are required as pharmacological tools to

investigate the physiological and pathophysiological role of the enzyme and its substrate hyaluronan.

1.4 Inhibitors of hyaluronidases

The first studies documenting the existence of hyaluronidase inhibitors emerged half a century ago[103-105]. Already in 1952, inhibitory effects on bovine testicular hyaluronidase were reported for iron, copper and zinc salts, heparin, polyphenols and flavonoids[106]. Based on the structural similarity to HA, heparin and heparan sulfate have been investigated as inhibitors of hyaluronidase but inhibition was observed only at concentrations much higher than physiological levels[107-109]. Inhibition of hyaluronidases was also described for other structurally related compounds, for instance, alginic acids comprising L-glucuronic acid and D-mannuronic acid[110], *O*-sulfated glycosaminoglycans in which fully sulfated substances showed the highest inhibitory activity[111], fully *O*-sulfated HA oligosaccharides[112] or dextran sulfate[113].

Further examples of inhibitors can be found in many classes of substances. For instance, some flavones and flavone analogs like apigenin and kaempferol inhibit hyaluronidase[114-119], but not selectively and only at millimolar concentrations[120]. Other natural products like saponins and sapogenins[121], norlignans[122] and extracts of plants or feces[109,123-128] reveal likewise weak inhibitory activity. Furthermore, it is well known that various anti-allergic drugs such as disodium cromoglycate (DSCG), tranilast and traxanox possess inhibitory effects on hyaluronidase[119,129]. Moreover, the anti-inflammatory drug indomethacin was found to inhibit hyaluronidase *in vivo*[130]. Other anti-inflammatory agents like glycyrrhizin[131], phenylbutazone and oxyphenbutazone[132] are also mentioned to weakly inhibit hyaluronidases. Recently, vitamin C[133], L-arginine derivatives[134] and *cis*-unsaturated fatty acids[135] were reported to inhibit a streptococcal hyaluronidase with IC_{50} values at (sub)millimolar concentration whereas lanostanoids[136] revealed IC_{50} values in the micromolar range. According to the literature, DSCG was one of the most potent inhibitors (IC_{50} value 29 µM[119]) of bovine testicular hyaluronidase. However, when we investigated DSCG as a

reference substance in our test system by using a turbidimetric assay (see chapter 3 and 5.5.3), the compound proved to be much less active on BTH with 50% inhibition at a concentration of 5.6 mM. Moreover, IC_{50} values of 1.5 mM (at pH 5) and 4.3 mM (at pH 7.4) were determined on the hyaluronate lyase of *S. agalactiae* strain 4755.

A comparison of the published data (% inhibition and IC_{50} values) of all aforementioned compounds is impossible because of differences in the applied test systems (*e.g*. incubation conditions, enzymes, enzyme concentrations and substrate concentrations). Owing to the increasing interest in hyaluronan and hyaluronidases, their physiological and pathophysiological role and their clinical applications selective and potent inhibitors are required as pharmacological tools and potential drugs as well, *e.g*. as new anti-fertility agent or as antimicrobial agent against penicillin-resistant bacteria like *S. pneumoniae*[137,138]. But it is apparent that the systematic search for inhibitors has barely begun.

Chapter 2

Objectives

To date, potent and selective inhibitors of hyaluronidases are not known. Such compounds are needed as pharmacological tools to study the physiological and pathophysiological role of hyaluronan and hyaluronidases. Moreover, such agents might be useful as drugs in the treatment of various diseases, *e.g*. cancer, arthroses or bacterial infections, or as contraceptives. The main goal of this thesis was the synthesis of hyaluronidase inhibitors starting either from structures suggested by molecular modeling or from already known putative inhibitors. The inhibitory effects of the considered molecules should be investigated on hyaluronate lyase from *S. agalactiae* and on the bovine testicular hyaluronidase as main representatives of the bacterial and mammalian enzymes, respectively.

Vitamin C, known to bind to the active site of bacterial hyaluronidase from *S. pneumoniae*, was envisaged as a core structure for potential hyaluronidase inhibitors. The first part of this doctoral project comprises the synthesis and pharmacological investigation of various ascorbic acid derivatives with increased lipophilicity. To gain insight into the enzyme-inhibitor interaction on a molecular level the crystal structure of vitamin C derivatives co-crystallized with the hyaluronate lyase of *S. pneumoniae* should be enlightened in cooperation with the group of Dr. Jedrzejas[†].

These days, one of the most promising strategies for the design and development of enzyme inhibitors is Computer-Aided Drug Design (CADD). CADD is

[†] The co-crystallization experiments have been carried out by Dr. Mark J. Jedrzejas (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA) and Dr. Daniel J. Rigden (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

accelerating the discovery of new lead compounds and their structural optimization. The elucidation of the three-dimensional structures of two bacterial hyaluronidases enabled a rational *de novo* design approach identifying 1,3-diacetylbenzimidazole-2-thione as a lead structure[‡]. Thus, the second part of this project deals with the optimization of the benzimidazole lead structure by structural modification and investigation of the inhibitory activities.

Recently, the binding mode of an alkyl-2-phenylindole, 1-decyl-2-(4-sulfamoyloxyphenyl)-1*H*-indol-6-yl-sulfamate, co-crystallized with *S. pneumoniae* hyaluronate lyase was determined by means of X-ray analysis[†]. By comparison of this binding mode with known structure-activity relationships of 2-phenylindoles, the putative binding mode of benzoxazole-2-thiones as bacterial hyaluronidase inhibitors was suggested[‡]. To verify this hypothesis a set of benzoxazole derivatives was synthesized and tested for inhibitory activity in the third part of this doctoral project. Additionally, the structure-activity relationships should be discussed in order to get more information for further developing inhibitors.

The fourth part of the project based on the binding mode of the 2-phenylindole as well. Derived from this model of molecular interaction substituted indole derivatives were prepared and pharmacologically investigated.

[‡] Molecular modeling studies have been carried out by *Alexander Botzki* as part of his PhD project, Universität Regensburg, 2004.

Chapter 3

Methods for the determination of hyaluronidase activity

3.1 Introduction

Since the discovery of the hyaluronidases in the year 1928[19], various methods for the determination of their enzymatic activity were devised. Many of them seem to be rarely used and are sparsely discussed in literature. Hynes and Ferretti[139] gave an overview on the applied methods, which should be applicable to hyaluronidases from either microbial or mammalian sources. They classified the different assays into spectrophotometric, radiochemical, fluorogenic, enzymo-immunological, plate (solid media) assays as well as chemical, physicochemical and zymographic analyses. Stern and Stern[140] discussed the advantages and limitations of the conventional methods in their article about an ELISA-like assay for hyaluronidase and hyaluronidase inhibitors. Recently, new assay methods were described for rapid determination of hyaluronidase activity. For instance, a combination of fluorescently labeled HA and gel filtration on high-performance liquid chromatography (HPLC) was used to examine the degree of digestion[141]. Moreover, a flow cytometric method detecting the decrease in fluorescence of substrate-coated beads[142], a fluorimetric Morgan-Elson assay[143] and quartz crystal impedance technique[144] were reported for measuring hyaluronidase activity.

All compounds synthesized within this doctoral project were investigated for their inhibitory effect on the enzymatic activity of *S.agalactiae* hyaluronate lyase strain 4755 and Neopermease®, a commercially available preparation of bovine

testicular hyaluronidase. A turbidimetric assay and/or a colorimetric assay were used to determine the inhibitory potency of the test compounds. The principles of both assays are described in the following.

3.2 Turbidimetric assay

According to the classification of Hynes and Ferretti[139] this assay is subsumed under the group of physicochemical assays. In general, the procedure is based on the turbidity, which results from addition of different reagents forming insoluble complexes with HA and their degradation products. Afterwards the optical density was photometrically determined.

In 1956 Di Ferrante[145] described a turbidimetric method for the determination of hyaluronidase activity. This procedure is based on the formation of insoluble complexes between cetyltrimethylammonium bromide (CTAB) and the residual high molecular weight substrate (mw > 8 kDa) after incubation with enzyme (Fig. 3.1).

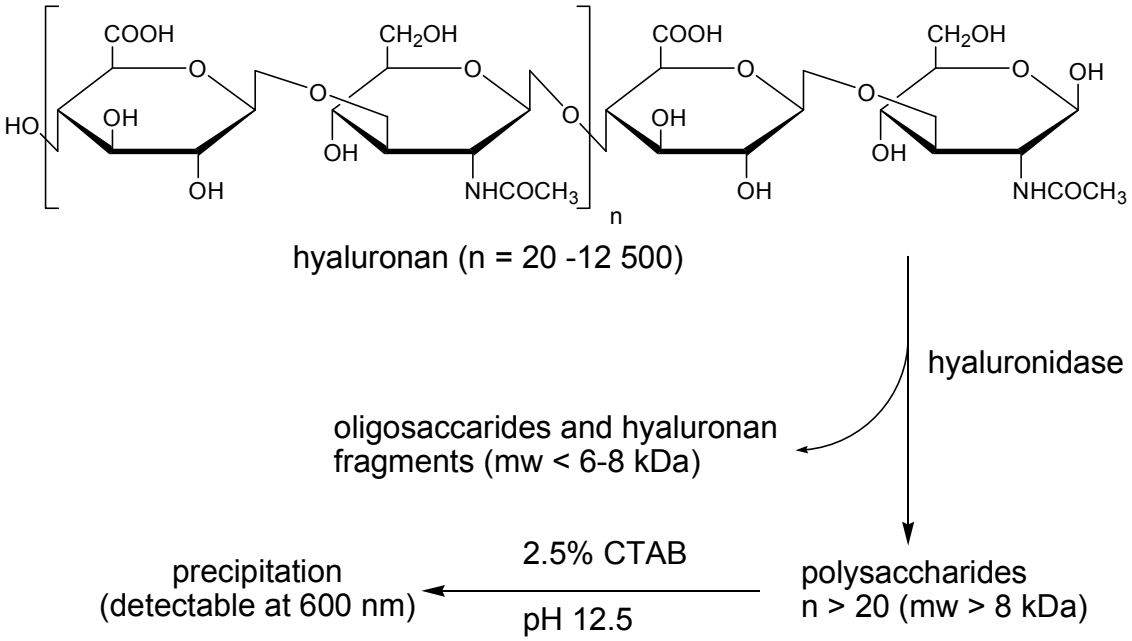


Fig. 3.1: Principle of the turbidimetric method described by Di Ferrante[145] for the determination of hyaluronidase activity

The turbidity is proportional to the amount of high molecular weight HA fragments. Therefore, the enzymatic activity can be quantified by turbidimetric measurements at 600 nm by means of reference samples. The assay is highly reproducible, fast and easy to perform. Moreover, the precipitation reagent is stable. This is in contrast to the classical turbidimetric method described by Kass and Seastone[146], who used unstable reagents including proteins. Furthermore, the CTAB reagent stops the enzymatic reaction and produces the turbidity at the same time. The detailed procedure of the modified method of Di Ferrante[145], which is established at our laboratory, as well as the calculation of the inhibitory activity of the examined compounds are described in the experimental part (see 5.5.3).

3.3 Morgan-Elson assay

For investigation of the potential enzyme inhibitors the hyaluronidase activity was quanitified using a colorimetric assay (Morgan-Elson assay). This assay, a chemical assay according to the classification by Hynes and Ferretti[139], is based on the methods of Gacesa *et al.*[147] and Reissig *et al.*[148] and reported in detail by Muckenschnabel *et al.*[149]. The reaction of terminal *N*-acetyl-D-glucosamine with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) results in a red colored product, which can be photometrically detected at 586 nm. Consequently, the hyaluronidase activity is determined by quantitation of the *N*-acetyl-D-glucosamine residues at the reducing ends of hyaluronan and HA fragments produced by enzymatic degradation.

Based on HPLC-MS studies Muckenschnabel *et al.*[149] suggested a mechanism for the Morgan-Elson reaction and a structure of the red colored product (Fig. 3.2). Under the reactions conditions (100 °C, pH 9) *N*-acetyl-D-glucosamine at the reducing end is cleaved off forming the chromogens I (α-configuration) and II (β-configuration). Subsequently, treatment with concentrated hydrochloric acid and glacial acid leads to an elimination of water. Finally, the chromogen III reacts with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to form the resonance-stabilized product possessing absorbance maxima at 545 nm and 586

nm. This red colored compound is sensitive to light and chemically rather unstable ($t_{1/2}$ < 1 min).

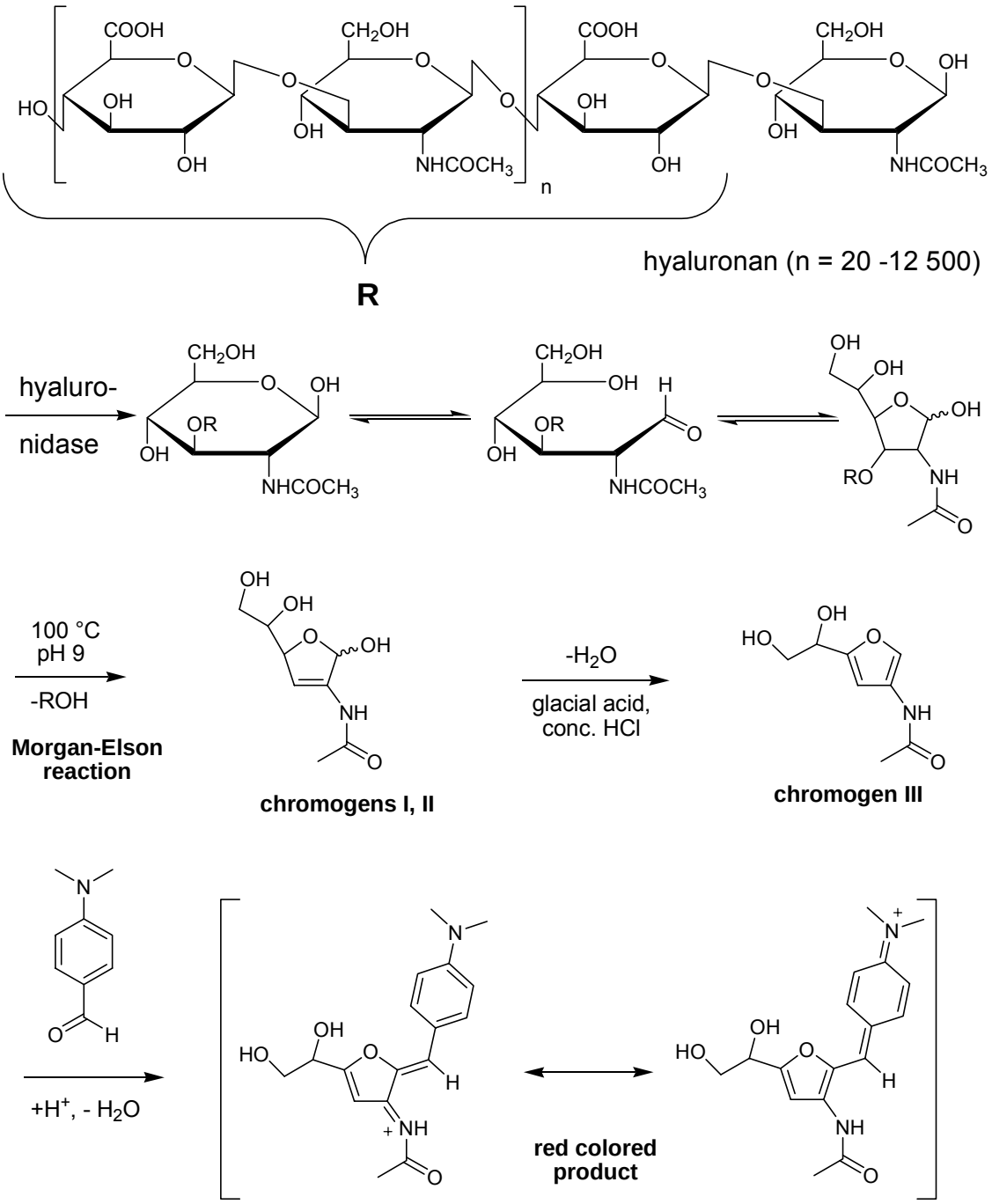


Fig. 3.2: Mechanism of the Morgan-Elson reaction – reaction of *N*-acetyl-D-glucosamine with *p*-dimethylaminobenzaldehyde resulting in the red colored product postulated by Muckenschnabel *et al.*[149]

This method is not applicable to the investigation of those hyaluronidase inhibitors which are reactive towards Ehrlich's reagent. For instance, the products

formed by reaction of indoles (cf. chapter 7.3) with dimethylaminobenzaldehyde are also detectable at 590 nm, so that the quantification of the red colored product of the Morgan-Elson assay is falsified. For details of the experimental procedure and the calculation of the inhibitory activity of the investigated compounds see section 4.5.3.

3.4 General conditions applied to the assays

3.4.1 Effect of organic solvent on enzymatic activity

Both the turbidimetric assay and the Morgan-Elson assay were performed in a buffered aqueous solution. Many of the tested potential inhibitors are poorly soluble under this conditions unless organic solvent is added. Therefore, different solvents like DMF, DMSO, ethanol or methanol, in which the compounds are readily soluble, were investigated for their influence on the enzymatic activity of the hyaluronidases. Salmen[120] determined the activity of hyaluronidase depending on the portion of organic solvent in the buffer. The effects of the aforementioned solvents on a bovine testicular hyaluronidase were negligible up to concentrations of 4%. Higher concentrations resulted in a weak to strong inhibition of the enzyme depending on the type of solvent. The effects of methanol, ethanol and DMSO on the bacterial enzyme $hylB_{4755}$ were also negligible at concentrations up to 4%, whereas the inhibitory effect of DMF was already significant at a concentration of 3%[120].

The effects of DMSO, DMF, methanol, ethanol and acetonitrile on the activity of hyaluronate lyase from *S. agalactiae* strain 4755 in the turbidimetric assay are illustrated in Figs. 3.3 and 3.4. At the pH optimum of $hylB_{4755}$ it is obvious that 3.7 % of DMSO or the two alcohols are well-tolerated as solvent (see Fig 3.3). By contrast, DMF and acetonitrile already exhibit a significant inhibition effect on the enzymatic activity at concentrations of 3%. The same tendency was observed for the influence of the five solvents on $hylB_{4755}$ at physiological pH as shown in Fig. 3.4.

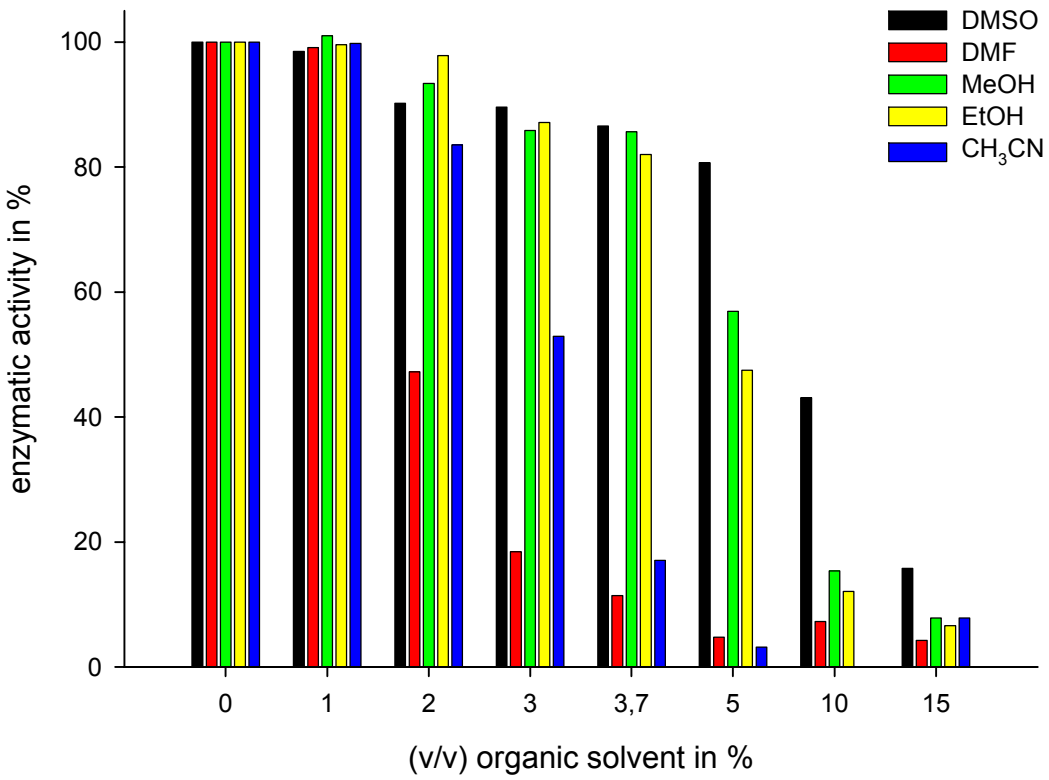


Fig. 3.3: Inhibitory effect of DMSO, DMF, methanol, ethanol and acetonitrile on $hylB_{4755}$ at pH 5

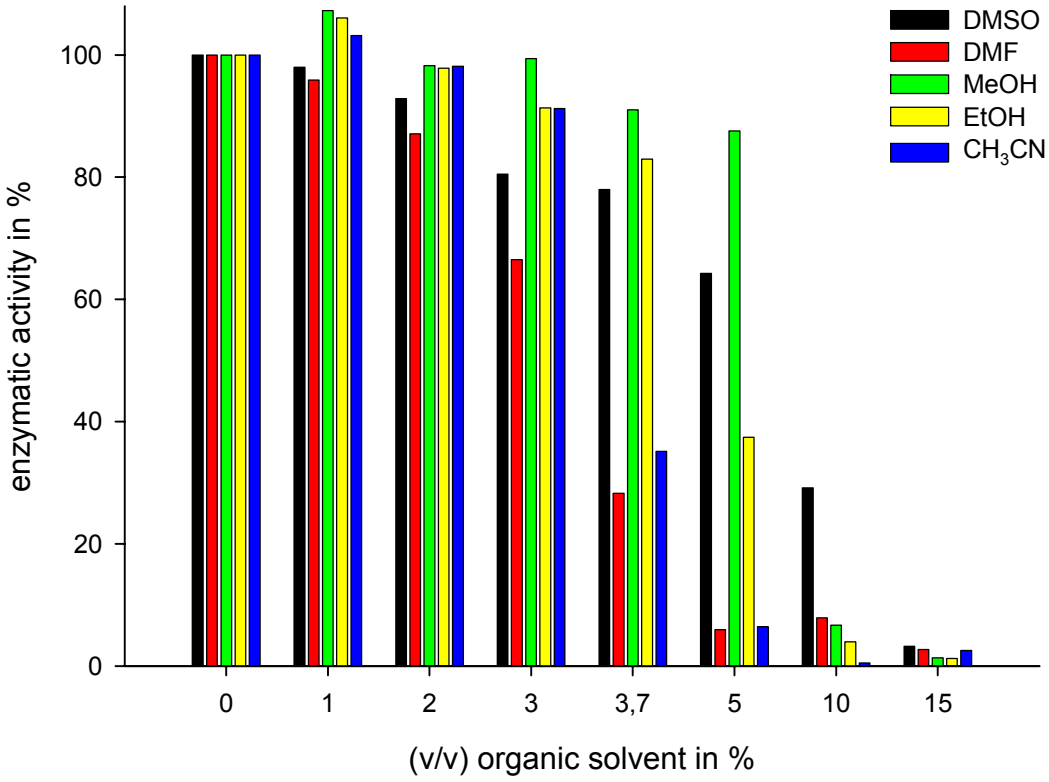


Fig. 3.4: Effect of DMSO, DMF, methanol, ethanol and acetonitrile on the activity of the bacterial hyaluronidase $hylB_{4755}$ at physiological pH (7.4)

With the exception of DMF, the investigated organic solvents were inactive on the activity of Neopermease®, a bovine testicular hyaluronidase, up to concen-

trations of 3.7 % as shown in Fig. 3.5. For the investigation of all hyaluronidase inhibitors described in this thesis, a percentage of 3.7 % of organic solvent in the buffer was used in the turbidimetric assay. DMSO was chosen as solvent since the test compounds were readily soluble in this solvent. Moreover, DMSO had no remarkable influence on the enzymatic activity of the two enzymes.

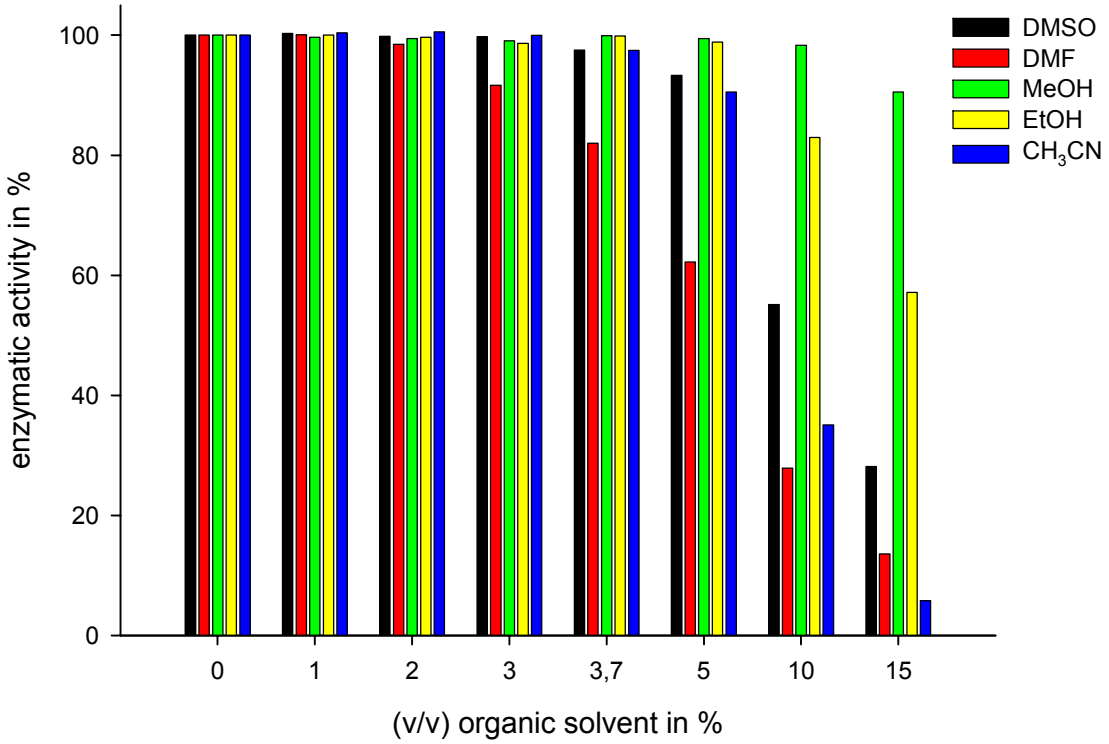


Fig. 3.5: Influence of DMSO, DMF, methanol, ethanol and acetonitrile on the activity of bovine testicular hyaluronidase (Neopermease®) at pH 5

In spite of the addition of DMSO to the buffer not all compounds remained completely dissolved during the assays at the applied concentrations. Hence, the results of the turbidimetric and colorimetric measurements to determine the enzymatic activity can be adulterated since the effective concentration of the test compounds may be lowered by precipitation, and undissolved compounds can cause a turbidity of the solution. Therefore, the solubilities of the inhibitors were tested in a separated experiment (cf. solubility tests in 5.5.3) and the maximum concentrations of the compounds in the assay were adapted accordingly to prevent precipitation.

3.4.2 Influence of the pH value on the enzymatic activity

The activities of the two hyaluronidases from different sources (bovine testis, *Streptococcus agalactiae*) are dependent on the pH value. Both aforementioned assays were applied to determine the enzyme activity under comparable conditions as a function of pH. As shown in Fig. 3.6, the results from both assays were in good agreement for the hyaluronate lyase. The bacterial enzyme exhibits its maximum activity at pH 5 and markedly reduced activity at physiological pH.

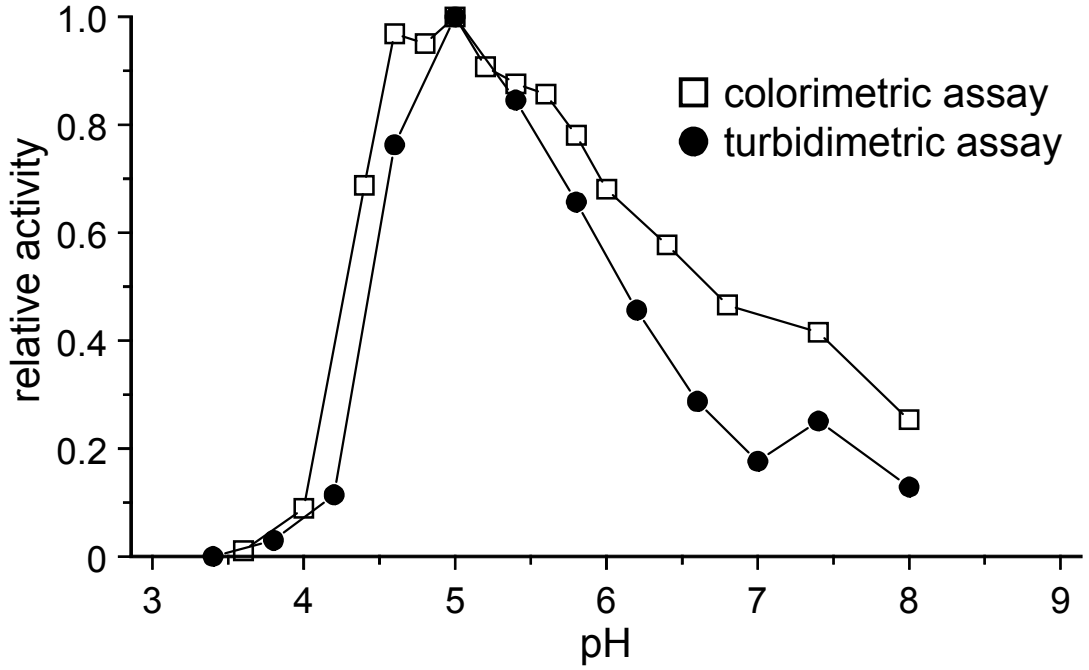


Fig. 3.6: pH-profile of hyaluronate lyase from *S.agalactiae* strain 4755 measured by the colorimetric and the turbidimetric assay[150]

By contrast, the pH profile of the bovine testicular hyaluronidase is almost inverted comparing the colorimetric and the turidimetric assay (see Fig. 3.7). This phenomenon can be ascribed to three enzymatically active fractions contained in the preparation Neopermease®[150].

To determine the enzyme selectivity the compounds were tested at the same pH value on the two enzymes: the inhibition was measured at pH 5, the pH optimum of $hylB_{4755}$, since both BTH and $hylB_{4755}$ exhibit a sufficiently high activity under these conditions. Additionally, the synthesized compounds were tested for inhibition of $hylB_{4755}$ at physiological pH.

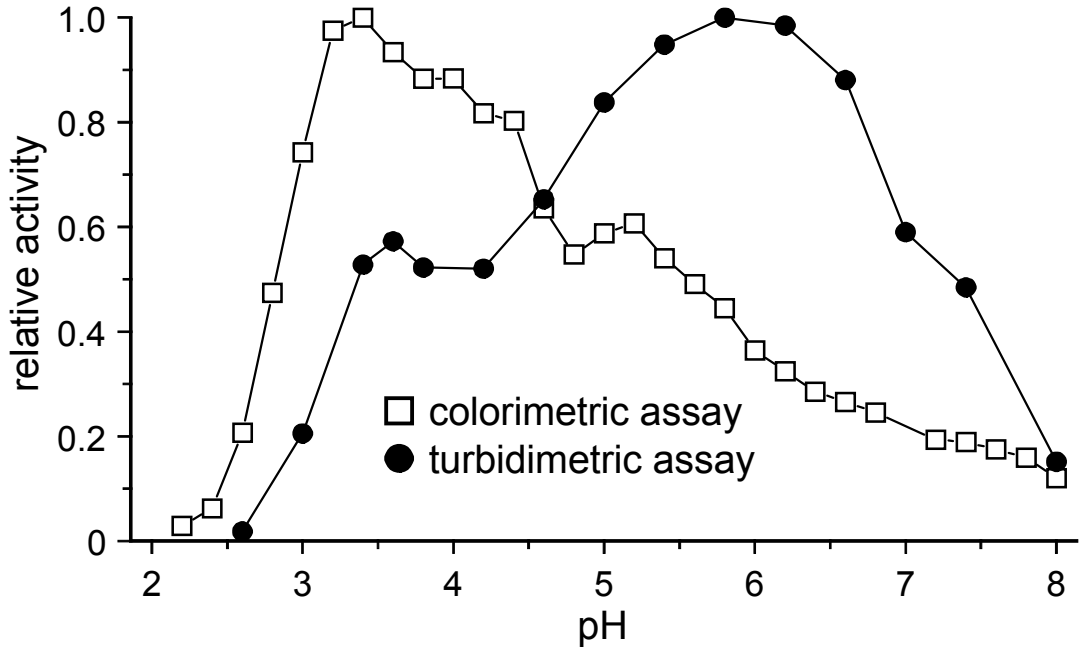


Fig. 3.7: Effect of pH on the activity of bovine testicular hyaluronidase (Neopermease®) as a function of the type of assay[150]

Chapter 4

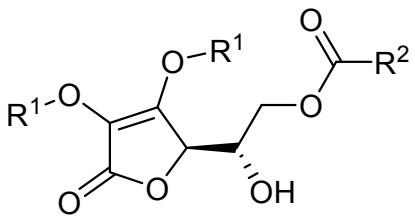
Vitamin C derivatives as hyaluronidase inhibitors

4.1 Introduction

The biological importance of L-ascorbic acid (vitamin C) was initially associated with scurvy, the symptoms of vitamin deficiency. Insights into the biochemistry of ascorbic acid revealed its key role in a variety of physiological processes including, for example, the production of collagen[151] or the function as an antioxidant and free radical scavenger. It plays also an important role in activating peptide hormones, regulating cell division and growth[133]. Furthermore, it acts as an electron donor for several enzymatic reactions, is implicated in host defense mechanisms[152] and is discussed to be involved in the prevention of various diseases[151]. Moreover, vitamin C and its derivatives are reported to possess antitumor and antiviral activities[153]. Abell *et al.*[154] found that L-ascorbic acid is an inhibitor of various α-amylases. But, the mechanisms of vitamin C interaction with proteins and enzymes are still largely unknown. The first observed protein-ascorbic acid interaction at the molecular level was found in the crystal structure of D-xylose isomerase (pdb-file: 1XID)[155].

Vitamin C was reported to be an inhibitor of bovine testicular hyaluronidase[51]. Recently, Li *et al.*[133] elucidated the crystal structure of the bacterial hyaluronidase hylSpn in complex with L-ascorbic acid (pdb-file: 1F9G). It was confirmed that vitamin C binds to the active site of the hylSpn. However, the hyaluronidase inhibitory activity is rather low (IC_{50} value of approximately 5.8 mM[133]). The bound compound provided 25 interactions with 7 amino acid residues of the

enzyme. Trp292 and Tyr408, which is one of the three key catalytic residues in the hyaluronan degradation, formed the most interactions confirming that the aromatic patch and the catalytic group of the active center are involved in binding of vitamin C. Moreover, it was indicated that hydrophobic interactions play an important role inside the active site. Thus, vitamin C derivatives with increased hydrophobicity could be stronger inhibitors of bacterial hyaluronidase. This prompted us to synthesize various ascorbic acid derivatives with lipophilic substituents (see Fig. 4.1).



R^1 = H, alkyl, benzyl
R^2 = alkyl, benzyl

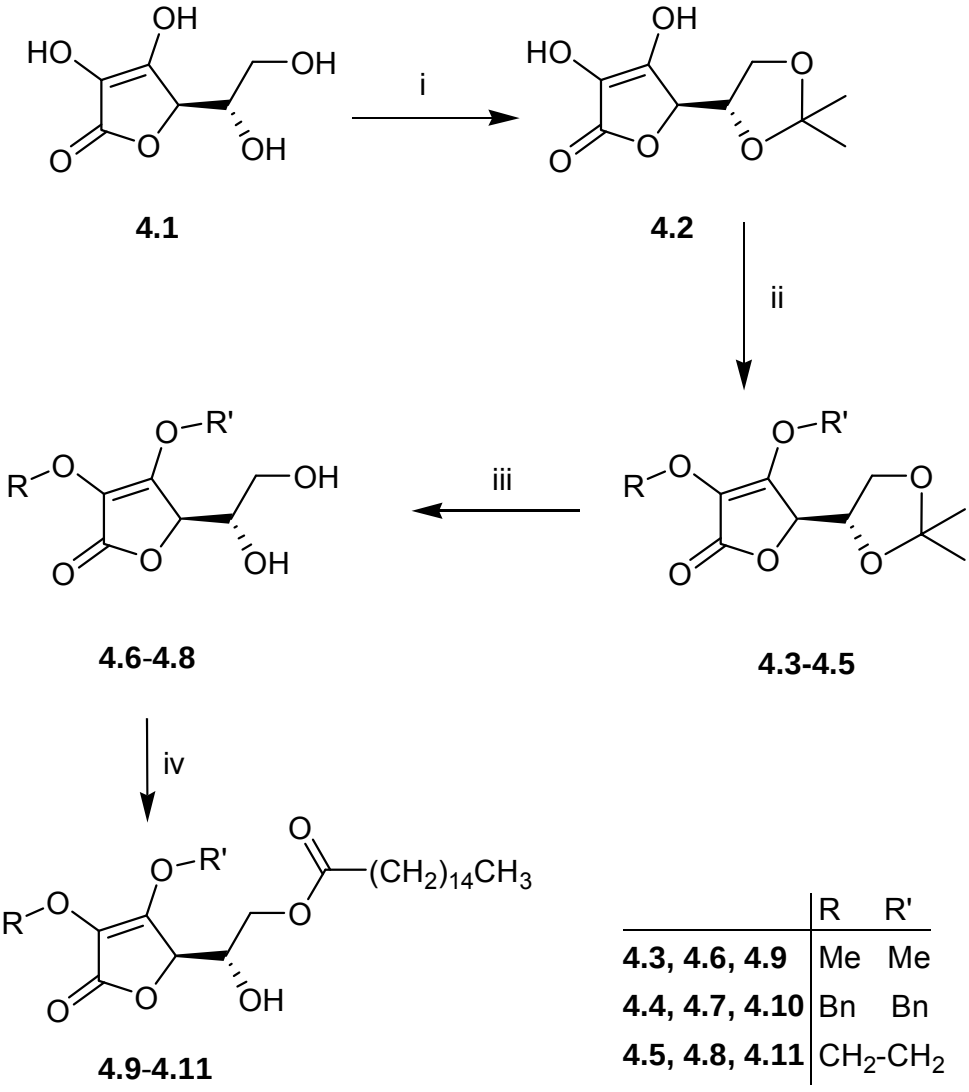
Fig. 4.1: Target compounds

4.2 Chemistry

The 6-*O*-acylated ascorbic acid derivatives **4.18-4.24** were accessible *via* the synthetic route illustrated in Schemes 4.1 and 4.2. Using L-ascorbic acid as starting material, the four hydroxyl groups must be protected in a way which allows selective deprotection followed by 6-*O*-acylation. Therefore, an inverse protecting strategy was chosen by initially protecting the diol moiety in positions 5 and 6 as acetonide. Afterwards, the enediol system was protected by etherification with protecting groups (PG), which are stable under the acidic conditions used to cleave the isopropylidene PG. In the next step, the ester group was selectively introduced by selective esterification at the primary alcohol group. At last, the PG of the enediol system was cleaved to obtain the various 6-*O*-acylated ascorbic acids.

Ascorbic acid derivatives **4.9**-**4.11** were synthesized according to a modified four-step reaction sequence following a procedure described by Sanders[156] as shown in Scheme 4.1. In the first step, ascorbic acid was converted into **4.2** by stirring in anhydrous acetone in the presence of catalytic amounts of acetyl chloride. The different PGs of the enediol system were introduced with the appropriate halo compounds using potassium carbonate as base in DMF. In the

case of **4.4**, the nucleophilic substitution worked only when using the more reactive benzyl bromide at 40 °C instead of benzyl chloride at 60 °C. By treatment with acetic acid (50 %) in methanol at 80 °C for several hours, the isopropylidene protecting group was removed yielding the vitamin C derivatives **4.6**-**4.8**.



	R	R'
4.3, 4.6, 4.9	Me	Me
4.4, 4.7, 4.10	Bn	Bn
4.5, 4.8, 4.11	CH_2-CH_2	

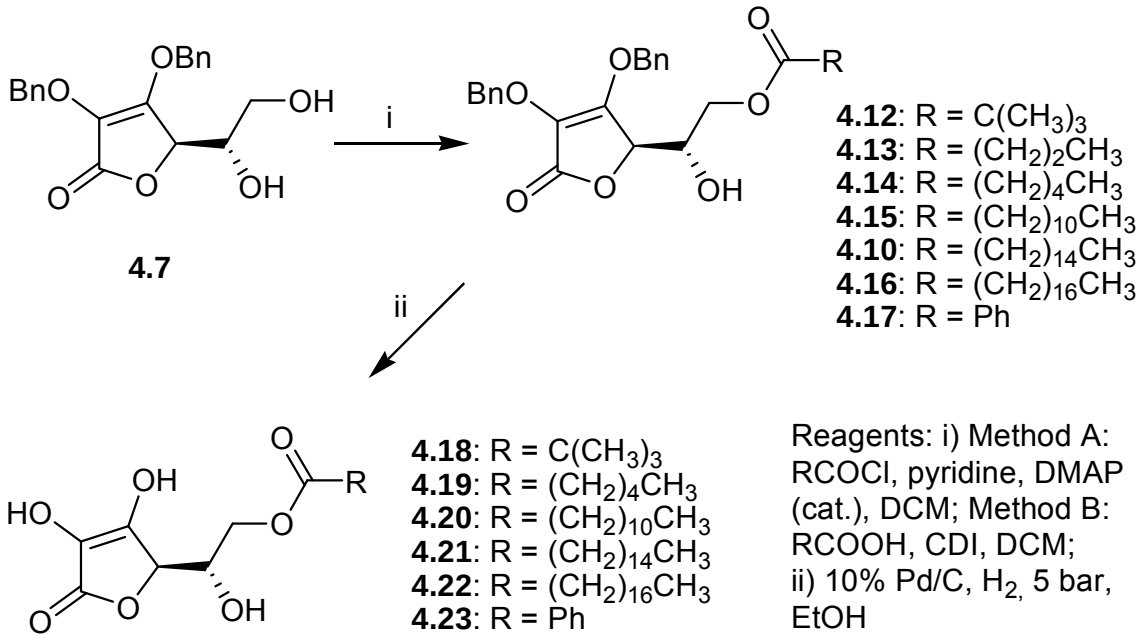
Reagents: i) abs. acetone, AcCl(cat.); ii) K_2CO_3, RI or RBr or 1,2-dibromoethane, DMF; iii) 50% HAc, MeOH, 80 °C; iv) pyridine, $CH_3(CH_2)_{14}COCl$, DMAP(cat.)

Scheme 4.1: Synthetic pathway to various 6-*O*-palmitoyl ascorbic acid derivatives

Finally, the *O*-acylation at the primary alcohol function was performed in dichloromethane with palmitoyl chloride and pyridine as base in the presence of the acylation catalyst DMAP resulting in the 6-*O*-palmitoyl ascorbic acid deriva-

tives **4.9**-**4.11**. For the synthesis of **4.11**, pyridine was used both as solvent and as base since **4.8** was only weakly soluble in dichloromethane.

Various 6-*O*-acylated ascorbic acids were accessible following the strategy depicted in Scheme 4.2. For this purpose, the enediol system was benzyl-protected since the solubility of the starting compound **4.7** in dichloromethane (DCM) was improved compared to **4.6** and **4.8**. Benzyl-protected ascorbic acid **4.7** was converted into 6-*O*-acylated derivatives **4.12**-**4.17** *via* two methods. Either **4.7** was treated with the pertinent acid chloride, pyridine (as base) and catalytic amounts of DMAP in anhydrous DCM, or the corresponding carboxylic acid was treated with **4.7** in anhydrous DCM using CDI as coupling reagent. The benzyl ether was cleaved by hydrogenolysis (H_2/Pd-C) resulting in high yields of the 2-*O*-,3-*O*-deprotected ascorbic acid derivatives **4.18-4.23**.



Scheme 4.2: Conversion of **4.7** into 6-*O*-acylated ascorbic acids

4.3 Results and discussion

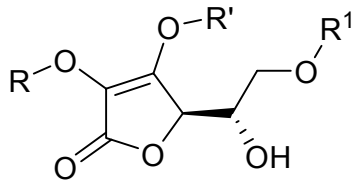
The inhibitory effect of the L-ascorbic acid derivatives on the activity of the hyaluronidases were examined both in a turbidimetric assay and in the Morgan-Elson assay (see chapter 3). To investigate the selectivity of these compounds both assays were performed with a bacterial hyaluronidase from *S.agalactiae*

($hylB_{4755}$) and either Hylase Dessau® or Neopermease®, which are hyaluronidase preparations from bovine testis.

4.3.1 Inhibitory activity of L-ascorbic acid derivatives on hyaluronidases determined with a turbidimetric assay

The turbidimetric method was used to determine the inhibitory activity of the vitamin C derivatives on $hylB_{4755}$ at pH optimum (pH 5) and at pH 7.4.

Table 4.1: Inhibitory effect of L-ascorbic acid derivatives on hyaluronate lyase from *S.agalactiae* strain 4755 and bovine testicular hyaluronidase (Neopermease®) (turbidimetric assay)



No.	R	R'	R^1	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
				pH = 5.0	pH = 7.4	pH = 5.0
4.1	H	H	H	6100 ± 100	inactive[b]	inactive[c]
4.6	CH_3	CH_3	H	inactive[d]	inactive[d]	inactive[d]
4.7	Bn	Bn	H	355.1 ± 59.2	1114 ± 72	inactive[d]
4.8	CH_2-CH_2		H	24 % (2000)	inactive[d]	inactive[d]
4.9	CH_3	CH_3	$CO(CH_2)_{14}CH_3$	5 % (160)	inactive[e]	inactive[e]
4.10	Bn	Bn	$CO(CH_2)_{14}CH_3$	inactive[e]	inactive[e]	inactive[e]
4.11	CH_2-CH_2		$CO(CH_2)_{14}CH_3$	32 % (190)	49.34±6.6	inactive[e]
4.18	H	H	$COC(CH_3)_3$	43 % (1100)	13 % (1100)	inactive[f]
4.19	H	H	$CO(CH_2)_4CH_3$	475 ± 16.1	1077 ± 27	inactive[d]
4.20	H	H	$CO(CH_2)_{10}CH_3$	6.71 ± 0.28	14.40 ± 1.37	219.0 ± 10.7
4.21	H	H	$CO(CH_2)_{14}CH_3$	4.22 ± 0.13	6.57 ± 0.07	56.5 ± 1.31
4.22	H	H	$CO(CH_2)_{16}CH_3$	0.93 ± 0.11	3.35 ± 0.25	38.6 ± 1.10
4.23	H	H	COPh	131.6 ± 5.9	459.6 ± 12.8	33 % (1430)

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at inhibitor concentration given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 13 mM, [c] at concentrations ≤ 100 mM, [d] at concentrations ≤ 2 mM, [e] at concentrations ≤ 200 µM, [f] at concentrations ≤ 1.1 mM.

Furthermore, in order to check the selectivity of the L-ascorbic acid derivatives for the bacterial enzyme, the inhibitory activity of the compounds was also tested on bovine testicular hyaluronidase using the preparation Neopermease® at pH 5.

Recently, vitamin C (**4.1**), reported to inhibit the bovine testicular hyaluronidase[52], was found to be also a weak inhibitor of the hyaluronate lyase from *S. pneumoniae* (IC_{50} value: 5.8 mM)[133]. It was confirmed by X-ray analysis that L-ascorbic acid binds to the active site of the bacterial hyaluronidase from *S. pneumoniae*. Hydrophobic interactions of **4.1** with two residues, Trp292 and Tyr408, within the catalytic cleft of *S. pneumoniae* play an important role for binding of **4.1**. Since hydrophobic interactions have a great influence on the substrate-enzyme interaction in aqueous solution as well[5] various L-ascorbic acid derivatives with increased lipophilicity were synthesized as potential hyaluronidase inhibitors (see Table 4.1).

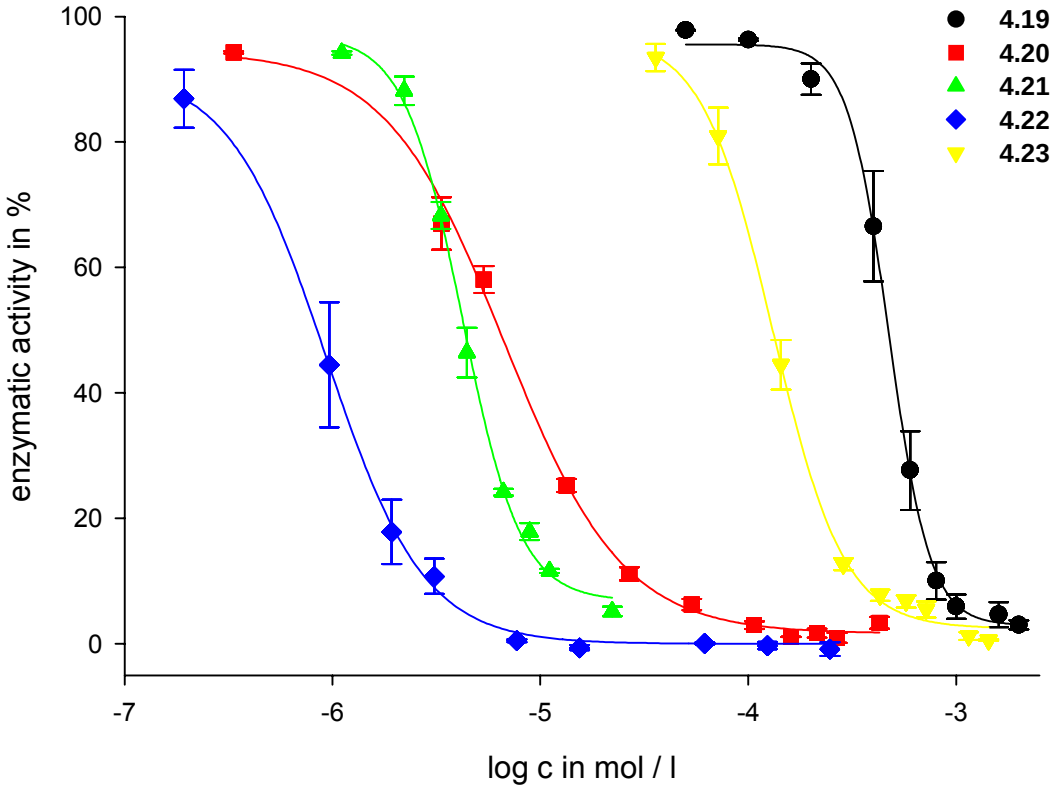


Fig. 4.2: Enzymatic activity of hyaluronate lyase of *S.agalactiae* in the presence of compounds **4.19**-**4.23** at optimum pH (5)

In contrast to Farr *et al*.[52], but in accordance with Li *et al*.[133]., we found that vitamin C at concentrations up to 100 mM did not inhibit the bovine testicular hyalu-

ronidase (BTH) in the turbidimetric assay. However, the bacterial enzyme ($hylB_{4755}$) was weakly inhibited by **4.1** at pH 5, whereas at physiological pH no inhibitory effect was found at concentrations ≤ 13 mM. Interestingly, vitamin C appeared to be an activator of $hylB_{4755}$ at concentrations higher than 4 mM at physiological pH. In opposition to results of Okorukwu and Vercruysse[157], vitamin C (**4.1**) did not affect the degradation of HA under our test conditions. Structural modifications of the enediol system as in **4.6** and **4.8** were not tolerated whereas the 2-*O*-,3-*O*-dibenzylated analog **4.7** was more active than **4.1**. **4.7** is an inhibitor of the hyaluronate lyase from *S. agalactiae* both at optimum pH and at physiological pH (IC_{50} values of 355 µM, and about 1.1 mM, respectively). This is in agreement with the hypothesis that the potency of hyaluronidase inhibitors derived from vitamin C may be increased by additional hydrophobic interactions with amino acids in the active site. By contrast, the bovine enzyme was not affected by **4.7** at concentrations up to 2 mM. The structurally related compounds **4.6** and **4.8** showed no notable inhibition of BTH and of $hylB_{4755}$ up to the denoted concentrations.

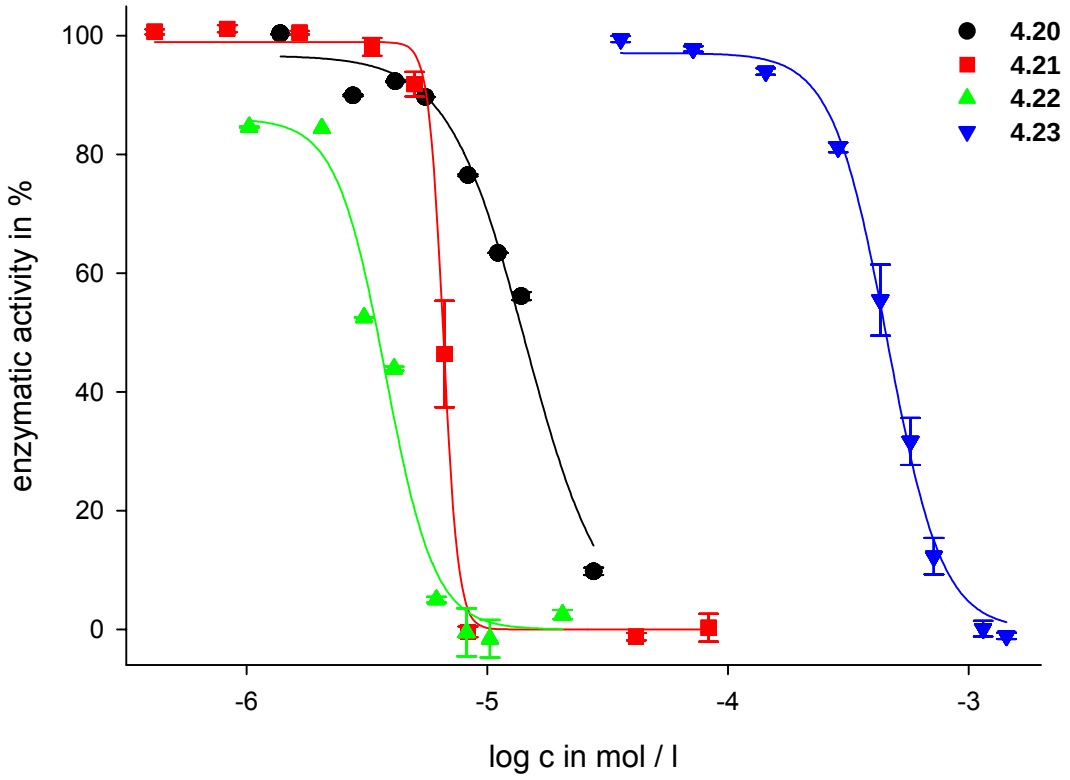


Fig. 4.3: Inhibitory effect of **4.20**-**4.23** on $hylB_{4755}$ at physiological pH (7.4)

At the optimum pH of $hylB_{4755}$, the L-ascorbic acid derivatives **4.19**-**4.23** proved to be inhibitors with IC_{50} values in the lower micromolar range (IC_{50} values of

475 µM (**4.19**), 6.7 µM (**4.20**), 4.2 µM (**4.21**), 0.9 µM (**4.22**) and 131 µM (**4.23**); see Fig. 4.2). Compound **4.18** induced 43 % inhibition at a concentration of 1.1 mM. The extension of the acyl residue in position 6 (**4.19-4.23**) further increased the inhibitory potency compared to vitamin C – presumably as the result of additional hydrophobic interactions with the enzyme. This tendency was confirmed at pH 7.4, although the inhibitory activity of the vitamin C derivatives **4.18**-**4.23** was a little weaker than at pH 5 (IC_{50} values: 1.1 mM (**4.19**), 14.4 µM (**4.20**), 6.6 µM (**4.21**), 3.4 µM (**4.22**) and 460 µM (**4.23**); see Table 4.1 and Fig. 4.3).

When tested on the bovine testicular hyaluronidase at pH 5 the vitamin C derivatives **4.18** and **4.19** did not affect the enzyme activity at the concentrations used. For compounds **4.20**-**4.22** bearing longer alkanoyl chains at position 6-*O* of the vitamin C skeleton, IC_{50} values of 219 µM (**4.20**), 57 µM (**4.21**) and 39 µM (**4.22**) were determined (see Table 4.1 and Fig. 4.4).

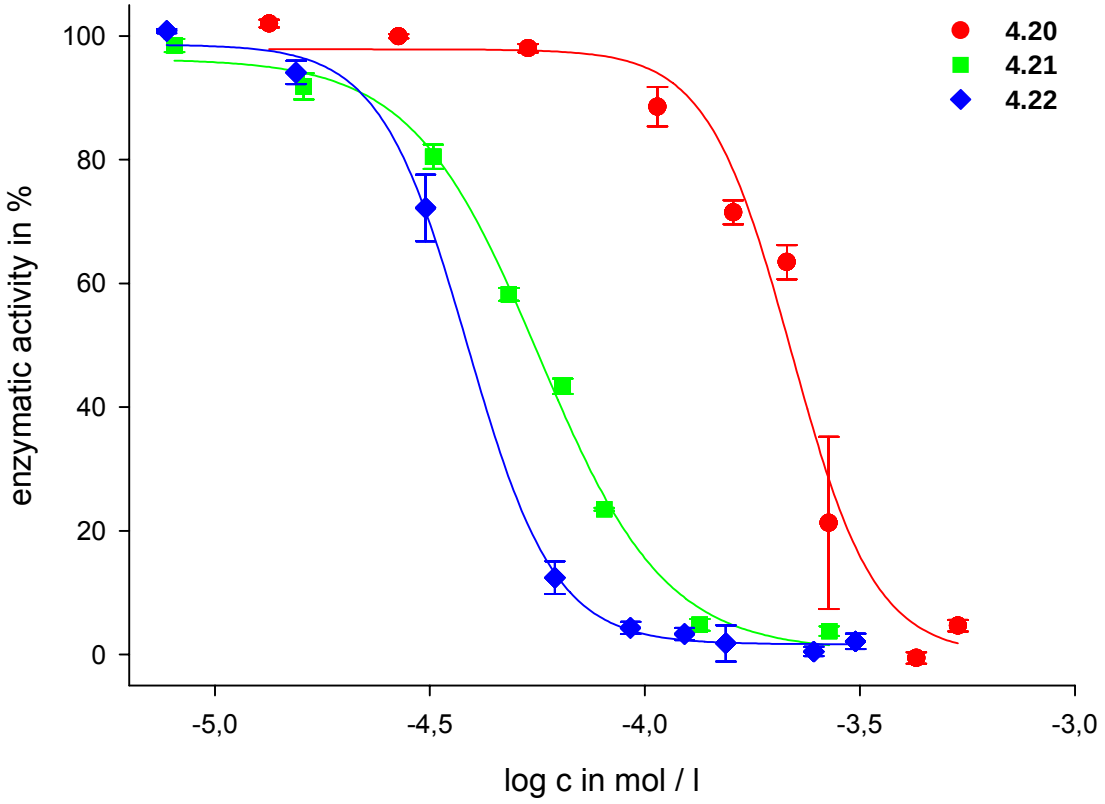


Fig. 4.4: Inhibition of bovine testicular hyaluronidase (Neopermease®) by compounds **4.20**-**4.22**

Obviously, the length of the alkanoyl chain can regulate the selectivity of the inhibitors for the bacterial enzyme vs. BTH. For instance, compound **4.20** was

about 33-fold more potent on $hylB_{4755}$ compared to BTH, whereas the shorter homologs were inactive at BTH. For vitamin C benzoate (**4.23**) only a rather weak inhibition by 33% was determined at a concentration of 1.4 mM.

Structural modifications at the hydroxy groups of the enediole system of the strong hyaluronidase inhibitor **4.21** (see **4.9**-**4.11**) abolished the inhibitory effect on BTH (the inhibitor concentrations were limited by the solubility of **4.9**-**4.11** in the buffer). Neither at optimum pH nor at physiological pH compounds **4.9** and **4.10** inhibited $hylB_{4755}$. Compound **4.11**, in which the oxygens of the enediole moiety are linked by an ethan-1,2-diyl group, represents an exception among the vitamin C derivatives listed in Table 4.1: **4.11** is the only compound with higher activity at pH 7.4 (IC_{50} value: 49 µM) than at pH 5 (32 % at a concentration of 190 µM). Compared to **4.21**, the inhibitory effect of **4.11** on $hylB_{4755}$ decreased, *e.g.* **4.11** showed a 7-fold weaker inhibitory activity against $hylB_{4755}$ at physiological pH.
Among the vitamin C derivatives compound **4.22** induced the most potent inhibition of $hylB_{4755}$ with an IC_{50} value of 0.9 µM at optimum pH. It was about 6500 times more active than the reference compound vitamin C and represents one of the most potent inhibitors of bacterial and bovine hyaluronidases described so far.

Bovine testicular hyaluronidase and $hylB_{4755}$ are very different enzymes, for instance, in terms of molecular mass, enzymatic mechanism and turnover. In order to obtain comparable data for the inhibition of the bacterial and the bovine enzyme and to gain information on the enzyme selectivity of the compounds the hyaluronidase assays had to be carried out at equimolar enzyme concentrations. Therefore, prior to the screening of the new hyaluronidase inhibitors, investigations were performed to identify equimolar concentrations of the bacterial enzyme ($hylB_{4755}$) and the bovine enzyme under the same reaction conditions. Moreover, at equimolar concentrations of the enzymes the incubation period was adjusted to obtain about the same substrate degradation over time. The assay was carried out at pH 5, the pH optimum of $hylB_{4755}$, since the BTH showed a sufficient activity at this pH (see chapter 3 for pH profiles). Equimolar amounts of $hylB_{4755}$ (11.4 ng) and Neopermease® (8 ng) were incubated at 7.5 min and 51 h, respectively, to give comparable substrate conversion. After set-

ting the test conditions of the turbidimetric assay to equiactive and equimolar amounts of BTH and $hylB_{4755}$ the potent inhibitor **4.21** was investigated. As shown in Table 4.2, the enzyme selectivity of compound **4.21** for $hylB_{4755}$ vs. BTH slightly dropped from a 13-fold to 6-fold selectivity under equimolar concentrations compared to equiactive enzyme concentrations.

Table 4.2: Inhibitory potency of **4.21** in the turbidimetric assay determined at equiactive enzyme concentrations of $hylB_{4755}$ and BTH or under equimolar concentrations of enzymes at pH 5

Enzyme concentration	$hylB_{4755}$, IC_{50} [µM][a]	BTH, IC_{50} [µM][a]
equiactive	4.22 ± 0.13	56.5 ± 1.31
equimolar	6.29 ± 0.18	36.7 ± 1.44

[a] inhibition of enzyme was expressed as $IC_{50} \pm$ SEM in µM, n = 4

The differences in the IC_{50} values obtained under equiactive and equimolar conditions were in good agreement. The more convenient assay under equiactive conditions was used in this doctoral thesis to determine enzyme selectivity.

4.3.2 Inhibition of hyaluronidases by L-ascorbic acid derivatives in the Morgan-Elson assay

In addition to the investigation of all L-ascorbic acid derivatives in the turbidimetric assay selected compounds were also investigated for inhibition of the hyaluronidases in the colorimetric assay to allow for a comparison of data from both experimental procedures.

In agreement with the results from the turbidimetric assay the purported inhibitory effect[51] of vitamin C (**4.1**) on the bovine enzyme was confirmed. At high concentrations (4.1 mM) compound **4.1** decreased the activity of the bacterial hyaluronate lyase only by approximately 25 % at pH 5. As also found for the turbidimetric assay, **4.1** acts as an activator of $hylB_{4755}$ at concentrations above 4 mM at physiological pH.

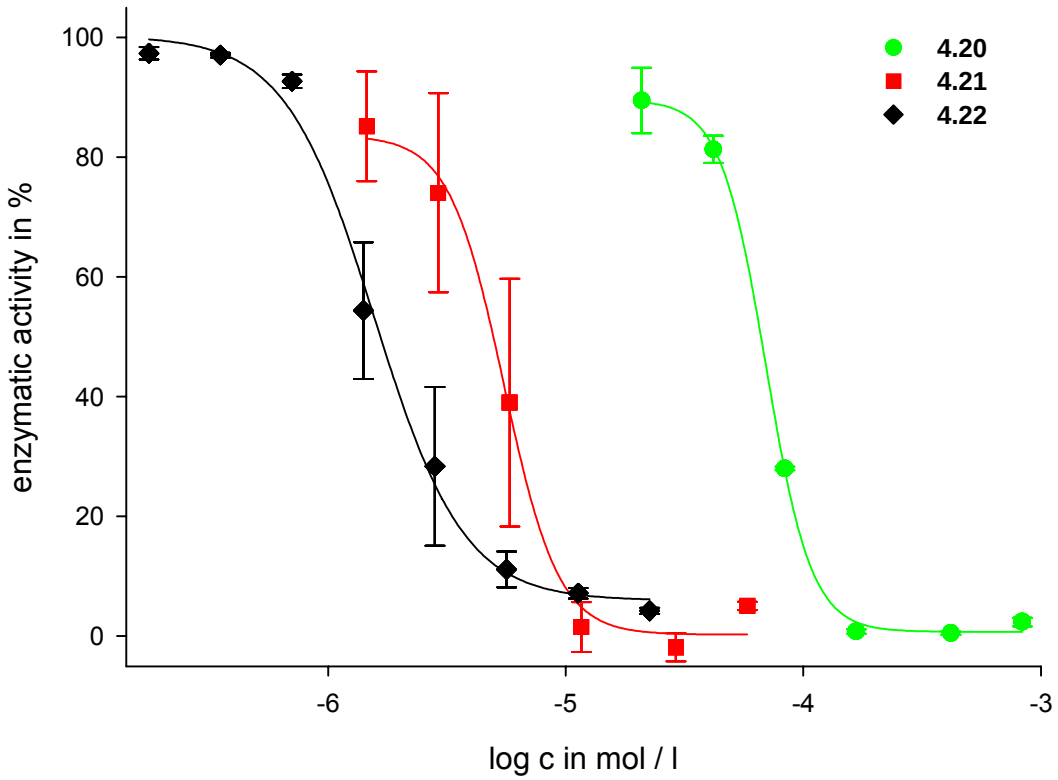


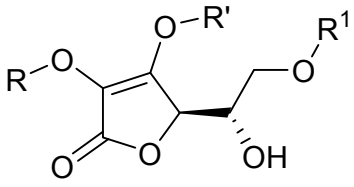
Fig. 4.5: Inhibitory activities of **4.20**-**4.22** determined on the bacterial enzyme ($hylB_{4755}$) at pH 5 (Morgan-Elson assay)

As summarized in Table 4.3, the inhibitory effect on $hylB_{4755}$ at optimum pH increased with the extension of the chain length from compound **4.20** to **4.22** (**4.20**: 69.1 µM, **4.21**: 5.5 µM, **4.22**: 1.6 µM; see Fig. 4.5). This is in accordance with the data from the turbidimetric assay. However, the IC_{50} values of **4.20**-**4.22** determined with the Morgan-Elson assay at pH 5 were higher than those found in the turbidimetric assay, whereas the opposite was found at physiological pH. The vitamin C derivatives **4.20**-**4.22** were about equipotent (IC_{50} values: 1.10 µM (**4.20**), 1.56 µM (**4.21**) and 1.50 µM (**4.22**)) at pH 7.4. Comparing the two assays at optimum pH, similar results were found for **4.18** and **4.23** which were nearly inactive in the Morgan-Elson assay on $hylB_{4755}$, whereas at physiological pH a stronger inhibition compared to the turbidimetric method was determined with IC_{50} values of 182 µM (**4.23**) and 188 µM (**4.18**).

The 6-*O*-acylated L-ascorbic acid derivatives **4.18** and **4.20**-**4.23** are much more potent inhibitors of the bacterial enzyme compared to the parent molecule,

for instance, the esters **4.20**-**4.22** are at least 1000 times more potent than vitamin C in the Morgan-Elson assay.

Table 4.3: Inhibitory activities in the Morgan-Elson assay determined on the *S. agalactiae* hyaluronate lyase at both physiological pH (7.4) and pH 5, and on bovine testicular hyaluronidase (Hylase Dessau®) at optimum pH (3.6) and physiological pH



No.	R	R'	R^1	$hylB_{4755}$, IC_{50} [µM] or % [a]		BTH, IC_{50} [µM][a]	
				pH = 5.0	pH = 7.4	pH = 3.6	pH = 7.4
4.1	H	H	H	25% (4100)	inactive[b]	inactive[b]	n.d.
4.9	CH_3	CH_3	$CO(CH_2)_{14}CH_3$	10% (160)	44% (160)	inactive[c]	inactive[c]
4.10	Bn	Bn	$CO(CH_2)_{14}CH_3$	inactive[d]	67% (150)	inactive[d]	inactive[c]
4.11	CH_2-CH_2		$CO(CH_2)_{14}CH_3$	n.d.	n.d.	n.d.	n.d.
4.18	H	H	$COC(CH_3)_3$	inactive[e]	187.9±28	inactive[e]	inactive[e]
4.20	H	H	$CO(CH_2)_{10}CH_3$	69.1±2.5	1.10±0.43	105±5	643±30
4.21	H	H	$CO(CH_2)_{14}CH_3$	5.50±0.90	1.56±0.84	60% (290)	120±60
4.22	H	H	$CO(CH_2)_{16}CH_3$	1.57±0.17	1.50±0.33	82.8±10	inactive[c]
4.23	H	H	COPh	inactive[f]	181.9±	inactive[f]	inactive[f]

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at inhibitor concentration given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 15 mM, [c] at concentrations ≤ 200 µM, [d] at concentrations ≤ 50 µM, [e] at concentrations ≤ 2.5 mM, [f] at concentrations ≤ 1.5 mM.

Neither **4.18** nor **4.23** induced an inhibitory effect on the activity of Hylase Dessau® at both physiological and optimum pH up to the denoted concentrations. Compounds **4.20**-**4.22** were equipotent at optimum pH of the BTH. Due to limited solubility complete inhibition of hyaluronidase could not be achieved by compound **4.21**. Therefore, only a percentage inhibition was indicated.

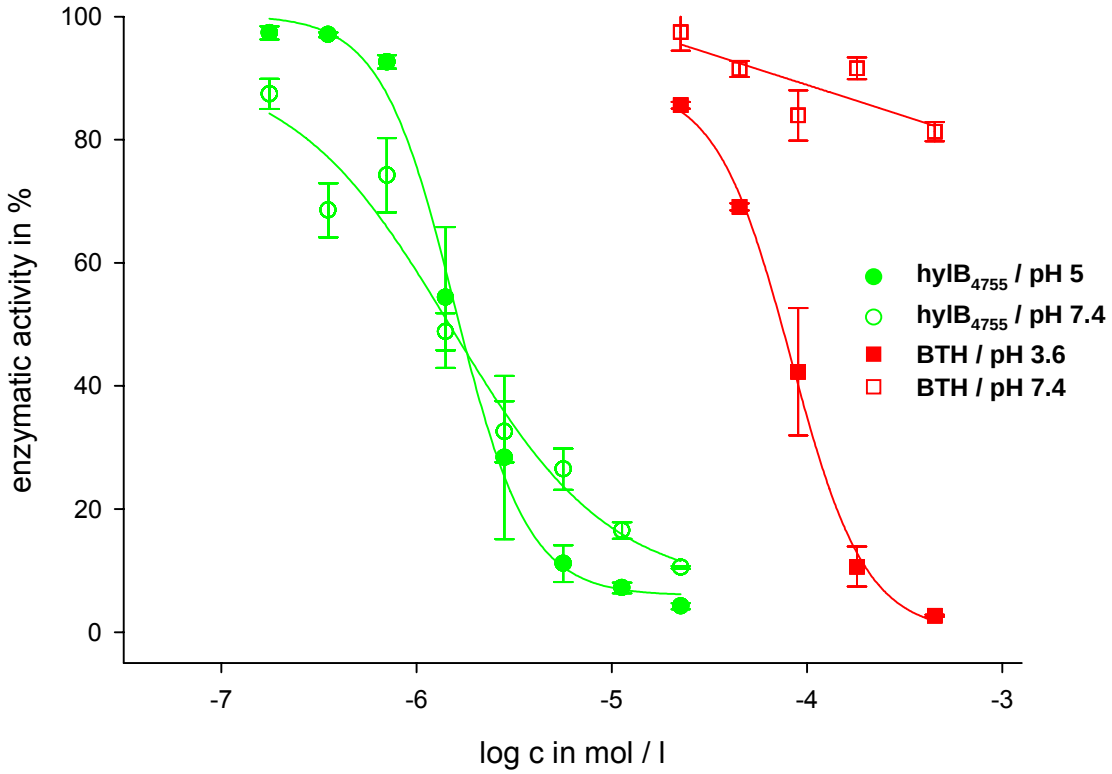


Fig. 4.6: Enzyme selectivity of **4.22**; inhibitory effect on BTH and $hylB_{4755}$ at physiological pH (7.4) and at optimum pH (BTH: pH 3.6; $hylB_{4755}$: pH 5.0) (data from Morgan-Elson assay)

At physiological pH, IC_{50} values of 643 µM (**4.20**) and 120 µM (**4.21**) were obtained. Surprisingly, compound **4.22** showed no inhibitory effect on Hylase Dessau® up to 200 µM indicating highest activity to reside in compounds bearing a hexadecanoyl residue in position 6-*O* of the vitamin C structure. The substances **4.9** and **4.10** were inactive on the BTH and exhibited only very weak and incomplete inhibition of $hylB_{4755}$ at pH 7.4 and pH 5.

The examined compounds **4.18** and **4.20**-**4.23** proved to be selective for $hylB_{4755}$ vs. BTH (see Table 4.3). As shown for compound **4.22** in Fig. 4.6, the selectivity is more pronounced when the assay is performed at pH 7.4 instead of optimum pH values.

In general, the results obtained with the two different assays were in good agreement and revealed the same tendencies, although one has to take into consideration the different principles of the two methods and the slightly differing enzyme concentration.

4.3.3 Structural elucidation of L-ascorbic acid-6-hexadecanoate – a potent hyaluronidase inhibitor

Most parts of this section were included in a recent publication by Botzki *et al*.[61].[§] Supported by X-ray analysis of the hylSpn-L-ascorbic acid complex[133] hydrophobic interactions with Trp292 and Tyr408 were supposed to play an important role for binding of vitamin C (**4.1**) in the catalytic site of the bacterial hyaluronidase from *S. pneumoniae* (hylSpn). Hence, vitamin C derivatives with increased hydrophobic properties should lead to stronger inhibitors. This hypothesis was confirmed as demonstrated in chapters 4.3.1 and 4.3.2. Subsequently, the potent inhibitor of $hylB_{4755}$ L-ascorbic acid-6-hexadecanoate (**4.21**), a highly effective antioxidant[158] and glutathione-S-transferase inhibitor[159], was investigated on its inhibitory effect on hylSpn and in cooperation with the work group of M. J. Jedrzejas a crystal structure of *S. pneumoniae* hyaluronate lyase, co-crystallized with **4.21** was determined at a 1.65 Å resolution (pdb-file: 1W3Y)[61]. The X-ray structure should shed light on the enzyme-inhibitor interactions resulting in proposals for more potent inhibitors. The crystallization experiments were performed by M. Nukui and M. J. Jedrzejas (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA). The X-ray structure of the complex was solved by D. Rigden (National Center of Genetic Resources and Biotechnology, Cenargen/Embrapa, Basília, D.F. 70770-900, Brazil).

4.3.3.1 Inhibition of hyaluronidases caused by L-ascorbic acid and L-ascorbic acid-6-hexadecanoate: a comparison

As already mentioned, it was reported that L-ascorbic acid inhibited the activitiy of hyaluronate lyase of *S. pneumoniae* (hylSpn) with an IC_{50} value of about 6 mM but no inhibitory effect could be observed on the bovine testicular hyaluronidase measured under the same reaction conditions[133]. These results were revised using our slightly different turbidimetric assay protocol using Neopermease®, a bovine testicular hyaluronidase, hylSpn and additionally, the bacte-

[§] Note: The author of this doctoral thesis made substantial contribution and is co-author of this publication by Botzki et al.

rial hyaluronidase from *S. agalactiae* strain 4755, the bacterial enzyme preferentially used in this doctoral project. At first, we had to standardize the experiments for all three enzymes by performing the turbidimetric assay with equiactive concentrations of all enzymes at pH 5. The enzyme activities in presence of **4.1** and **4.21** were determined as described elsewhere[61].

In agreement with the result described in the previous sections, the bacterial enzymes were weakly inhibited by vitamin C (**4.1**) with IC_{50} values of 6 mM for $hylB_{4755}$ and 35 mM for hylSpn, respectively, and **4.1** was inactive on the bovine enzyme up to concentrations of 100 mM. Consequently, vitamin C turned out to be a weak and selective inhibitor of the bacterial enzymes under the used reaction conditions. As depicted in Fig. 4.7, vitamin C ester **4.21** displayed a strong inhibitory effect on $hylB_{4755}$ with an IC_{50} value of 4 µM. On the related bacterial enzyme hylSpn and the bovine testicular hyaluronidase IC_{50} values of 100 µM and 56 µM, respectively, were determined. Compared to the parent compound **4.1**, 6-*O*-palmitoyl-L-ascorbic acid (**4.21**) is an up to 1500 times more potent inhibitor of bacterial and bovine hyaluronidases (see Table 4.4).

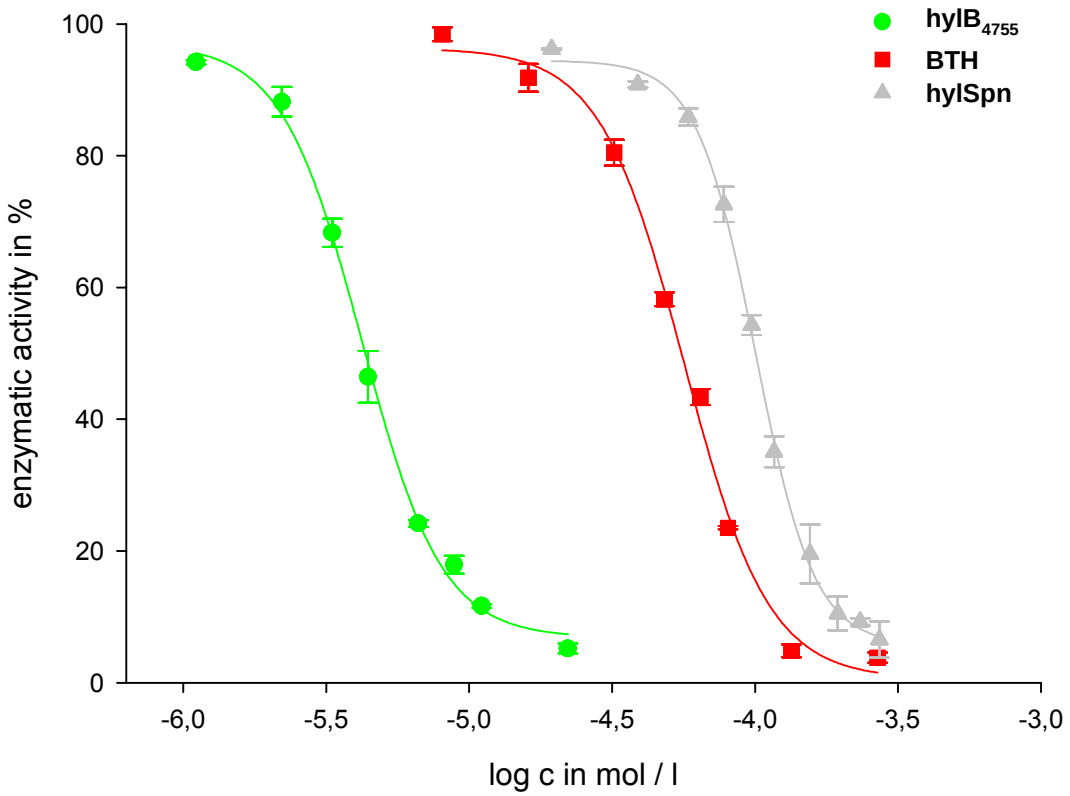


Fig. 4.7: Inhibition of $hylB_{4755}$, hylSpn and Neopermease® (BTH) by L-ascorbic acid-6-hexadecanoate (**4.21**)

Table 4.4: IC_{50} values ± SEM of vitamin C (**4.1**) and L-ascorbic acid-6-hexadecanoate (**4.21**) determined on equiactive concentrations of *S. pneumoniae* hyaluronate lyase, *S. agalactiae* hyaluronate lyase strain 4755 and BTH (Neopermease®) using a turbidimetric assay at pH 5

Compound	$hylB_{4755}$, IC_{50} [µM]	hylSpn, IC_{50} [µM]	BTH, IC_{50} [µM]
4.1	6100±100	34800±300	inactive (< 100 mM)
4.21	4.2±0.13	100.0±2.00	56.5±0.13

Furthermore, **4.21** is about 25-fold more active on $hylB_{4755}$ than on hylSpn whereas **4.1** revealed only a 6-fold selectivity. Moreover, the selectivity of **4.21** for the bacterial enzyme vs. BTH is only weak. With respect to the selectivity for the enzymes further explorations are necessary since the obvious selectivity cannot be quantitatively confirmed without the data for the K_m values of the applied HA substrate.

Finally, it can be concluded that additional hydrophobic enzyme-inhibitor interactions enhance the inhibitory effect as confirmed by the distinctly higher inhibitory activity of **4.21** on all investigated enzymes compared to **4.1**.

4.3.3.2 The binding mode of 6-*O*-palmitoyl-L-ascorbic acid to *S. pneumoniae hyaluronidase*

In order to determine the binding mode of compound **4.21** the X-ray structure of hylSpn co-crystallized with L-ascorbic acid-6-hexadecanoate was determined at 1.65 Å resolution (pdb-file: 1W3Y)[61]. This information should be useful to create a model for the interactions of the inhibitor with amino acid residues inside the active site of the enzyme and should facilitate the further development of more potent inhibitors. The hylSpn part of the complex structure contains an N-terminal α-domain and a C-terminal β-domain connected by a 10-residue linker and has only slight changes when compared with the native hylSpn[68] and the vitamin C-hylSpn[133] crystal structures. The active site of the enzyme is situated in the predominant cleft between α- and the β-domains. The catalytic mechanism as proposed[79,160-162] has been supported by structural determinations[66,67,71,160] and mutagenesis studies[67,71,163]. Catalysis is implemented at the narrowest part of the catalytic cleft in which Asn349, His399 and Tyr408 are mainly responsible

for it. Additionally, a hydrophobic patch consisting of Trp291, Trp292 and Phe343 contributes to precise positioning of the substrate.

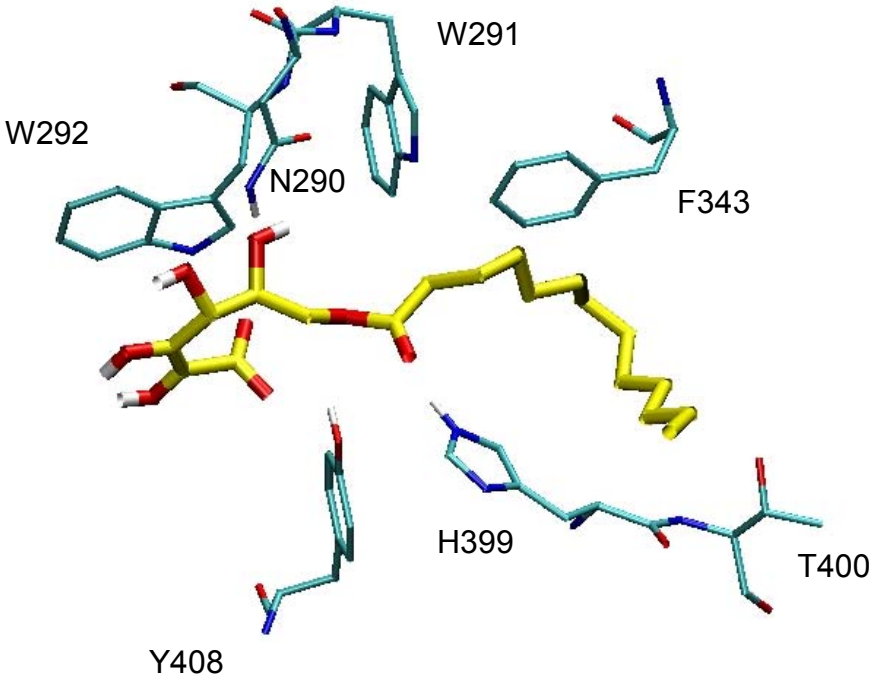


Fig. 4.8: Binding mode of L-ascorbic acid-6-hexadecanoate (**4.21**) at the active site of hylSpn. Distances for possible Hydrogen Bonds: N290-O5: 1.65 Å; Y408-COO: 1.78 Å

The electron density at the binding site enabled the satisfactory localization of **4.21** within the active site of hylSpn as shown in Fig. 4.8. The final X-ray structure offered additional difference electron density and elevated B-factors of the inhibitor (Table 4.3) suggesting the possibility of alternative binding modes of **4.21**. However, despite repeated attempts, only the conformation shown was well supported by the density. Surprisingly, the lactone ring of the inhibitor seems to bind in a ring-opened form which was evident from electron density, although the vitamin C portion of **4.21** binds to the same region as intact vitamin C[133]. Possibly, the ring-opened form was emerged during the crystallization time (Fig. 4.8). It is well-known that oxidation of L-ascorbic acid (**4.1**) gives L-dehydroascorbic acid, which is rapidly degraded under physiological conditions (pH 7.4) *via* L-diketogulonate to yield L-erythrulose and oxalate[164]. It is suggested that **4.21** is modified in a similar manner, but only the hydrolyzed lactone moiety was modeled into the electron density. However, it is unlikely that ring-opening

occurs during the performance of the enzymatic assay with respect to the much shorter time needed for the assay.

As shown in Fig. 4.8, **4.21** binds within the catalytic site of hylSpn explaining the inhibitory activity. With the exceptions of a hydrogen bond between the carboxylate group of the inhibitor and the hydroxyl of Tyr408 and others between the hydroxy groups at C-4 and C-5 and Asn290 the majority of protein-inhibitor interactions are hydrophobic. The hydrophobic face of the vitamin C portion lies flat on the side chain of Trp292, a member of the hydrophobic patch. Such an arrangement is quite common in complexes of carbohydrate-binding proteins with their ligands[165]. The palmitoyl moiety fits in a mainly hydrophobic surface crevice. With the exception of three terminal carbon atoms for which density did not permit modeling the aliphatic chain matches very well with the existent electron density. The palmitoyl group forms hydrophobic interactions with Trp291 and Phe343, both contributing to the hydrophobic patch along with His399 and Thr400.

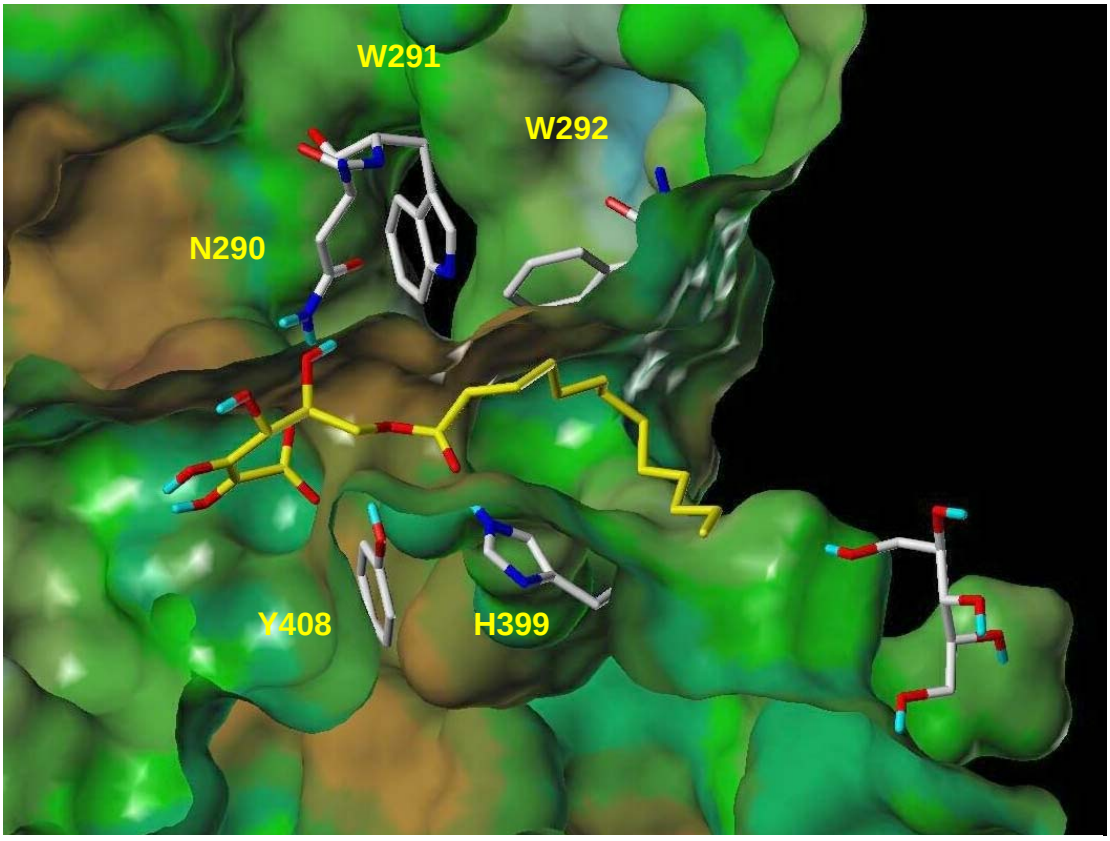


Fig. 4.9: Illustration of the binding site of **4.21** (carbon atoms are colored in yellow, oxygen atoms are colored in red and nitrogen atoms are illustrated in white) with Connolly surface (sample radius: 1.4 Å). The color encoding range from brown (high lipophilicity) to blue (polar).

None of the interactions is sufficiently close and geometrically suitable to be considered as a weak hydrogen bond involving methylene carbons of the palmitoyl group. Comparing the binding modes of **4.21** with that of a substrate-based hexasaccharide (pdb-file:1LOH)[70] there is an obvious overlapping of the binding sites. But the surface cleft that accommodates the palmitoyl group is not directly involved in substrate binding. Interestingly, one of the three well-ordered cryoprotectant xylitol molecules bound to hylSpn fits close to the end of the palmitoyl moiety. The distance between the penultimate resolved carbon of the palmitoyl group and atom O5 of xylitol is only 4.0 Å (Fig. 4.9). This position of the bound xylitol suggests that the affinity of the present inhibitor might be enhanced through addition of matching groups at the aliphatic end of the palmitoyl moiety.

4.4 Conclusions

L-Ascorbic acid (**4.1**), reported to inhibit the bovine testicular hyaluronidase, was described recently as a weak inhibitor of hyaluronate lyase of *S. pneumoniae* (hylSpn). It was confirmed by X-ray analysis that vitamin C binds to the active site of the bacterial enzyme suggesting that additional hydrophobic interactions might increase the inhibitory effect. Thus, a series of L-ascorbic acid derivatives, including compounds with increased lipophilic properties, was successfully synthesized and investigated for its inhibitory activity against hyaluronate lyase of *S. agalactiae* ($hylB_{4755}$) and bovine testicular hyaluronidase.

The 6-*O*-acyl derivatives of L-ascorbic acid, compounds **4.19**-**4.23**, exhibit a substantial inhibitory potency against hyaluronidases with preference for the bacterial enzyme. At optimum pH the IC_{50} values for inhibition of $hylB_{4755}$ range from 0.9 µM to 475 µM with highest activity residing in the esters of the long chain alkanoic acids. Compared with the inhibition at optimum pH, the IC_{50} values at physiological pH are slightly weaker but they reveal the same inhibitory tendency. Since the inhibitory effect increases by lengthening the alkyl chains of the inhibitors the hypothesis that additional hydrophobic interactions with the enzyme are crucial for enhancing the inhibitory affinity could be approved. Con-

sidering the bovine testicular hyaluronidase only the vitamin C derivatives **4.20**-**4.22** displayed a high inhibitory activity on the enzyme with IC_{50} values in the lower micromolar range. Among the studied vitamin C derivatives L-ascorbic acid-6-octadecanoat (**4.22**) proved to be the most potent inhibitor of hyaluronidases. **4.22** is up to 6500 times more potent than the parent compound **4.1** and is the most potent hyaluronate lyase inhibitor known to date. The aforementioned inhibitory data was obtained by using a turbidimetric assay for determining the enzyme activity. When using a colorimetric method which based on the Morgan-Elson reaction similar inhibitory effects were found for the vitamin C derivatives. Additionally, the elucidation of the X-ray structure of compound **4.21** provided new insights into enzyme-inhibitor interactions at the molecular level. This information should be useful for further development of more potent and selective inhibitors of hyaluronidases.

4.5 Experimental section

4.5.1 General conditions

Starting materials and solvents were purchased from Acros Organics (Belgium), Lancaster Synthesis GmbH (Germany), Sigma-Aldrich Chemie GmbH (Germany) or Merck (Germany). THF was distilled from NaH. Commercially available petroleum ether (60-80 °C) was distilled before use and dichloromethane was either predried over $CaCl_2$ or distilled from P_2O_5. All other solvents used were of analytical grade.

Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded on a Bruker AC-250, ARX-300 or ARX-400 NMR spectrometer with per-deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$). The chemical shift δ is given in parts per million (ppm) with reference to the chemical shift of the residual protic solvent compared to tetramethylsilane (TMS, δ = 0 ppm). “s” indicates a singlet, “d” a doublet, “dd” a doublet of doublet, “t” a triplet, “q” a quartet, “m” a multiplet and “br” a broad peak. 2D-NMR (H-H and C-H) COSY techniques were used in a few cases to support interpretation of 1D

spectra. The multiplicity of carbon atoms (^{13}C-NMR) were determined by DEPT 135 and DEPT 90 (distortionless enhancement by polarization transfer): "+" primary and tertiary carbon atom (positive DEPT 135 signal), "-" secondary carbon atom (negative DEPT 135 signal), "C_{quart}" quaternary carbon atom. Mass spectrometry analysis (MS) was performed on a Finnigan MAT 95 (PI-EIMS 70 eV, HR-MS), a Finnigan SSQ 710A (PI-EIMS 70 eV, CI-MS (NH_3)) and on a Finnigan ThermoQuest TSQ 7000 (ESI-MS, APCI-MS) spectrometer. The peak-intensity is indicated relatively to the strongest signal in %. Infrared spectra (IR) were recorded on a BRUKER TENSOR 27 spectrophotometer. The wave number is given in cm^{-1}. Melting points (Mp) were measured on a BÜCHI 530 electrically heated copper block apparatus using an open capillary and are uncorrected. Column chromatography was carried out using Merck silica gel SI 60 (0.063-0.200) and Merck silica gel 60 (0.040-0.063) for flash column chromatography. The department of microanalysis Regensburg carried out elemental analysis. Compounds were dried *in vacuo* (0.1-1Torr) at room temperature or with heating up to 50 °C for at least 24 h prior to submission for elemental analysis. Reactions were routinely monitored by thin layer chromatography (TLC) on Merck silica gel 60 F_{254} aluminum sheets and spots were visualized with UV light at 254 nm.

4.5.2 Chemistry

5-*O*-,6-*O*-Isopropylidene-L-ascorbic acid (4.2)

To L-ascorbic acid (**4.1**) (25.0 g, 141.95 mmol), dissolved in absolute acetone (160 ml), was added acetyl chloride (2.35 ml, 32.93 mmol). After stirring for 4.5 h at room temperature in a flask closed with a drying tube, the mixture was stored for 20 h in the refrigerator to get fully crystallization. The precipitated product was collected and washed thoroughly with cold acetone for three times. Drying over potassium carbonate remains a white solid.

Yield: 22.96 g (106.20 mmol, 75 %, white solid)

Mp: 193-195 °C (Lit. 201-203 °C[166])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.26 (s, 6H, $C(CH_3)_2$), 3.88 (dd, 1H, ^2J = 8.3 Hz, ^3J = 6.3 Hz, CH_2O-), 4.10 (dd, 1H, ^2J = 8.3 Hz, ^3J = 7.1 Hz, CH_2O-), 4.22-4.30 (m, 1H, CHO-), 4.70 (d, 1H, ^3J = 3.0 Hz, H-5), 8.47 (br, 1H, OH), 11.28 (br, 1H, OH)

$C_9H_{12}O_6$ (216.19)

2-*O*-,3-*O*-Dimethyl-(5-*O*-,6-*O*-isopropylidene)-L-ascorbic acid (4.3)

A solution of 5-*O*-,6-*O*-isopropylidene-L-ascorbic acid (**4.2**) (10.65 g, 49.26 mmol), potassium carbonate (7.6 g, 54.99 mmol) and iodomethane (6.8 ml, 109.23 mmol) in anhydrous DMF (150 ml) was heated under stirring at 60 °C for 7 h. The solvent was removed and the oily residue was picked up in dichloromethane (300 ml). The organic phase was washed with water (60 ml and 30 ml), dried over magnesium sulfate and evaporated *in vacuo*. The orange-brown oil was recrystallized from ethanol. After slow crystallization in the refrigerator, the precipitated product was collected, washed with a little amount of cold ethanol and dried *in vacuo*.

Yield: 4.66 g (19.08 mmol, 39 %, white, yellow shimmering needles)

Mp: 94 °C (Lit. 97 °C[156])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.25 (s, 6H, $C(CH_3)_2$), 3.71 (s, 3H, OCH_3), 3.88 (dd, 1H, ^2J = 8.3 Hz, ^3J = 5.6 Hz, CH_2O-), 4.08 (s, 3H, OCH_3), 4.10 (dd, 1H, ^2J = 8.3 Hz, ^3J = 7.1 Hz, CH_2O-), 4.27 (ddd, 1H, ^3J = 7.1 Hz, ^3J = 5.6 Hz, ^3J = 2.5 Hz, CHO-), 4.87 (d, 1H, ^3J = 2.5 Hz, H-5)

MS (CI-MS, NH_3): m/z (%) = 262 ($[M + NH_4]^+$, 100), 245 ($[M + H]^+$, 7)

$C_{11}H_{16}O_6$ (244.25)

2-*O*-,3-*O*-Dibenzyl-(5-*O*-,6-*O*-isopropylidene)-L-ascorbic acid (4.4)

A solution of 5-*O*-,6-*O*-isopropylidene-L-ascorbic acid (**4.2**) (5.48 g, 25.35 mmol), potassium carbonate (3.88 g, 28.07 mmol), benzyl bromide (6.6 ml, 55.56 mmol) and DMF (75 ml) was stirred at 40 °C for 4 h. The reaction mixture was diluted with water (120 ml) and extracted three times with di-

chloromethane (60 ml). The combined organic layers were dried over magnesium sulfate and concentrated *in vacuo*. The red-brown oil was recrystallized from methanol. After slow crystallization in the refrigerator, the precipitated product was collected, washed with a little cold methanol and dried under reduced pressure to obtain white needles.

Yield: 6.14 g (15.49 mmol, 61 %, white needles)

Mp: 118-120 °C (Lit. 123-125 °C[156])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.28 (s, 3H, C(CH_3)$_2$), 1.29 (s, 3H, C(CH_3)$_2$), 3.87 (dd, 1H, ^{2}J = 8.5 Hz, ^{3}J = 5.9 Hz, CH_2O-), 4.08 (dd, 1H, ^{2}J = 8.5 Hz, ^{3}J = 7.1 Hz, CH_2O-), 4.27 (ddd, 1H, ^{3}J = 7.1 Hz, ^{3}J = 5.9 Hz, ^{3}J = 2.5 Hz, CHO-), 4.94 (d, 1H, ^{2}J = 11.2 Hz, C$\underline{H}_2$Ph), 4.95 (d, 1H, ^{3}J = 2.5 Hz, H-5), 5.01 (d, 1H, ^{2}J =11.2 Hz, C$\underline{H}_2$Ph), 5.16 (d, 1H, ^{2}J = 11.8 Hz, C$\underline{H}_2$Ph), 5.25 (d, 1H, ^{2}J = 11.8 Hz, C$\underline{H}_2$Ph), 7.27-7.43 (m, 10H, Ar-H)

MS (CI-MS, NH_3): m/z (%) = 414 ([M + NH_4] $^+$, 100), 397 ([M + H] $^+$, 10)

$C_{23}H_{24}O_6$ (396.44)

2-*O*-,3-*O*-Ethan-1,2-diyl-(5-O-,6-*O*-isopropylidene)-L-ascorbic acid (4.5)

Potassium carbonate (3.04 g, 21.99 mmol) was provided on a three-neck flask together with DMF (20 ml). 5-*O*-,6-*O*-Isopropylidene-L-ascorbic acid (**4.2**) (4.32 g, 19.98 mmol), dissolved in DMF (20 ml), and 1,2-dibromoethane (1.80 ml, 20.89 mmol), dissolved in DMF (20 ml), were dropped simultaneously during a period of 1h at 60 °C under continuously stirring. After further stirring at 60 °C for 2 h, water (120 ml) was added and the reaction mixture was extracted with dichloromethane (3x60 ml). The combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The yellow-brown colored oil was recrystallized from methanol to give a white solid.

Yield: 1.96 g (8.09 mmol, 40 %, white solid)

Mp: 125-126 °C (Lit. 133-134 °C[156])

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 1.27 (s, 3H, C(CH_3)$_2$), 1.28 (s, 3H, C(CH_3)$_2$), 3.89 (dd, 1H, ^{2}J = 8.5 Hz, ^{3}J = 5.8 Hz, CH_2O-), 4.11 (dd, 1H, ^{2}J = 8.5 Hz, ^{3}J =

7.1 Hz, CH_2O-), 5.07 (d, 1H, ^3J = 3.6 Hz, H-5), 4.14-4.30 (m, 3H, $OC\underline{H}_2CH_2O$, C<u>H</u>O-), 4.32-4.45 (m, 2H, $OC\underline{H}_2CH_2O$)

MS (CI-MS, NH_3): m/z (%) = 260 ($[M + NH_4]^+$, 100), 243 ($[M + H]^+$, 4)

$C_{11}H_{14}O_6$ (242.23)

2-*O*-,3-*O*-Dimethyl-L-ascorbic acid (4.6)

2-*O*-,3-*O*-Dimethyl-(5-*O*-,6-*O*-isopropylidene)-L-ascorbic acid (**4.3**) (5.72 g, 23.42 mmol) was dissolved in methanol (58 ml) and aqueous acetic acid (50%, 144 ml) at 60 °C for 9.5 h. The solvent was removed and the yellow oil was re-crystallized from ethyl acetate/ether to give a hygroscopic white solid.

Yield: 3.60 g (17.63 mmol, 75 %, white solid)

Mp: 56-58 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 3.35-3.49 (m, 2H, C<u>H</u>$_2$OH), 3.61-3.68 (m, 1H, C<u>H</u>OH), 3.70 (s, 3H, OCH_3), 4.08 (s, 3H, OCH_3), 4.81 (d, 1H, ^3J = 1.3 Hz, H-5), 4.88 (dd, 1H, ^3J = 6.3 Hz, ^3J = 5.1 Hz, $CH_2O\underline{H}$), 5.06 (d, 1H, ^3J = 6.1 Hz, CHO<u>H</u>)

MS (CI-MS, NH_3): m/z (%) = 222 ($[M + NH_4]^+$, 100), 205 ($[M + H]^+$, 6)

$C_8H_{12}O_6$ (204.18)

2-*O*-,3-*O*-Dibenzyl-L-ascorbic acid (4.7)

A solution of 2-*O*-,3-*O*-dibenzyl-(5-*O*-,6-*O*-isopropylidene)-L-ascorbic acid (**4.4**) (6.14 g, 15.49 mmol), methanol (62 ml) and aqueous acetic acid (50 %, 155 ml) was stirred at 90 °C for 4 h. The solvent was evaporated and the oily yellow residue was absorbed in ethyl acetate (125 ml). After extraction with a saturated solution of sodium carbonate (3x10 ml), the combined organic layers were washed with water (10 ml). The organic phase was dried over magnesium sulfate and removed under reduced pressure. This crude product was purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of ethyl acetate and petroleum ether (60-80 °C) to obtain a yellow oil which gave after a few days a white crystalline solid.

Yield: 5.34 g (14.98 mmol, 97 %, white solid)

Mp: 70-72 °C (Lit. 67-69 °C[156])

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 3.36-3.51 (m, 2H, C<u>H</u>OH, C<u>H$_2$</u>OH), 3.66-3.73 (m, 1H, C<u>H$_2$</u>OH), 4.88 (dd, 1H, ^{3}J = 6.1 Hz, ^{3}J = 4.8 Hz, CH$_2$O<u>H</u>), 4.90 (d, 1H, ^{3}J = 1.4 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.3 Hz, C<u>H$_2$</u>Ph), 4.98 (d, 1H, ^{2}J = 11.3 Hz, C<u>H$_2$</u>Ph), 5.15 (d, 1H, ^{3}J = 6.3 Hz, CHO<u>H</u>), 5.20 (d, 1H, ^{2}J = 11.5 Hz, C<u>H$_2$</u>Ph), 5.26 (d, 1H, ^{2}J = 11.5 Hz, C<u>H$_2$</u>Ph), 7.29-7.45 (m, 10H, Ar-H)

MS (APCI-MS, CH$_3$CN/H$_2$O+1%HOOCH): m/z (%) = 357 ([M + H]$^+$, 100), 180 ([2C$_6$H$_5$ - CH]$^+$, 65)

$C_{20}H_{20}O_6$ (356.38)

2-*O*-,3-*O*-Ethan-1,2-diyl-L-ascorbic acid (4.8)

A solution of 2-*O*-,3-*O*-ethan-1,2-diyl-(5-*O*-,6-*O*-isopropylidene)-L-ascorbic acid (**4.5**) (1.00 g, 4.13 mmol), methanol (17 ml) and aqueous acetic acid (50%, 43 ml) was stirred at 80 °C for 5 h. The solvent was removed and the remaining light-yellow oil was treated with ethyl acetate (10 ml). The resulting solid was analytically pure.

Yield: 0.80 g (3.96 mmol, 96 %, white solid)

Mp: 126.5-127.5 °C (Lit. 127-128 °C[156])

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 3.41-3.52 (m, 2H, C<u>H$_2$</u>OH), 3.63-3.71 (m, 1H, C<u>H</u>OH), 4.17-4.22 (m, 2H, CH$_2$), 4.31-4.44 (m, 2H, CH$_2$), 4.90 (t, 1H, ^{3}J = 5.6 Hz, CH$_2$O<u>H</u>), 5.02 (d, 1H, ^{3}J = 1.9 Hz, H-5), 5.13 (d, 1H, ^{3}J = 6.0 Hz, CHO<u>H</u>)

MS (CI-MS, NH$_3$): m/z (%) = 220 ([M + NH$_4$]$^+$, 100), 203 ([M + H]$^+$, 4)

$C_8H_{10}O_6$ (202.16)

2-*O*-,3-*O*-Dimethyl-6-*O*-hexadecanoyl-L-ascorbic acid (4.9)

2-*O*-,3-*O*-Dimethyl-L-ascorbic acid (**4.6**) (0.60 g, 2.94 mmol), dissolved in dichloromethane (20 ml, dried over $CaCl_2$), and pyridine (0.31 ml, 3.84 mmol) were annealed with an ice bath. After adding catalytic amounts of DMAP (40 mg), palmitoyl chloride (1.15 ml, 3.81 mmol) was slowly added. The temperature of the solution was maintained at 0 °C for 1h under stirring. Afterwards,

the solution was stirred at room temperature for 26 h. The reaction was quenched with a saturated solution of sodium bicarbonate (20 ml). The mixture was extracted with dichloromethane (3x15 ml) and the combined organic layers were dried over magnesium sulfate. After filtration, the solvent was removed under reduced pressure. The oily crude product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of ethyl acetate and petroleum ether (60-80 °C). To get analytical pure substance the obtained product was recrystallized with methanol.

Yield: 0.68 g (1.54 mmol, 52 %, white solid)

Mp: 73-73.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.85 (t, 3H, ^{3}J = 6.5 Hz, $(CH_2)_{14}C\underline{H}_3$), 1.19-1.30 (m, 24H, $(C\underline{H}_2)_{12}CH_3$), 1.44-1.59 (m, 2H, $COCH_2C\underline{H}_2$-), 2.31 (t, 2H, ^{3}J = 7.3 Hz, $COC\underline{H}_2$-), 3.70 (s, 3H, OCH_3), 3.85-4.12 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.08 (s, 3H, OCH_3), 4.80 (d, 1H, ^{3}J = 1.6 Hz, H-5), 5.47 (d, 1H, ^{3}J = 5.5 Hz, CHO<u>H</u>)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, CH_3), 22.1 (-, CH_2), 24.3 (-, CH_2), 28.4 (-, CH_2), 28.7 (-, $2CH_2$), 28.9 (-, CH_2), 29.0 (-, $6CH_2$), 31.3 (-, CH_2), 33.3 (-, CH_2), 59.2 (+, OCH_3), 60.3 (+, OCH_3), 64.0 (-, CH_2), 65.6 (+, CHOH), 74.8 (+, CH), 122.4 (C_{quart}, C2), 157.8 (C_{quart}, C3), 168.9 (C_{quart}, lactone CO), 172.7 (C_{quart}, $\underline{C}O(CH_2)_{14}$-)

MS (CI-MS, NH_3): m/z (%) = 460 ($[M + NH_4]^+$, 100)

$C_{24}H_{42}O_7$ (442.59)

2-*O*-,3-*O*-Dibenzyl-6-*O*-hexadecanoyl-L-ascorbic acid (4.10)

To an ice-cold solution of 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1.00 g, 2.81 mmol), pyridine (0.25 ml, 3.10 mmol) and previously dried dichloromethane (25 ml) were added catalytic amounts of DMAP (55 mg). Palmitoyl chloride (0.93 ml, 3.08 mmol) was added dropwise and the solution was stirred at 0 °C for 1 h. After stirring at room temperature for additional 23 h, the reaction was quenched with a solution of saturated sodium bicarbonate (20 ml). The mixture was extracted with dichloromethane (3x15 ml) and the combined organic phases were dried over magnesium sulfate. The solvent was removed *in vacuo*.

The product was purified by chromatography on a silica gel column eluting with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.99 g (1.66 mmol, 59 %, white crystalline solid)

Mp: 62.5-63.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.85 (t, 3H, ^{3}J = 6.5 Hz, CH_3), 1.15-1.30 (m, 24H, $(C\underline{H}_2)_{12}CH_3$), 1.44-1.56 (m, 2H, $COCH_2C\underline{H}_2$-), 2.30 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 3.88-4.15 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.90 (d, 1H, ^{3}J = 1.2 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.5 Hz, $C\underline{H}_2Ph$), 4.99 (d, 1H, ^{2}J = 11.5 Hz, $C\underline{H}_2Ph$), 5.19 (d, 1H, ^{2}J = 11.9 Hz, $C\underline{H}_2Ph$), 5.25 (d, 1H, ^{2}J = 11.9 Hz, $C\underline{H}_2Ph$), 5.55 (d, 1H, ^{3}J = 6.3 Hz, CHO<u>H</u>), 7.28-7.44 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, CH_3), 22.1 (-, CH_2), 24.3 (-, CH_2), 28.4 (-, CH_2), 28.7 (-, $2CH_2$), 28.8 (-, CH_2), 29.0 (-, $6CH_2$), 31.3 (-, CH_2), 33.3 (-, CH_2), 64.1 (-, CH_2O-), 65.8 (+, CHOH), 72.7 (-,C3O<u>C</u>H$_2$), 73.6 (-,C2O<u>C</u>H$_2$), 75.1 (+, CH), 120.8 (C_{quart}, C2), 127.7 (+, 2Ar-C), 128.4 (+, 6Ar-C), 128.7 (+, 2Ar-C), 135.6 (C_{quart}, $C3OCH_2Ph$), 136.1 (C_{quart}, $C2OCH_2Ph$), 157.4 (C_{quart}, C3), 169.0 (C_{quart}, lactone CO), 172.7 (C_{quart}, <u>C</u>O$(CH_2)_{14}$-)

MS (ES-MS, DCM/MeOH+10mmol/l NH_4Ac): m/z (%) = 612 ($[M + NH_4]^+$, 100), 595 ($[M + H]^+$, 72)

$C_{36}H_{50}O_7$ (594.78)

2-*O*-,3-*O*-Ethan-1,2-diyl-6-*O*-hexadecanoyl-L-ascorbic acid (4.11)

A solution of 2-*O*-,3-*O*-ethan-1,2-diyl-L-ascorbic acid (**4.8**) (0.40 g, 1.98 mmol) and pyridine (10 ml) was cooled down to 0 °C with an ice bath. Catalytic amounts of DMAP (40 mg) and palmitoyl chloride (0.66 ml, 2.18 mmol) were added under a nitrogen atmosphere. The solution was stirred overnight and diluted with dichloromethane (25 ml). Then, the mixture was extracted with hydrochloric acid (3 M, 3x25 ml) and washed with water (10 ml). The combined organic layers were dried over magnesium sulfate, filtrated and concentrated *in vacuo*. The product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of ethyl acetate and petroleum ether (60-80 °C) to obtain a white powder.

Yield: 0.54 g (1.23 mmol, 62 %, white solid)

Mp: 96-97 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.85 (t, 3H, ^{3}J = 6.8 Hz, CH_3), 1.18-1.33 (m, 24H, $(C\underline{H}_2)_{12}CH_3$), 1.46-1.58 (m, 2H, $COCH_2C\underline{H}_2$-), 2.31 (t, 2H, ^{3}J = 7.4 Hz, $COCH_2$-), 3.87-3.96 (m, 1H, C<u>H</u>OH), 4.06-4.10 (m, 2H, CH_2), 4.18-4.22 (m, 2H, CH_2), 4.32-4.44 (m, 2H, $C\underline{H}_2O$-), 5.02 (d, 1H, ^{3}J = 2.3 Hz, H-5), 5.53 (d, 1H, ^{3}J = 6.2 Hz, CHO<u>H</u>)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.8 (+, CH_3), 22.0 (-, CH_2), 24.3 (-, CH_2), 28.4 (-, CH_2), 28.6 (-, $2CH_2$), 28.8 (-, CH_2), 28.9 (-, $6CH_2$), 31.2 (-, CH_2), 33.3 (-, CH_2), 64.0 (-, CH_2), 64.7 (-, CH_2), 65.8 (+, CHOH), 67.2 (-, CH_2), 75.1 (+, CH), 122.7 (C_{quart}, C2), 153.6 (C_{quart}, C3), 165.2 (C_{quart}, lactone CO), 172.6 (C_{quart}, $\underline{C}O(CH_2)_{14}$-)

MS (ES-MS, DCM/MeOH+10mmol/l NH_4Ac): m/z (%) = 458 ($[M + NH_4]^+$, 100)

$C_{24}H_{40}O_7$ (440.58)

Preparation of 2-*O*-,3-*O*-dibenzyl-6-*O*-acyl-L-ascorbic acid derivatives 4.12-4.17

General procedures

Method A. To an ice-cold solution of 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1 eq), pyridine (1.1 eq) and previously dried dichloromethane (20 ml) were added catalytic amounts of DMAP (40-55 mg) under an inert atmosphere. Acid chloride (1.1 eq) was added dropwise and the solution was stirred at 0 °C for 1 h. After stirring at room temperature for additional 24 h, the reaction was quenched with a solution of saturated sodium bicarbonate (20 ml). The mixture was extracted with dichloromethane (3x15 ml) and the combined organic phases were dried over magnesium sulfate. The solvent was removed *in vacuo*. The product was purified by column chromatography on a silica gel.

Method B. Carboxylic acid (1 eq) was dissolved in dry dichloromethane (8-15 ml) under a nitrogen atmosphere and CDI (1 eq) was added at ambient temperature. After the development of gas was not recognizable any more (approximately 10 min) the solution was chilled in an ice bath. 2-*O*-,3-*O*-Dibenzyl-L-

ascorbic acid (**4.7**) (1 eq), dissolved in dry dichloromethane (8-10 ml), was added dropwise and the mixture was stirred at room temperature for further 24 h. After concentrating the mixture *in vacuo*, water (10 ml) was added and the aqueous phase was extracted with dichloromethane (3x10 ml). The combined organic phases were dried over magnesium sulfate, the solvent was removed and the residue was purified by column chromatography on a silica gel.

2-*O*-,3-*O*-Dibenzyl-6-*O*-(2,2-dimethylpropanoyl)-L-ascorbic acid (4.12)

Method A: Reaction of 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1.20 g, 3.37 mmol), pyridine (0.30 ml, 3.72 mmol) and pivaloyl chloride (0.46 ml, 3.74 mmol); chromatography on a silica gel column, elution with a 3:1 (v/v) mixture of chloroform and ethyl acetate.

Yield: 0.89 g (2.02 mmol, 60 %, white solid)

Mp: 88.5-90 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 1.14 (s, 9H, C(C$\underline{H}_3$)$_3$), 3.94-3.97 (m, 1H, C$\underline{H}$OH), 4.02-4.13 (m, 2H, C$\underline{H}_2$O-), 4.89 (d, 1H, ^{3}J = 1.6 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.1 Hz, C2OC$\underline{H}_2$Ph), 4.98 (d, 1H, ^{2}J = 11.1 Hz, C2OC$\underline{H}_2$Ph), 5.20 (d, 1H, ^{2}J = 11.8 Hz, C3OC$\underline{H}_2$Ph), 5.27 (d, 1H, ^{2}J = 11.8 Hz, C3OC$\underline{H}_2$Ph), 5.56 (d, 1H, ^{3}J = 6.3 Hz, CHO$\underline{H}$), 7.29-7.44 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 26.7 (+, C($\underline{C}$H$_3$)$_3$), 64.3 (-, CH$_2$), 65.8 (+, CHOH), 72.7 (-, C3O$\underline{C}$H$_2$), 73.5 (-, C2O$\underline{C}$H$_2$), 75.1 (+, CH), 120.9 (C$_{quart}$, C2), 127.6 (+, 2Ar-C), 128.3 (+, 2Ar-C), 128.4 (+, 4Ar-C), 128.6 (+, 2Ar-C), 135.6 (C$_{quart}$, C3OCH$_2$Ph), 136.1 (C$_{quart}$, C2OCH$_2$Ph), 157.3 (C$_{quart}$, C3), 169.0 (C$_{quart}$, lactone CO), 177.1 (C$_{quart}$, $\underline{C}$OC(CH$_3$)$_3$)

MS (ES-MS, DCM/MeOH+10mmol/l NH$_4$Ac): m/z (%) = 458 ([M + NH$_4$]$^+$, 100), 441 ([M + H]$^+$, 77)

$C_{25}H_{28}O_7$ (440.49)

2-*O*-,3-*O*-Dibenzyl-6-*O*-butanoyl-L-ascorbic acid (4.13)

Method A: Reaction of 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1.20 g, 3.37 mmol), pyridine (0.30 ml, 3.72 mmol) and butyryl chloride (0.39 ml, 3.77 mmol); chromatography on a silica gel column, elution with a 3:1 (v/v) mixture of chloroform and ethyl acetate.

Yield: 0.99 g (2.32 mmol, 69 %, colorless oil)

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.87 (t, 3H, ^{3}J = 7.1 Hz, CH_3), 1.46-1.62 (m, 2H, $CH_2C\underline{H}_2CH_3$), 2.30 (t, 2H, ^{3}J = 7.2 Hz, $COCH_2$-), 3.88-4.15 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.91 (d, 1H, ^{3}J = 1.6 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.5 Hz, $C\underline{H}_2Ph$), 4.99 (d, 1H, ^{2}J = 11.5 Hz, $C\underline{H}_2Ph$), 5.19 (d, 1H, ^{2}J = 11.9 Hz, $C\underline{H}_2Ph$), 5.26 (d, 1H, ^{2}J = 11.9 Hz, $C\underline{H}_2Ph$), 5.55 (d, 1H, ^{3}J = 6.3 Hz, CHO<u>H</u>), 7.29-7.44 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.4 (+, CH_3), 17.8 (-, CH_2), 35.2 (-, CH_2), 64.1 (-, CH_2O-), 65.8 (+, CHOH), 72.7 (-, C3O<u>C</u>H$_2$), 73.6 (-, C2O<u>C</u>H$_2$), 75.1 (+, CH), 120.8 (C_{quart}, C2), 127.7 (+, 2Ar-C), 128.4 (+, 6Ar-C), 128.7 (+, 2Ar-C), 135.6 (C_{quart}, C3OCH_2Ph), 136.1 (C_{quart}, C2OCH_2Ph), 157.4 (C_{quart}, C3), 169.0 (C_{quart}, lactone CO), 172.6 (C_{quart}, <u>C</u>O$(CH_2)_2$-)

MS (CI-MS, NH$_3$): m/z (%) = 444 ([M + NH_4] $^+$, 100), 427 ([M + H] $^+$, 1), 354 ([M + NH_4 - C_7H_6] $^+$, 10)

$C_{24}H_{26}O_7$ (426.46)

2-*O*-,3-*O*-Dibenzyl-6-*O*-hexanoyl-L-ascorbic acid (4.14)

Method B: Reaction of hexanoic acid (0.29 ml, 2.32 mmol), CDI (0.38 g, 2.34 mmol) and 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (0.84 g, 2.36 mmol); purification by chromatography on a silica gel column, elution with a 3:1 (v/v) mixture of chloroform and ethyl acetate.

Yield: 0.55 g (1.21 mmol, 52 %, colorless oil)

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.84 (t, 3H, ^{3}J = 6.7 Hz, CH_3), 1.15-1.32 (m, 4H, $CH_2(C\underline{H}_2)_2CH_3$), 1.45-1.59 (m, 2H, $COCH_2C\underline{H}_2$-), 2.31 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 3.88-4.15 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.91 (d, 1H, ^{3}J = 1.6 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.1 Hz, $C\underline{H}_2Ph$), 4.99 (d, 1H, ^{2}J = 11.1 Hz, $C\underline{H}_2Ph$), 5.19 (d, 1H, ^{2}J

= 11.9 Hz, C<u>H</u>$_2$Ph), 5.25 (d, 1H, ^{2}J = 11.9 Hz, C<u>H</u>$_2$Ph), 5.55 (d, 1H, ^{3}J = 6.3 Hz, CHO<u>H</u>), 7.28-7.44 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.7 (+, CH_3), 21.7 (-, CH_2), 24.0 (-, CH_2), 30.6 (-, CH_2), 33.3 (-, CH_2), 64.1 (-, CH_2O-), 65.8 (+, CHOH), 72.7 (-,C3O<u>C</u>H$_2$), 73.6 (-,C2O<u>C</u>H$_2$), 75.1 (+, CH), 120.8 (C_{quart}, C2), 127.7 (+, 2Ar-C), 128.4 (+, 6Ar-C), 128.7 (+, 2Ar-C), 135.6 (C_{quart}, C3OCH$_2$Ph), 136.1 (C_{quart}, C2OCH$_2$Ph), 157.4 (C_{quart}, C3), 169.0 (C_{quart}, lactone CO), 172.7 (C_{quart}, <u>C</u>O(CH$_2$)$_4$-)

$C_{26}H_{30}O_7$ (454.51)

2-*O*-,3-*O*-Dibenzyl-6-*O*-dodecanoyl-L-ascorbic acid (4.15)

Method B: Reaction of lauric acid (0.57 g, 2.85 mmol), CDI (0.46 g, 2.84 mmol) and 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1.00 g, 2.81 mmol); purification by chromatography on a silica gel column, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate; product was crystallized with methanol to get an analytically pure sample as a white solid.

Yield: 0.75 g (1.39 mmol, 50 %, white solid)

Mp: 49.5-50 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.85 (t, 3H, ^{3}J = 6.9 Hz, CH_3), 1.21-1.25 (m, 16H, (C<u>H</u>$_2$)$_8$CH$_3$), 1.48-1.53 (m, 2H, COCH$_2$C<u>H</u>$_2$-), 2.30 (t, 2H, ^{3}J = 7.4 Hz, COCH$_2$-), 3.90-4.13 (m, 3H, C<u>H</u>$_2$O-, C<u>H</u>OH), 4.90 (d, 1H, ^{3}J = 1.6 Hz, H-5), 4.95 (d, 1H, ^{2}J = 11.2 Hz, C<u>H</u>$_2$Ph), 4.99 (d, 1H, ^{2}J = 11.2 Hz, C<u>H</u>$_2$Ph), 5.20 (d, 1H, ^{2}J = 11.8 Hz, C<u>H</u>$_2$Ph), 5.25 (d, 1H, ^{2}J = 11.8 Hz, C<u>H</u>$_2$Ph), 5.54 (d, 1H, ^{3}J = 6.5 Hz, CHO<u>H</u>), 7.30-7.43 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, CH_3), 22.1 (-, CH_2), 24.3 (-, CH_2), 28.4 (-, CH_2), 28.6 (-, 2CH_2), 28.9 (-, 3CH_2), 31.3 (-, CH_2), 33.3 (-, CH_2), 64.1 (-, CH_2O-), 65.8 (+, CHOH), 72.7 (-,C3O<u>C</u>H$_2$), 73.6 (-,C2O<u>C</u>H$_2$), 75.1 (+, CH), 120.8 (C_{quart}, C2), 127.7 (+, 2Ar-C), 128.3 (+, 2Ar-C), 128.4 (+, 4Ar-C), 128.6 (+, 2Ar-C), 135.6 (C_{quart}, C3OCH$_2$Ph), 136.1 (C_{quart}, C2OCH$_2$Ph), 157.3 (C_{quart}, C3), 169.0 (C_{quart}, lactone CO), 172.7 (C_{quart}, <u>C</u>O(CH$_2$)$_{10}$-)

MS (CI-MS, NH$_3$): m/z (%) = 556 ([M + NH_4] $^+$, 100), 466 ([M + NH_4 - C_7H_6] $^+$, 9)

$C_{32}H_{42}O_7$ (538.67)

2-*O*-,3-*O*-Dibenzyl-6-*O*-octadecanoyl-L-ascorbic acid (4.16)

Method B: Reaction of stearic acid (0.80 g, 2.81 mmol), CDI (0.46 g, 2.84 mmol) and 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (1.00 g, 2.81 mmol); purification by chromatography on a silica gel column, elution with a 3:1 (v/v) mixture of chloroform and ethyl acetate.

Yield: 0.66 g (1.06 mmol, 38 %, white solid)

Mp: 69-70 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 0.85 (t, 3H, ^{3}J = 6.9 Hz, CH_3), 1.18-1.28 (m, 28H, $(C\underline{H}_2)_{14}CH_3$), 1.44-1.56 (m, 2H, $COCH_2C\underline{H}_2$-), 2.30 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 3.89-4.14 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.90 (d, 1H, ^{3}J = 1.6 Hz, H-5), 4.94 (d, 1H, ^{2}J = 11.2 Hz, $C\underline{H}_2Ph$), 4.98 (d, 1H, ^{2}J = 11.2 Hz, $C\underline{H}_2Ph$), 5.19 (d, 1H, ^{2}J = 11.6 Hz, $C\underline{H}_2Ph$), 5.25 (d, 1H, ^{2}J = 11.6 Hz, $C\underline{H}_2Ph$), 5.55 (d, 1H, ^{3}J = 6.4 Hz, CHO<u>H</u>), 7.29-7.44 (m, 10H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, CH_3), 22.0 (-, CH_2), 24.2 (-, CH_2), 28.3 (-, CH_2), 28.6 (-, $2CH_2$), 28.8 (-, CH_2), 28.9 (-, $8CH_2$), 31.2 (-, CH_2), 33.2 (-, CH_2), 64.0 (-, CH_2O-), 65.7 (+, CHOH), 72.6 (-,C3O<u>C</u>H$_2$), 73.5 (-,C2O<u>C</u>H$_2$), 75.0 (+, CH), 120.8 (C_{quart}, C2), 127.6 (+, 2Ar-C), 128.3 (+, 2Ar-C), 128.4 (+, 4Ar-C), 128.6 (+, 2Ar-C), 135.5 (C_{quart}, C3OCH_2Ph), 136.0 (C_{quart}, C2OCH_2Ph), 157.3 (C_{quart}, C3), 169.0 (C_{quart}, lactone CO), 172.6 (C_{quart}, <u>C</u>O$(CH_2)_{16}$-)

MS (ES-MS, DCM/MeOH+10mmol/l NH_4Ac): m/z (%) = 640 ([M + NH_4] $^{+}$, 100), 623 ([M + H] $^{+}$, 83)

$C_{38}H_{54}O_7$ (622.84)

2-*O*-,3-*O*-Dibenzyl-6-*O*-benzoyl-L-ascorbic acid (4.17)

Method A: Reaction of 2-*O*-,3-*O*-dibenzyl-L-ascorbic acid (**4.7**) (0.80 g, 2.24 mmol), pyridine (0.25 ml, 3.10 mmol) and benzoyl chloride (0.34 ml, 2.93 mmol); chromatography on a silica gel column eluting with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate; product was crystallized with methanol to get an analytically pure sample as a white solid.

Yield: 0.50 g (1.09 mmol, 48 %, colorless oil)

Mp: 89.5-92 °C

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 4.08-4.17 (m, 1H, C<u>H</u>OH), 4.29-4.42 (m, 2H, C<u>H</u>$_2$O-), 4.96 (d, 1H, ^{2}J = 11.1 Hz, C<u>H</u>$_2$Ph), 5.01 (d, 1H, ^{2}J = 11.1 Hz, C<u>H</u>$_2$Ph), 5.07 (d, 1H, ^{3}J = 1.6 Hz, H-5), 5.21 (d, 1H, ^{2}J = 11.9 Hz, C<u>H</u>$_2$Ph), 5.27 (d, 1H, ^{2}J = 11.9 Hz, C<u>H</u>$_2$Ph), 5.70 (d, 1H, ^{3}J = 6.3 Hz, CHO<u>H</u>), 7.30-7.45 (m, 10H, Ar-H), 7.49-7.57 (m, 2H, Ar-H), 7.63-7.72 (m, 1H, Ar-H), 7.99-8.06 (m, 2H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 65.2 (-, CH_2), 65.9 (+, CHOH), 72.8 (-,C3O<u>C</u>H$_2$), 73.6 (-,C2O<u>C</u>H$_2$), 75.3 (+, CH), 120.9 (C_{quart}, C2), 127.7 (+, 2Ar-C), 128.4 (+, 2Ar-C), 128.5 (+, 4Ar-C), 128.7 (+, 4Ar-C), 129.4 (+, 2Ar-C), 129.5 (C_{quart}, Ar-C), 133.4 (+, Ar-C), 135.6 (C_{quart}, C3OCH$_2$Ph), 136.1 (C_{quart}, C2OCH$_2$Ph), 157.5 (C_{quart}, C3), 165.5 (C_{quart}, <u>C</u>OPh), 169.1 (C_{quart}, lactone CO)

MS (CI-MS, NH$_3$): m/z (%) = 478 ([M + NH_4] $^{+}$, 100), 388 ([M + NH_4- C_7H_6] $^{+}$, 26)

$C_{27}H_{24}O_7$ (460.48)

Synthesis of 6-*O*-acyl-L-ascorbic acid derivatives 4.18-4.23

General procedure

The appropriate 2-*O*-,3-*O*-dibenzyl-6-*O*-acyl-L-ascorbic acid derivative (1 eq) was dissolved in ethanol (8-10 ml) and treated with 10% Pd-C catalyst (55-65 mg). Hydrogenolysis was performed in an autoclave under a pressure of 5 bar at room temperature for 24 h. Insoluble material was filtered off and was washed with ethanol, and the filtrate was concentrated *in vacuo*.

6-*O*-(2,2-Dimethylpropanoyl)-L-ascorbic acid (4.18)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-(2,2-dimethylpropanoyl)-L-ascorbic acid (**4.12**) (0.39 g, 0.89 mmol); crystallization from ethanol/chloroform/hexane.

Yield: 0.20 g (0.77 mmol, 87 %, white solid)

Mp: >167 °C (decomposition)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.15 (s, 9H, C(CH_3)$_3$), 3.92-4.12 (m, 3H, C<u>H</u>$_2$O-, C<u>H</u>OH), 4.65 (d, 1H, ^{3}J = 1.4 Hz, H-5), 5.33 (d, 1H, ^{3}J = 5.9 Hz, CHO<u>H</u>), 8.41 (br, 1H, OH), 11.12 (br, 1H, OH)

MS (CI-MS, NH_3): m/z (%) = 278 ([M + NH_4]$^+$, 100)

$C_{11}H_{16}O_7$ (260.24)

6-*O*-Hexanoyl-L-ascorbic acid (4.19)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-hexanoyl-L-ascorbic acid (**4.14**) (0.39 g, 0.86 mmol); crystallization from chloroform/hexane.

Yield: 0.16 g (0.58 mmol, 68 %, pale yellow oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86 (t, 3H, ^{3}J = 7.0 Hz, CH_3), 1.21-1.32 (m, 4H, $COCH_2CH_2(C\underline{H}_2)_2CH_3$), 1.47-1.60 (m, 2H, $COCH_2C\underline{H}_2$-), 2.32 (t, 2H, ^{3}J = 7.4 Hz, $COCH_2$-), 3.92-4.11 (m, 3H, C<u>H</u>$_2$O-, C<u>H</u>OH), 4.68 (d, 1H, ^{3}J = 1.8 Hz, H-5), 5.31 (d, 1H, ^{3}J = 5.5 Hz, CHO<u>H</u>), 8.40 (br, 1H, OH), 11.11 (br, 1H, OH)

MS (ESI-MS, DCM/MeOH+10mmol/l NH_4Ac): m/z (%) = 273 ([M - H]$^+$, 100)

$C_{12}H_{18}O_7$ (274.27)

6-*O*-Dodecanoyl-L-ascorbic acid (4.20)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-dodecanoyl-L-ascorbic acid (**4.15**) (0.30 g, 0.56 mmol); crystallization from chloroform/hexane.

Yield: 0.13 g (0.50 mmol, 66 %, white solid)

Mp: 95-97 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86 (t, 3H, ^{3}J = 6.5 Hz, CH_3), 1.15-1.33 (m, 16H, $(C\underline{H}_2)_8CH_3$), 1.43-1.60 (m, 2H, $COCH_2C\underline{H}_2$-), 2.32 (t, 2H, ^{3}J = 7.1 Hz, $COCH_2$-), 3.92-4.15 (m, 3H, C<u>H</u>$_2$O-, C<u>H</u>OH), 4.67 (d, 1H, ^{3}J = 1.6 Hz, H-5), 5.32 (br, 1H, OH), 8.42 (br, 1H, OH), 11.15 (br, 1H, OH)

MS (CI-MS, NH_3): m/z (%) = 376 ([M + NH_4]$^+$, 100)

HR-MS (CI-MS, NH_3): calculated for [M+H]$^+$: 359.20698

found 359.20705

$C_{18}H_{30}O_7$ (358.43)

6-*O*-Hexadecanoyl-L-ascorbic acid (4.21)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-hexadecanoyl-L-ascorbic acid (**4.10**) (0.46 g, 0.77 mmol); crystallization from hexane.

Yield: 0.24 g (0.58 mmol, 74 %, white solid)

Mp: 109.5 °C-111.5 °C (Lit. 111-112 °C[167])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.85 (t, 3H, ^{3}J = 6.7 Hz, CH_3), 1.18-1.34 (m, 24H, $(C\underline{H}_2)_{12}CH_3$), 1.44-1.61 (m, 2H, $COCH_2C\underline{H}_2$-), 2.31 (t, 2H, ^{3}J = 7.4 Hz, $COCH_2$-), 3.91-4.15 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.67 (d, 1H, ^{3}J = 1.6 Hz, H-5), 5.32 (br, 1H, OH), 8.39 (br, 1H, OH), 11.14 (br, 1H, OH)

$C_{22}H_{38}O_7$ (414.53)

6-*O*-Octadecanoyl-L-ascorbic acid (4.22)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-octadecanoyl-L-ascorbic acid (**4.16**) (0.31 g, 0.50 mmol); crystallization from hexane.

Yield: 0.17 g (0.38 mmol, 77 %, white solid)

Mp: 105-106.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.85 (t, 3H, ^{3}J = 6.9 Hz, CH_3), 1.18-1.31 (m, 28H, $(C\underline{H}_2)_{14}CH_3$), 1.46-1.58 (m, 2H, $COCH_2C\underline{H}_2$-), 2.31 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 3.92-4.12 (m, 3H, $C\underline{H}_2O$-, C<u>H</u>OH), 4.67 (d, 1H, ^{3}J = 1.6 Hz, H-5), 5.32 (br, 1H, OH), 8.40 (br, 1H, OH), 11.11 (br, 1H, OH)

MS (CI-MS, NH_3): m/z (%) = 460 ($[M + NH_4]^+$, 100), 442 ($[M + H]^+$, 43)

$C_{24}H_{42}O_7$ (442.59)

6-*O*-Benzoyl-L-ascorbic acid (4.23)

Reaction of 2-*O*-,3-*O*-dibenzyl-6-*O*-benzoyl-L-ascorbic acid (**4.17**) (0.29 g, 0.63 mmol); recrystallization from chloroform.

Yield: 0.14 g (0.50 mmol, 79 %, white-grey solid)

Mp: >140 °C (decomposition)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 4.11-4.15 (m, 1H, CH_2O-), 4.28-4.39 (m, 2H, C<u>H</u>$_2$O-, C<u>H</u>OH), 4.82 (d, 1H, ^{3}J = 1.8 Hz, H-5), 5.44 (d, 1H, ^{3}J = 6.4 Hz, CHO<u>H</u>), 7.51-7.56 (m, 2H, Ar-H), 7.65-7.69 (m, 1H, Ar-H), 8.01-8.05 (m, 2H, Ar-H), 8.40 (br, 1H, OH), 11.10 (br, 1H, OH)

MS (CI-MS, NH_3): m/z (%) = 298 ($[M + NH_4]^+$, 100)

HR-MS (CI-MS, NH_3): calculated for $[M+H]^+$: 281.06613

found 281.06601

$C_{13}H_{12}O_7$ (280.23)

4.5.3 Pharmacological methods

Materials

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 hyaluronate lyase, *i.e.* 200,000 units** (0.572 mg from *S.agalactiae* strain 4755 plus 2.2 mg of BSA and 37 mg of Tris-HCl per vial) of lyophilized hyaluronate lyase, was kindly provided by id-Pharma (Jena, Germany). Lyophilized hyaluronidase from bovine testis (Neopermease®) (200,000 units**; 4 mg plus 25 mg of gelatine per vial) was a gift from Sanabo (Vienna, Austria). Hylase Dessau®, 1 500 units** of lyophilized bovine testicular hyaluronidase (stabilized with gelafusal (partially hydrolyzed gelatin)), was kindly provided by Pharma Dessau (Dessau, Germany). *Streptococcus pneumoniae* hyaluronate lyase[67,161,168] has been produced as described previously[169].The enzyme was concentrated to 5 mg/ml in 10 mM Tris-HCl buffer, 150 mM NaCl, 1 mM DTT, pH 7.4 using centrifugal spin devices with 50 kDa molecular weight cutoff (Millipore, Billerica, USA) and was used for the inhibition studies and for crystallization experiments. The enzyme

** according to the declaration of the supplier

concentration was determined photometrically at 280 nm using a calculated molar absorption coefficient of 127,090 $L \cdot mol^{-1} \cdot cm^{-1}$ [68,169] according to Pace *et al*.[170]. All other chemicals were of analytical grade and were received from Merck or Sigma.

Estimation of the aqueous solubility of the test compounds

To determine the solubility of the test compounds, a sample containing 600 µl of citrate-phosphate buffer (pH 5 and pH 7.4), 396 µl of water, 300 µl of BSA solution (0.2 mg/ml in water) and a solution of 54 µl of the respective test compound (dissolved in DMSO) of various concentrations was measured at 600 nm. A cuvette filled with water served as reference. Compounds were tested for inhibitory activity at concentrations, where no turbidity was measured.

Determination of enzyme inhibition

The inhibitory potency of the prepared vitamin C derivatives on the enzymatic activities of hyaluronidases was determined both in a turbidimetric assay and in the Morgan-Elson assay (see chapter 3). The turbidimetric method was developed in our laboratory, based on the method of Di Ferrante[145] as described in section 5.5.3. Following a procedure of Muckenschnabel *et al*.[149], which is based on the method of Gacesa *et al*.[147] and Reissig *et al*.[148], a modified Morgan-Elson test was implemented. The reported protocol from Muckenschnabel *et al*.[149] was scaled down to minimize the required amounts of test compound and enzyme as described in the following.

The test compounds (0.2 µM - 100 mM), dissolved in DMSO (7 µl), were incubated at 37 °C in an incubation mixture containing 80 µl of McIlvaine's buffer (solution A: 0.2 M Na_2HPO_4, 0.1 M NaCl, solution B: 0.1 M citric acid, 0.1 M NaCl; solution A and B were mixed in appropriate portions to adjust the required pH), 20 µl of BSA solution (0.2 mg/ml in water), 20 µl of HA solution (5 mg/ml in water), 33 µl of water and 20 µl of an enzyme solution (equiactive concentration: 8 IU Hylase Dessau® (pH 3.6), 40 IU Hylase Dessau® (pH 7.4), $hylB_{4755}$ (1.14 ng, pH 5.0 and 7.4)). According to the different applied pH conditions in the assay equiactive concentrations of BTH and $hylB_{4755}$ were incubated for 2 h at optimum pH (BTH: pH 3.6, $hylB_{4755}$: pH 5) whereas at physiological pH the

samples were incubated for 4 h ($hylB_{4755}$) or 48 h (BTH). The final DMSO concentration was 3.9% (v/v).

The enzyme reaction was stopped by addition of 45 µl of alkaline borate solution and subsequent heating for 4.5 min at 100 °C. The alkaline borate solution was prepared immediately before use from a borate solution (17.3 g H_3BO_4 and 7.8 g KOH in 100 ml water) and a potassium carbonate solution (8.0 g K_2CO_3 in 10 ml water). After cooling on ice for 1 min 600 µl of *N*,*N*-dimethylaminobenzaldehyde (20.0 g *N*,*N*-dimethylaminobenzaldehyde dissolved in 25 ml concentrated and 75 ml glacial acid; the solution was diluted with 4 volumes of glacial acid immediately before use) was added and the mixture was incubated at 37 °C for 20 min. The samples (sample solution was separated into 2 plate holes (in each case 300 µl)) were transferred to a 96-well microtiter plate and the absorbance of the colored product was measured photometrically with an automatized EL312E (BIO-TEK^R Instruments INC., Highland Park, Winooski) microplate reader at 590 nm. As reference for 100% enzyme activity the formation of the red colored product of a sample without inhibitor (7 µl of DMSO was used instead) was quantified. In absence of both enzyme and inhibitor (20 µl of BSA solution and 7 µl of DMSO, respectively, was used instead) a reference for 0% enzyme activity could be measured.

Quantification of inhibitory activity

The enzyme activity was calculated from the formation of the red colored product measured at 590 nm. The effect of the test compounds on the enzymatic activity was calculated according to the equation:

$$\text{relative activity [in \%]} = \frac{(A_{sample} - A_{enzyme})}{(A_{inhibitor} - A_{both})} \cdot 100\ \%$$

where A_{enzyme} is the absorbance of the reference sample without enzyme (20 µl of BSA (0.2 mg/ml) solution was used instead), $A_{inhibitor}$ is the absorbance of the reference sample without inhibitor (7 µl of DMSO was used instead) and A_{both} is the absorbance without both enzyme and inhibitor.

The enzyme activities were plotted against the logarithm of the inhibitor concentration. The IC_{50} values (50% inhibition of equiactive enzyme concentrations)

± SEM were calculated fitting the experimental data with the standard curves analysis of Sigma Plot 8.0 (SPSS Inc., Chicago, IL) in a sigmoidal concentration-dependent manner and are the means of at least two independent experiments performed in duplicate.

4.5.4 Crystallization of the L-ascorbic acid-6-hexadecanoate-hylSpn complex

The crystallization experiments were carried out by Masatoshi Nukui and Mark J. Jedrzejas (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA). For crystallization experiments, L-ascorbic acid-6-hexadecanoate was dissolved in 5% DMSO (v/v) and 10 mM Tris-HCl buffer, 150 mM NaCl, 11mM DTT, pH=7.4 to the final concentration of 50 mM. The hanging drop vapour diffusion[171] using Linbro culture plates (Hampton Research Inc.) at room temperature was used to grow the crystals of the enzyme-inhibitor complex. Equal volumes of protein, reservoir solution (1µl each) and various amounts of inhibitor solutions (0.1, 0.5 and 1.0 µl) were mixed and equilibrated against 1 ml of the reservoir solution. The reservoir solution contained saturated ammonium sulphate, 0.2 M NaCl, 2% dioxane and 0.1 M sodium citrate buffer (pH 6.0) as reported for the native enzyme crystallization[68].

4.5.5 X-ray diffraction

The X-ray diffraction experiments were performed by Masatoshi Nukui and Mark J. Jedrzejas (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA). The crystals of the enzyme-inhibitor complex were cryoprotected using 30% xylitol (w/v), 3.5 M ammonium sulphate and 0.1 M sodium citrate buffer (pH 6.0) as described for the native crystals[67] and frozen in liquid nitrogen. Standard fiber loops (Hampton Research Inc.) of a suitable size were used to pick up and mount the frozen crystals under a nitrogen flow at -180 °C. The X-ray diffraction data for enzyme-inhibitor complex was collected using rotation (oscillation) photography and a Quantum 4u CCD detector. The crystallographic setup of beamline 5.0.1 of the Berkeley Center for Structural

Biology, Advanced Light Source, Lawrence Berkeley National Laboratory was used. The collected data was analyzed, indexed, integrated and scaled using the HKL2000 software package[172]. The crystals were isomorphous to the native *S. pneumoniae* hyaluronate lyase crystals[68]. The statistics of the native diffraction data were analyzed.

4.5.6 Structure solution and refinement

Structure solution and refinement were carried out by Daniel J. Rigden (National Center of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

The structure was solved by isomorphous replacement and rigid body-refinement in CNS[173] using the 1.56 Å crystal structure of *S. pneumoniae* hyaluronate lyase (pdb-file: 1EGU[133]) as a starting point. Refinement employed alternating cycles of computational refinement with CNS[173] and manual rebuilding using the program O[174]. All data was used throughout with no intensity or sigma-based cut-offs applied. SigmaA-weighted map coefficients[175] were used throughout.

An R_{free} value[173], calculated from 5% of reflections set aside at the outset, was used to monitor the progress of refinement. Water molecules were placed into 3σ positive peaks in $|F_o\text{-}F_c|$ maps when density was also evident in $|2F_o\text{-}F_c|$ maps and suitable hydrogen bonding partners were available. The ligand L-ascorbic acid-6-hexadecanoate (**4.21**) was modeled into the catalytic site according to the strong, immediately evident difference density. Sulfate and xylitol molecules, deriving from the crystallization and cryo-cooling solutions, respectively, were modeled into suitably shaped regions of electron density. Final statistics for the model are shown in Table 4.5.

Programs of the CCP4 package[176] were applied to manipulations and structural superpositions made with LSQMAN[177]. PyMOL[178] was used to create illustrations.

Table 4.5: Crystallographic data and refinement statistics

Space group		$P2_12_12_1$
Unit Cell (Å)		
	a	84.13
	b	102.71
	c	102.4
Low resolution diffraction limit (Å)		45.9
High resolution diffraction limit (Å)		1.65
Completeness[a] (%)		99.9 (99.0)
I/σ (I)[a]		22.8 (1.3)
Multiplicity[a] (%)		5.8 (5.7)
R_{merge}[a] (%)		7.3 (87.5)
Non-hydrogen protein atoms		5835
Sulfate atoms		25
Non-hydrogen atoms xylitol		30
Non-hydrogen atoms compound **4.21**		27
Non-hydrogen atoms solvent		564
Number of reflections[a]		106633 (9620)
R (%)[a]		19.2 (30.5)
R_{free} (%)[a]		21.1 (31.4)
Mean temperature all atoms		23.9
B-factor (Å^2)		
	Protein	22.2
	Protein main chain	21.2
	Protein side chain	22.5
	Sulfate	46.4
	Xylitol	38.1

	Compound **4.21**		54.9
		Vitamin C moiety	56.5
		Palmitoyl moiety	46.8
	Solvent		31.1
r.m.s.[b] deviation from ideal values			
	Bond lengths (Å)		0.005
	Bond angles (°)		1.2

[a]Values in parentheses are for the highest resolution shell, 1.65-1.71 Å.

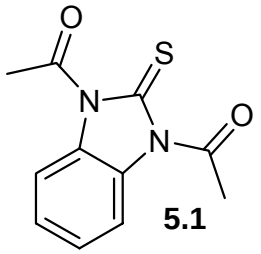
[b] Root mean square.

Chapter 5

Benzimidazole-type hyaluronate lyase inhibitors: Synthesis and pharmacological investigation

5.1 Introduction

The recent elucidation of the three-dimensional X-ray crystal structures of two hyaluronate lyases from Streptococcus species, one from *S. pneumoniae* (pdb-file: 1EGU)[68] and the other one from *S. agalactiae* strain 3502 (pdb-file: 1F1S)[63], enabled the rational design of new ligands for *S. agalactiae* hyaluronate lyase from strain 4755 ($hylB_{4755}$) by creating a model by means of partially superimposing corresponding domains of the aforementioned crystal structures using Sybyl 6.8 (Tripos Inc., St. Louis). Subsequent LUDI calculations based on this model resulted in different proposals. The aim of this *de novo* design approach of new hyaluronate lyase inhibitors was to identify promising leads, which should be small molecules and easily accessible. 1,3-Diacetylbenzimidazol-2-thione (**5.1**, Fig. 5.1) was discovered as a hit[83]. The investigation of **5.1** for inhibition of $hylB_{4755}$ revealed IC_{50} values of 5 µM (at physiological pH 7.4) and 160 µM (at optimum pH 5.0) in a turbidimetric assay (see chapter 3)[120]. Starting from lead compound **5.1**, we decided to investigate structurally diverse benzimidazole derivatives for inhibition of the bacterial hyaluronate lyase $hylB_{4755}$ in order to obtain more information about the binding mode. Therefore, benzimi-



5.1

Fig. 5.1: Lead structure

dazole derivatives, as depicted in Figure 5.2, were defined as target compounds.

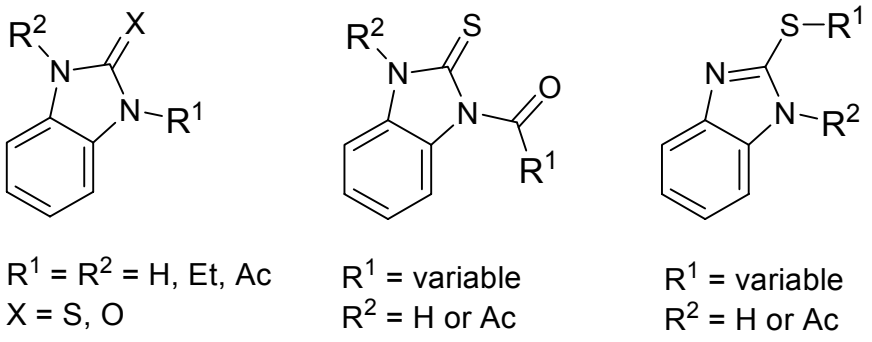
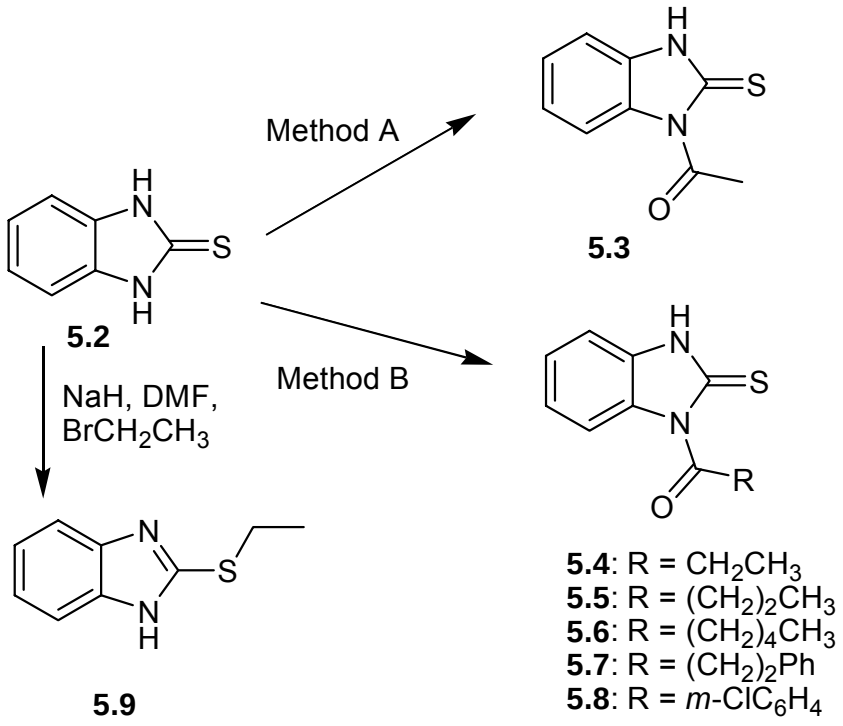


Fig. 5.2: Various benzimidazole derivatives as target compounds

5.2 Chemistry

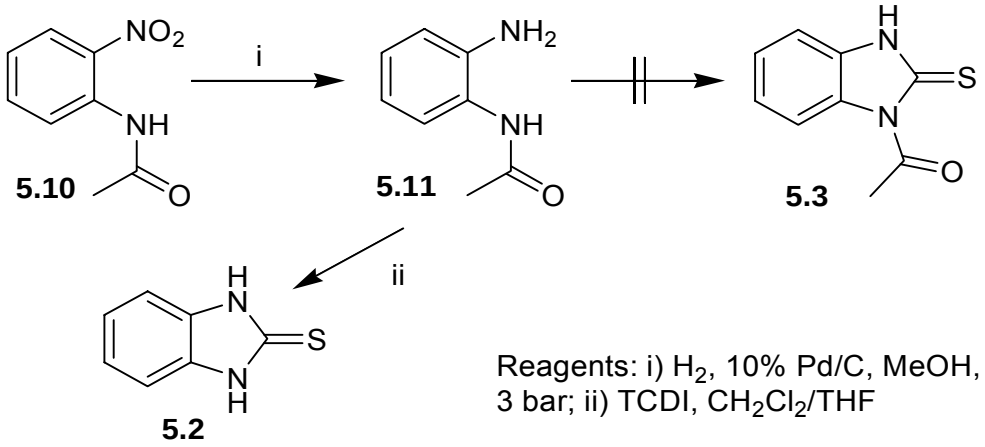
The preparation of 1-(2-sulfanyl-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) was performed according to the procedure described by Saxena *et al.*[179] (method A), whereas the *N*-monoacylated benzimidazol-2-thione derivatives **5.4-5.8** were accessible by a slightly modified protocol (method B) using benzimidazol-2-thione **5.2** as starting material and acid chlorides or acid anhydrides as acylating reagents (Scheme 5.1). Pyridine was used as solvent and base and the workup process was altered.



Scheme 5.1: Synthesis of *N*-monoacylated benzimidazol-2-thione derivatives

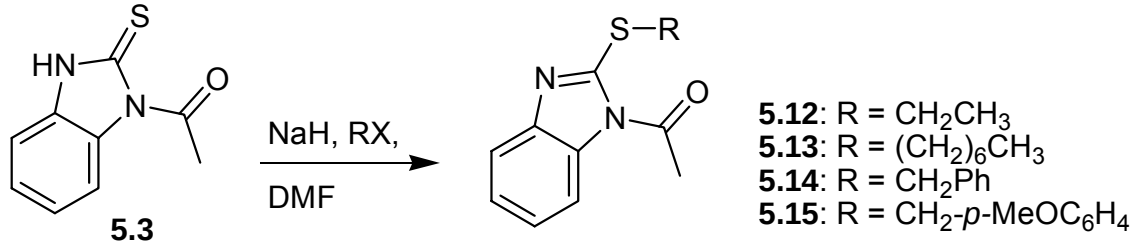
The benzimidazol-2-thione structure offers two reactive positions due to the two possible tautomeric forms. Both the sulfur atom and the nitrogen atom can be deprotonated by base. According to the HSAB concept an alkylation with bromoalkanes should preferentially take place at the soft sulfur atom whereas the acylation should be favored at the hard nitrogen atom. Actually, the alkylation of **5.2** with bromoethane and sodium hydride gave compound **5.9** and no alkylation at the nitrogen atom.

The attempt to prepare **5.3** by hydrogenation of 2-nitroacetanilide over 10 % Pd-C catalyst under pressure, followed by ring closure with 1,1'-thiocarbonyldiimidazole (TCDI) failed as the acetyl group was cleaved off resulting in 2-sulfanylbenzimidazole (Scheme 5.2).



Scheme 5.2: Attempted alternative synthesis of compound **5.3**

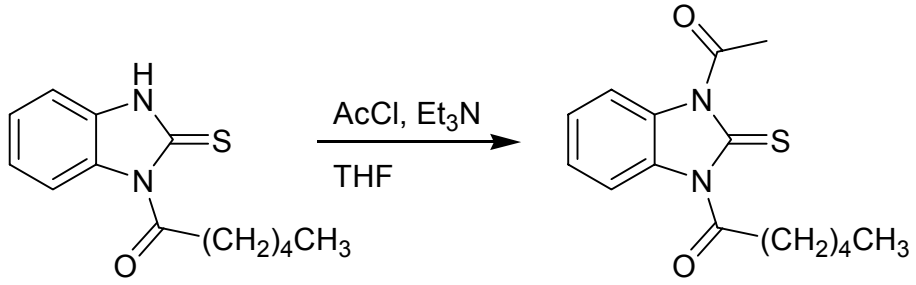
Various *S*-alkylated benzimidazole derivatives **5.12-5.15** were synthesized from *N*-monoacetylated **5.3** by treatment with sodium hydride (1.5 eq) and alkyl halide (1 eq) in DMF (Scheme 5.3).



Scheme 5.3: Preparation of the 2-alkylsulfanylbenzimidazoles

As expected according to the HSAB concept and confirmed by NMR studies (NOESY, HMBC) the alkylation took place preferentially at the soft sulfur atom of **5.3** under these reaction conditions, yielding compounds **5.12-5.15**.

The diacylated compound **5.16** was obtained by treating **5.6** with acetyl chloride and triethylamine in dry THF (Scheme 5.4). Isolation and purification of the product is difficult because of the low stability against hydrolysis during the workup. Therefore, the yield was very low.



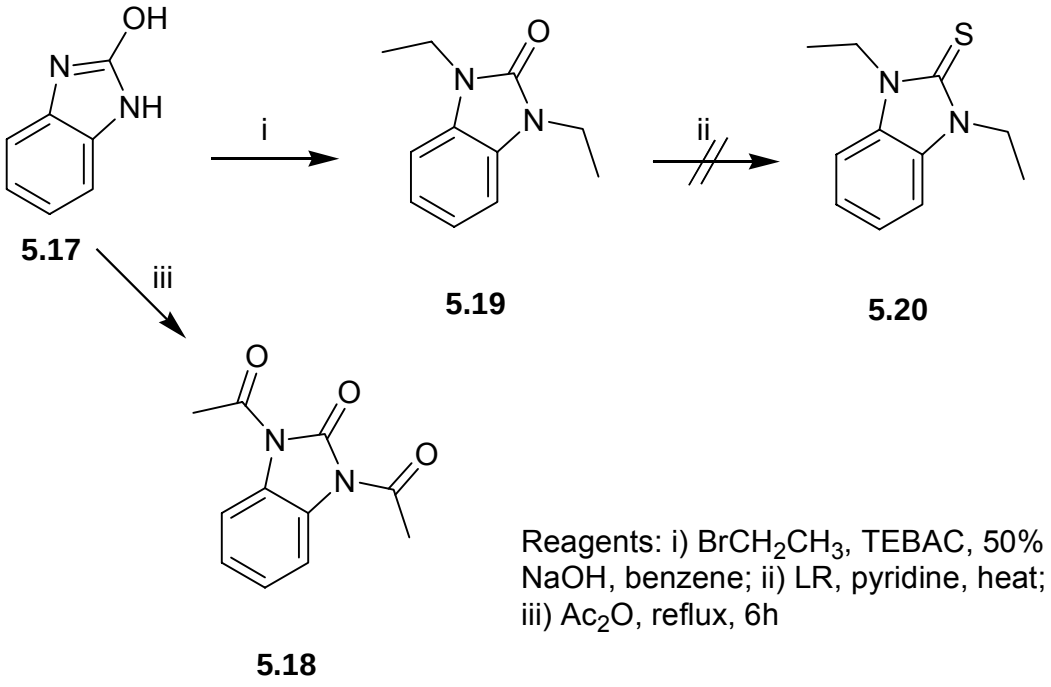
5.6 **5.16**

Scheme 5.4: Synthesis of 1-(3-acetyl-1,2-dihydro-2-thioxobenzo[*d*]imidazol-1-yl)hexan-1-one (**5.16**)

Simple variations of the functional groups of the lead structure were realized as depicted in Schemes 5.5 and 5.6. The oxo analog of the lead, 1,3-diacetyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (**5.18**), was synthesized according to the method described by Chung *et al*.[180]. Compound **5.18** is described as "a versatile and selective acetylating agent with many advantages due to its facile preparation, good reactivity, stability and easy work-up process"[180]. From the chemical point of view **5.18** is an interesting reagent, but probably, it cannot be used as an enzyme inhibitor due to its high reactivity towards nucleophiles, although it is more stable than the corresponding thio analogs.

In order to increase the chemical stability of the molecule and to investigate the role of the carbonyl groups in binding to the enzyme, the acetyl groups of the lead compound should be replaced with ethyl residues. Treating 2-sulfanylbenzimidazole with two equivalents of bromoethane is inappropriate to synthesize 1,3-diethyl-1*H*-benzo[*d*]imidazol-2(3*H*)-thione (**5.19**) as, according to the HSAB concept, the halo compound reacts preferentially with the soft sulfur atom instead of the hard nitrogen atom. Therefore, a different strategy was chosen. In the first step, 2-hydroxybenzimidazole (**5.17**) was converted to the oxo analog of **5.20** following a well established procedure described by Vernin *et al*.[181] for the preparation of symmetrically and unsymmetrically 1,3-dialkylated benzimidazol-2-ones. The alkylation was performed under phase transfer catalysis conditions with an excess of alkyl halide, 50 % aqueous sodium hydroxide,

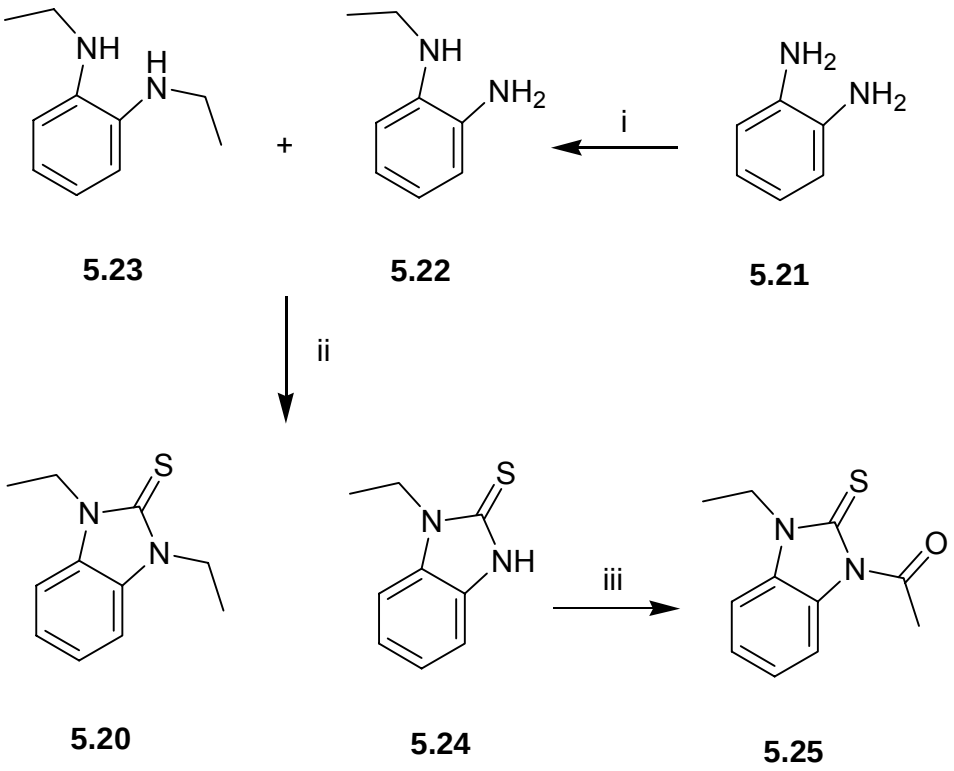
benzene as the organic solvent and tetrabutylammonium chloride (TEBAC) as the catalyst. When treated with Lawesson's reagent in pyridine as solvent 1,3-diethyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (**5.19**) was not converted to the desired thio analog. Variation of the reaction conditions (reaction temperature up to 140 °C, longer reaction time (2 days)) remained unsuccessful: the starting material was always nearly quantitatively recovered.



Scheme 5.5: Attempted synthetic route to 1,3-diethyl-1*H*-benzo[*d*]imidazol-2(3*H*)-thione (**5.20**) and synthesis of 1,3-diacetyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (**5.18**)

This required a new synthetic route. Therefore, a two step synthesis was chosen, in which the first step should be the *N,N'*-dialkylation of *o*-phenylendiamine followed by ring closure to obtain the corresponding benzimidazol-2-thione. *o*-Phenylenediamine (**5.21**) was alkylated with bromoethane (2 eq), sodium hydride (2.2 eq) as base in DMF at 60 °C for 2 hours yielding both the diethylated compound (21 %) and the monoethylated substance (38 %). According to the literature the cyclisation of 1,2-diaminobenzene to benzimidazoles is often performed with carbon disulphide[182,183] under heat in an alkaline alcoholic medium[184,185,186]. A convenient and mild procedure can be envisaged with the reagent TCDI. Therefore, **5.22** and **5.23** were stirred with TCDI in dry THF overnight at ambient temperature resulting in moderate and high yields of **5.20**

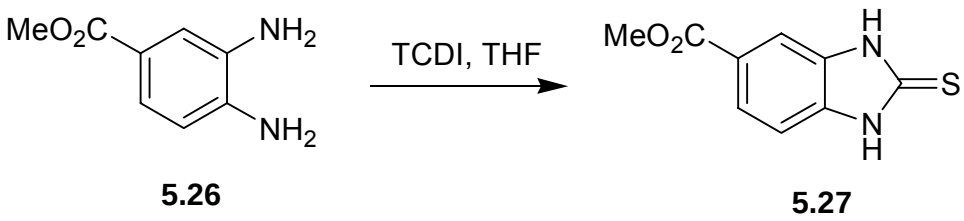
(37%) and **5.24** (93%), respectively. **5.24** was converted to **5.25** by acylation with acetyl chloride in anhydrous THF in the presence of triethylamine.



Reagents: i) $BrCH_2CH_3$ (2 eq), NaH (2.2 eq), DMF, 60 °C, 2h;
ii) TCDI (1.1 eq), THF; iii) AcCl, Et_3N, THF

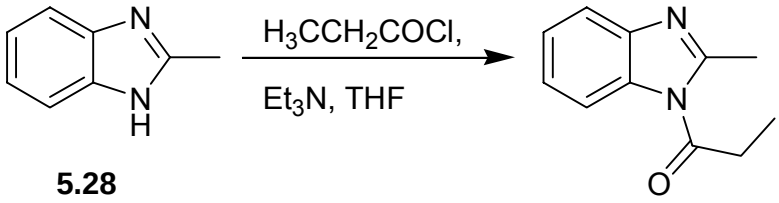
Scheme 5.6: Synthetic pathway to mono- and dialkylated benzimidazol-2-thione derivatives

The introduction of an additional substituent at the benzene ring of the benzimidazole scaffold was achieved by a ring closing reaction of methyl 3,4-diaminobenzoate (**5.26**) with TCDI in dry THF. Compound **5.27** was obtained in high yield (Scheme 5.7).



Scheme 5.7: Synthesis of methyl 2-thioxo-3*H*-benzo[*d*]imidazole-5-carboxylate (**5.27**)

The new 2-methylbenzimidazole derivative **5.29** was synthesized by analogy with the aforementioned procedures as illustrated in Scheme 5.8.



5.28 **5.29**

Scheme 5.8: Acylation of 2-methylbenzimidazole (**5.28**)

5.3 Results and Discussion

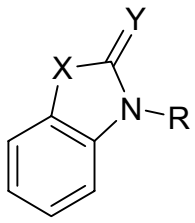
The synthesized compounds were investigated for inhibitory activity on the bacterial hyaluronidase from *S. agalactiae* strain 4755 at both pH 7.4 and pH 5, and on a hyaluronidase from bovine testis at pH 5 by using a turbidimetric assay according to a modified protocol from Di Ferrante[145] (see chapter 3).

In general, all compounds inhibited the bacterial enzyme but were inactive on the bovine testicular hyaluronidase. Therefore, only the inhibitory effects on $hylB_{4755}$ are elaborated on below. At the physiological pH, the affinity of the substrate HA is lower than at pH optimum probably facilitating the displacement of HA. In agreement with the pH dependency of the substrate affinity[150] the benzimidazoles were more potent at physiological pH.

5.3.1 N-Acylated benzimidazol-2-thiones

Starting from the lead structure **5.1** that bears two acetyl groups at the nitrogen atoms, a series of mono- and diacylated benzimidazol-2-thiones was synthesized and tested to prove the role of the acyl groups (Table 5.1). All monoacylated compounds (**5.3**-**5.8**) showed only weak inhibition of $hylB_{4755}$ at pH 5 at highest possible concentrations (dependent on the solubility of the compounds). Higher inhibitory potency was found at physiological pH.

Table 5.1: Inhibitory activities of acylated benzimidazoles on hyaluronidase from *S. agalactiae* at pH 5 and pH 7.4, respectively, and bovine testicular hyaluronidase at pH 5 (data determined in a turbidimetric assay)



Compd.	R	X	Y	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
				pH = 5.0	pH = 7.4	pH = 5.0
5.1[b]	$COCH_3$	$NCOCH_3$	S	160	5	n.d.
5.2	H	NH	S	1148 ± 50	1862±117	25 % (4000)
5.3	$COCH_3$	NH	S	20 % (460)	17 ± 1	inactive[c]
5.4	$COCH_2CH_3$	NH	S	4 % (180)	26 ± 3	inactive[d]
5.5	$CO(CH_2)_2CH_3$	NH	S	7 % (190)	20 ± 2	inactive[d]
5.6	$CO(CH_2)_4CH_3$	NH	S	14 % (170)	16 ± 1	inactive[d]
5.7	$CO(CH_2)_2Ph$	NH	S	5 % (100)	29 ± 5	inactive[e]
5.8	CO-*m*-ClC_6H_4	NH	S	16 % (400)	70 ± 10	inactive[f]
5.16	$CO(CH_2)_4CH_3$	$NCOCH_3$	S	50 % (49)	12 ± 1	inactive[g]
5.18	$COCH_3$	$NCOCH_3$	O	inactive[d]	10 % (200)	inactive[d]

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as percent inhibition at the inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] data according to Salmen[120], [c] at concentrations ≤ 460 µM, [d] at concentrations ≤ 200 µM, [e] at concentrations ≤ 100 µM, [f] at concentrations ≤ 400 µM, [g] at concentrations ≤ 50 µM.

Compounds **5.3**-**5.7** were about equipotent: IC_{50} values of 17 µM (**5.3**), 26 µM (**5.4**), 20 µM (**5.5**), 16 µM (**5.6**) and 29 µM (**5.7**) were determined (see Fig. 5.3). Compound **5.8** revealed a weaker inhibition with an IC_{50} of 70 µM. This may be interpreted as a hint that the benzoyl residue of **5.8** is too rigid. In contrast to the ascorbic acid derivatives described in chapter 4 an increase in hydrophobicity did not result in an increase in inhibitory activity; the extension of the alkanoyl chain of monoacylated benzimidazol-2-thiones had no influence on the inhibitory activity. However, for *N*,*N'*-diacylated compounds derived from **5.1** an extension of one of the alkanoyl chains by 4 methylene groups (**5.16**) resulted in

about 3-fold higher inhibitory activity compared to **5.1** (IC_{50} = 160 µM) at optimum pH.

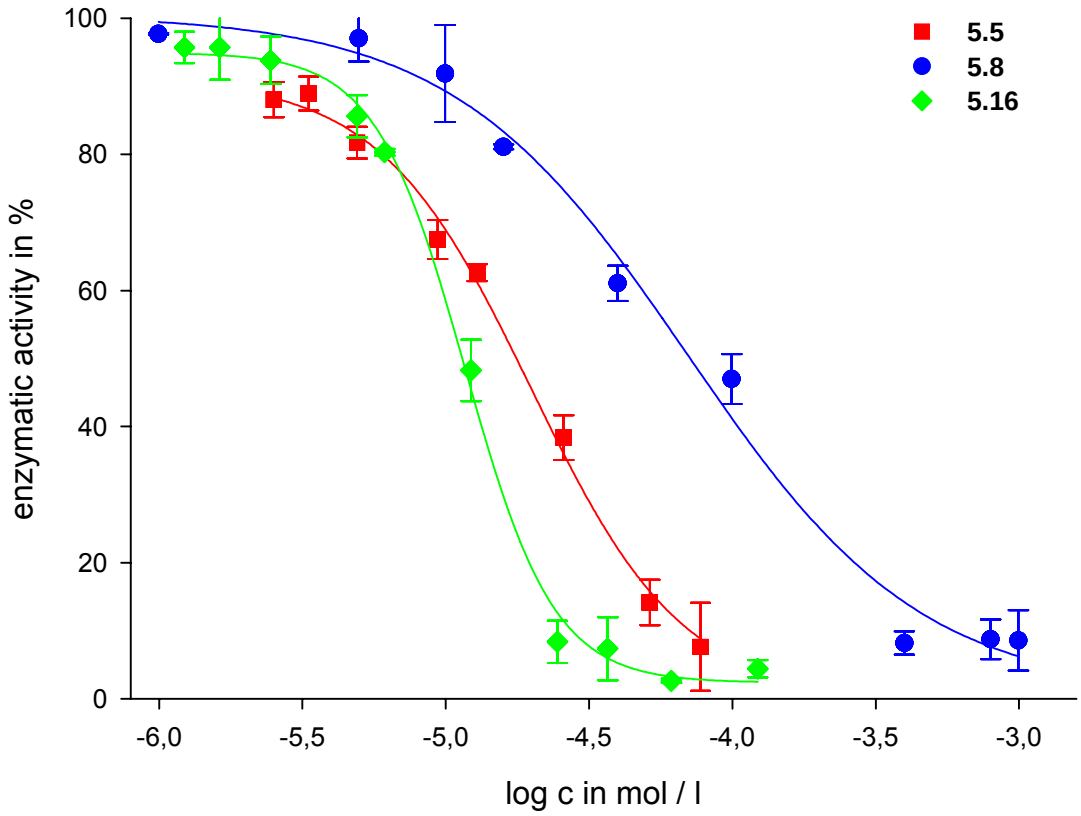


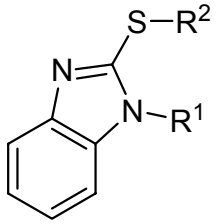
Fig. 5.3: Enzyme activity of $hylB_{4755}$ in the presence of the acylated benzimidazol-2-thiones **5.5**, **5.8** and **5.16** at physiological pH (7.4)

Due to the low solubility of **5.16** no IC_{50} value could be determined, but 50 % inhibition of the enzyme activity at a concentration of 49 µM could be measured. At physiological pH, the compounds **5.1** and **5.16** were the most potent ones (e.g., **5.16**: IC_{50} = 12 µM) within the whole benzimidazole set. The difference in activity depending on pH was most pronounced for the monoacylated benzimidazol-2-thiones. This could be explained by the protonated or unprotonated state of amino acid residues in the active site of $hylB_{4755}$ and of the inhibitors as well as the pH dependency of the substrate affinity. Lead structure **5.1** was significantly more potent than the corresponding oxo analog **5.18**. Further investigations of the promising *N,N'*-diacylated benzimidazol-2-thiones were not carried out because of the instability of such compounds resulting in a difficult synthesis with very low yields and doubtful results from pharmacological investigations due to (partial or total) decomposition during incubation in the turbidimetric assay.

5.3.2 S-Alkylated benzimidazole derivatives

Based upon the results shown in Table 5.1, we decided to continue with more stable *S*-substituted benzimidazole derivatives selecting either 2-sulfanylbenzimidazole or compound **5.3** as core skeleton.

Table 5.2: Inhibitory activities of *S*-alkylated benzimidazole derivatives on $hylB_{4755}$ at pH 5 and pH 7.4, respectively and on BTH at pH 5



Compd.	R^1	R^2	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
			pH = 5.0	pH = 7.4	pH = 5.0
5.9	H	CH_2CH_3	18 % (2000)	8 % (2000)	inactive[b]
5.12	$COCH_3$	CH_2CH_3	4 % (420)	5 % (420)	inactive[c]
5.13	$COCH_3$	$(CH_2)_6CH_3$	inactive[d]	inactive[e]	inactive[d]
5.14	$COCH_3$	CH_2Ph	4 % (100)	12 % (100)	inactive[f]
5.15	$COCH_3$	CH_2-*p*$MeOC_6H_4$	inactive[f]	inactive[g]	inactive[f]

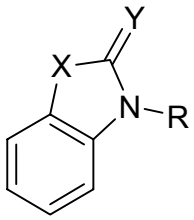
[a] inhibition of enzyme expressed as $IC_{50} \pm$ SEM in µM or as percent inhibition at the inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 2 mM, [c] at concentrations ≤ 420 µM, [d] at concentrations ≤ 140 µM, [e] at concentrations ≤ 270 µM, [f] at concentrations ≤ 100 µM, [g] at concentrations ≤ 120 µM.

Therefore, several 2-(alkylsulfanyl)benzimidazole derivatives were prepared and tested for inhibitory activity on $hyalB_{4755}$ and bovine testicular hyaluronidase. As summarized in Table 5.2 the inhibitory activity of compounds **5.9** and **5.12**-**5.15** on the bacterial hyaluronidase range from very weak to inactive. At optimum pH and at physiological pH a decrease in inhibitory activity on $hylB_{4755}$ was found for compound **5.9** compared to **5.2** and for compounds **5.12**-**5.15** compared to **5.3**.

5.3.3 N-Alkylated benzimidazole derivatives

Since the *S*-alkylation of *N*-monoacylated **5.3** and non-acylated benzimidazole **5.2** did not result in higher inhibitory potency it was attempted to stabilize the lead structure **5.1** by replacing the acetyl groups with ethyl groups. Considering compounds **5.1**, **5.20** and **5.25** the stepwise replacement of the acetyl groups by alkyl residues resulted in a gradual loss of inhibitory activity on $hylB_{4755}$ to the point of complete inactivity (at physiological pH, Table 5.3).

Table 5.3: Inhibitory activities of various benzimidazole derivatives determined on $hylB_{4755}$ at both pH optimum and physiological pH and on BTH at pH 5



Compd.	R	X	Y	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
				pH = 5.0	pH = 7.4	pH = 5.0
5.1[b]	$COCH_3$	$NCOCH_3$	S	160	5	n.d.
5.19	CH_2CH_3	NCH_2CH_3	O	40 % (4900)	inactive[c]	inactive[c]
5.20	CH_2CH_3	NCH_2CH_3	S	inactive[d]	inactive[d]	inactive[d]
5.24	H	NCH_2CH_3	S	19 % (4200)	28 % (4200)	inactive[e]
5.25	$COCH_3$	NCH_2CH_3	S	10 % (380)	19 ± 1	inactive[f]

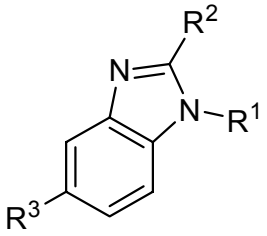
[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as percent inhibition at the inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] data from Salmen[120], [c] at concentrations ≤ 4.9 mM, [d] at concentrations ≤ 100 µM, [e] at concentrations ≤ 4.2 mM, [f] at concentrations ≤ 380 µM.

Moreover, the oxo analog **5.19** of **5.20** did not show an improved inhibitory effect. As found for the *N,N'*-diacetylated lead **5.1** in comparison to the alkylated analogs, the *N*-monoacetylated benzimidazol-2-thione **5.3** was likewise much more active than its corresponding ethyl analog **5.24**. At least one amide bond seems to be essential for the inhibitory effect of this class of compounds.

5.3.4 Substituted benzimidazole derivatives

An additional functional group was introduced at the anellated benzene moiety of benzimidazole to broaden the possibilities for derivatization and structural variation, respectively, and to improve the solubility of these compounds at the same time. This was achieved with the synthesis of compound **5.27** bearing a methoxycarbonyl group in position 5.

Table 5.4: Enzymatic activity of bovine testicular hyaluronidase and hyaluronate lyase of *S. agalactiae* in the presence of compounds **5.2** and **5.27**-**5.29**



No.	R^1	R^2	R^3	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
				pH = 5.0	pH = 7.4	pH = 5.0
5.2	H	SH	H	1148 ± 50	1862 ± 117	25 % (4000)
5.27	H	SH	CO_2CH_3	25 % (4500)	53 % (4500)	inactive[b]
5.28	H	CH_3	H	inactive[c]	6 % (4000)	10 % (4000)
5.29	$COCH_2CH_3$	CH_3	H	12 % (2000)	inactive[d]	inactive[d]

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as percent inhibition at the inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 4.5 mM, [c] at concentrations ≤ 4 mM, [d] at concentrations ≤ 2 mM.

Actually, the solubility in aqueous buffer was improved, however, the benzimidazol-5-carboxylic acid ester **5.27** was a less active hyaluronidase inhibitor than compound **5.2** (Table 5.4). Finally, the thione group in position 2 of **5.2** was replaced by a methyl group (**5.28**). This modification caused a distinct loss of inhibitory activity on the bacterial enzyme both at physiological and optimum pH. Moreover, in contrast to 2-sulfanylbenzimidazole the introduction of a propanoyl residue in position 1 of 2-methylbenzimidazole was inappropriate to increase the inhibitory potency (see **5.28** vs. **5.29**, Table 5.4, and **5.2** vs. **5.4**, Table 5.1). Thus, the thione group in position 2 of the benzimidazole moiety seems to be crucial for inhibition of the bacterial hyaluronidase.

5.4 Summary

The synthesized *N*-monoacylated and *N,N'*-diacylated benzimidazol-2-thiones proved to be inhibitors of hyaluronate lyase from *S. agalactiae* strain 4755 ($hylB_{4755}$). The compounds are selective for the bacterial enzyme, whereas bovine testicular hyaluronidase is not affected. Compared to the lead compound 1,3-diacetylbenzimidazol-2-thione (**5.1**) only 1-acetyl-3-hexanoylbenzimidazol-2-thione (**5.16**) displayed IC_{50} values in low micromolar range. Although the *N,N'*-diacylated derivatives **5.1** and **5.16** are potent inhibitors of $hylB_{4755}$ at both pH optimum and physiological pH they cannot be used for further investigations owing to their very high instability against hydrolysis. The much more stable *N*-monoacylated derivatives **5.3**-**5.8** revealed likewise IC_{50} values in the low µM range but only at physiological pH. The difference in activity depending on pH was most obvious for the monoacylated benzimidazoles. An exchange of the acyl residues by ethyl groups resulted in a strong decrease in inhibitory activity. Thus, an amide bond appears to be essential for binding to the active site of the enzyme. Moreover, the thioxo function in position 2 of the benzimidazoles is favorable to increase the inhibitory activity. The thiones were significantly more potent than the oxo analogs, and a methyl group in position 2 led to a marked decrease in inhibitory activity.

5.5 Experimental section

5.5.1 General conditions

See section 4.5.1 for general methods.

5.5.2 Chemistry

Synthesis of the monoacylated benzimidazoles 5.3-5.8

General procedures

Method A. 2-Sulfanylbenzimidazole (**5.2**) (1 eq) and acid anhydride (1.1 eq) were dissolved in pyridine (4.4 eq) under a nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. The formed precipitate was removed by suction and washed with a small amount of dichloromethane. The crude product was purified as stated below.

Method B. 2-Sulfanylbenzimidazole (**5.2**) (1 eq) and acid chloride or acid anhydride (1.1 eq) were dissolved in pyridine (4.4 eq) under a nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. After quenching with water (10-25 ml), ethyl acetate (30-45 ml) was added. The organic phase was extracted with 3M hydrochloric acid (3 x 25 ml), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified as stated below.

1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (5.3)

Method A: Reaction of 2-sulfanylbenzimidazole (**5.2**) (4.02 g, 26.76 mmol), acetic anhydride (3.00 ml, 31.74 mmol) and pyridine (9.70 ml, 120.18 mmol); recrystallization from ethyl acetate.

Yield: 3.80 g (19.77 mmol, 74 %, white solid)

Mp: 193-194 °C (Lit. 198-200 °C[179])

^{1}H-NMR (DMSO-d_6): δ [ppm] = 3.00 (s, 3H, CH_3), 7.16-7.33 (m, 3H, Ar-H), 7.97-8.02 (m, 1H, Ar-H), 13.31 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 192 ([$M^{\bullet+}$], 20), 150 ([M - CH_2CO] $^+$, 100)

Analysis: $C_9H_8N_2OS$ (192.24)

calculated	C: 56.23	H: 4.19	N: 14.57
found	C: 56.27	H: 4.23	N: 14.89

1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)propan-1one (5.4)

Method B: Reaction of 2-sulfanylbenzimidazole (**5.2**) (1.03 g, 6.86 mmol), propanoyl chloride (0.64 ml, 7.33 mmol) and pyridine (2.40 ml, 29.73 mmol); recrystallization from ethyl acetate.

Yield: 0.78 g (3.79 mmol, 55 %, white flakes)

Mp: 176-177.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.19 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H}_3$), 3.56 (q, 2H, ^{3}J = 7.1 Hz, $C\underline{H}_2CH_3$), 7.17-7.34 (m, 3H, Ar-H), 7.97-8.02 (m, 1H, Ar-H), 13.29 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 206 ([$M^{\bullet+}$], 6), 150 ([M - CH_3CHCO]$^+$, 100)

Analysis: $C_{10}H_{10}N_2OS$ (206.05)

calculated	C: 58.23	H: 4.89	N: 13.58	S: 15.55
found	C: 58.43	H: 5.09	N: 13.26	S: 15.20

1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)butan-1-one (5.5)

Method B: Reaction of 2-sulfanylbenzimidazole (**5.2**) (1.00 g, 6.66 mmol), butyric anhydride (1.20 ml, 7.32 mmol) and pyridine (2.40 ml, 29.73 mmol); crude product was treated with a 2:1 (v/v) mixture of petroleum ether (boiling point range 60-80 °C) and ethyl acetate to separate pure product; analytically pure product was obtained by column chromatography on silica gel eluting with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.98 g (4.45 mmol, 67 %, white solid)

Mp: 152-153 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.98 (t, 3H, ^{3}J = 7.4 Hz, $(CH_2)_2C\underline{H}_3$), 1.67-1.81 (m, 2H, $CH_2C\underline{H}_2CH_3$), 3.56 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 7.17-7.34 (m, 3H, Ar-H), 7.95-8.00 (m, 1H, Ar-H), 13.29 (br, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.3 (+, CH_3), 17.6 (-, CH_2), 40.7 (-, CH_2), 109.4 (+, Ar-C), 115.0 (+, Ar-C), 123.3 (+, Ar-C), 125.2 (+, Ar-C), 130.9 (C_{quart}, Ar-C), 131.0 (C_{quart}, Ar-C), 169.4 (C_{quart}, CS), 175.1 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 220 ([$M^{\bullet+}$], 13), 150 ([M - C_4H_9CHCO]$^+$, 100)

Analysis: $C_{11}H_{12}N_2OS$ (220.30)

calculated	C: 59.97	H: 5.49	N: 12.72	S: 14.56
found	C: 60.06	H: 5.50	N: 12.56	S: 14.66

1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)hexan-1-one (5.6)

Method B: Reaction of 2-sulfanylbenzimidazole (**5.2**) (1.02 g, 6.79 mmol), hexanoic anhydride (1.80 ml, 7.81 mmol) and pyridine (2.40 ml, 29.73 mmol); purification by column chromatography on silica gel, elution with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.58 g (2.34 mmol, 34 %, white solid)

Mp: 132.5-133 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.89 (t, 3H, ^{3}J =7.0 Hz, $(CH_2)_4C\underline{H}_3$), 1.28-1.40 (m, 4H, $(C\underline{H}_2)_2CH_3$), 1.67-1.78 (m, 2H, $COCH_2C\underline{H}_2$-), 3.57 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 7.17-7.33 (m, 3H, Ar-H), 7.94-7.99 (m, 1H, Ar-H), 13.29 (br, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.7 (+, CH_3), 21.8 (-, CH_2), 23.7 (-, CH_2), 30.5 (-, CH_2), 38.7 (-, CH_2), 109.5 (+, Ar-C), 115.0 (+, Ar-C), 123.3 (+, Ar-C), 125.2 (+, Ar-C), 130.9 (C_{quart}, Ar-C), 131.1 (C_{quart}, Ar-C), 169.4 (C_{quart}, CS), 175.2 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 248 ([$M^{\bullet+}$], 9), 150 ([M - C_4H_9 - CHCO]$^+$, 100)

Analysis: $C_{13}H_{16}N_2OS$ (248.35)

calculated	C: 62.87	H: 6.49	N: 11.28	S: 12.91
found	C: 62.91	H: 6.49	N: 11.05	S: 12.60

1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)-3-phenylpropan-1-one (5.7)

Method B: Reaction of 2-sulfanylbenzimidazole (**5.2**) (0.50 g, 3.33 mmol), 3-phenylpropanoyl chloride (0.50 ml, 3.33 mmol) and pyridine (2.20 ml, 26.98 mmol); purification by flash column chromatography on silica gel, elution with a 5:2 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.46 g (1.63 mmol, 49 %, white solid)

Mp: 161-162.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 3.05 (t, 2H, ^{3}J = 7.8 Hz, $COC\underline{H}_2CH_2Ph$), 3.90 (t, 2H, ^{3}J = 7.8 Hz, $COCH_2C\underline{H}_2Ph$), 7.16-7.34 (m, 8H, Ar-H), 7.96-7.99 (m, 1H, Ar-H), 13.33 (br, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 30.0 (-, CH_2), 40.7 (-, CH_2), 109.5 (+, Ar-C), 115.1 (+, Ar-C), 123.3 (+, Ar-C), 125.3 (+, Ar-C), 126.0 (+, Ar-C), 128.3 (+, 4Ar-C), 130.9 (C_{quart}, Ar-C), 131.1 (C_{quart}, Ar-C), 140.5 (C_{quart}, Ar-C), 169.4 (C_{quart}, CS), 174.4 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 282 ([$M^{\bullet+}$], 6), 249 ([M - SH]$^+$, 19), 150 ([M - $C_6H_5CH_2CHCO$] $^+$, 100), 91 ([C_7H_7]$^+$, 28)

Analysis: $C_{16}H_{14}N_2OS$ (282.37)

calculated	C: 68.06	H: 5.00	N: 9.92	S: 11.36
found	C: 67.95	H: 5.07	N: 9.92	S: 11.18

(3-Chlorophenyl)(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)methanone (5.8)

Method B: Reaction of 2-sulfanylbenzimidazole (**5.2**) (0.30 g, 2.00 mmol), *m*-chlorobenzoyl chloride (0.26 ml, 2.00 mmol) and pyridine (3.50 ml, 42.92 mmol); purification by flash column chromatography on silica gel, elution with dichloromethane.

Yield: 0.13 g (0.45 mmol, 23 %, pale yellow solid)

Mp: 170-172 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 7.21-7.35 (m, 3H, benzimidazole-H), 7.45-7.48 (m, 1H, benzimidazole-H), 7.55 (ddd, 1H, ^{3}J = 8.1 Hz, ^{3}J = 7.7 Hz, ^{5}J = 0.4 Hz, H-5), 7.75 (ddd, 1H, ^{3}J = 8.1 Hz, ^{4}J = 2.1 Hz, ^{5}J = 1.1 Hz, H-4/6), 7.80 (ddd, 1H, ^{3}J = 7.7 Hz, ^{4}J = 1.7 Hz, ^{5}J = 1.1 Hz, H-4/6), 7.91 (ddd, 1H, ^{4}J = 2.1 Hz, ^{4}J = 1.7 Hz, ^{5}J = 0.4 Hz, H-2), 13.29 (br, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 110.0 (+, Ar-C), 112.1 (+, Ar-C), 123.2 (+, Ar-C), 124.9 (+, Ar-C), 128.8 (+, Ar-C), 129.7 (+, Ar-C), 130.5 (+, Ar-C), 131.3 (C_{quart}, Ar-C), 131.9 (C_{quart}, Ar-C), 133.1 (C_{quart}, Ar-C), 133.5 (+, Ar-C), 134.8 (C_{quart}, Ar-C), 168.5 (C_{quart}, CO), 169.5 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 288 ([$M^{\bullet+}$], 41), 139 ([ClC_6H_4CO]$^+$, 100), 111 ([ClC_6H_4]$^+$, 47)

$C_{14}H_9ClN_2OS$ (288.75)

2-(Ethylsulfanyl)-1*H*-benzo[*d*]imidazole (5.9)

A solution of 2-sulfanylbenzimidazole (**5.2**) (1.03 g, 6.86 mmol), bromoethane (0.50 ml, 6.70 mmol) and DMF (15 ml) was cooled down in an ice-bath under an inert atmosphere. Sodium hydride (0.24 g, 10.0 mmol) was added in small portions and the reaction was stirred overnight. After quenching the reaction with methanol (20 ml), the mixture was stirred for 30 min and concentrated under reduced pressure. The residue was taken up in ethyl acetate. After addition of water (20 ml), the phases were separated and the aqueous phase was extracted with ethyl acetate (2x15 ml). The combined organic phases were washed with water (15 ml) and dried over sodium sulfate. Then the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel eluting with a 2:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.60 g (3.37 mmol, 49 %, white flakes)

Mp: 165-166 °C (Lit. 172-173 °C[187])

^{1}H-NMR (DMSO-d_6): δ [ppm] = 1.36 (t, 3H, ^{3}J = 7.3 Hz, $CH_2C\underline{H}_3$), 3.27 (q, 2H, ^{3}J = 7.3 Hz, $C\underline{H}_2CH_3$), 7.07-7.14 (m, 2H, Ar-H), 7.31-7.39 (m, 1H, Ar-H), 7.47-7.54 (m, 1H, Ar-H), 12.49 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 178 ([$M^{\bullet+}$], 96), 163 ([M - CH_3]$^+$, 28), 150 ([M - C_2H_4]$^+$, 100), 145 ([M-SH]$^+$, 72)

Analysis: $C_9H_{10}N_2S$ (178.25)

calculated	C: 60.64	H: 5.65	N: 15.72
found	C: 60.69	H: 5.56	N: 15.83

***N*-(2-Aminophenyl)acetamide (5.11)**

To 2-nitroacetanilide (**5.10**) (1.80 g, 10.0 mmol), dissolved in absolute methanol (40 ml), 10 % Pd-C catalyst (100 mg) and 15 drops of glacial acetic acid were added. After hydrogenation in an autoclave at 3 bar at room temperature for 25 h, the solution was filtrated, washed with methanol and concentrated *in vacuo*. The crude product was recrystallized from methanol.

Yield: 0.76 g (5.06 mmol; 51%, creamy solid)

Mp: 127-128 °C (Lit. 130-131 °C[188])

^{1}H-NMR (DMSO-*d*$_6$): δ (ppm) = 2.03 (s, 3H, CH_3), 4.84 (br, 2H, NH_2), 6.52 (ddd, 1H, ^3J = 7.8 Hz, ^3J = 7.2 Hz, ^4J = 1.5 Hz, H-5), 6.70 (dd, 1H, ^3J = 7.9 Hz, ^4J = 1.5 Hz, H-3), 6.88 (ddd, 1H, ^3J = 7.9 Hz, ^3J = 7.2 Hz, ^4J = 1.5 Hz, H-4), 7.14 (dd, 1H, ^3J =7.8 Hz, ^4J = 1.5 Hz, H-6), 9.11 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 150 ([$M^{\bullet+}$], 64), 108 ([M - CH_2CO]$^+$, 100)

$C_8H_{10}N_2O$ (150.18)

Synthesis of the 1-(2-alkylsulfanyl-1*H*-benzo[*d*]imidazol-1-yl)ethanones 5.12-5.15

General procedure. 1-(2-Thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) (1 eq) was dissolved in DMF (10-15 ml) and treated with the pertinent alkyl halide (1 eq) under a nitrogen atmosphere. After cooling with an ice-bath, sodium hydride (60 % suspension in paraffin oil) (1.5 eq) was added in small portions. The reaction mixture was stirred overnight at room temperature. Afterwards the reaction was stopped with methanol (10 ml) to destroy excess sodium hydride. After stirring for a few minutes, water (20-40 ml) and ethyl acetate (20-35 ml) were added. The separated aqueous phase was extracted twice with ethyl acetate (15 ml). The combined organic layers were dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified as stated below.

1-[2-(Ethylsulfanyl)-1*H*-benzo[*d*]imidazol-1-yl]ethanone (5.12)

Reaction of 1-(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) (0.70 g, 3.64 mmol), bromoethane (0.28 ml, 3.75 mmol) and sodium hydride (0.24 g, 6.00 mmol); purification by column chromatography on silica gel, elution with a 2:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.50 g (2.27 mmol, 62 %, white solid)

Mp: 108-109 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.38 (t, 3H, ^{3}J = 7.4 Hz, $SCH_2C\underline{H}_3$), 2.81 (s, 3H, $COCH_3$), 3.23 (q, 2H, ^{3}J = 7.4 Hz, $SC\underline{H}_2CH_3$), 7.26-7.36 (m, 2H, Ar-H), 7.59-7.65 (m, 1H, Ar-H), 7.77-7.84 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 220 ([$M^{\bullet+}$], 65), 178 ([M - CH_2CO] $^+$, 51), 163 ([M - CH_2CO - CH_3] $^+$, 27), 150 ([M - CH_2CO - C_2H_4] $^+$, 100)

Analysis: $C_{11}H_{12}N_2OS$ (220.30)

calculated	C: 59.97	H: 5.49	N: 12.72
found	C: 60.19	H: 5.58	N: 12.30

1-[2-(Heptylsulfanyl)-1*H*-benzo[*d*]imidazol-1-yl]ethanone (5.13)

Reaction of 1-(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) (0.72 g, 3.75 mmol), 1-bromoheptane (0.58 ml, 3.69 mmol) and sodium hydride (0.25 g, 6.25 mmol); purification by column chromatography on silica gel, elution with a 7:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.35 g (1.21 mmol, 33 %, white solid)

Mp: 42-43 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86 (t, 3H, ^{3}J = 6.9 Hz, $(CH_2)_6C\underline{H}_3$), 1.21-1.48 (m, 8H, $(C\underline{H}_2)_4CH_3$), 1.67-1.79 (m, 2H, $SCH_2C\underline{H}_2$-), 2.81 (s, 3H, $COCH_3$), 3.22 (t, 2H, ^{3}J = 7.3 Hz, SCH_2-), 7.26-7.36 (m, 2H, Ar-H), 7.57-7.63 (m, 1H, Ar-H), 7.77-7.83 (m, 1H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, CH_3), 22.0 (-, CH_2), 26.2 (+, $CO\underline{C}H_3$), 28.0 (-, CH_2), 28.2 (-, CH_2), 28.3 (-, CH_2), 31.1 (-, CH_2), 31.4 (-, CH_2), 114.1 (+, Ar-C), 118.1 (+, Ar-C), 123.1 (+, Ar-C), 124.1 (+, Ar-C), 132.9(C_{quart}, Ar-C), 143.5 (C_{quart}, Ar-C), 154.7 (C_{quart}, Ar-C), 169.4 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 290 ([$M^{\bullet+}$], 15), 248 ([M - CH_2CO]$^+$, 3), 150 ([M - CH_2CO - C_7H_{14}]$^+$, 100)

Analysis: $C_{16}H_{22}N_2OS$ (290.42)

calculated	C: 66.17	H: 7.64	N: 9.65
found	C: 66.36	H: 7.34	N: 9.42

1-(2-(Benzylsulfanyl)-1*H*-benzo[*d*]imidazol-1-yl)ethanone (5.14)

Reaction of 1-(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) (0.70 g, 3.64 mmol), benzyl bromide (0.45 ml, 3.79 mmol) and sodium hydride (0.24 g, 6.00 mmol); purification by column chromatography on silica gel, elution with dichloromethane.

Yield: 0.45 g (1.59 mmol, 44 %, white solid)

Mp: 119-120 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.81 (s, 3H, $COCH_3$), 4.51 (s, 2H, CH_2), 7.24-7.38 (m, 5H, Ar-H), 7.47-7.52 (m, 2H, Ar-H), 7.63-7.67 (m, 1H, Ar-H), 7.78-7.84 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 282 ($[M^{\bullet+}]$, 54), 240 ($[M - CH_2CO]^+$, 80), 207 ($[M - CH_2CO - SH]^+$, 48), 91 ($[C_6H_5CH_2]^+$, 100)

Analysis: $C_{16}H_{14}N_2OS$ (282.36)

calculated	C: 68.06	H: 5.00	N: 9.92
found	C: 68.26	H: 5.17	N: 9.81

1-[2-(4-Methoxybenzylsulfanyl)-1*H*-benzo[*d*]imidazol-1-yl]ethanone (5.15)

Reaction of 1-(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)ethanone (**5.3**) (0.50 g, 2.60 mmol), 4-methoxybenzylchloride (0.37 ml, 2.73 mmol; stabilized in 1% K_2CO_3) and sodium hydride (0.16 g, 4.00 mmol); extraction with dichloromethane instead of ethyl acetate; purification by column chromatography on silica gel, elution with dichloromethane; analytical pure sample was obtained by recrystallization from ethanol.

Yield: 0.25 g (0.80 mmol, 31 %, white needles)

Mp: 121-122 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.80 (s, 3H, $COCH_3$), 3.73 (s, 3H, OCH_3), 4.45 (s, 2H, SCH_2), 6.85-6.92 (m, 2H, Ar-H), 7.28-7.44 (m, 4H, Ar-H), 7.61-7.67 (m, 1H, Ar-H), 7.77-7.84 (m, 1H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 26.0 (+, CH_3), 35.3 (-, CH_2), 55.0 (+, CH_3), 113.8 (+, 2Ar-C), 114.0 (+, Ar-C), 118.1 (+, Ar-C), 123.2 (+, Ar-C), 124.2 (+, Ar-

C), 128.3 (C_{quart}, Ar-C), 130.4 (+, 2Ar-C), 132.7 (C_{quart}, Ar-C), 143.4 (C_{quart}, Ar-C), 154.3 (C_{quart}, Ar-C), 158.4 (C_{quart}, Ar-C), 169.5 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 312 ($[M^{\bullet+}]$, 20), 270 ($[M - CH_2CO]^+$, 12), 121 ($[MeOC_6H_4CH_2]^+$, 100)

Analysis: $C_{17}H_{16}N_2O_2S$ (312.4)

calculated	C: 65.36	H: 5.16	N: 8.97	S: 10.26
found	C: 65.24	H: 5.38	N: 8.94	S: 10.23

1-(3-Acetyl-1,2-dihydro-2-thioxobenzo[*d*]imidazol-1-yl)hexan-1-one (5.16)

To a solution of 1-(2-thioxo-1*H*-benzo[*d*]imidazol-1-yl)hexan-1-one (**5.6**) (0.29 g, 1.17 mmol), triethylamine (0.19 ml, 1.37 mmol) and absolute THF (10 ml) acetyl chloride (0.10 ml, 1.40 mmol) was added. After stirring for 1 h at room temperature, the reaction mixture was concentrated *in vacuo*. Water (10 ml) was added and the aqueous phase was extracted with ethyl acetate (3x10 ml). The combined organic layers were washed with a solution of saturated sodium carbonate (10 ml), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel eluting with a 8:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 20 mg (0.07 mmol, 6 %, white solid)

^{1}H-NMR (DMSO-d_6): δ [ppm] = 0.88 (t, 3H, ^3J = 7.1 Hz, $(CH_2)_4C\underline{H}_3$), 1.29-1.40 (m, 4H, $(C\underline{H}_2)_2CH_3$), 1.70-1.80 (m, 2H, $COCH_2C\underline{H}_2$-), 2.98 (s, 3H, $COCH_3$), 3.45 (t, 2H, ^3J = 7.3 Hz, $COCH_2$-), 7.34-7.40 (m, 2H, Ar-H), 7.69-7.74 (m, 1H, Ar-H), 7.86-7.90 (m, 1H, Ar-H)

$C_{15}H_{18}N_2O_2S$ (290.40)

1,3-Diacetyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (5.18)

2-Hydroxybenzimidazole (**5.17**) (0.50 g, 3.73 mmol) was suspended in acetic anhydride (15 ml) and heated under reflux for 6 h. Water (20 ml) and ethyl acetate (30 ml) were added and the separated organic phase was extracted with a solution of saturated sodium carbonate (3x20 ml). The organic phase was

washed with water (20 ml), dried over magnesium sulfate and concentrated under reduced pressure. The crude product was recrystallized from a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.59 g (2.70 mmol, 73 %, white needles)

Mp: 145.5-146.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.66 (s, 6H, 2COCH$_3$), 7.27-7.34 (m, 2H, Ar-H), 8.09-8.16 (m, 2H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 218 ([M$^{\bullet+}$], 15), 176 ([M - CH_2CO]$^+$, 18), 134 ([M - 2CH_2CO]$^+$, 100)

Analysis: $C_{11}H_{10}N_2O_3$ (218.21)

calculated	C: 60.55	H: 4.62	N: 12.84
found	C: 60.42	H: 4.53	N: 12.98

1,3-Diethyl-1*H*-benzo[*d*]imidazol-2(3*H*)-one (5.19)

2-Hydroxybenzimidazole (**5.17**) (0.50 g, 3.73 mmol), dissolved in benzene (10 ml), a solution of sodium hydroxide (50%, 5 ml), TEBAC (0.06 g, 0.20 mmol) and bromoethane (0.85 ml, 11.39 mmol) were stirred at 60 °C for 4 h. The mixture was cooled down and the separated organic phase was washed with water (3x10 ml). After drying the organic phase over magnesium sulfate, the solvent was removed under reduced pressure. The pale yellow crude product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of chloroform and ethyl acetate.

Yield: 0.40 g (2.10 mmol, 56 %, white solid)

Mp: 67-68 °C (Lit. 68-69 °C[189])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 1.34 (t, 6H, ^{3}J = 7.3 Hz, 2$CH_2C\underline{H_3}$), 3.95 (q, 4H, ^{3}J = 7.3 Hz, 2$C\underline{H_2}CH_3$), 6.97-7.12 (m, 4H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 190 ([M$^{\bullet+}$], 100), 175 ([M - CH_3]$^+$, 87), 162 ([M - C_2H_4]$^+$, 20), 147 ([M - C_2H_4 - CH_3]$^+$, 38)

Analysis: $C_{11}H_{14}N_2O$ (190.24)

calculated	C: 69.45	H: 7.42	N: 14.73

found C: 69.40 H: 7.07 N: 14.45

1,3-Diethyl-1*H*-benzo[*d*]imidazole-2(3*H*)-thione (5.20)

N^1,N^2-Diethylbenzene-1,2-diamine (**5.23**) (0.15 g, 0.91 mmol) was dissolved in absolute THF (10 ml). This solution was treated with TCDI (0.18 g, 1.01 mmol) under a nitrogen atmosphere. After stirring at room temperature for 22 h, the mixture was concentrated *in vacuo*. Ethyl acetate (15 ml) and water (15 ml) were added to the residue and the organic phase was separated. The aqueous phase was extracted three times with ethyl acetate (15 ml). The combined organic phases were dried over magnesium sulfate and the solvent was removed. The crude product was purified by column chromatography on silica gel eluting with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.07 g (0.34 mmol, 37 %, pale yellow oil)

^{1}H-NMR (DMSO-d_6): δ [ppm] = 1.26 (t, 6H, ^{3}J = 7.1 Hz, $2CH_2C\underline{H}_3$), 4.33 (q, 4H, ^{3}J = 7.1 Hz, $2C\underline{H}_2CH_3$), 7.23-7.31 (m, 2H, Ar-H), 7.44-7.53 (m, 2H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 207 ([$M^{\bullet+}$], 83), 164 ([M – CS + 2H]$^+$, 100), 119 ([$C_6H_5NCH_2CH_2$]$^+$, 53)

$C_{11}H_{14}N_2S$ (206.31)

N^1-Ethylbenzene-1,2-diamine (5.22) and N^1,N^2-diethylbenzene-1,2-diamine (5.23)

A solution of *o*-phenylendiamine (**5.21**) (0.50 g, 4.62 mmol) and bromoethane (0.68 ml, 9.11 mmol) in DMF (10 ml) was treated with sodium hydride (0.25 g, 6.25 mmol) in small portions under an inert atmosphere. After stirring for 2 h at 60 °C, the reaction was quenched with methanol (10 ml) and stirred for further 30 min. The solution was concentrated under reduced pressure and water (20 ml) and ethyl acetate (20 ml) were added. The separated aqueous phase was extracted with ethyl acetate (5x20 ml). The combined organic layers were dried over magnesium sulfate and the solvent was removed under reduced pressure. The mixture was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

The first fraction yielded the dialkylated product **5.23** (0.16 g, 0.97 mmol, 21 %, red-brown oil) whereas the second fraction provided the monoalkylated compound **5.22** (0.24 g, 1.76 mmol, 38 %, red-brown oil).

***N*^{1}-Ethylbenzene-1,2-diamine** (**5.22**):

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.20 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H}_3$), 3.02 (q, 2H, ^{3}J = 7.1 Hz, $C\underline{H}_2CH_3$), 4.46 (br, 3H, NH), 6.36-6.43 (m, 2H, Ar-H), 6.45-6.55 (m, 2H, Ar-H)

$C_8H_{12}N_2$ (136.19)

***N*^{1},*N*^{2}-Diethylbenzene-1,2-diamine** (**5.23**):

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.21 (t, 6H, ^{3}J = 7.1 Hz, $2CH_2C\underline{H}_3$), 3.03 (dq, 4H, ^{3}J = 7.1 Hz, ^{3}J = 5.0 Hz, $NHC\underline{H}_2CH_3$), 4.41 (t, 2H, ^{3}J = 5.0 Hz, $N\underline{H}CH_2$), 6.38-6.45 (m, 2H, Ar-H), 6.48-6.56 (m, 2H, Ar-H)

$C_{10}H_{16}N_2$ (164.25)

1-Ethyl-1*H*-benzo[*d*]imidazole-2(3*H*)-thione (5.24)

N^1-Ethylbenzene-1,2-diamine (**5.22**) (0.23 g, 1.69 mmol) was dissolved in absolute THF (10 ml). This solution was treated with TCDI (0.33 g, 1.85 mmol) under a nitrogen atmosphere. After stirring at room temperature for 22 h, the mixture was concentrated *in vacuo*. Ethyl acetate (15 ml) and water (15 ml) were added to the residue and the organic phase was separated. The aqueous phase was extracted three times with ethyl acetate (15 ml). The combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel eluting with a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.28 g (1.57 mmol, 93 %, white needles)

Mp: 161.5-162.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.24 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H_3}$), 4.27 (q, 2H, ^{3}J = 7.1 Hz, $C\underline{H}_2CH_3$), 7.17-7.24 (m, 3H, Ar-H), 7.38-7.45 (m, 1H, Ar-H), 12.74 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 178 ([$M^{\bullet+}$], 100), 150 ([M - C_2H_4]$^+$, 91)

Analysis: $C_9H_{10}N_2S$ (178.25)

calculated	C: 60.64	H: 5.65	N: 15.72
found	C: 60.55	H: 5.68	N: 15.68

1-(3-Ethyl-1,2-dihydro-2-thioxobenzo[*d*]imidazole-1-yl)ethanone (5.25)

A solution of 1-ethyl-1*H*-benzo[*d*]imidazole-2(3*H*)-thione (**5.24**) (0.14 g, 0.79 mmol), dissolved in absolute THF (5 ml), triethylamine (0.11 ml, 0.79 mmol) and acetyl chloride (0.07 ml, 0.98 mmol) was stirred at room temperature for 1 h under an inert atmosphere. Afterwards, the reaction mixture was concentrated under reduced pressure and water (5 ml) was added. After extraction with ethyl acetate (3x10 ml), the combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel eluting with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.07 g (0.32 mmol, 40 %, white solid)

Mp: 81.5-82.5 °C

^{1}H-NMR (DMSO-d_6): δ [ppm] = 1.27 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H_3}$), 3.03 (s, 3H, $COCH_3$), 4.37 (q, 2H, ^{3}J = 7.1 Hz, $C\underline{H_2}CH_3$), 7.28-7.43 (m, 2H, Ar-H), 7.52-7.57 (m, 1H, Ar-H), 8.03-8.08 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 220 ([$M^{\bullet+}$], 42), 178 ([M - CH_2CO]$^+$, 97), 150 ([M - C_2H_4 - CH_2CO]$^+$, 100), 43 ([H_3C - CO]$^+$, 24)

Analysis: $C_{11}H_{12}N_2OS$ (220.30)

calculated	C: 59.97	H: 5.49	N: 12.72
found	C: 59.77	H: 5.53	N: 13.03

Methyl 2-thioxo-3*H*-benzo[*d*]imidazole-5-carboxylate (5.27)

To a solution of methyl 3,4-diaminobenzoate (**5.26**) (0.20 g, 1.20 mmol), dissolved in absolute THF (10 ml), TCDI (0.23 g, 1.29 mmol) was added under inert atmosphere. After stirring at room temperature for 4 h, the reaction mixture

was concentrated under reduced pressure. The residue was treated with water (30 ml) and ethyl acetate (50 ml) and stirred for 10 min. After extraction of the isolated aqueous phase with ethyl acetate (3x20 ml), the combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The crude product was resumed in a small amount of THF and purified by column chromatography on silica gel eluting with ethyl acetate.

Yield: 0.23 g (1.10 mmol, 92 %, beige solid)

Mp: 285 °C (decomposition)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 3.85 (s, 3H, $CO_2C\underline{H_3}$), 7.23 (dd, 1H, ^3J = 8.4 Hz, ^5J = 0.5 Hz, H-7), 7.66 (dd, 1H, ^4J = 1.5 Hz, ^5J = 0.5 Hz, H-4), 7.78 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.5 Hz, H-6), 12.84 (br, 2H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 208 ([$M^{\bullet+}$], 100), 177 ([M - OCH_3]$^+$, 63), 149 ([M - CH_3OCO]$^+$, 21)

Analysis: $C_9H_8N_2O_2S$ (208.24)

calculated	C: 51.91	H: 3.87	N: 13.45	S: 15.40
found	C: 51.82	H: 4.03	N: 13.20	S: 15.12

1-(2-Methyl-1*H*-benzo[*d*]imidazol-1-yl)propan-1-one (5.29)

2-Methylbenzimidazole (**5.28**) (0.40 g, 3.03 mmol) was dissolved in dry THF (20 ml) and mixed with triethylamine (0.46 ml, 3.33 mmol). After cooling down to 0 °C, propionyl chloride (0.29 ml, 3.33 mmol) was added and the mixture was allowed to stir for further 30 min. Afterwards, water (20 ml) was added and the mixture was extracted with ethyl acetate (3x20 ml). The combined organic phases were extracted with a solution of saturated sodium bicarbonate (20 ml), dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel eluting with a 3:1 (v/v) mixture of ethyl acetate and petroleum ether (60-80 °C).

Yield: 0.41 g (2.18 mmol, 72 %, white solid)

Mp: 74-76 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.20 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H_3}$), 2.78 (s, 3H, CH_3), 3.17 (q, 2H, ^{3}J = 7.1 Hz, $C\underline{H_2}CH_3$), 7.28-7.36 (m, 2H, Ar-H), 7.57-7.64 (m, 1H, Ar-H), 7.95-8.02 (m, 1H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.4 (+, CH_3), 18.9 (+, CH_3), 31.1 (-, CH_2), 114.9 (+, Ar-C), 118.8 (+, Ar-C), 123.8 (+, Ar-C), 123.9 (+, Ar-C), 132.8 (C_{quart}, Ar-C), 142.0 (C_{quart}, Ar-C), 152.5 (C_{quart}, Ar-C), 173.6 (C_{quart}, $\underline{C}OCH_2CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 188 ([$M^{\bullet+}$], 21), 132 ([M - CH_3CHCO]$^{+}$, 100)

$C_{11}H_{12}N_2O$ (188.23)

5.5.3 Pharmacological methods

Materials

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 hyaluronate lyase, *i.e.* 200,000 units[††] (0.572 mg from *S.agalactiae* strain 4755 plus 2.2 mg of BSA and 37 mg of Tris-HCl per vial) of lyophilized hyaluronate lyase, was kindly provided by id-Pharma (Jena, Germany). Lyophilized hyaluronidase from bovine testis (Neopermease®) (200,000 units[††]; 4 mg plus 25 mg of gelatin per vial) was a gift from Sanabo (Vienna, Austria). All other chemicals were of analytical grade and were received from Merck or Sigma.

Estimation of the aqueous solubility of the test compounds
see section 4.5.3.

Determination of enzyme inhibition

The inhibitory effect of benzimidazole derivatives on the activities of hyaluronidases was determined by a turbidimetric assay according to the method of Di Ferrante[145] described in chapter 3.

[††] according to the declaration of the supplier

10 µl of the test compounds (0.2 µM-2 mM, dissolved in DMSO) were incubated at 37 °C in a mixture composed of 120 µl of McIlvaine's buffer (solution A: 0.2 M Na_2HPO_4, 0.1 M NaCl, solution B: 0.1 M citric acid, 0.1 M NaCl; solution A and B were mixed in the appropriate proportions to reach pH 5.0 and pH 7.4), 30 µl of BSA solution (0.2 mg/ml in water), 50 µl of water, 30 µl of HA solution (2 mg/ml in water) and 30 µl of an enzyme solution (bacterial enzyme was dissolved in BSA solution (0.2 mg/ml in water); bovine enzyme dissolved in 1 ml H_2O and further diluted with BSA solution (0.2 mg/ml in water)). Equiactive concentrations of bovine testicular hyaluronidase (54 ng) or of *S.agalactiae* hyaluronate lyase (2.9 ng) were either incubated for 30 min at pH 5.0 or for 3 h at pH 7.4 (just used for the bacterial enzyme). Equimolar concentrations of *S.agalactiae* hyaluronate lyase (11.4 ng) or of BTH (8 ng) were either incubated at pH 5 for 7.5 min or for 51 h, respectively. The final DMSO concentration was 3.7% (v/v). After incubation of the assay mixture, the residual high molecular weight HA was precipitated by adding 700 µl of a 2.5% (w/v) cetyltrimethylammonium bromide (CTAB) solution (2.5 g of CTAB dissolved in 100 ml of 0.5 M sodium hydroxide solution, pH 12.5). The stopped enzyme reaction was again incubated at 25 °C for 20 min and the turbidity of each sample was visualised at 600 nm with a Uvikon 930 UV spectrophotometer (Kontron, Eching, Germany).

Quantification of inhibitory activity

The turbidity of the sample without inhibitor (10 µl of DMSO was used instead) was taken as reference for 100% enzyme activity, whereas the turbidity of the sample without both enzyme and inhibitor (30 µl of BSA solution and 10 µl of DMSO, respectively, was used instead) was taken as reference for 0% enzyme activity.

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 8.0 (SPSS Inc., Chicago, IL) and are the means of at least two independent experiments performed in duplicate.

To exclude factors affecting turbidity (interactions of the test compound with the substrate, absorption by the test compound etc.) controls containing the aforementioned incubation mixture without enzyme (30 µl of BSA solution was used instead) were run in parallel.

Chapter 6

Structure-activity relationships of benzoxazole-2-thiones as inhibitors of bacterial hyaluronidase

6.1 Introduction

In the previous chapter 1,3-diacylated benzimidazole-2-thiones were described as the most potent inhibitors of bacterial hyaluronidase $hylB_{4755}$. However, such compounds are very unstable in solution owing to their susceptibility to hydrolysis and, furthermore, they possess a poor shelf life. The drawbacks of these compounds stimulated us to replace the benzimidazole skeleton by a benzoxazole structure.

For the design of *S. agalactiae* hyaluronate lyase ($hylB_{4755}$) inhibitors, the binding mode of benzoxazole derivatives was derived from the X-ray structure of the related *S. pneumoniae* hyaluronidase (hylSpn) co-crystallized with an alkyl-2-phenylindole derivative[120], 1-decyl-2-(4-sulfamoyloxyphenyl)-1*H*-indol-6-yl-sulfamate **5.1** (Fig. 6.1).

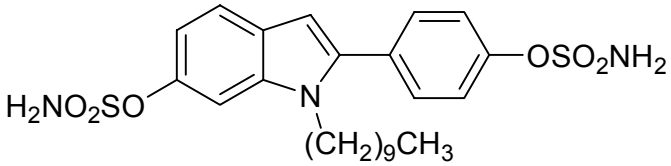
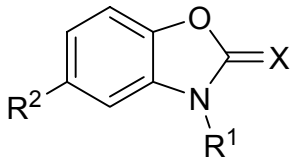


Fig. 6.1: Structure of 1-decyl-2-(4-sulfamoyloxy-phenyl)-1*H*-indol-6-yl-sulfamate **5.1**

By superposition of the crystal structure of hylSpn in complex with either compound **5.1** or a hexasaccharide substrate novel benzoxazole-2-thiones bearing

3-substituted *N*-propanoyl residues were proposed. Therefore, a series of benzoxazole-2-thione derivatives and benzoxazole-2-one derivatives with different substituents at the N atom was synthesized. Moreover, introducing an additional functional group should increase the solubility of such compounds and enable further structural variations. Position 5 was chosen for the introduction of a functional group (Fig 6.2). This appeared reasonable considering the structural features of substance **5.1** with a sulfamate residue at position 6 of the indole moiety. All synthesized benzoxazole-2-thione derivatives were tested for inhibition of hyaluronidases in a turbidimetric assay (see chapter 3).

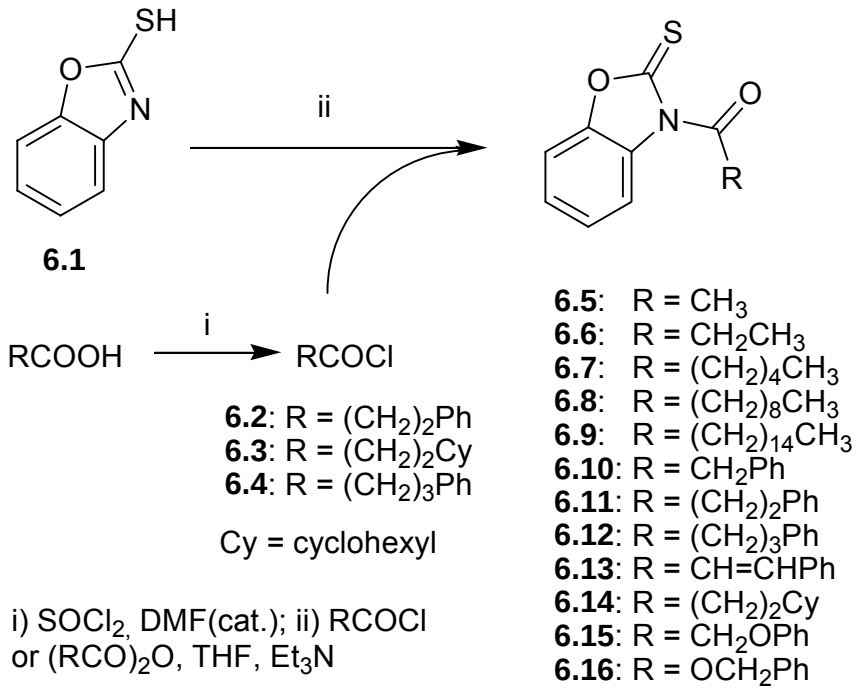


X = O, S
R^1 = variable
R^2 = H, CO_2Me, SO_3H

Fig. 6.2: Target compounds

6.2 Chemistry

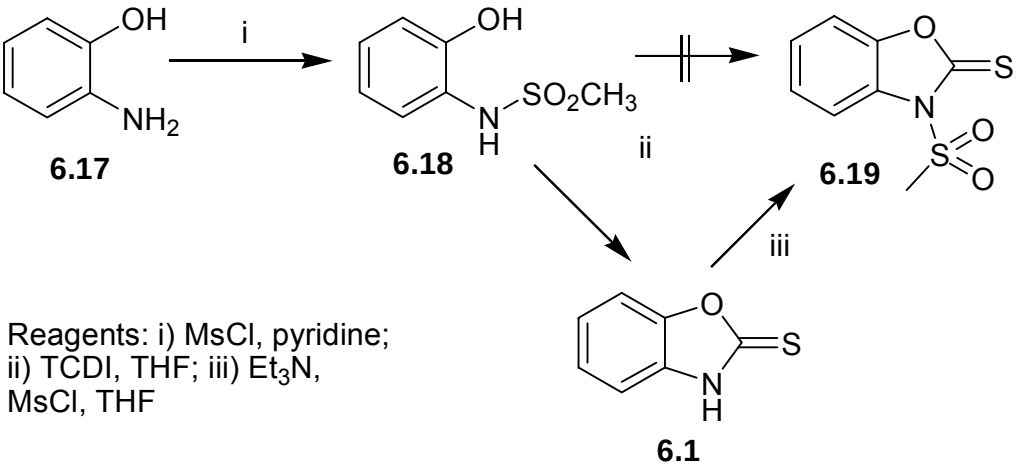
The preparation of the *N*-acylated benzoxazole-2-thiones **6.5-6.16** was performed by reaction of 2-mercaptobenzoxazole (**6.1**) with either acid chloride or



Scheme 6.1: Synthetic pathway to *N*-acylated benzoxazole-2-thiones

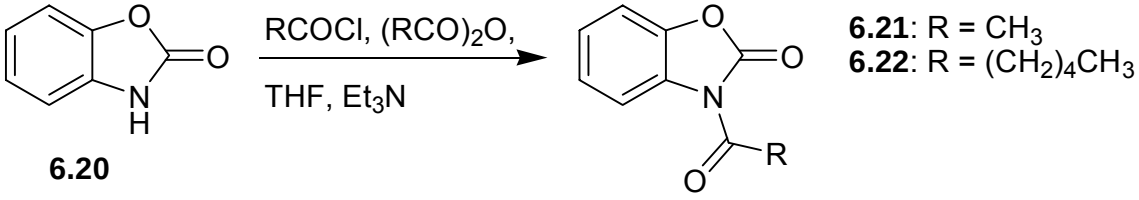
acid anhydride in anhydrous THF using triethylamine as base according to a slightly modified protocol of Ueda *et al.*[190]. Some acid chlorides were first prepared from the corresponding carboxylic acids with thionyl chloride and one drop of DMF as catalyst before reacting with 2-mercaptobenzoxazole (Scheme 6.1).

The synthesis of the sulfonylated benzoxazole-2-thione derivative **6.19** was tried *via* *N*-sulfonylation of *o*-aminophenol (**6.17**), yielding **6.18**, and cyclisation with 1,1'-thiocarbonyldiimidazole (TCDI). However, the ring closing reaction of **6.18** with TCDI yielded 2-mercaptobenzoxazole (**6.1**) as main product indicating that the sulfonamide bond of the possibly generated product **6.19** was cleaved under these reaction conditions. Probably, the reaction time (24 h) for the ring closure was too long. However, **6.19** was accessible by *N*-sulfonylation of **6.1** (Scheme 6.2) by analogy with the method applied for the *N*-acylation of benzoxazole-2-thiones.



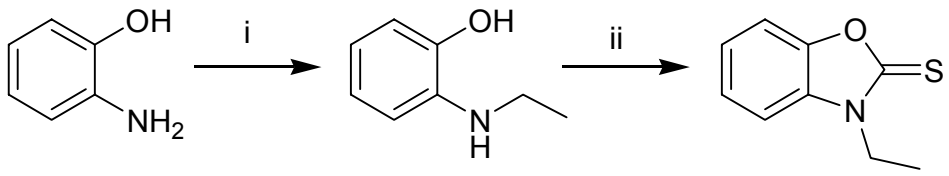
Scheme 6.2: Preparation of **6.19** by different pathways

The benzoxazole-2-one derivatives **6.21** and **6.22** were synthesized according to a modified procedure described by Ucar *et al*.[191] by an acylation reaction using acid chloride or acid anhydride and triethylamine in dry THF (Scheme 6.3).



Scheme 6.3: Synthesis of *N*-acylated benzoxazole-2-ones

For the synthesis of the *N*-alkylated benzoxazole-2-thione **6.24** a different synthetic strategy was chosen. In compliance with the HSAB concept a direct alkylation at the nitrogen atom with alkyl halides should not be favored. Therefore, the oxazole ring was build up in the last step as shown in Scheme 6.4. In the first step, the *N*-alkylation of *o*-aminophenol (**6.17**) was achieved by using bromoethane and sodium hydride in DMF. Afterwards, 2-(ethylamino)phenol (**6.23**) was cyclized with TCDI in anhydrous THF.

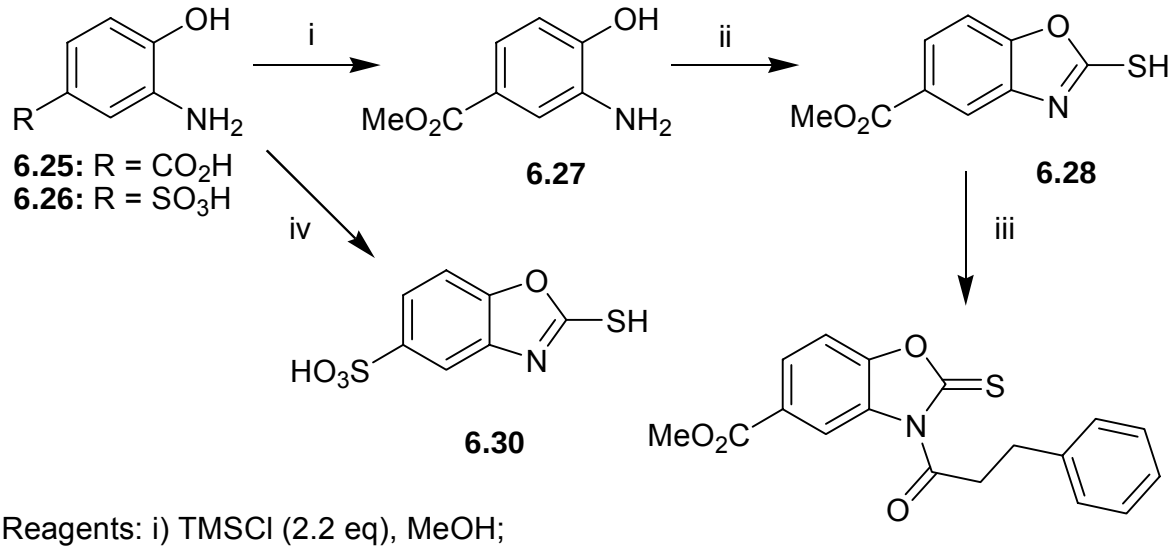


6.17 **6.23** **6.24**

Reagents: i) NaH, $BrCH_2CH_3$, DMF; ii) TCDI, THF

Scheme 6.4: Conversion of *o*-aminophenol into *N*-alkylated benzoxazole-2-thione **6.24**

5-Substituted benzoxazole-2-thiones were prepared as illustrated in Scheme 6.5. In a first step the carboxylic acid of 3-amino-4-hydroxybenzoic acid (**6.25**) was protected by esterification according to a slightly modified method described by Brook *et al.*[192]. These authors had pointed out a simple procedure for

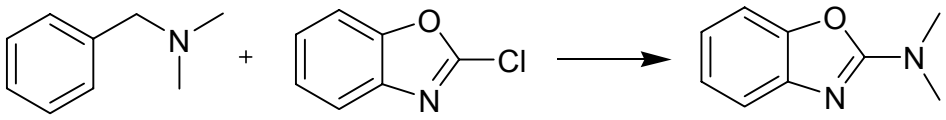


Reagents: i) TMSCl (2.2 eq), MeOH;
ii) TCDI, THF; iii) $Ph(CH_2)_2COCl$,
Et_3N, THF; iv) NaOH, CS_2, H_2O

Scheme 6.5: Synthetic route to various 5-substituted benzoxazole-2-thione derivatives

the esterification of carboxylic acids under mild conditions by using TMSCl both as acid catalyst and dehydrating agent. In case of 3-amino-4-hydroxybenzoic acid, the methyl ester **6.27** was smoothly formed in presence of an amino group. The cyclisation of **6.27** to methyl 2-sulfanylbenzo[*d*]oxazole-5-carboxylate **6.28** was achieved with TCDI in anhydrous THF. Compound **6.29** was synthesized by analogy with the procedure for the acylation of benzoxazole-2-thiones **6.5-6.16** (see Scheme 6.1). Following a procedure described by Matsuyama *et al*.[193], 2-sulfanylbenzo[*d*]oxazole-5-sulfonic acid (**6.30**) was obtained in moderate yields.

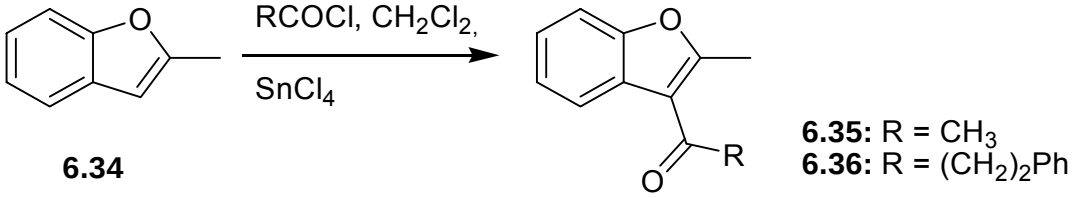
N,*N*-Dimethylbenzo[*d*]oxazol-2-amine (**6.33**) was synthesized according to the procedure described by Khalaf *et al*.[194] by refluxing *N*,*N*-dimethylbenzylamine (**6.31**) with 2-chlorobenzoxazole (**6.32**) without solvent at 130 °C for 3 h (see Scheme 6.6).



6.31 **6.32** **6.33**

Scheme 6.6: Preparation of compound **6.33**

Due to the poor stability of 3-acylated benzoxazole-2-thiones, the more stable 2-methylbenzofurane analogs **6.35** and **6.36**, which should mimic the benzoxazole scaffold of **6.5** and **6.11**, were synthesized in a Friedel-Crafts reaction as depicted in Scheme 6.7.



6.34

6.35: R = CH_3
6.36: R = $(CH_2)_2Ph$

Scheme 6.7: Synthesis of 3-acylated 2-methyl-benzofuranes **6.35** and **6.36**

For this purpose, 2-methylbenzofurane was transformed in high yields to the desired 3-acylated benzofuranes following a procedure described by Carvalho *et al.*[195] for acetylation of methyl benzofuran-2-yl-acetate.

6.3 Results and discussion

All synthesized compounds were tested in a turbidimetric assay (see chapter 3) to determine their inhibitory activities on the bacterial hyaluronidase $hylB_{4755}$ at pH 7.4 and pH 5, the pH optimum of the bacterial hyaluronidase[150]. In addition, the inhibition of the bovine hyaluronidase at pH 5 was investigated to check the selectivity of these substances.

6.3.1 Structure-activity relationships of benzoxazole derivatives

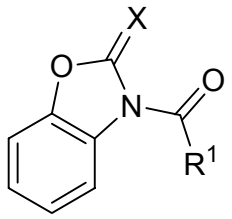
Stimulated by the elucidation of the binding mode of **5.1**, the introduction of lipophilic *N*-substituents at benzoxazoles was considered a promising approach to obtain potent hyaluronidase inhibitors. The results of the investigations on the bacterial enzyme $hylB_{4755}$ and bovine testicular hyaluronidase are summarized in Table 6.1.

Considering the bovine testicular hyaluronidase, all listed compounds in Table 6.1 were inactive on the bovine enzyme except for benzoxazoles **6.5** and **6.6**, which very weakly inhibited the BTH. Hence, in the following only the inhibitory influence on the bacterial enzyme $hylB_{4755}$ is discussed.

Generally, the compounds were more potent inhibitors of hyaluronate lyase $hylB_{4755}$ at physiological pH (IC_{50} values in the µM range), however, the marked differences in activity at pH 5 are more suitable for structure-activity considerations. One reason for the disagreement of inhibition depending on the pH could be the different affinity of the substrate to the enzyme depending on the pH of the test system[150]. At physiological pH, the affinity is lower than at pH optimum probably facilitating the displacement of HA.

The inhibitory effects of benzoxazoles **6.5**-**6.9** determined on the hyaluronate lyase at pH 7.4 were in the lower micromolar range with IC_{50} values of 42 µM (**6.5**), 48 µM (**6.6**), 29 µM (**6.7**), 25 µM (**6.8**) and 17 µM (**6.9**) (see Table 6.1 and Fig. 6.3).

Table 6.1: Inhibitory activities (IC_{50} values) of benzoxazole derivatives determined on the hyaluronidase from *S. agalactiae* ($hylB_{4755}$) at pH 5.0 and pH 7.4 and the bovine testicular hyaluronidase (BTH) at pH 5.0, respectively.



Compd.	R	X	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
			pH = 5.0	pH = 7.4	pH = 5.0
6.5	CH_3	S	125 ± 5	42 ± 3	21 % (455)
6.6	CH_2CH_3	S	128 ± 5	48 ± 2	19 % (400)
6.7	$(CH_2)_4CH_3$	S	51 % (130)	29 ± 1	inactive[b]
6.8	$(CH_2)_8CH_3$	S	41 % (100)	25 ± 4	inactive[c]
6.9	$(CH_2)_{14}CH_3$	S	23 % (63)	17 ± 1	inactive[d]
6.10	CH_2Ph	S	260 ± 41	69 ± 2	inactive[e]
6.11	$(CH_2)_2Ph$	S	15 ± 1	24 ± 1	inactive[f]
6.12	$(CH_2)_3Ph$	S	47 % (100)	19 ± 1	inactive[f]
6.13	CH=CHPh	S	9 % (80)	36 % (200)	inactive[f]
6.14	$(CH_2)_2Cy$	S	227 ± 122	20 ± 1	inactive[e]
6.15	CH_2OPh	S	213 ± 30	62 ± 2	inactive[g]
6.16	OCH_2Ph	S	inactive[f]	556 ± 20	inactive[f]
6.21	CH_3	O	inactive[h]	1466 ± 76	inactive[h]
6.22	$(CH_2)_4CH_3$	O	inactive[b]	inactive[i]	inactive[b]

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at the concentration of inhibitor given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 150 µM, [c] at concentrations ≤ 125 µM, [d] at concentrations ≤ 80 µM, [e] at concentrations ≤ 220 µM, [f] at concentrations ≤ 100 µM, [g] at concentrations ≤ 200 µM, [h] at concentrations ≤ 2 mM, [i] at concentrations ≤ 300 µM

Obviously, there is a tendency towards stronger inhibition with increasing alkanoyl chain length at the nitrogen atom of the benzoxazole moiety. This correlates with the presence and topology of hydrophobic residues in the catalytic cleft recently described by Li *et al.*[133]. Hydrophobic interactions also play an important role for the substrate HA since it has been shown to form a 2-fold helical

structure with an extensive hydrophobic patch of about eight CH units in aqueous solution[5].

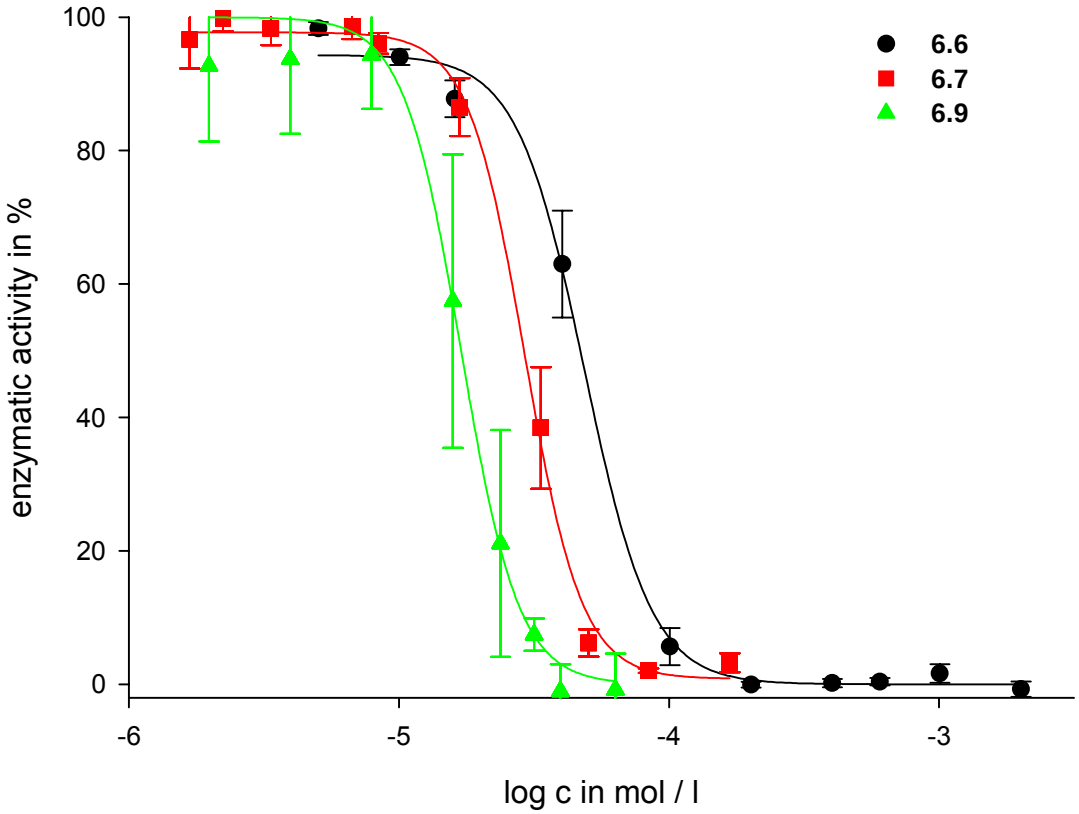


Fig. 6.3: Enzymatic activity of hylB$_{4755}$ at physiological pH in presence of **6.6**, **6.7** and **6.9**

At the pH optimum of hylB$_{4755}$, benzoxazoles **6.5** and **6.6** were equipotent (IC_{50} values of 125 µM (**6.5**) and 128 µM (**6.6**), respectively). For compounds **6.7**-**6.9** only a very weak inhibition of hylB$_{4755}$ at pH 5 was found. This might be associated with poor solubility of these compounds in the buffer used for the turbidimetric assay. The inhibitory activity was also very weak at physiological pH, regardless of further elongation of the side chain.

Among the benzoxazoles, compound **6.11** proved to be the most potent derivative. **6.11** was about 17 times more active than the lower homolog **6.10** at optimum pH of hylB$_{4755}$. The calculated IC_{50} values on hylB$_{4755}$ at pH 5 were 15 µM (**6.11**) and 260 µM (**6.10**). The higher homolog **6.12** exhibited an inhibition of hylB$_{4755}$ by about 47 % at a concentration of 100 µM (see Fig. 6.4). The obvious difference between the inhibitory effects of **6.10**, **6.11** and **6.12**, can be possibly

explained by the aforementioned hydrophobic patch in the active site of the enzyme.

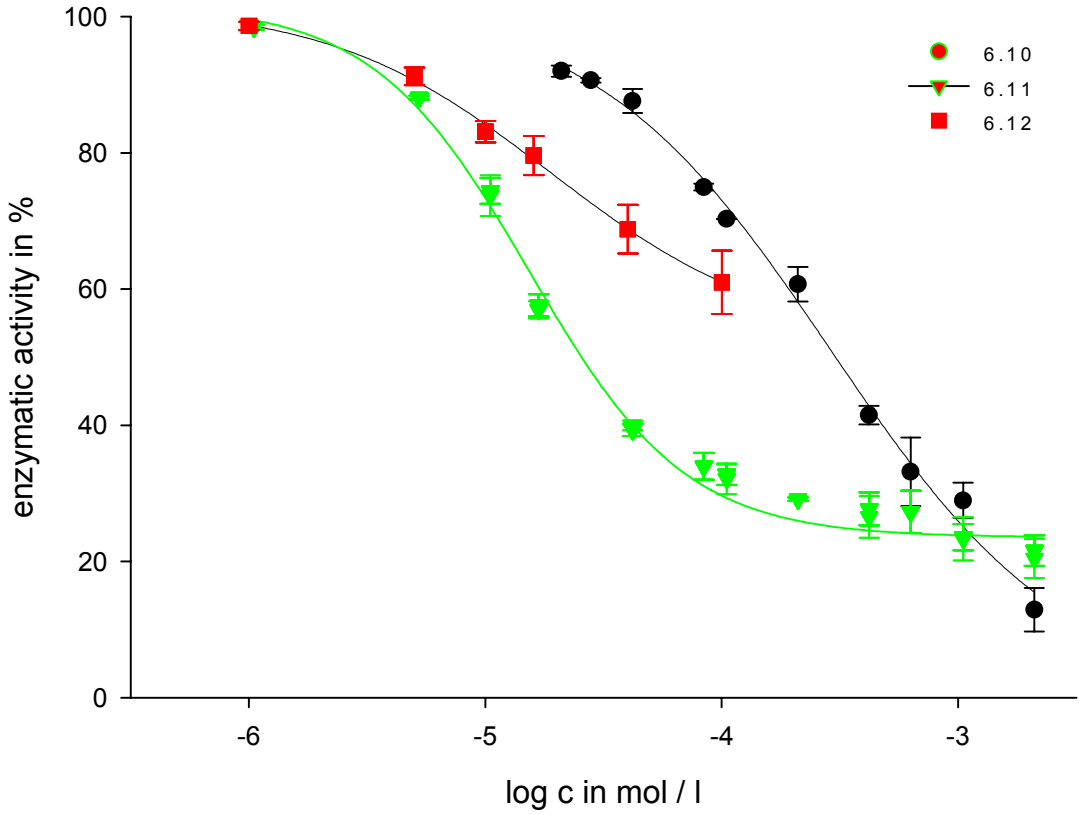


Fig. 6.4: Effects of **6.10**-**6.12** on hyaluronate lyase $hylB_{4755}$ at pH optimum

On the basis of the IC_{50} values we concluded that the phenyl ring of **6.11** with a spacer of 2 methylene groups is most favorably interacting with the hydrophobic patch within the crevice in the active site. This will be discussed in detail in section 6.3.2. At physiological pH, the preference for the 3-phenylpropanoyl substituted benzoxazole **6.11** is only detectable compared to compound **6.10**, but not for **6.12**, which is an equipotent inhibitor (IC_{50} of 19 µM) as illustrated in Fig. 6.5. This discrepancy could be explained by the low stability of the benzoxazoles against hydrolysis (see 6.3.3.). Hydrolytic cleavage is more relevant at pH 7.4 as a longer incubation time is required. To explore the influence of the side chain of **6.11**, hydrocinnamoic acid was tested for inhibitory potency on hyaluronidases. An inhibition of $hylB_{4755}$ by 15 % at 2 mM was achieved at pH 5, whereas no influence of hydrocinnamoic acid (concentrations ≤ 2 mM) on enzyme activity of $hylB_{4755}$ was detectable at pH 7.4. Thereby, it can be excluded

that the high inhibitory potency of **6.11** stems from its degradation products formed by hydrolysis (cf. 6.3.3.).

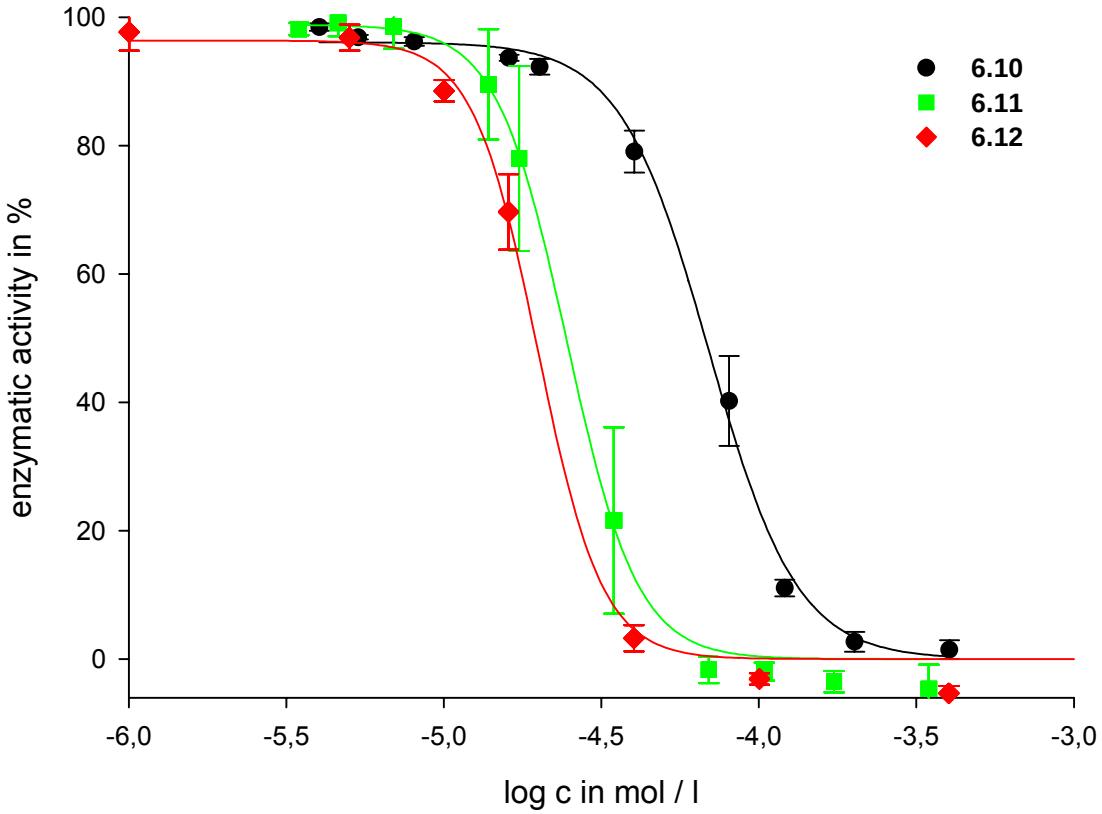


Fig. 6.5: Inhibition of $hylB_{4755}$ by compounds **6.10**-**6.12** at physiological pH

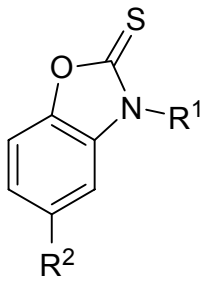
Replacement of the phenyl in **6.11** by a cyclohexyl ring (**6.14**) or the introduction of a C=C double bond (**6.13**) resulted in a strong decrease in inhibitory activity on $hylB_{4755}$ at pH optimum compared to **6.11**. The C=C double bond in **6.13** reduces the flexibility of the side chain which is supposed to bind in a small crevice of the enzyme[83]. Therefore, this side chain appears to be too rigid. The large difference in inhibitory activity (at pH 5) between **6.11** and **6.14** could possibly be ascribed to cation-π interactions between inhibitor **6.11** and the enzyme. At physiological pH, however, **6.14** was about as active as **6.11** (IC_{50} values of 20 µM and 24 µM, respectively)

Replacing one of the methylene groups of the ethylene spacer in **6.11** by oxygen (**6.15**, **6.16**) resulted in a considerable decrease in inhibitory activity both at pH 5 and at pH 7.4. Among the series of benzoxazoles, the thiones were sig-

nificantly more potent than the corresponding oxo analogs (see, *e.g.* **6.5** vs. **6.21**).

To increase the solubility of the benzoxazole-2-thione derivatives and to have additional options for structural variations a polar group was introduced in position 5. Moreover, this substituent could confer additional affinity by interactions with functional groups in the active site of the enzyme. The 5-substituted benzoxazoles **6.28** and **6.30** displayed moderately stronger inhibitory activity on $hylB_{4755}$ at pH 5 compared to 2-mercaptobenzoxazole (**6.1**) with IC_{50} values of 1299 µM (**6.28**) and 781 µM (**6.30**).

Table 6.2: Inhibitory activities of 2-mercaptobenzoxazole (**6.1**) and compounds **6.24**-**6.26** on BTH and on *S. agalactiae* hyaluronate lyase



Compd.	R^1	R^2	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
			pH = 5.0	pH = 7.4	pH = 5.0
6.1	H	H	42 % (3800)	2542 ± 125	inactive[b]
6.28	H	CO_2CH_3	1299 ± 223	27 % (2000)	inactive[c]
6.29	$CO(CH_2)_2Ph$	CO_2CH_3	56 % (200)	21 ± 1	inactive[d]
6.30	H	SO_3H	781 ± 100	46 % (2000)	inactive[c]

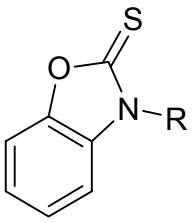
[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at concentration of inhibitor given in parentheses; [b] at concentrations ≤ 4.9 mM, [c] at concentrations ≤ 2 mM, [d] at concentrations ≤ 200 µM.

According to the suggested binding mode of benzoxazoles (see 6.3.2.) additional hydrogen bonds between the polar substituent in position 5 of the benzoxazole and the amino acids Arg466/Ser463 of hylSpn appear possible. This assumption is also in accordance with the structure-activity relationships of 2-phenylindoles described by Salmen[120]: polar substituents in position 6 of the indole, for instance a hydroxy group, were found found to enhance the inhibition of $hylB_{4755}$. Surprisingly, the activity-enhancing effect was not confirmed for the

benzoxazole-5-sulfonic acid **6.30** when the compound was tested at the physiological pH. In this case, the pH-dependent ratio of charged and uncharged state of the test compound and the amino acids in the active site may play a role. By contrast, benzoxazole **6.29** revealed a strong inhibition on $hylB_{4755}$ at physiological pH (IC_{50} = 21 µM) and an inhibition by about 56 % at a concentration of 200 µM at pH 5. Compared to the compound **6.11** (IC_{50} values: 15 µM at pH 5, 24 µM at pH 7.4), the striking pH-dependent difference in inhibitory effect found for the methoxycarbonyl-substitued analog **6.29** and the sulfonic acid **6.30** suggests that ionic interactions as well as H-bonds of the functional group with amino acids in the active site could play a role. Unfortunately, the benzoxazole bearing a sulfonic acid group in position 5 and the 3-phenylpropanoyl side chain was not accessible by acylation of **6.30** to flesh out the hypothesis. All compounds listed in Table 6.2 are selective inhibitors of the bacterial enzyme since they were all inactive on the bovine testicular hyaluronidase.

By reason of the labile amide bond of *N*-acylated benzoxazole-2-thiones (see 6.3.3.), compound **6.19** and **6.24** were synthesized with the aim to stabilize the bond between the nitrogen atom and the substituent without loosing inhibitory effect. Unfortunately, on the bacterial enzyme, both at pH 5 and at pH 7.4, a distinct decrease in inhibitory potency was found compared to the *N*-acylated substance **6.5** (see Table 6.3).

Table 6.3: Inhibitory activities of benzoxazole-2-thiones on BTH at pH 5 and $hylB_{4755}$ at optimum and physiological pH



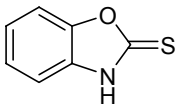
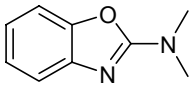
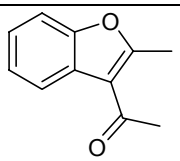
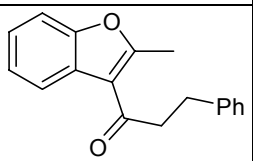
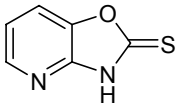
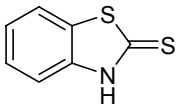
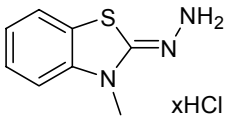
Compound	**R**	**$hylB_{4755}$, IC_{50} [µM] or %[a]**		**BTH[a]**
		pH = 5.0	pH = 7.4	pH = 5.0
6.5	$COCH_3$	125 ± 5	42 ± 3	21 % (455)
6.19	SO_2CH_3	inactive[b]	inactive[b]	inactive[b]
6.24	CH_2CH_3	46 % (400)	17 % (1000)	inactive[c]

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at concentration of inhibitor given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 400 µM, [c] at concentrations ≤ 1 mM.

The decrease in activity may be ascribed to the missing interaction between Tyr488, a key residue in the catalytic mechanism of *S. agalactiae* hyaluronate lyase[69,66], and the carbonyl oxygen of the acylated benzoxazoles[83].

At last, the benzoxazole scaffold was replaced by several analogous heterocyclic systems. The results of the investigation for hyaluronidase inhibition are summarized in Table 6.4.

Table 6.4: Inhibitory activities of various substituted benzoxazoles and related heterocycles on the bovine testicular hyaluronidase and *S. agalactiae* hyaluronate lyase

Compd.	Structure	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
		pH = 5.0	pH = 7.4	pH = 5.0
6.1		42 % (3800)	2542 ± 125	inactive (4900)
6.33		20 % (5700)	inactive (5700)	inactive (5700)
6.35		43 % (2000)	20 % (2000)	inactive (2000)
6.36		inactive (150)	inactive (200)	inactive (150)
6.37		10 % (2200)	inactive (2200)	inactive (2200)
6.38		45 % (3700)	1623 ± 56	10 % (3700)
6.39		12 % (2000)	inactive (2000)	inactive (2000)

[a] inhibition of enzyme was expressed as IC_{50} ± SEM in µM or as % inhibition at concentration of inhibitor given in parentheses; highest tested concentrations were dependent on the solubility of the compounds.

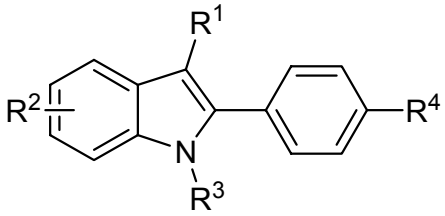
With exception of 2-mercaptobenzothiazole **6.38** the tested compounds (**6.33**-**6.39**) were either inactive or displayed only negligible inhibitory activity on BTH as well as on hyaluronate lyase $hylB_{4755}$. Benzothiazole **6.38** may be considered a possible alternative to the benzoxazole as a scaffold for the preparation of hyaluronidase inhibitors.

Within the series of the aforementioned benzoxazoles, all compounds showed a marked selectivity for the bacterial *versus* the bovine enzyme. Due to poor solubility the compounds could not be investigated at higher concentrations than denoted.

6.3.2 Putative binding mode of the benzoxazoles at $hylB_{4755}$

For the design of bacterial hyaluronidase inhibitors, the binding mode of benzoxazole derivatives was derived from X-ray structure of hyaluronate lyase from *S. pneumoniae* (hylSpn) co-crystallized with compound **5.1**. Previously, Botzki[83] has shown that structure-activity relationships (SAR) of analogous 2-phenylindoles (see Table 6.5) correlate with the binding mode of compound **5.1** at hylSpn[83] (see Fig. 6.6). The increase in $hylB_{4755}$ inhibition with increasing chain length of the alkyl substituents at the nitrogen atom of the indole moiety clearly indicates that the binding mode of **6.40-6.42** is similar to that found for **5.1**.

Table 6.5: IC_{50}values of 2-phenylindole derivatives (data from Salmen[120])



Compd.	Substitution				IC_{50} [µM]	
	R^1	**R^2**	**R^3**	**R^4**	pH 5.0	pH 7.4
5.1	H	6-OSO_2NH_2	$C_{10}H_{21}$	OSO_2NH_2	11	16
6.40	CH_3	5-OH	H	OH	740	280
6.41	CH_3	5-OH	C_3H_7	OH	220	160
6.42	CH_3	5-OH	C_7H_{15}	OH	26	12

Probably, the aliphatic chains of the 3-acylbenzoxazoles occupy the same crevice like the decyl substituent of **5.1**. The SAR of the benzoxazoles are similar to those of the 2-phenylindoles since $hylB_{4755}$ inhibition increases with chain length of R (see Table 6.1).

It may be concluded that benzoxazole-2-thione derivatives have a binding mode similar to that of compound **5.1**, *i.e.* the benzoxazole moiety overlays with the indole moiety, and the alkoxy substituents point into the same direction as the aliphatic chain of **5.1**. Additionally, the amide oxygen might interact with Tyr408. These considerations were complemented by comparing the binding mode of **5.1** and a hexasaccharide unit of the substrate hyaluronic acid with crystallographic hylSpn data[83] allowing the rational prediction of more potent benzoxazoles. As shown in Fig. 6.6, the depicted trisaccharide overlaps with compound **5.1**.

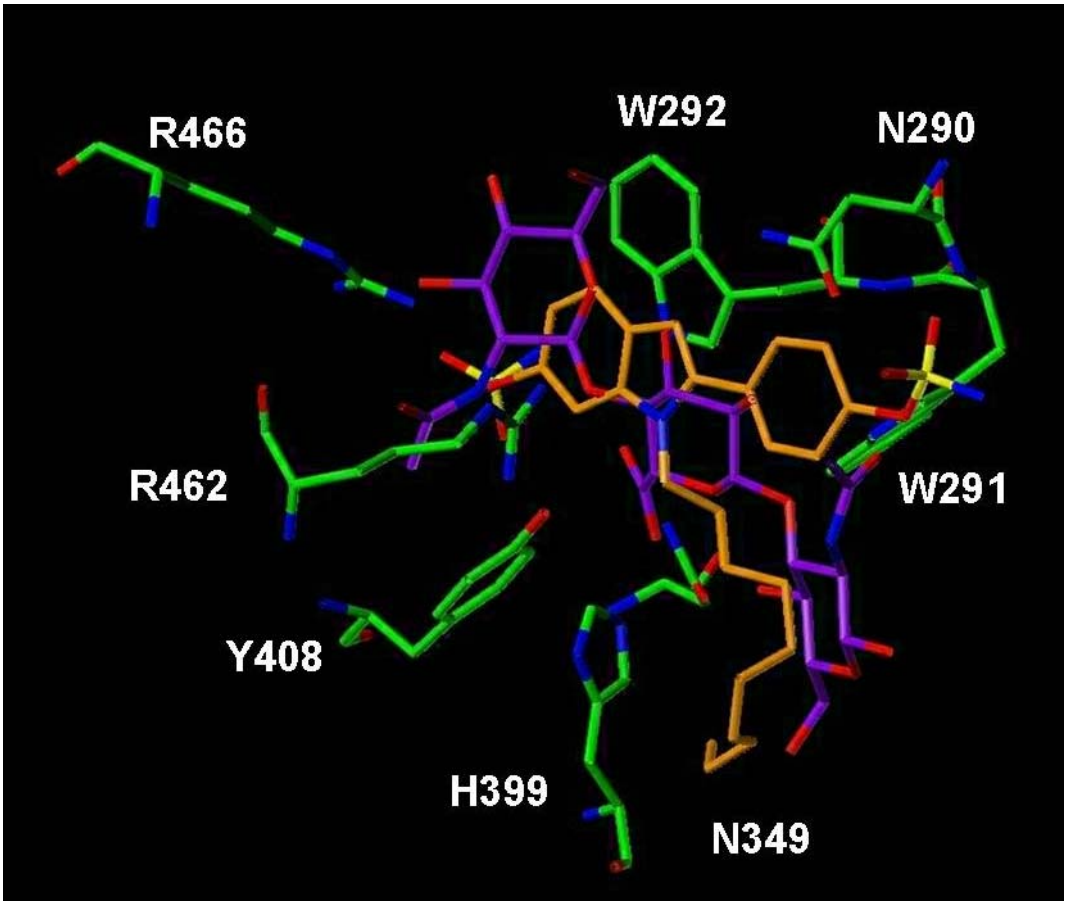
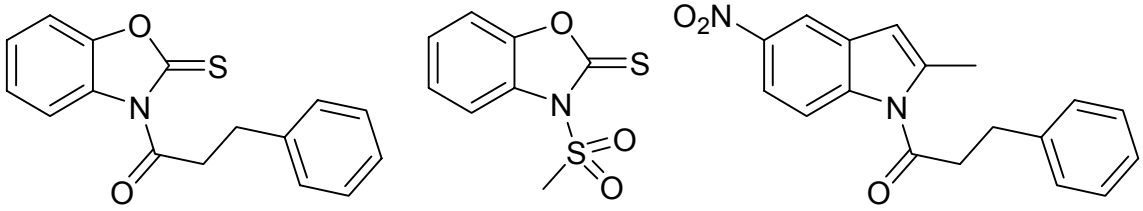


Fig. 6.6: Comparison of binding modes of hylSpn inhibitor **5.1** (carbon atoms orange) and a HA hexasaccharide (carbon atoms purple, for clarity only a trisaccharide moiety is shown).

With respect to substituents at the nitrogen atom of the benzoxazole moiety, 3-substituted propionic acid derivatives are promising, since a *N*-acetylglucosamine residue of the HA substrate is in the same location as the methylene groups 4 and 5 of the decyl substituent of **5.1** (Fig. 6.6). Moreover, there is a hydrophobic patch in this crevice of hylB$_{4755}$, which is assumed to interact with the lipophilic sugar ring of *N*-acetylglucosamine. Therefore, the substituent at the nitrogen atom of the benzoxazole moiety should be a propanoyl residue with a lipophilic subsituent at the end. This hypothesis is supported by the 17-fold (pH 5) and 3-fold (pH 7.4) higher inhibitory activity of compound **6.11** compared to compound **6.10** and the 8-fold (pH 5) and 2-fold (pH 7.4) higher inhibition of **6.11** compared to compound **6.6**.

6.3.3. Stability measurement

While performing the turbidimetric assay for compound **6.11** fluctuations in the determined IC_{50} values occurred. It turned out that the inhibitor, was not stable in the DMSO solutions used in the assay. As we assumed that the amide bond is labile against hydrolysis, stability investigations were carried out with the strong hylB$_{4755}$ inhibitor **6.11**, the sulfonamide **6.19**, and compound **7.16**, having an indole scaffold instead of the benzoxazole moiety (Fig. 6.7).



6.11 **6.19** **7.16**

Fig. 6.7: Structures, investigated on their stability against hydrolysis

At first, UV spectra of all three compounds and the supposable decomposition products, 2-mercaptobenzoxazole (**6.1**) and 2-methyl-5-nitroindole (**7.1**), were recorded (see Appendix). The stability investigations were performed by HPLC analysis with detection at 305 nm corresponding to the absorption maximum of **6.11**. In the chromatograms of **6.11** and **6.19** only one additional peak was de-

tected at 305 nm. This peak was assigned to the decomposition product 2-mercaptobenzoxazole.

For kinetic studies solutions (1 mM) of **6.11**, **6.19** and **7.16** were prepared in a 80:20 (v/v) mixture of DMSO and water. Subsequently, samples were analyzed by HPLC at different time intervals. The hydrolysis rate was determined by the temporal decrease in absorbance at the characteristic wavelength of 305 nm. The extinction area of 2-MBO was normalized on the area of the particular benzoxazole peak by multiplying the area of 2-MBO peak with a factor resulting from the division of the molar extinction coefficient of 2-MBO and the particular benzoxazole. Then, the extinction areas were summed up and the percentage portion of **6.11**, **6.19** can be given. As depicted in Fig. 6.8, the decomposition of **6.11** was plotted against the time.

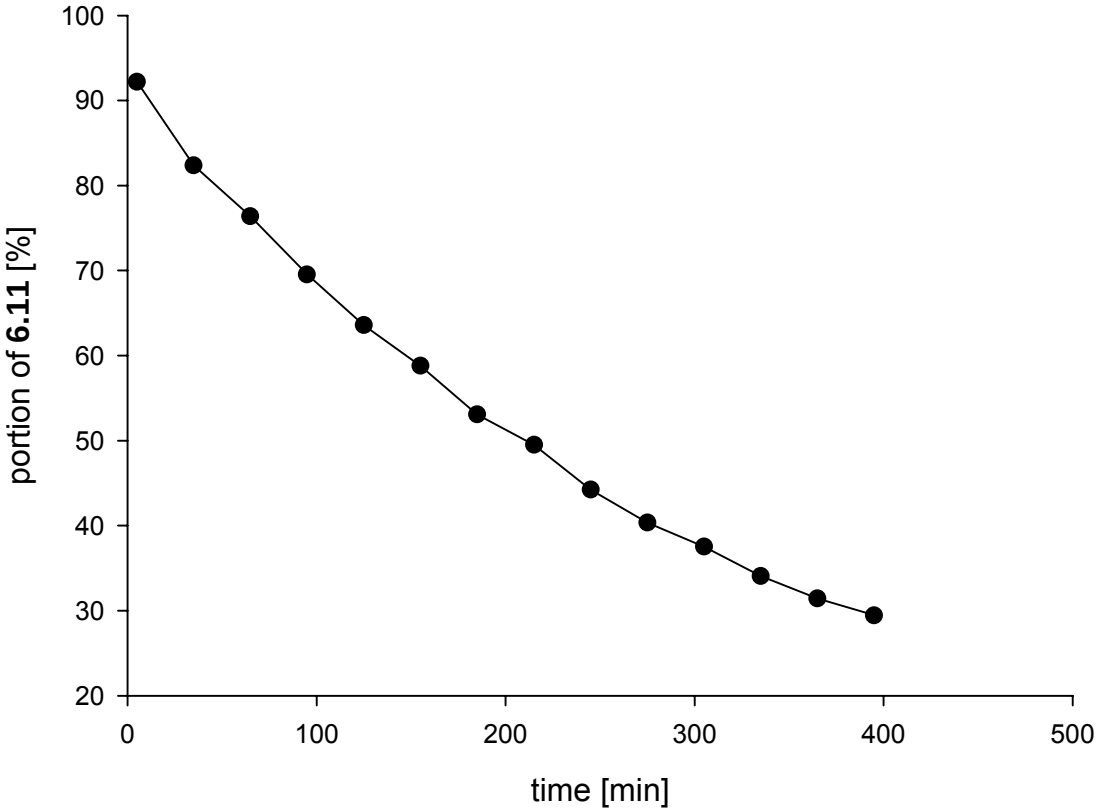


Fig. 6.8: Hydrolysis of compound **6.11** plotted against time

The hydrolysis obeyed a first order kinetic within very close borders. According to the kinetic measurement of **6.11**, the calculated half-life is 235 minutes with a rate constant (first order) of $2.9 \cdot 10^{-3}$ min^{-1}. The rate constant was determined corresponding to the following equation from the graph illustrated in Fig. 6.9, where k is equivalent to the slope of the linear plot.

$$\ln c(t) = -k \cdot t + \ln c(0)$$

c: portion of **6.11**

k: rate constant

Given that **6.11** is following a first order kinetic, the half-life is independent of the starting concentration of **6.11**. It was calculated according to the equation:

$$t_{1/2} = \ln 2 / k$$

$t_{1/2}$: half-life

k : rate constant

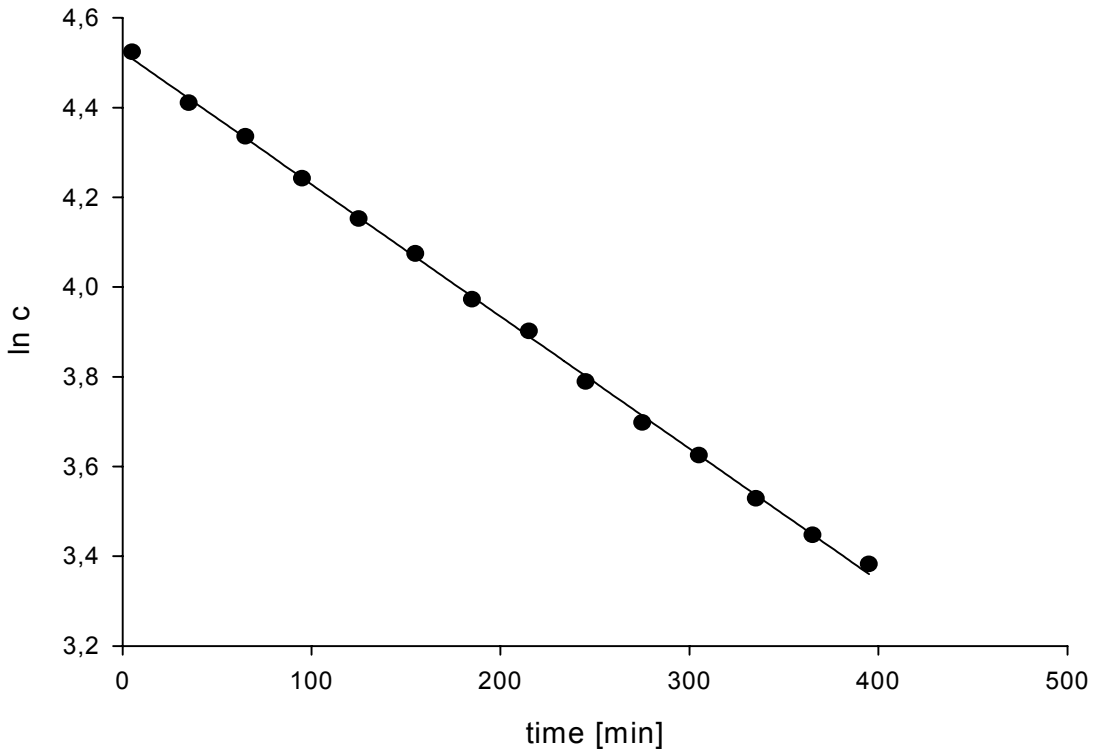


Fig. 6.9: First order kinetics of **6.11**

As expected, the hydrolysis of the sulfonamide analog **6.19** was much slower than for **6.11**. After 475 minutes just 13 % of **6.19** was decomposed. Since the decomposition was only tracked as far as 13 %, no decomposition constant could be calculated.

In contrast to the benzoxazoles **6.11** and **6.19** the indole **7.16** showed no significant decrease in absorbance over a period of 400 min. This is in good agreement with results reported by Staab[196] for *N*-acetylindole, which was stable in aqueous solution at room temperature for a couple of days.

6.4 Conclusions

The presented benzoxazole-2-thione derivatives are among the most potent inhibitors of bacterial hyaluronidase known so far. All examined compounds are highly selective for the hyaluronate lyase of *S. agalactiae* strain 4755 ($hylB_{4755}$) *versus* the bovine enzyme. IC_{50} values in the µM range were determined for 3-acylated benzoxazole-2-thiones (*e.g.* **6.5**-**6.12**) at physiological pH, whereas at optimum pH 5 the calculated IC_{50} values of 3-acylated benzoxazole-2-thiones reveal slightly decreased inhibitory activity. Within this series of compounds 3-propanoylbenzoxazole-2-thione (**6.11**) was the most potent inhibitor of $hylB_{4755}$ with an IC_{50} value of 15 µM at optimum pH. Hence, a carbonyl group in 3-position linked to a phenyl group *via* an ethylene spacer appears to be optimal for inhibition of $hylB_{4755}$.

With respect to the limited stability of the 3-acylbenzoxazoles in aqueous solutions the obtained inhibition data are more reliable at pH 5 owing to the much shorter incubation time necessary at optimum pH. Hence, it has to be taken into account that the small amounts of water contained in the solvent DMSO may be sufficient to hydrolyze the *N*-acylated benzoxazoles during storage of stock solution.

In order to enhance the solubility, substituents were successfully introduced in position 5 of the benzoxazole scaffold. The results are promising with respect to the design of new structurally related inhibitors. Due to the low stability of **6.11** against hydrolysis of the amide bond various modifications were attempted. However, replacing the amide by a sulfonamide bond or a *N*-alkyl bond resulted in a marked decrease in inhibitory activity on $hylB_{4755}$, and the exchange of the benzoxazole scaffold by other heterocyclic systems such as benzofuranes was not tolerated.

In summary, the structure-activity relationships of the benzoxazole-2-thione derivatives strengthen the structure-based design strategy, which had started from the 3D structure of hylSpn.

6.5 Experimental section

6.5.1 General conditions

See section 4.5.1 for general methods.
HPLC measurement was performed by injecting 30 µl of the corresponding sample in mobile phase into a LUNA C18 column (150 x 4.6 mm, 3 µ) at room temperature with a flow rate of 0.75 ml/min. The UV absorbance was determined at 305 nm and the following gradient was used:
0 min: 30:70 (v/v) mixture of acetonitrile and water
4 min: 30:70 (v/v) mixture of acetonitrile and water 14 min: 80:20 (v/v) mixture of acetonitrile and water
20 min: 80:20 (v/v) mixture of acetonitrile and water
22 min: 30:70 (v/v) mixture of acetonitrile and water

6.5.2 Chemistry

Preparation of acid chlorides 6.2-6.4

General procedure. Carboxylic acid (1 eq) was treated with thionyl chloride (1.5 eq) and one drop of DMF under a nitrogen atmosphere. After stirring for 1 h at ambient temperature (development of gas was not observable any more!), the mixture was evaporated with a water-jet pump at room temperature to separate surplus thionyl chloride. The obtained crude product was used without purification for further reaction.

3-Phenylpropanoyl chloride (6.2)

Reaction of 3-phenylpropanoic acid (0.54 g, 3.60 mmol) and thionyl chloride (0.39 ml, 5.38 mmol).

Yield: 0.66 g of oily compound

3-Cyclohexylpropanoyl chloride (6.3)

Reaction of 3-cyclohexylpropanoic acid (0.62 ml, 3.60 mmol) and thionyl chloride (0.40 ml, 5.51 mmol).

Yield: 0.80 g of colorless oil

4-Phenylbutanoyl chloride (6.4)

Reaction of 4-phenylbutanoic acid (0.59 g, 3.59 mmol) and thionyl chloride (0.40 ml, 5.51 mmol).

Yield: 0.87 g of colorless oil

Synthesis of *N*-acylated benzoxazole-2-thiones 6.5-6.16 and 6.19

General procedures

Method A. 2-Mercaptobenzoxazole (**6.1**) (1 eq) was dissolved in absolute THF (5 ml) under an inert atmosphere. Triethylamine (1.1 eq) was added and the solution was cooled to 0 °C. The particular acid chloride (1 eq), dissolved in 5 ml absolute THF, was added dropwise. After stirring for 30 minutes at room temperature, the reaction mixture was poured into ice water (35 ml). The precipitated product was collected, washed with water, dried *in vacuo* and recrystallized.

Method B. 2-Mercaptobenzoxazole (**6.1**) (1 eq) was dissolved in absolute THF (5 ml) and mixed with triethylamine (1.1 eq) under a nitrogen atmosphere. Alkanoic anhydride (1 eq) was added dropwise and the mixture was heated under reflux (7 h). After diluting with water (30 ml), the mixture was extracted three times with ethyl acetate (10 ml). The combined organic phases were washed with a saturated solution of sodium carbonate (10 ml), dried over magnesium sulfate and the solvent was removed under reduced pressure. The product was purified by column chromatography on a silica gel.

1-(2-Thioxobenzo[*d*]oxazol-3(2*H*)-yl)ethanone (6.5)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and acetyl chloride (0.24 ml, 3.36 mmol); recrystallization from cyclohexane.

Yield: 0.46 g (2.38 mmol, 72 %, beige solid)

Mp: 113-114 °C (Lit. 120-121 °C[190])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 3.06 (s, 3H, $COCH_3$), 7.28-7.38 (m, 3H, Ar-H), 8.07-8.12 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 193 ([$M^{\bullet+}$], 17), 151 ([M - CH_2CO]$^+$, 100)

Analysis: $C_9H_7NO_2S$ (193.22)

calculated	C: 55.94	H: 3.65	N: 7.25	S: 16.60
found	C: 55.89	H: 3.73	N: 7.27	S: 16.64

1-(2-Thioxobenzo[*d*]oxazol-3(2*H*)-yl)propan-1-one (6.6)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and propanoyl chloride (0.30 ml, 3.44 mmol); recrystallization from ethanol.

Yield: 0.30 g (1.45 mmol, 44 %, white needles)

Mp: 81-82 °C

^{1}H-NMR (DMSO-d_6): δ [ppm] = 1.19 (t, 3H, ^{3}J = 7.1 Hz, CH_3), 3.44 (q, 2H, ^{3}J = 7.1 Hz, CH_2), 7.37-7.47 (m, 2H, Ar-H), 7.56-7.61 (m, 1H, Ar-H), 8.00-8.05 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 207 ([$M^{\bullet+}$], 22), 179 ([M - C_2H_4]$^+$, 14), 151 ([M - CH_3CHCO]$^+$, 100)

Analysis: $C_{10}H_9NO_2S$ (207.25)

calculated	C: 57.95	H: 4.38	N: 6.76
found	C: 57.97	H: 4.42	N: 6.77

1-(2-Thioxobenzo[*d*]oxazol-3(2*H*)-yl)hexan-1-one (6.7)

Method B: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and hexanoic anhydride (0.76 ml, 3.30 mmol); purification by chromatography on a

silica gel column, elution with chloroform; analytically pure product was obtained by recrystallization from methanol.

Yield: 0.30 g (1.20 mmol, 36 %, white solid)

Mp: 46-47.5 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 0.94 (t, 3H, ^{3}J = 7.1 Hz, $(CH_2)_4C\underline{H}_3$), 1.32-1.48 (m, 4H, $(C\underline{H}_2)_2CH_3$), 1.77-1.88 (m, 2H, $COCH_2C\underline{H}_2$-), 3.51 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 7.27-7.35 (m, 3H, Ar-H), 8.06-8.12 (m, 1H, Ar-H)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 14.0 (+, CH_3), 22.5 (-, CH_2), 24.0 (-, CH_2), 31.1 (-, CH_2), 39.2 (-, CH_2), 109.7 (+, Ar-C), 116.5 (+, Ar-C), 125.5 (+, Ar-C), 126.1 (+, Ar-C), 130.0 (C_{quart}, Ar-C), 146.6 (C_{quart}, Ar-C), 174.3 (C_{quart}, CO), 178.8 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 249 ([$M^{\bullet+}$], 8), 221 ([M - CO]$^{+}$, 10), 151 ([M - C_4H_9CHCO]$^{+}$, 100)

$C_{13}H_{15}NO_2S$ (249.33)

1-(2-Thioxobenzo[*d*]oxazol-3(2*H*)-yl)decan-1-one (6.8)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and decanoyl chloride (0.69 ml, 3.35 mmol); stirring for 3 h; recrystallization from methanol.

Yield: 0.51 g (1.67 mmol, 51 %, white solid)

Mp: 64.5-65 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 0.88 (t, 3H, ^{3}J = 6.9 Hz, $(CH_2)_8C\underline{H}_3$), 1.25-1.42 (m, 12H, $(C\underline{H}_2)_6CH_3$), 1.76-1.87 (m, 2H, $COCH_2C\underline{H}_2$-), 3.51 (t, 2H, ^{3}J = 7.3 Hz, $COCH_2$-), 7.29-7.36 (m, 3H, Ar-H), 8.05-8.12 (m, 1H, Ar-H)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 14.1 (+, CH_3), 22.7 (-, CH_2), 24.3 (-, CH_2), 29.0 (-, CH_2), 29.3 (-, CH_2), 29.4 (-, $2CH_2$), 31.9 (-, CH_2), 39.2 (-, CH_2), 109.7 (+, Ar-C), 116.5 (+, Ar-C), 125.5 (+, Ar-C), 126.1 (+, Ar-C), 130.0 (C_{quart}, Ar-C), 146.6 (C_{quart}, Ar-C), 174.3 (C_{quart}, CO), 178.8 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 305 ([$M^{\bullet+}$], 2), 277 ([M - CO]$^{+}$, 2), 151 ([M - $C_8H_{17}CHCO$]$^{+}$, 100)

Analysis: $C_{17}H_{23}NO_2S$ (305.44)

calculated	C: 66.85	H: 7.59	N: 4.59
found	C: 66.81	H: 7.81	N: 4.39

1-(2-Thioxobenzo[*d*]oxazol-3(2*H*)-yl)hexadecan-1-one (6.9)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.51 g, 3.37 mmol) and hexadecanoyl chloride (1.00 ml, 3.31 mmol); stirring for 3 h; recrystallization from methanol.

Yield: 0.65 g (1.67 mmol, 50 %, white solid)

Mp: 81-81.5 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 0.88 (t, 3H, ^3J = 6.7 Hz, $(CH_2)_{14}C\underline{H}_3$), 1.23-1.50 (m, 24H, $CH_2(C\underline{H}_2)_{12}CH_3$), 1.76-1.87 (m, 2H, $COCH_2C\underline{H}_2$-), 3.51 (t, 2H, ^3J = 7.3 Hz, $COCH_2$-), 7.28-7.37 (m, 3H, Ar-H), 8.05-8.11 (m, 1H, Ar-H)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 14.1 (+, CH_3), 22.7 (-, CH_2), 24.3 (-, CH_2), 28.9 (-, CH_2), 29.4 (-, $2CH_2$), 29.5 (-, CH_2), 29.6 (-, CH_2), 29.7 (-, $5CH_2$), 31.9 (-, CH_2), 39.2 (-, CH_2), 109.6 (+, Ar-C), 116.5 (+, Ar-C), 125.5 (+, Ar-C), 126.0 (+, Ar-C), 130.0 (C_{quart}, Ar-C), 146.5 (C_{quart}, Ar-C), 174.3 (C_{quart}, CO), 178.8 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 389 ([$M^{\bullet+}$], 25), 361 ([M - CO] $^+$, 83), 151 ([M - $C_{14}H_{29}CHCO]^+$, 100)

Analysis: $C_{23}H_{35}NO_2S$ (389.60)

calculated	C: 70.91	H: 9.06	N: 3.60
found	C: 70.69	H: 9.54	N: 3.33

2-Phenyl-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)ethanone (6.10)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) with phenylacetyl chloride (0.44 ml, 3.33 mmol) in 10 ml dry acetone; recrystallization from ethanol.

Yield: 0.28 g (1.04 mmol, 31 %, white solid)

Mp: 132.5-134 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 4.91 (s, 2H, CH_2), 7.26-7.42 (m, 8H, Ar-H), 8.02-8.09 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 269 ([$M^{\bullet+}$], 26), 151 ([M - C_6H_5CHCO] $^+$, 28), 118 ([C_6H_5 - CHCO]$^+$, 85), 91 ([$C_6H_5CH_2$]$^+$, 100)

Analysis: $C_{15}H_{11}NO_2S$ (269.32)

calculated	C: 66.89	H: 4.12	N: 5.20
found	C: 66.84	H: 4.12	N: 5.20

3-Phenyl-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)propan-1-one (6.11)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and 3-phenylpropionyl chloride (**6.2**) (0.66 g , 3.91 mmol); recrystallization from ethanol.

Yield: 0.33 g (1.16 mmol, 35 %, yellow solid)

Mp: 73-75 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 3.16 (t, 2H, ^{3}J = 7.5 Hz, $COCH_2CH_2Ph$), 3.87 (t, 2H, ^{3}J = 7.5 Hz, $COCH_2CH_2Ph$), 7.19-7.25 (m, 1H, Ar-H), 7.27-7.35 (m, 7H, Ar-H), 8.05-8.10 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 283 ([$M^{\bullet+}$], 15), 250 ([M - SH]$^+$, 64), 151 ([M - $C_6H_5CH_2CHCO$] $^+$, 100), 105 ([$C_6H_5CH_2CH_2$]$^+$, 69), 91 ([$C_6H_5CH_2$]$^+$, 78)

Analysis: $C_{16}H_{13}NO_2S$ (283.35)

calculated	C: 67.82	H: 4.62	N: 4.94
found	C: 67.67	H: 4.24	N: 4.99

4-Phenyl-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)butan-1-one (6.12)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and 4-phenylbutanoyl chloride (**6.4**) (0.87 g, 4.76 mmol); reaction mixture was extracted two times with chloroform (15 ml); the combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure; purification by column chromatography on silica gel, elution with chloroform.

Yield: 0.13 g (0.44 mmol, 13 %, brown solid)

Mp: 145-146.5 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 2.10-2.21 (m, 2H, $COCH_2C\underline{H}_2CH_2Ph$), 2.78 (t, 2H, ^3J = 7.8 Hz, $COC\underline{H}_2$-), 3.54 (t, 2H, ^3J = 7.3 Hz, $CO(CH_2)_2C\underline{H}_2Ph$), 7.16-7.37 (m, 8H, Ar-H), 8.04-8.12 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 264 ($[M - SH]^+$, 55), 151 ($[M - C_6H_5(CH_2)_2CHCO]^+$, 58), 147 ($[C_6H_5CH_2CH_2CH_2CO]^+$, 65), 91 ($[C_6H_5CH_2]^+$, 100)

Analysis: $C_{17}H_{15}NO_2S$ (297.38)

calculated	C: 68.66	H: 5.08	N: 4.71
found	C: 68.41	H: 5.08	N: 4.70

(*E*)-3-Phenyl-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)prop-2-en-1-one (6.13)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and cinnamoyl chloride (0.55 g, 3.30 mmol); recrystallization from ethanol.

Yield: 0.53 g (1.88 mmol, 57 %, yellow powder)

Mp: 150.5-151.5 °C (Lit. 148-149 °C[197])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 7.32-7.36 (m, 3H, Ar-H), 7.43-7.48 (m, 3H, Ar-H), 7.65-7.70 (m, 2H, Ar-H), 7.97-8.02 (m, 1H, Ar-H), 7.98 (d, 1H, ^3J = 15.5 Hz, =CH), 8.26 (d, 1H, ^3J = 15.5 Hz, =CH)

MS (PI-EIMS, 70 eV): m/z (%) = 281 ($[M^{\bullet+}]$, 16), 253 ($[M - CO]^+$, 3), 151 ($[M - C_6H_5CHCHCO]^+$, 9), 131 ($[C_6H_5CHCHCO]^+$, 100), 103 ($[C_6H_5 - CH{=}CH]^+$, 34), 77 ($[C_6H_5]^+$, 18)

Analysis: $C_{16}H_{11}NO_2S$ (281.33)

calculated	C: 68.31	H: 3.94	N: 4.98
found	C: 68.32	H: 4.02	N: 4.99

3-Cyclohexyl-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)propan-1-one (6.14)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.5 g, 3.31 mmol) and 3-cyclohexylpropanoyl chloride (**6.3**) (0.80 g, 4.58 mmol); recrystallization from ethanol.

Yield: 0.54 g (1.87 mmol, 56 %, white solid)

Mp: 76-77.5 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 0.89-1.44 (m, 5H, $2CH_2$+CH), 1.62-1.83 (m, 8H, $4CH_2$), 3.53 (t, 2H, ^3J = 7.7 Hz, $COCH_2$-), 7.27-7.36 (m, 3H, Ar-H), 8.05-8.10 (m, 1H, Ar-H)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 26.2 (-, $2CH_2$), 26.5 (-, CH_2), 31.6 (-, CH_2), 33.1 (-, $2CH_2$), 36.9 (-, CH_2), 37.1 (+, CH), 109.6 (+, Ar-C), 116.5 (+, Ar-C), 125.5 (+, Ar-C), 126.0 (+, Ar-C), 130.0 (C_{quart}, Ar-C), 146.5 (C_{quart}, Ar-C), 174.6 (C_{quart}, CO), 178.8 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 289 ([$M^{\bullet+}$], 5), 151 ([M - $C_6H_{11}CH_2CHCO$] $^+$, 100), 121 (benzoxazole, 56)

Analysis: $C_{16}H_{19}NO_2S$ (289.39)

calculated	C: 66.40	H: 6.62	N: 4.84
found	C: 65.86	H: 6.49	N: 5.04

2-Phenoxy-1-(2-thioxobenzo[*d*]oxazol-3(2*H*)-yl)ethanone (6.15)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and phenoxyacetyl chloride (0.46 ml, 3.33 mmol); recrystallization from ethanol.

Yield: 0.32 g (1.12 mmol, 34 %, light-brown solid)

Mp: 138-139 °C

^{1}H-NMR (DMSO-d_6): δ [ppm] = 5.73 (s, 2H, CH_2), 6.96-7.08 (m, 3H, Ar-H), 7.25-7.50 (m, 4H, Ar-H), 7.61-7.66 (m, 1H, Ar-H), 8.03-8.08 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 285 ([$M^{\bullet+}$], 36), 192 ([M - OC_6H_5]$^+$, 46), 164 ([M - OC_6H_5CO]$^+$, 100), 151 ([M - C_6H_5OCHCO] $^+$, 34), 77 ([C_6H_5]$^+$, 71)

Analysis: $C_{15}H_{11}NO_3S$ (285.32)

calculated	C: 63.14	H: 3.89	N: 4.91
found	C: 63.07	H: 3.99	N: 4.92

Benzyl 2-thioxobenzo[*d*]oxazol-3(2*H*)-carboxylate (6.16)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.50 g, 3.31 mmol) and benzyl chloroformate (0.48 ml, 3.36 mmol); different workup: reaction mixture was concentrated under reduced pressure and ethyl acetate (15 ml) and water (10 ml) were added; the organic phase was dried over magnesium sulfate and the solvent was removed under reduced pressure; crystallization from petroleum ether (60-80 °C) and subsequent recrystallization from methanol.

Yield: 0.30 g (1.05 mmol, 32 %, white needles)

Mp: 86-87 °C (Lit. 89-92 °C[198])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 5.55 (s, 2H, CH_2), 7.20-7.47 (m, 6H, Ar-H), 7.50-7.58 (m, 2H, Ar-H), 7.69-7.75 (m 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 285 ($[M^{\bullet+}]$, 5), 91 ($[C_6H_5CH_2]^+$, 100)

Analysis: $C_{15}H_{11}NO_3S$ (285.32)

calculated	C: 63.14	H: 3.89	N: 4.91
found	C: 63.25	H: 3.74	N: 4.83

***N*-Methanesulfonyl-2-aminophenol (6.18)**

o-Aminophenol (**6.17**) (1.1 g, 10.08 mmol) was dissolved in pyridine (10 ml) and cooled with an ice-bath. Methanesulfonyl chloride (0.85 ml, 10.94 mmol) was added and the solution was stirred over night at ambient temperature. The reaction mixture was poured into an ice-cold solution of hydrochloric acid (10 %, 70 ml) and extracted with ethyl acetate (3x30 ml). The combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure. Recrystallization from benzene yielded the desired product.

Yield: 1.19 g (6.36 mmol, 63 %, pale pink solid)

Mp: 105-106 °C (Lit. 110-111 °C[199])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.93 (s, 3H, SO_2CH_3), 6.78 (ddd, 1H, ^{3}J = 7.9 Hz, ^{3}J = 7.3 Hz, ^{4}J = 1.5 Hz, H-5), 6.88 (dd, 1H, ^{3}J = 8.1 Hz, ^{4}J = 1.5 Hz, H-3), 7.04 (ddd, 1H, ^{3}J = 8.1 Hz, ^{3}J = 7.3 Hz, ^{4}J = 1.7 Hz, H-4), 7.18 (dd, 1H, ^{3}J = 7.9 Hz, ^{4}J = 1.7 Hz, H-6), 8.69 (br, 1H, NH), 9.84 (br, 1H, OH)

MS (PI-EIMS, 70 eV): m/z (%) = 187 ([$M^{\bullet+}$], 31), 108 ([M - SO_2CH_3] $^+$, 100), 80 ([M - SO_2CH_3 - CO] $^+$, 46)

$C_7H_9NO_3S$ (187.20)

3-Methanesulfonylbenzoxazole-2-thione (6.19)

Method A: Reaction of 2-mercaptobenzoxazole (**6.1**) (0.51 g, 3.37 mmol) and methanesulfonyl chloride (0.27 ml, 3.49 mmol); reaction mixture was extracted three times with ethyl acetate (15 ml); the combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure; purification by column chromatography on silica gel, elution with chloroform.

Yield: 0.14 g (0.61 mmol, 18 %, light-yellow solid)

Mp: 131-132 °C (Lit. 136 °C[200])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 3.74 (s, 3H, CH_3), 7.28-7.37 (m, 3H, Ar-H), 7.80-7.86 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 229 ([$M^{\bullet+}$], 81), 150 ([M - SO_2CH_3] $^+$, 100), 122 ([M - SO_2CH_3 - CO] $^+$, 63)

Analysis: $C_8H_7NO_3S_2$ (229.28)

calculated	C: 41.91	H: 3.08	N: 6.11
found	C: 42.07	H: 3.18	N: 6.09

3-Acetylbenzo[*d*]oxazol-2(3*H*)-one (6.21)

Benzoxazol-2-one (**6.20**) (0.67 g, 4.96 mmol), dissolved in dry THF (10 ml), and triethylamine (2.0 ml ,14.43 mmol) were mixed under a nitrogen atmosphere. After cooling in an ice bath, acetyl chloride (0.43 ml, 6.03 mmol) was added dropwise. The reaction mixture was heated under reflux for 2 h and then poured

into ice-cold water (100 ml). After stirring for further 1 h, the precipitate was collected, washed with water and recrystallized from ethanol.

Yield: 0.28 g (1.58 mmol, 32 %, white solid)

Mp: 90.5-91.5 °C (Lit. 95-96 °C[191])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.61 (s, 3H, CH_3), 7.25-7.35 (m, 2H, Ar-H), 7.39-7.46 (m, 1H, Ar-H), 7.91-7.99 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 177 ([$M^{\bullet+}$], 26), 135 ([M - CH_2CO]$^+$, 100), 43 ([CH_3CO]$^+$, 84)

Analysis: $C_9H_7NO_3$ (177.16)

calculated	C: 61.02	H: 3.98	N: 7.91
found	C: 60.79	H: 4.34	N: 7.79

3-Hexanoylbenzo[*d*]oxazol-2(3*H*)-one (6.22)

Benzoxazol-2-one (**6.20**) (0.50 g, 3.70 mmol) was dissolved in dry THF (10 ml) under an inert atmosphere and treated with triethylamine (0.63 ml, 4.54 mmol). After addition of hexanoic anhydride (1.03 ml, 4.47 mmol), the mixture was heated under reflux for 4 h. Then water (15 ml) was added and the mixture was extracted three times with ethyl acetate (15 ml). The combined organic layers were washed with a saturated solution of sodium carbonate (15 ml), dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel eluting with chloroform.

Yield: 0.60 g (2.57 mmol, 70 %, pale light brown solid)

Mp: 72.5-73.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86-0.92 (m, 3H, CH_3), 1.29-1.39 (m, 4H, ($C\underline{H}_2$)$_2$$CH_3$), 1.60-1.71 (m, 2H, $COCH_2C\underline{H}_2$-), 3.02 (t, 2H, ^{3}J =7.4 Hz, $COCH_2$-), 7.24-7.34 (m, 2H, Ar-H), 7.36-7.46 (m, 1H, Ar-H), 7.93-8.02 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 233 ([$M^{\bullet+}$], 29), 135 ([M - C_4H_9CHCO]$^+$, 100), 99 ([C_4H_9CHCO]$^+$, 60), 71 ([C_4H_9 - CH_2]$^+$, 44)

Analysis: $C_{13}H_{15}NO_3$ (233.26)

calculated	C: 66.94	H: 6.48	N: 6.00

found C: 66.80 H: 6.73 N: 5.85

2-(Ethylamino)phenol (6.23)

To a solution of *o*-aminophenol (**6.17**) (0.50 g, 4.58 mmol) and bromoethane (0.35 ml, 4.69 mmol), dissolved in DMF (15 ml), sodium hydride (0.12 g, 5.03 mmol) was added in small portions at 0 °C. After stirring for 3 h, the reaction was quenched with methanol (10 ml). The solvent was removed under reduced pressure, and water (20 ml) and ethyl acetate (20 ml) were added. Subsequently, the organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x20 ml). The combined organic layers were dried over magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.17 g (1.24 mmol, 27 %, brown solid)

Mp: 105.5 °C (decomposition) (Lit. 110-111 °C[201])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.16 (t, 3H, ^{3}J = 7.1 Hz, CH_3), 3.05 (q, 2H, ^{3}J = 7.1 Hz, CH_2), 4.40 (br, 1H, NH), 6.36-6.49 (m, 2H, Ar-H), 6.60-6.66 (m, 2H, Ar-H), 9.15 (br, 1H, OH)

MS (CI-MS, NH_3): m/z (%) = 155 ($[M+NH_4]^+$, 6), 138 ($[M+H]^+$, 100)

$C_8H_{11}NO$ (137.18)

3-Ethylbenzo[*d*]oxazole-2(3*H*)-thione (6.24)

Compound **6.23** (0.15 g, 1.09 mmol) was dissolved in dry THF (5 ml) under an inert atmosphere. Subsequently, TCDI (0.21 g, 1.20 mmol) was added in small portions and the mixture was stirred for 3 h at ambient temperature. The reaction mixture was concentrated under reduced pressure and water (15 ml) was added. After extraction with ethyl acetate (3x15 ml), the combined organic layers were dried over magnesium sulfate and reduced *in vacuo*. The crude product was purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.07 g (0.39 mmol, 36 %, beige solid)

Mp: 105-107 °C (Lit. 96-98 °C[202])

^{1}H-NMR (DMSO-d_6): δ [ppm] = 1.30 (t, 3H, ^{3}J = 7.2 Hz, CH_3), 4.25 (q, 2H, ^{3}J = 7.2 Hz, CH_2), 7.30-7.43 (m, 2H, Ar-H), 7.54-7.60 (m, 2H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 179 ([$M^{\bullet+}$], 100), 151 ([M - C_2H_4] $^+$, 93)

C_9H_9NOS (179.24)

Methyl 3-amino-4-hydroxybenzoate (6.27)

3-Amino-4-hydroxybenzoic acid (**6.25**) (0.40 g, 2.61 mmol) was dissolved in dry methanol (10 ml) and treated with TMSCl (0.75 ml, 5.94 mmol). The mixture was stirred at 55 °C for 2 days. After evaporation of the solvent, the obtained residue was purified by column chromatography on silica gel eluting with ethyl acetate.

Yield: 0.25 g (1.50 mmol, 57 %, white solid)

^{1}H-NMR (DMSO-d_6): δ [ppm] = 3.74 (s, 3H, CO_2CH_3), 4.77 (br, 2H, NH_2), 6.70 (d, 1H, ^{3}J = 8.2 Hz, H-5), 7.09 (dd, 1H, ^{3}J = 8.2 Hz, ^{4}J = 2.2 Hz, H-6), 7.23 (d, 1H, ^{4}J = 2.2 Hz, H-2), 9.92 (br, 1H, OH)

MS (PI-EIMS, 70 eV): m/z (%) = 167 ([$M^{\bullet+}$], 100), 136 ([M - OCH_3] $^+$, 99), 108 ([M - CO_2CH_3] $^+$, 32)

$C_8H_9NO_3$ (167.16)

Methyl 2-sulfanylbenzo[*d*]oxazole-5-carboxylate (6.28)

Methyl 3-amino-4-hydroxybenzoate (**6.27**) (0.20 g, 1.20 mmol), dissolved in anhydrous THF (10 ml), was mixed with TCDI (0.26 g, 1.46 mmol) under an inert atmosphere. The solution was stirred overnight at room temperature. After evaporation of the solvent the residue was treated with water (15 ml) and extracted with ethyl acetate (3x15 ml). The combined organic layers were dried over magnesium sulfate and concentrated under reduced pressure. The remaining crude product was recrystallized from ethanol.

Yield: 0.22 g (1.05 mmol, 88 %, brown solid)

Mp: 203-205 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 3.87 (s, 3H, CO_2CH_3), 7.62 (d, 1H, ^3J = 8.5 Hz, H-7), 7.68 (d, 1H, ^4J = 1.5 Hz, H-4), 7.88 (dd, 1H, ^3J = 8.5 Hz, ^4J = 1.5 Hz, H-6), 14.08 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 209 ([$M^{\bullet+}$], 87), 178 ([M - OCH_3] $^+$, 100), 150 ([M - CO_2CH_3] $^+$, 13)

$C_9H_7NO_3S$ (209.22)

Methyl 3-(3-phenylpropanoyl)-2,3-dihydro-2-thioxobenzo[*d*]oxazole-5-carboxylate (6.29)

Methyl 2-sulfanylbenzo[*d*]oxazole-5-carboxylate (**6.28**) (0.20 g, 0.96 mmol) was dissolved in dry THF (5 ml) and treated with triethylamine (0.15 ml, 1.05 mmol). The mixture was cooled in an ice bath and 3-phenylpropanoyl chloride (0.15 ml, 0.96 mmol) was added dropwise. After stirring for 30 min at 0 °C, the reaction mixture was poured into ice water (35 ml). The resulting precipitate was collected, washed with water and dried *in vacuo*. The remaining crude product was recrystallized from ethanol.

Yield: 0.17 g (0.50 mmol, 52 %, beige solid)

Mp: 112.5-115.5 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 3.17 (t, 2H, ^3J = 7.5 Hz, $COC\underline{H}_2CH_2Ph$), 3.85 (t, 2H, ^3J = 7.5 Hz, $COCH_2C\underline{H}_2Ph$), 3.95 (s, 3H, CO_2CH_3), 7.22-7.36 (m, 6H, Ar-H), 8.09 (dd, 1H, ^3J = 8.5 Hz, ^4J = 1.7 Hz, H-6), 8.74 (dd, 1H, ^4J = 1.7 Hz, ^5J = 0.6 Hz, H-4)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 30.1 (-, CH_2), 40.7 (-, CH_2), 52.6 (+, CH_3), 109.4 (+, Ar-C), 117.9 (+, Ar-C), 126.5 (+, Ar-C), 127.9 (C_{quart}, Ar-C), 128.4 (+, Ar-C), 128.5 (+, 2Ar-C), 128.7 (+, 2Ar-C), 130.1 (C_{quart}, Ar-C), 139.7 (C_{quart}, Ar-C), 149.4 (C_{quart}, Ar-C), 165.9 (C_{quart}, $\underline{C}O_2Me$), 173.0 (C_{quart}, CO), 178.6 (C_{quart}, CS)

MS (PI-EIMS, 70 eV): m/z (%) = 341 ([$M^{\bullet+}$], 8), 308 ([M - SH] $^+$, 36), 209 ([M - $C_6H_5CH_2CHCO$] $^+$, 87), 178 ([M - $C_6H_5CH_2CHCOOCH_3$] $^+$, 35), 104 ([C_6H_5cyclopropyl] $^+$, 100), 91 ([C_7H_7] $^+$, 89)

Analysis: $C_{18}H_{15}NO_4S$ (341.38)

calculated	C: 63.33	H: 4.43	N: 4.10
found	C: 63.27	H: 4.40	N: 3.99

2-Sulfanylbenzo[*d*]oxazole-5-sulfonic acid (6.30)

3-Amino-4-hydroxybenzenesulfonic acid hydrate (**6.26**) (4.0 g, 21.14 mmol), sodium hydroxide (1.60 g, 40.0 mmol), carbon disulfide (3.5 ml, 50.9 mmol) and water were heated under reflux for 8 h. After cooling the mixture was acidified with concentrated hydrochloric acid. The resulting precipitate was removed by suction and recrystallized from ethanol.

Yield: 0.99 g (4.28 mmol, 20 %, white solid)

Mp: >300 °C

^{1}H-NMR (DMSO-d_6): δ [ppm] = 7.40 (dd, 1H, ^{4}J = 1.6 Hz, ^{5}J = 0.6 Hz, H-4), 7.43 (dd, 1H, ^{3}J = 8.4 Hz, ^{5}J = 0.6 Hz, H-7), 7.51 (dd, 1H, ^{3}J = 8.4 Hz, ^{4}J = 1.6 Hz, H-6), 13.91 (br, 1H, NH)

MS (PI-EIMS, 70 eV): m/z (%) = 231 ([$M^{\bullet+}$], 44), 151 ([M - SO_3H] $^+$, 82), 76 ([C_6H_4] $^+$, 100)

$C_7H_5NO_4S_2$ (231.25)

N,*N*-Dimethylbenzo[*d*]oxazol-2-amine (6.33)

N,*N*-Dimethylbenzylamine (**6.31**) (0.78 g, 5.77 mmol) and 2-chlorobenzoxazole (**6.32**) (0.86 g, 5.60 mmol) were heated at 130 °C for 3 h. After cooling the reaction mixture was dissolved in a small amount of chloroform and purified by column chromatography on silica gel eluting with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate and then with chloroform.

Yield: 0.80 g (4.93 mmol, 88 %, white-grey solid)

Mp: 89-90 °C (Lit. 89-91 °C[194])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 3.30 (s, 6H, $N(CH_3)_2$), 7.04-7.11 (m, 1H, Ar-H), 7.18-7.24 (m, 1H, Ar-H), 7.25-7.31 (m, 1H, Ar-H), 7.44-7.48 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 162 ([$M^{\bullet+}$], 100), 147 ([M - CH_3]$^+$, 79)

Analysis: $C_9H_{10}N_2O$ (162.19)

calculated	C: 66.65	H: 6.21	N: 17.27
found	C: 66.43	H: 6.45	N: 17.30

Synthesis of 2-methylbenzofuranes 6.35-6.36

General procedure. To an ice-cold solution of 2-methylbenzofuran (**6.34**) (1 eq), acid chloride (1 eq) and previously dried dichloromethane (5 ml), tin(IV) chloride (1 eq) was added dropwise. The solution changed color to dark red and purple, respectively. After stirring the reaction mixture at room temperature for 3.5 h, the mixture was poured into ice water (30 ml) and extracted three times with ethyl acetate (15 ml). The combined organic layers were dried over magnesium sulfate, filtrated and the solvent was removed *in vacuo*. The products were purified by column chromatography.

1-(2-Methylbenzofuran-3-yl)ethanone (6.35)

Reaction of 2-methylbenzofuran (**6.34**) (0.30 g, 2.27 mmol), acetyl chloride (0.16 ml, 2.27 mmol) and tin(IV) chloride (0.28 ml, 2.38 mmol); purification by chromatography on a silica gel column, elution with a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate to obtain a yellow oil which gave a yellow crystalline solid after cooling in the refrigerator.

Yield: 0.35 g (2.01 mmol, 89 %, yellow solid)

Mp: 48-49 °C (Lit. 50-52 °C[203])

^{1}H-NMR ($CDCl_3$): δ [ppm] = 2.65 (s, 3H, $COCH_3$), 2.79 (s, 3H, CH_3), 7.27-7.36 (m, 2H, Ar-H), 7.42-7.48 (m, 1H, Ar-H), 7.91-7.98 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 174 ([$M^{\bullet+}$], 44), 159 ([M - CH_3]$^+$, 100), 131 ([M - CH_3CO]$^+$, 8)

Analysis: $C_{11}H_{10}O_2$ (174.20)

calculated	C: 75.84	H: 5.79
found	C: 75.74	H: 5.85

1-(2-Methylbenzofuran-3-yl)-3-phenylpropan-1-one (6.36)

Reaction of 2-methylbenzofuran (**6.34**) (0.31 g, 2.34 mmol), hydrocinnamoyl chloride (0.35 ml, 2.34 mmol) and tin(IV) chloride (0.28 ml, 2.38 mmol); purification by chromatography on a silica gel column, elution with chloroform; recrystallization from methanol yielded analytically pure product.

Yield: 0.61 g (2.30 mmol, 98 %, white solid)

Mp: 83-84 °C

^{1}H-NMR ($CDCl_3$): δ [ppm] = 2.77 (s, 3H, CH_3), 3.11 (t, 2H, ^{3}J = 7.8 Hz, $COCH_2$-), 3.28 (t, 2H, ^{3}J = 7.8 Hz, $PhCH_2$-), 7.19-7.35 (m, 7H, Ar-H), 7.42-7.48 (m, 1H, Ar-H), 7.87-7.93 (m, 1H, Ar-H)

^{13}C-NMR ($CDCl_3$): δ [ppm] = 15.5 (+, CH_3), 29.7 (-, CH_2), 44.9 (-, CH_2), 111.1 (+, Ar-C), 117.1 (C_{quart}, Ar-C), 121.4 (+, Ar-C), 124.0 (+, Ar-C), 124.4 (+, Ar-C), 125.8 (C_{quart}, Ar-C), 126.2 (+, Ar-C), 128.5 (+, 2Ar-C), 128.6 (+, 2Ar-C), 141.3 (C_{quart}, Ar-C), 153.5 (C_{quart}, Ar-C), 163.0 (C_{quart}, Ar-C), 195.7 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 264 ([$M^{\bullet+}$], 46), 159 ([M - $C_6H_5CH_2CH_2$]$^{+}$, 100), 132 ([M - $C_6H_5CH_2CHCO$] $^{+}$, 18)

Analysis: $C_{18}H_{16}O_2$ (264.32)

calculated	C: 81.79	H: 6.10
found	C: 81.84	H: 6.00

6.5.3 Pharmacological methods

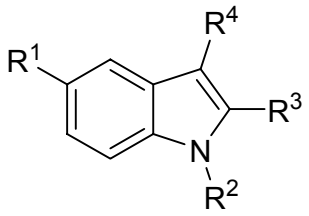
The inhibitory effect of the benzoxazole derivatives on the enzymatic activities of hyaluronidases were determined in a turbidimetric assay, developed in our laboratory, based on the method of Di Ferrante[145] as described in section 5.5.3.

Chapter 7

The indole scaffold as a core structure for hyaluronidase inhibitors

7.1 Introduction

Salmen[120] found that 1-decyl-2-(4-sulfamoyloxyphenyl)-1*H*-indol-6-yl-sulfamate (**5.1**) inhibits the bacterial hyaluronidase from *Streptococcus agalactiae* ($hylB_{4755}$) with an IC_{50} value of 11 µM at pH optimum, whereas the compound was inactive on the bovine testicular hyaluronidase. Subsequently, this 2-phenylindole derivative was co-crystallized with the related hyaluronate lyase from *Streptococcus pneumoniae*. The elucidation of the X-ray structure resulted in new suggestions for the development of more potent inhibitors. As described in chapter 6 one approach was the design and synthesis of 3-acyl-benzoxazoles, which proved to be rather potent inhibitors of bacterial hyaluronidases. However, the stability of these compounds was unsatisfying. Therefore, indole derivatives, which are structurally similar but more stable under the same conditions (cf. 6.3.3), were considered a promising alternative. The aim of the investigations described in this chapter was to find out whether the structure-activity relationships of the benzoxazoles can be transferred to the indole series or not. Hence, *N*-alkylated and *N*-acylated indole derivatives (see Fig. 7.1) were prepared. Moreover, as indometha-



R^1 = H, NO_2, NH_2, NHR
R^2 = H, alkyl, acyl
R^3 = H, CH_3
R^4 = H, acyl

Fig. 7.1: Target compounds

cin is a weak inhibitor of $hylB_{4755}$ (IC_{50} = 350 µM at pH 5)[120], a series of 1,3-disubstituted 2-methyl-indoles was synthesized (see Fig. 7.1). All synthesized indoles were investigated for inhibition of hyaluronidases from bacterial source and from bovine testis.

7.2 Chemistry

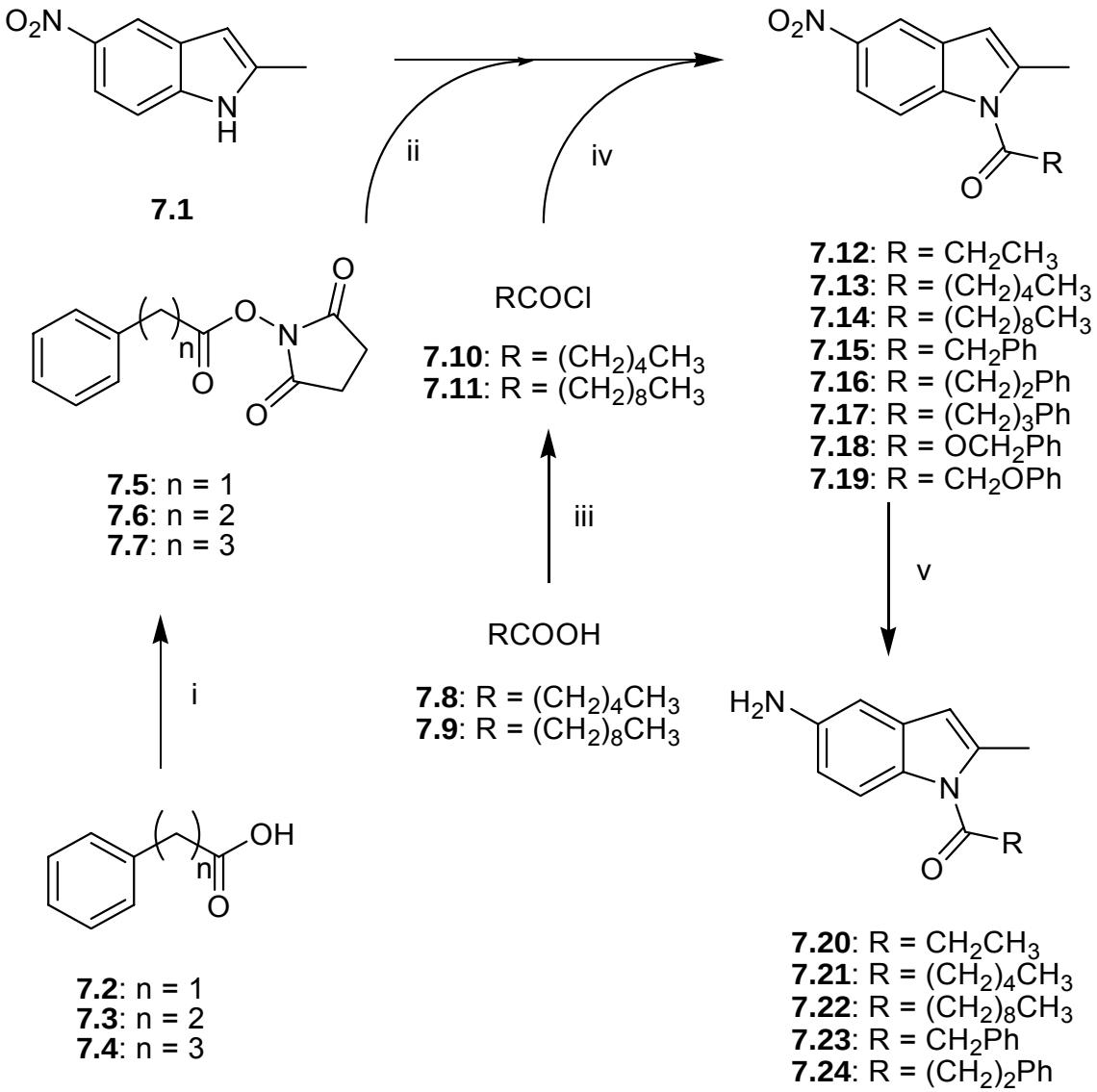
The indole skeleton is an important synthetic building block for the synthesis of a variety of natural products and biologically active compounds. A large number of preeminent methods for the synthesis of indoles have been well documented. But there is still a need for simple methods for the synthesis of highly functionalized indoles. The Fischer indole reaction is a quite versatile indolization procedure although it often suffers from low yields. Methods for the preparation of selectively substituted indoles were reported by Pindur and Adam[204]. Recently, a general approach for a one-pot synthesis of highly functionalized indoles from simple aryl hydrazines and cyclic enol ethers was developed[205]. This method was pointed out in the efficient synthesis of the anti-migraine drug sumitriptan and the anti-inflammatory drug indomethacin. Another interesting pathway has been described by Dai *et al*.[206] starting from 2-aminophenols including a Sonogashira cross-coupling reaction as key step and a subsequent alkoxide-mediated cyclisation to provide a number of indoles possessing substituents at the positions 4, 5, 6 and 7. A similar indole synthesis was reported by Rodriguez *et al.*[207].

As part of this doctoral project *N*-acylated and *N*-alkylated indoles with an additional functionality in position 5 at the indole moiety had to be prepared. In general, the regioselective *N*-acylation of indoles is accompanied by concomitant formation of 3-acyl and /or 1,3-diacyl derivatives depending on the associated metal cation, the solvent, the other substituents, the temperature and on the electrophilic reagent used[208]. A number of methods for the *N*-acylation of indoles are outlined in a review by Pindur *et al.*[209]. A simple acylation of the indole nitrogen under neutral conditions was described by Macor *et al.*[210] for the synthesis of indole-1-carboxylates, indole-1-thiocarboxylates and indole-1-carb-

oxamides: treatment of the indole with CDI and subsequent reaction with the corresponding alcohol, thiol or amine yielded the indole-1-carboxylic acid derivatives. Most often the sodium salts of the indoles, prepared in anhydrous DMF[211], are used as starting material for *N*-acylation. High yields of *N*-acylindoles were obtained by using the phase transfer catalyst tetrabutylammonium hydrogen sulfate and the two-phase system dichloromethane/powdered sodium hydroxide[212]. Another convenient procedure for the synthesis of *N*-acylated indoles was described by Kikugawa[213] who used powdered potassium hydroxide in dimethoxyethane at room temperature. Moderate yields of acylindoles were obtained when carboxylic acids instead of acyl chlorides were used for direct *N*-acylation in the presence of boric acid as catalyst at a long reaction time[214].

The starting material for both the *N*-alkylated and *N*-acylated indole derivatives was 2-methyl-5-nitroindole (**7.1**). As depicted in Scheme 7.1 the *N*-acylated 2-methyl-5-nitroindoles **7.12-7.19** were accessible *via* two synthetic routes. One possible pathway is the regioselective acylation of **7.1** with the particular acyl chloride after deprotonation with sodium hydride as base in DMF as solvent. Some of the acid chlorides (**7.10** and **7.11**) were prepared from the corresponding carboxylic acids as shown in Scheme 7.1. Another way is the treatment of **7.1** with an activated ester like *e.g.* a succinimidyl ester. The utilized succinimidyl esters **7.5**-**7.7** were prepared according to a standard method by coupling the corresponding carboxylic acids **7.2**-**7.4** with *N*-hydroxysuccinimide using DCC as coupling reagent and DMF as solvent. After the deprotonation of the weakly nucleophilic nitrogen atom of **7.1** with the strong base sodium hydride the succinimidyl esters were added to yield **7.15**-**7.17**.
The conversion of the nitro group of the *N*-acylated indoles to an amino group required a selective reduction system not affecting the pyrrole moiety of the indole scaffold. Furthermore, the amide bond at the nitrogen atom of the indoles should remain unchanged. Numerous methods for the reduction of aromatic nitro groups are reported in the literature. However, there are limitations considering safety, handling and selectivity[215]. Catalytic hydrogenation[216,217] of nitro compounds, the most common procedure for the synthesis of aromatic amines, is inappropriate for the synthesis of the compounds of interest, as partial reduction of the indole system may occur under these conditions. Recently, Banik[218]

highlighted reducing systems containing samarium. For instance, an easy, fast and highly efficient ultrasound-promoted reduction of aromatic nitro compounds was achieved by samarium/ammonium chloride mediated reaction[215].

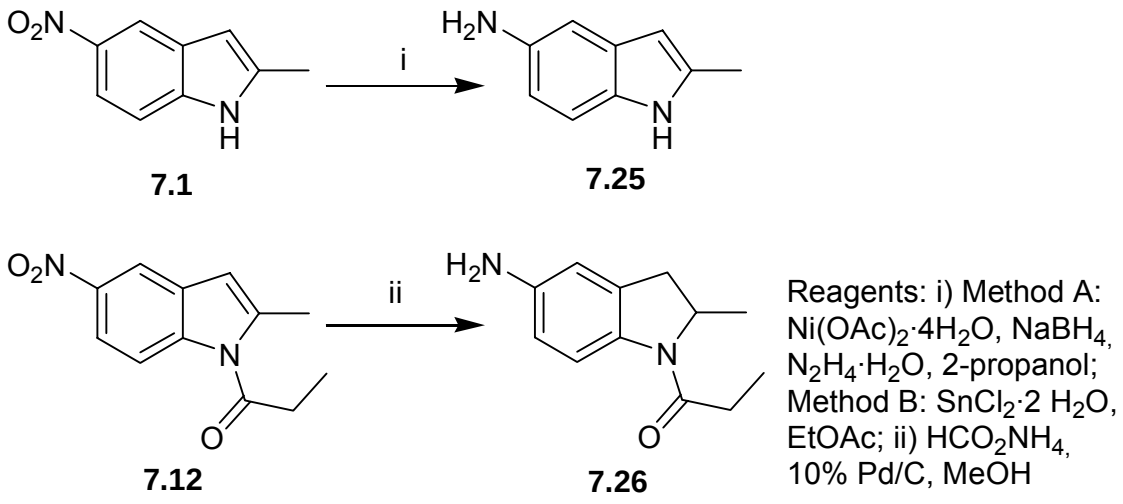


Reagents: i) DCC, N-hydroxysuccinimide, DMF; ii) NaH, DMF; iii) $SOCl_2$, DMF(cat.); iv) RCOCl, NaH, DMF, 0 °C; v) $SnCl_2 \cdot 2\ H_2O$, EtOAc

Scheme 7.1: Pathway to *N*-acylated indole derivatives **7.8**-**7.19**

With the aim to find a mild, fast and selective method different reducing systems were tried out. In the last years an Indian working group described a facile and selective reduction by using magnesium as catalyst and hydrazinium monoformate as hydrogen donor[219]. Reducible substituents such as alkenes, nitriles, carboxylic acids, phenols, halogens, esters, amides should remain unaffected. A reaction time of about 5 min at room temperature was reported to result in

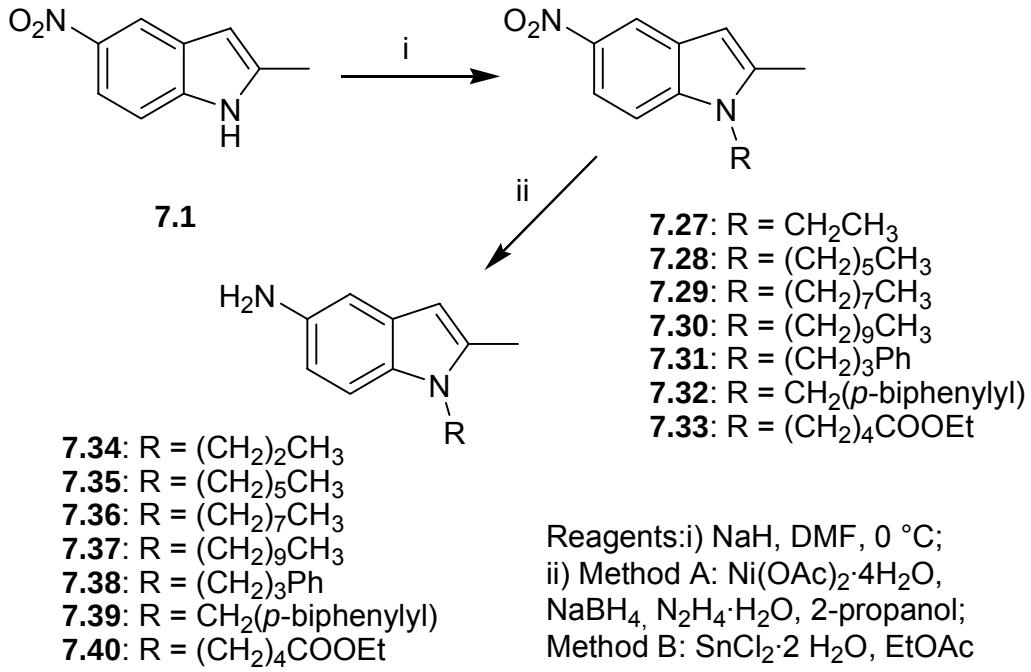
high yields. Unfortunately, when this method was applied to the reduction of **7.1**, no conversion to 5-amino-2-methyl-1*H*-indole **7.25** was detected after 10 minutes or after longer reaction times. The use of ammonium formate instead of hydrazinium formate as hydrogen donor was unsuccessful, too. The attempt to reduce the aromatic nitro group of **7.1** by transfer hydrogenation with Pd-C (10 %) and formic acid according to a protocol described by Entwistle *et al*.[220] resulted in a black slurry. According to the literature the reduction of nitroarenes using the system of Ram and Ehrenkaufer[221], containing anhydrous ammonium formate as a hydrogen transfer agent and Pd-C (10%) in methanol at room temperature, should occur without affecting functional groups such as carboxylic acids, esters, ethers, nitriles, guanidino and amide groups. However, the application of this method to the reduction of **7.12** resulted in **7.26**, i. e. the indole scaffold was partially hydrogenated (see Scheme 7.2).



Scheme 7.2: Reduction of nitro compounds **7.1** and **7.12**

At last, two methods afforded the selective reduction of **7.1**. The first was the use of nickel boride/hydrazine hydrate which was successfully applied by Lloyd and Nichols[222] for the synthesis of 4-(benzyloxy)indole and α-alkyltryptamines. The catalyst is of nonpyrophoric nature and is easy to prepare. The reduction of nitroindole **7.1** gave compound **7.25** in 71 % yield. However, under the same conditions this method failed to reduce the *N*-acylated 2-methyl-5-nitroindoles **7.12**-**7.19**, since the acyl residue was cleaved off. Nose and Kudo[223,224] described nickel boride in methanol/THF or in basic or acidic medium as an effective system for the conversion of aromatic nitro compounds containing reducible groups such as olefins, aldehydes, ketones, nitriles, amides, carboxylic acids

and esters. But keeping the reaction conditions exactly as reported by Nose and Kudo the desired product was not formed. Presumably, the catalyst was inactivated. This could be explained by the difficult preparation of the catalyst and its susceptibility against air. As an alternative the reduction of the nitro groups with stannous chloride in non acidic and non aqueous medium was tried out by analogy with the method reported by Bellamy and Ou[225]. Ethyl acetate or alcohols were used as solvents, and sensitive groups like aldehyde, ketone, ester, cyano, halogen and *O*-benzyl remained unaffected. According to this protocol all *N*-acylated compounds **7.20**-**7.24** were accessible in moderate to good yields.

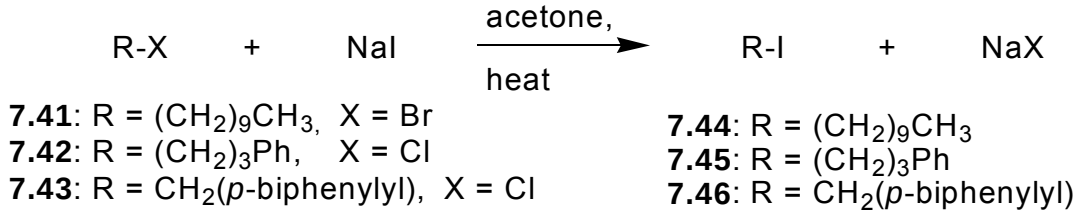


Scheme 7.3: Synthetic route to *N*-alkylated indole derivatives

For the synthesis of *N*-alkylindols with a hydrophilic group in position 5 a similar strategy was selected as that applied for the *N*-acylated indoles (see Scheme 7.3). 2-Methyl-5-nitroindole (**7.1**) was converted into the corresponding alkylated compounds **7.27**-**7.33** *via* a nucleophilic substitution reaction using the particular iodoalkane and sodium hydride as base. Compound **7.33** was obtained by refluxing **7.1** with ethyl 5-bromopentanoate in the presence of potassium carbonate in acetonitrile for 48 h. In contrast to our results Gray *et al*.[226] described a procedure for the regioselective alkylation of ethyl indole-2-carboxylate at position 3. The reduction of **7.27**-**7.33** to the corresponding amines was performed either with the nickel boride/hydrazine hydrate system or with stannous chloride

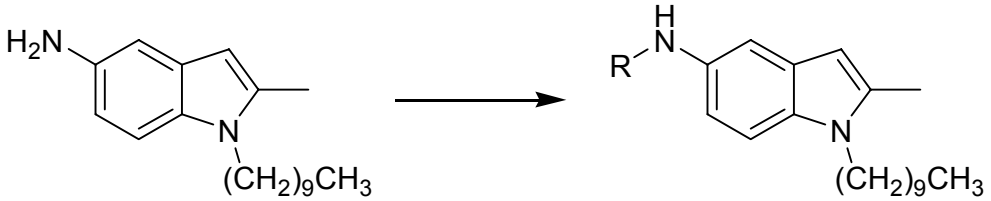
in ethyl acetate as explained above for the reduction of the *N*-acylated substances. Compared to the 1-acylindoles the 1-alkylindoles are much more stable against hydrogenolytic cleavage, and in contrast to the former the *N*-alkylated 2-methyl-5-nitroindoles **7.27**-**7.33** could be successfully reduced to the corresponding aminoindoles by nickel boride/hydrazine hydrate.

The iodo compounds **7.44**-**7.46** used for the *N*-alkylation of **7.1** were not commercially available and were prepared by the Finkelstein reaction (Scheme 7.4).



Scheme 7.4: Finkelstein reaction

Several derivatives of the amine **7.37** were synthesized as shown in Scheme 7.5. Sulfonamides **7.47** and **7.48** were prepared by a standard method using pyridine as solvent and either methanesulfonyl chloride or *N*,*N*-dimethylsulfamoyl chloride as reagent. Urea **7.49** was obtained in excellent yields by treatment of **7.37** with ethyl isocyanate in acetonitrile as solvent.



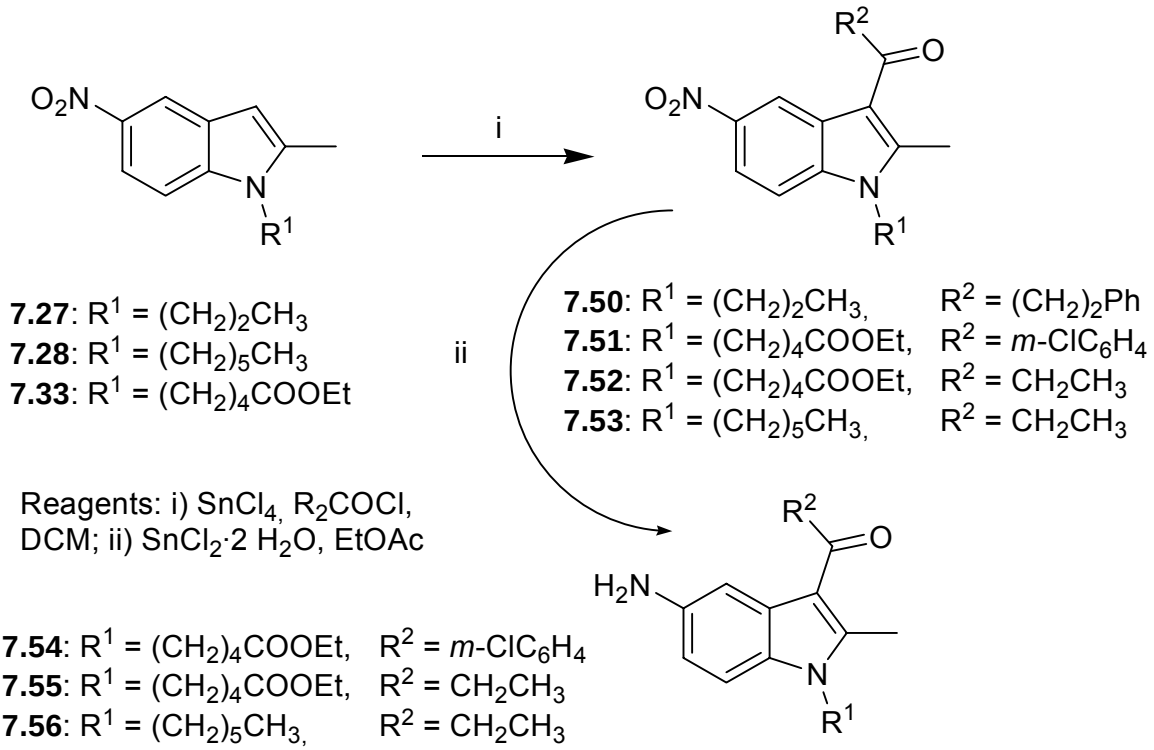
7.37

7.47: R = SO_2CH_3
7.48: R = $SO_2N(CH_3)_2$
7.49: R = $CONHC_2H_5$

Scheme 7.5: Conversion of **7.37** into sulfonamide derivatives **7.47** and **7.48** and urea derivative **7.49**

The 1,3-disubstituted 5-amino-2-methylindole derivatives **7.54**-**7.56** were obtained in a two-step reaction sequence as illustrated in Scheme 7.6. In the first step, the indole derivatives **7.27**, **7.28** and **7.33** were acylated in position 3 of the indole skeleton in a Friedel-Crafts like reaction using the corresponding acid chloride and tin(IV) chloride by analogy with a procedure described by Carvalho *et al*.[195] for acylation of benzofuranes. Subsequently, the obtained 1,3-disubstituted compounds **7.50**-**7.53** were treated with stannous chloride according to

the aforementioned procedure described by Bellamy and Ou[225] resulting in **7.54**-**7.56**.



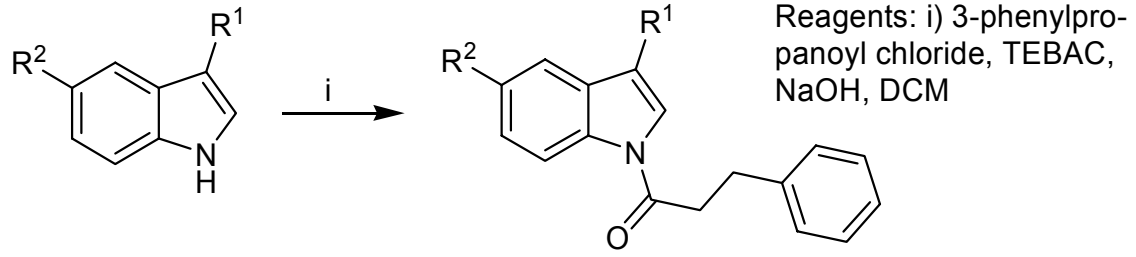
7.27: $R^1 = (CH_2)_2CH_3$
7.28: $R^1 = (CH_2)_5CH_3$
7.33: $R^1 = (CH_2)_4COOEt$

7.50: $R^1 = (CH_2)_2CH_3$, $R^2 = (CH_2)_2Ph$
7.51: $R^1 = (CH_2)_4COOEt$, $R^2 = m\text{-}ClC_6H_4$
7.52: $R^1 = (CH_2)_4COOEt$, $R^2 = CH_2CH_3$
7.53: $R^1 = (CH_2)_5CH_3$, $R^2 = CH_2CH_3$

7.54: $R^1 = (CH_2)_4COOEt$, $R^2 = m\text{-}ClC_6H_4$
7.55: $R^1 = (CH_2)_4COOEt$, $R^2 = CH_2CH_3$
7.56: $R^1 = (CH_2)_5CH_3$, $R^2 = CH_2CH_3$

Scheme 7.6: Synthetic pathway for the preparation of 1,3-disubstituted indole derivatives

As depicted in Scheme 7.7, a 3-phenylpropanoyl side chain was introduced at the indole-N of **7.57**-**7.59** by a nucleophilic substitution reaction utilizing TEBAC as phase transfer catalyst according to a protocol described by Illi[212].



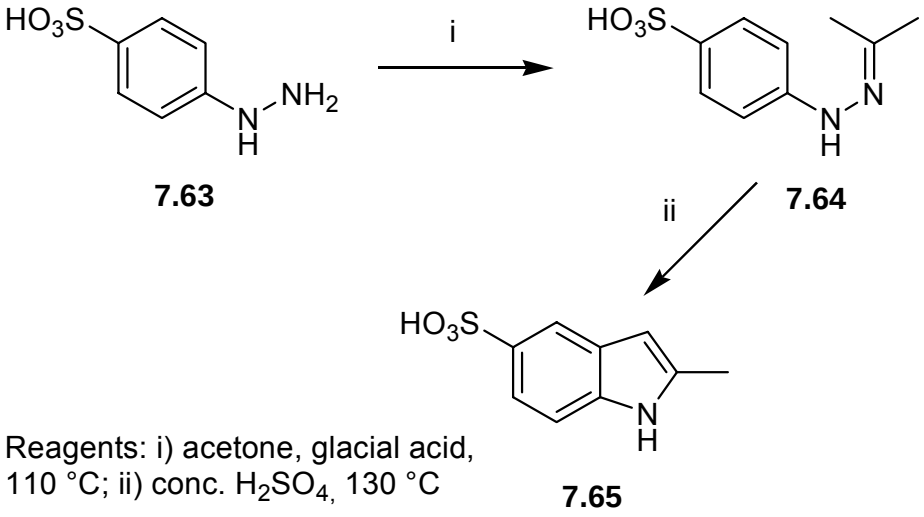
7.57: $R^1 = H$, $R^2 = H$
7.58: $R^1 = COCH_3$, $R^2 = H$
7.59: $R^1 = H$, $R^2 = COOH$

7.60: $R^1 = H$, $R^2 = H$
7.61: $R^1 = COCH_3$, $R^2 = H$
7.62: $R^1 = H$, $R^2 = COOH$

Scheme 7.7: *N*-acylation of indoles **7.57**-**7.59** using phase transfer conditions

For the introduction of a sulfonic acid group in position 5 of the indole scaffold a Fischer indole reaction protocol[227] was carried out as illustrated in Scheme 7.8. In the first step, the hydrazone **7.64** was prepared from 4-hydrazinobenzene-

sulfonic acid **7.63** and acetone. In contrast to the direct transformation of **7.64** into **7.65** described in the patent, the hydrazone had to be isolated and the following diaza-cope rearrangement succeeded by treatment with concentrated sulfuric acid as promoter under reflux for several hours. The acylation of **7.65** by acid chlorides or activated esters such as succinimidyl esters in DMF failed, presumably, due to insufficient solubility of the starting material.



Scheme 7.8: Preparation of 2-methyl-1*H*-indole-5-sulfonic acid (**7.65**) according to a Fischer indole synthesis

7.3 Results and discussion

To investigate the synthesized indoles for hyaluronidase inhibition a turbidimetric assay was carried out using either the bacterial hyaluronate lyase from *S. agalactiae* at pH 5 and pH 7.4, respectively, or bovine testicular hyaluronidase at pH 5. The Morgan-Elson assay is not suitable as the indole derivatives and Ehrlich's reagent give colored compounds which are detectable at 590 nm, the wavelength used for the quantification of the red-colored product of the Morgan-Elson reaction.

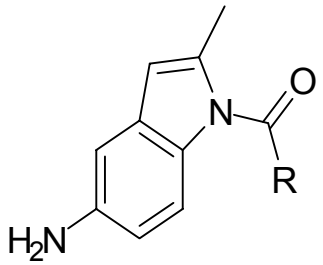
At highest concentrations tested the synthesized 5-nitroindoles, which are poorly soluble in aqueous buffer, had only negligible inhibitory effects on both hyaluronidases (data not shown). All other investigated indoles except for **7.25** and **7.65** were inactive on the bovine testicular hyaluronidase but proved to be

weak to moderate inhibitors of $hylB_{4755}$ indicating selectivity for the bacterial enzyme (results see Tables 7.1-7.5).

7.3.1 N-acylated 5-amino-2-methylindole derivatives

In accordance with the results from the X-ray structure of **5.1** co-crystallized with the bacterial hyaluronidase from *S. pneumoniae* long alkyl chains were introduced at the nitrogen atom of the indole scaffold in order to increase the inhibitory activity (see Table 7.1). Assuming similar binding modes of benzoxazoles and indole **5.1** arylalkanoyl residues such as the 3-phenylpropanoyl group were suggested as activity-enhancing substituents.

Table 7.1: Inhibitory activities of *N*-acylated indoles determined on the hyaluronidase from *S. agalactiae* at pH 5 and pH 7.4 and on the bovine testicular hyaluronidase at pH 5



Compound	R	**$hylB_{4755}$, % inhibition[a]**		**BTH[a]**
		pH = 5.0	pH = 7.4	pH = 5.0
7.20	CH_2CH_3	20 % (2000)	12 % (2000)	inactive (2000)
7.21	$(CH_2)_4CH_3$	42 % (600)	42 % (600)	inactive (600)
7.22	$(CH_2)_8CH_3$	12 % (100)	8 % (100)	inactive 100)
7.23	$(CH_2)_2Ph$	3 % (200)	4 % (200)	inactive (200)
7.24	$(CH_2)_3Ph$	10 % (200)	9 % (200)	inactive (200)

[a] inhibition of enzyme expressed as percent inhibition at inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds.

As this structural modification led to the most potent benzoxazole-type hyaluronidase inhibitors (see chapter 6) the optimized 3-phenylpropanoyl side chain was attached to the indole-N as well. Compounds **7.20**-**7.22** each induced about the same percentage inhibition of $hylB_{4755}$ at pH 5 and pH 7.4, respec-

tively. Due to different solubilities **7.20**-**7.22** were tested at different concentrations. Therefore, the comparability of the data presented in Tab 7.1 is restricted in terms of structure-activity considerations. The very weak inhibitory activity of compound **7.23** demonstrates that the optimized side chain of the strong benzoxazole-type inhibitor **6.11** is not suitable for the 5-amino-2-methyl-indole scaffold suggesting different binding modes of indoles and benzoxazoles and lower affinity of the indole compared to the benzoxazole-2-thione scaffold. Nevertheless, due to the aforementioned unfavorable physicochemical properties of the acylindoles, these results should not be over interpreted.

7.3.2 N-alkylated 5-amino-2-methylindole derivatives

The hyaluronidase inhibitory activities of the *N*-alkylindoles **7.35**-**7.37** are summarized in Table 7.2.

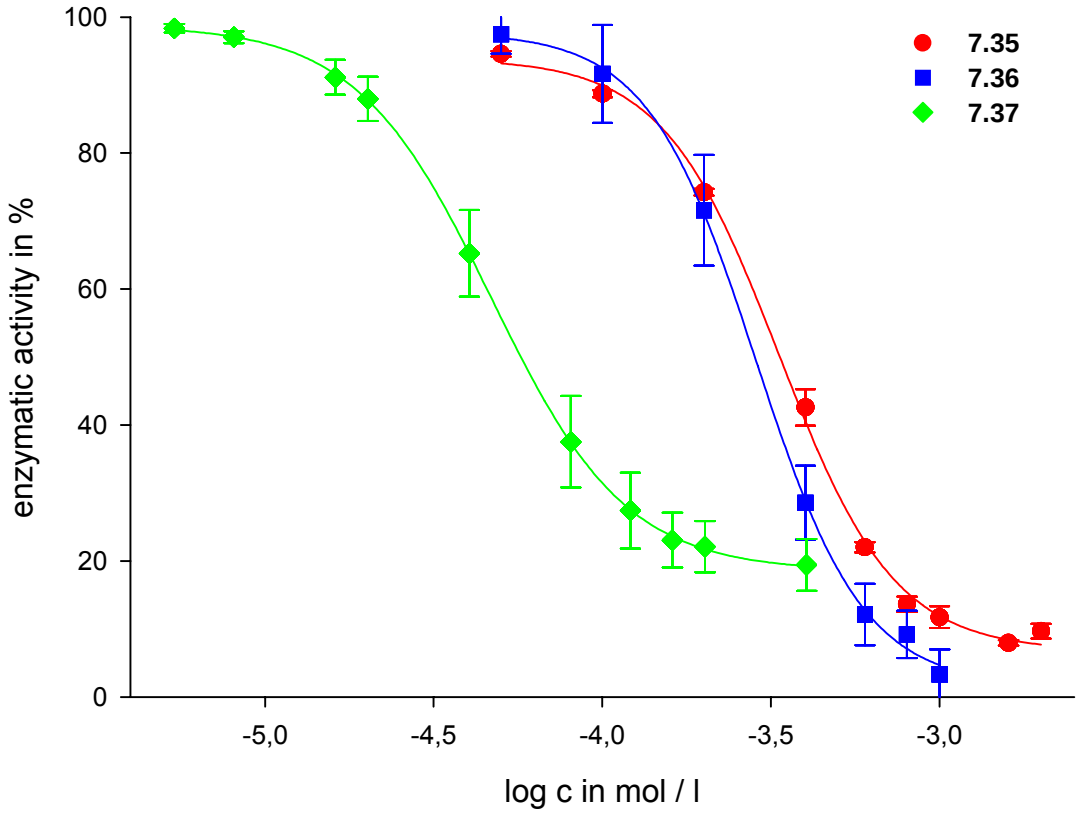
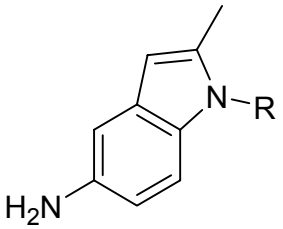


Fig. 7.2: Enzymatic activity of hyaluronate lyase of *S. agalactiae* in the presence of compounds **7.35**-**7.37** at pH 5

At pH optimum, IC_{50} values in the micromolar range were determined for the inhibition of the hyaluronate lyase from *S. agalactiae* strain 4755: 334 µM (**7.35**), 285 µM (**7.36**) and 47 µM (**7.37**) (see Fig. 7.2). Compared to the 1-unsubstituted parent compound **7.25** the inhibitory activity of the alkylindoles

7.34-**7.37** was considerably higher, and there was a remarkable increase in potency with elongation of the alkyl chain. This result is in good agreement with the structure-activity relationships discussed in the previous chapters and underlines the contribution of hydrophobic interactions to ligand binding in the active site of the enzyme. However, at physiological pH there was no clear increase in activity depending on the chain length of the substituent, in fact the hexyl group proved to be the optimum.

Table 7.2: Inhibitory activities of *N*-alkylated indole derivatives on hyaluronate lyase from *S. agalactiae* at pH 5 and pH 7.4 and on bovine testicular hyaluronidase at pH 5



Compd.	R	hylB$_{4755}$, IC$_{50}$ [µM] or % inhibition[a]		BTH[a]
		pH = 5.0	pH = 7.4	pH = 5.0
7.25	H	17 % (5000)	27 % (5000)	25 % (5000)
7.34	$(CH_2)_2CH_3$	15 % (2000)	32 % (2000)	inactive[b]
7.35	$(CH_2)_5CH_3$	334 ± 6	376 ± 24	inactive[c]
7.36	$(CH_2)_7CH_3$	285 ± 12	21 % (200)	inactive[d]
7.37	$(CH_2)_9CH_3$	47 ± 2	7 % (160)	inactive[e]
7.38	$(CH_2)_3Ph$	31 % (420)	13 % (420)	inactive[f]
7.39	CH_2-*p*-biphenylyl	13 % (200)	inactive[g]	inactive[g]
7.40	$(CH_2)_4COOEt$	25 % (1600)	51 % (1600)	inactive[h]

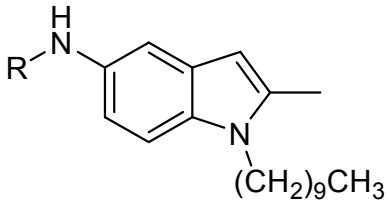
[a] inhibition of enzyme expressed as IC$_{50}$ ± SEM in µM or as percent inhibition at inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 2 mM, [c] at concentrations ≤ 150 µM, [d] at concentrations ≤ 350 µM, [e] at concentrations ≤ 160 µM, [f] at concentrations ≤ 420 µM, [g] at concentrations ≤ 200 µM, [h] at concentrations ≤ 1.6 mM.

A phenyl group as in compound **7.38** slightly increased the inhibitory potency (**7.38** vs. **7.34**) whereas a *p*-biphenylylmethyl group (**7.39**) was not tolerated. An ester group (**7.40**) led to improved solubility but the inhibitory activity of compound **7.40** on hylB$_{4755}$ was considerably reduced (25 % inhibition at a concen-

tration of 1.6 mM). Obviously, the ester group is not tolerated due to its electronic properties, whereas methylene groups are favorable. Therefore, it is conceivable that homologisation of the alkyl alkanoate moiety could compensate or even over-compensate the decrease in activity caused by the ester function. However, this approach could not be further elaborated within this doctoral project.

Compound **7.37**, the strongest inhibitor within the *N*-alkylindole series, was taken for further structural modifications. In consideration of the structure-activity relationships of **5.1**[120] interactions of a polar substituent in position 5 of the indole moiety with amino acids in the active site of $hylB_{4755}$ are conceivable. Salmen proposed two possible binding modes for the sulfamoyl substituent in position 5 of the indole **5.1**: hydrogen bonds between sulfamoyl group, water molecules and either Arg466/Ser463 or Asn349/Asp352 of hylSpn.

Table 7.3: Inhibitory potency of 5-amino-1-decyl-2-methyl-1*H*-indole (**7.37**) and its derivatives **7.47**-**7.49** determined on the hyaluronidase from *S. agalactiae* at pH 5 and pH 7.4 and on the bovine testicular hyaluronidase at pH 5



Compd.	R	$hylB_{4755}$, IC_{50} [µM] or % inhibition[a]		BTH[a]
		pH = 5.0	pH = 7.4	pH = 5.0
7.37	H	47 ± 2	7 % (160)	inactive[b]
7.47	SO_2CH_3	16 % (50)	12 % (200)	inactive[c]
7.48	$SO_2N(CH_3)_2$	6 % (100)	6 % (200)	inactive[d]
7.49	$CONHC_2H_5$	inactive[c]	8 % (200)	inactive[d]

[a] inhibition of enzyme expressed as IC_{50} ± SEM in µM or as percent inhibition at inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 160 µM, [c] at concentrations ≤ 50 µM, [d] at concentrations ≤ 100 µM.

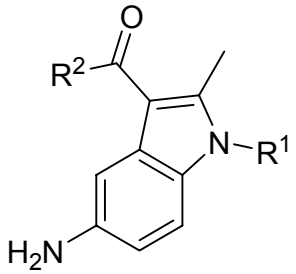
Hence, the amino group in **7.37** was derivatized in order to increase the N-H acidity (see Table 7.3). As summarized in Table 7.3, neither an urea nor a sulfamoyl group did enhance the inhibitory effect on the bacterial enzyme at pH 5.

H-bonds as well as ionic interactions might play an important role in this part of enzyme-ligand interaction.

7.3.3 1,3-disubstituted 5-amino-2-methylindole derivatives

Starting from the inhibition data for indomethacin (IC_{50} values of 350 µM at pH 5 and of 1.1 mM at pH 7.4 for inhibition of $hylB_{4755}$) determined by Salmen[120], a series of 1,3-disubstituted 2-methylindole derivatives with an additional amino function in position 5 were investigated for inhibition of $hylB_{4755}$ at pH 5 and pH 7.4 (see Table 7.4). Compounds **7.55** and **7.56** showed a moderate percentage inhibition of hyaluronate lyase of *S. agalactiae* strain 4755 at both physiological pH and optimum pH. The poorly soluble **7.54** revealed only a low percentage inhibition at the highest tested concentration.

Table 7.4: Inhibitory activities of 1,3-disubstituted 5-amino-2-methyl-1*H*-indole derivatives **7.54**-**7.56** determined on the hyaluronidase from *S. agalactiae* at pH 5 and pH 7.4 and on the bovine testicular hyaluronidase at pH 5



Compd.	R^1	R^2	$hylB_{4755}$, % inhibition[a]		BTH[a]
			pH = 5.0	pH = 7.4	pH = 5.0
7.54	$(CH_2)_4COOEt$	*m*-ClC_6H_4	20 % (100)	8 % (100)	inactive[b]
7.55	$(CH_2)_4COOEt$	CH_2CH_3	52 % (1600)	59 % (1600)	inactive[c]
7.56	$(CH_2)_5CH_3$	CH_2CH_3	60 % (400)	31 % (400)	inactive[d]

[a] inhibition of enzyme expressed as percent inhibition at inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 100 µM, [c] at concentrations ≤ 1.6 mM, [d] at concentrations ≤ 400 µM.

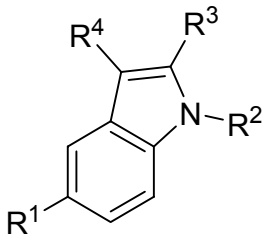
The 3-propanoylindoles **7.55** and **7.56** were about equipotent with the 3-unsubstituted analogs **7.40** and **7.35**, respectively. Thus, the propanoyl substituent in

position 3 does neither result in an increase nor in a decrease in inhibitory activity.

7.3.4 1-Phenylpropanoylindole derivatives

According to the hypothesis that the phenylpropanoyl-substituted benzoxazole-2-thiones (discussed in chapter 6) and the corresponding indoles have a similar binding mode comparable inhibitory activities on the bacterial hyaluronidase were expected. However, compounds **7.60** and **7.61**, tested at highest possible concentrations, were inactive on $hylB_{4755}$. Presumably, due to poor solubility at pH 5 compound **7.62** could not be tested at concentrations higher than 200 µM. Under these conditions only a low percentage inhibition of 20 % was determined, whereas at physiological pH **7.62** displayed a moderate inhibitory effect on $hylB_{4755}$ with an IC_{50} value of 906 µM.

Table 7.5: Inhibitory activities of indoles determined on the bacterial hyaluronidase from *S. agalactiae* at pH 5 and pH 7.4 and on the bovine hyaluronidase from testis at pH 5



No.	R^1	R^2	R^3	R^4	$hylB_{4755}$, IC_{50} [µM] or %[a]		BTH[a]
					pH = 5.0	pH = 7.4	pH = 5.0
7.60	H	$CO(CH_2)_2Ph$	H	H	inactive[b]	inactive[b]	inactive[b]
7.61	H	$CO(CH_2)_2Ph$	H	$COCH_3$	inactive[b]	inactive[b]	inactive[b]
7.62	COOH	$CO(CH_2)_2Ph$	H	H	12 % (200)	906 ± 18	inactive[c]
7.65	SO_3H	H	CH_3	H	2310 ± 66	28 % (7000)	2352 ± 107

[a] inhibition of enzyme expressed as IC_{50} ± SEM in µM or as percent inhibition at inhibitor concentration (µM) given in parentheses; highest tested concentrations were dependent on the solubility of the compounds, [b] at concentrations ≤ 100 µM, [c] at concentrations ≤ 200 µM.

Obviously, an acidic functional group like a carboxylic acid in position 5 of the indole structure is favorable to increase both the solubility and the inhibitory activity. The sulfonic acid **7.65** induced an inhibition of $hylB_{4755}$, too, but the level of activity was rather low. As compound **7.65** is lacking the *N*-substituent, it is conceivable that an increase in activity can be achieved by lipophilic substituents in 1-position. For the sulfonic acid **7.65** the IC_{50} values for inhibition of $hylB_{4755}$ and BTH were in the same range (about 2.3 mM).

7.4 Summary

The indoles presented in this study showed a preference for the bacterial hyaluronidase of *S. agalactiae* strain 4755 ($hylB_{4755}$). Among the examined indole derivatives the *N*-alkylated 5-amino-2-methylindole derivatives **7.35**-**7.37** displayed the strongest inhibitory potency (in the micromolar range) against $hylB_{4755}$ at optimum pH. Modifications of the amino group in position 5 of the potent inhibitor **7.37** (IC_{50} value of 47 µM at pH 5) resulted in a strong decrease in inhibitory activity. The *N*-acylated indoles revealed only very weak inhibitory effects indicating a missing interaction of the carbonyl oxygen with Tyr488 in the active site of $hylB_{4755}$. Within the series of 1,3-disubstituted 5-amino-2-methylindole derivatives, compounds bearing a hexyl group at the nitrogen atom and a 3-propanoyl substituent are about as potent as indomethacin and compound **7.35**. Different substituents such as nitro, amino, urea, sulfonamide, carboxylic acid and sulfonic acid groups were introduced in position 5 of the indole scaffold. Only the amino group and the acidic groups confer additional affinity resulting in increased inhibitory potency against the bacterial enzyme. This suggests that ionic interactions as well as hydrogen bonds may play a role in the interactions between the 5-substituent and the enzyme. In summary, compared to benzoxazole-2-thione the indole structure is less promising as a scaffold for the development of hyaluronidase inhibitors.

7.5 Experimental section

7.5.1 General conditions

For general methods see section 4.5.1.

7.5.2 Chemistry

Synthesis of succinimidyl esters 7.5-7.7

General procedure. *N*-Hydroxysuccinimide (1 eq) was dissolved in DMF (10-20 ml). After cooling in an ice-bath, DCC (1.1 eq) and the corresponding carboxylic acid (1 eq) was added. Then, the reaction mixture was stirred for further 2 h at 0 °C and, subsequently, stirring was continued overnight at room temperature. The resulting precipitate was filtered off using a sintered glass crucible and the filtrate was treated with a solution of sodium bicarbonate (5 %, 20 ml). After extraction with ethyl acetate (3x30 ml), the combined organic phases were washed with water (20 ml) and dried over magnesium sulfate. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel eluting with ethyl acetate.

1-(2-Phenylethanoyl)pyrrolidine-2,5-dione (7.5)

Reaction of *N*-hydroxysuccinimide (0.58 g, 5.04 mmol), DCC (1.13 g, 5.48 mmol) and 2-phenylacetic acid (**7.2**) (0.68 g, 4.99 mmol).

Yield: 0.68 g (2.91 mmol, 58 %, white solid)

Mp: 109.5-112.5 °C (Lit. 113-117 °C[228])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.81 (s, 4H, CH_2), 4.11 (s, 2H, PhC$\underline{H}_2$CO-), 7.28-7.41 (m, 5H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 233 ([$M^{\bullet+}$], 5), 119 ([$C_6H_5CH_2CO$] $^+$, 17), 91 ([C_7H_7] $^+$, 100)

$C_{12}H_{11}NO_4$ (233.22)

1-(3-Phenylpropanoyl)pyrrolidine-2,5-dione (7.6)

Reaction of *N*-hydroxysuccinimide (0.58 g, 5.04 mmol), DCC (1.15 g, 5.57 mmol) and 3-phenylpropanoic acid (**7.3**) (0.76 g, 5.06 mmol).

Yield: 0.84 g (3.40 mmol, 67 %, white solid)

Mp: 115.5-117.5 °C (Lit. 111-113 °C[229])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.81 (s, 4H, CH_2), 2.91-3.05 (m, 4H, Ph($C\underline{H}_2$)$_2$-), 7.18-7.33 (m, 5H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 247 ([$M^{\bullet+}$], 1), 149 ([$C_6H_5(CH_2)_2CO_2$]$^+$, 10), 133 ([$C_6H_5(CH_2)_2CO$]$^+$, 33), 105 ([C_8H_9]$^+$, 100), 91 ([C_7H_7]$^+$, 69)

$C_{13}H_{13}NO_4$ (247.25)

1-(4-Phenylbutanoyl)pyrrolidine-2,5-dione (7.7)

Reaction of *N*-hydroxysuccinimide (0.58 g, 5.04 mmol), DCC (1.14 g, 5.53 mmol) and 4-phenylbutanoic acid (**7.4**) (0.82 g, 4.99 mmol).

Yield: 0.88 g (3.37 mmol, 67 %, white solid)

Mp: 55.5-57 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.86-1.98 (m, 2H, $PhCH_2C\underline{H}_2CH_2$-), 2.63-2.71 (m, 4H, $PhC\underline{H}_2CH_2C\underline{H}_2$-), 2.82 (s, 4H, CH_2), 7.17-7.34 (m, 5H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 261 ([$M^{\bullet+}$], 6), 163 ([$C_6H_5(CH_2)_3CO_2$]$^+$, 20), 147 ([$C_6H_5(CH_2)_3CO$]$^+$, 100), 104 ([$C_6H_5CHCH_2$]$^+$, 27), 91 ([C_7H_7]$^+$, 82)

$C_{14}H_{15}NO_4$ (261.27)

Preparation of acid chlorides 7.10 and 7.11

General procedure. Carboxylic acid (1 eq) was treated with thionyl chloride (1.5 eq) and one drop of DMF under a nitrogen atmosphere. After stirring for 1 h at ambient temperature until the liberation of gas had ceased the mixture was evaporated with a water-jet pump at room temperature to separate surplus thionyl chloride. The obtained crude product was used for further reaction without purification.

Hexanoyl chloride (7.10)

Reaction of hexanoic acid (**7.8**) (0.23 ml, 1.87 mmol) and thionyl chloride (0.20 ml, 2.76 mmol).

Yield: colorless liquid

Decanoyl chloride (7.11)

Reaction of decanoic acid (**7.9**) (0.32 g, 1.86 mmol) and thionyl chloride (0.20 ml, 2.76 mmol).

Yield: colorless liquid

Synthesis of the *N*-acylated 2-methyl-5-nitro-1*H*-indoles 7.12-7.19

General procedures

Method A. 2-Methyl-5-nitro-1*H*-indole (**7.1**) (1 eq) was dissolved in DMF (10 ml) and cooled down to 0 °C under a nitrogen atmosphere. Sodium hydride (1.5 eq) was added and the reaction mixture was allowed to stir for about 20 min until gas generation ceased. After dropwise addition of the pertinent acid chloride (1 eq), the mixture was stirred for further 30 min at 0 °C. The reaction was quenched with a solution of hydrochloric acid (1 N, 20 ml). After extraction with ethyl acetate (3x30 ml) the combined organic phases were washed with a solution of saturated sodium bicarbonate (20 ml) and dried over magnesium sulfate. The solvent was removed under reduced pressure.

Method B. 2-Methyl-5-nitro-1*H*-indole (**7.1**) (1 eq) was dissolved in DMF (10 ml) and cooled down to 0 °C under a nitrogen atmosphere. Sodium hydride (1.5 eq) was added and the reaction mixture was allowed to stir for about 30 min until gas generation ceased. After dropwise addition of the pertinent succinimidyl ester (1 eq) the mixture was stirred for further 2 h at room temperature. The reaction was quenched with a solution of hydrochloric acid (1 N, 20 ml). After extraction with ethyl acetate (3x 30 ml) the combined organic phases were washed with a solution of saturated sodium bicarbonate (20 ml), dried over magnesium sulfate and the solvent was removed under reduced pressure.

2-Methyl-5-nitro-1-propanoyl-1*H*-indole (7.12)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (105 mg, 2.62 mmol) and propanoyl chloride (0.15 ml, 1.72 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.34 g (1.46 mmol, 86 %, yellow solid)

Mp: 121 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.20 (t, 3H, ^{3}J = 7.1 Hz, $CH_2C\underline{H}_3$), 2.68 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 3.12 (q, 2H, ^{3}J = 7.1 Hz, $COC\underline{H}_2CH_3$), 6.73 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.8 Hz, H-3), 8.09 (dd, 1H, ^{3}J = 9.2 Hz, ^{4}J = 2.5 Hz, H-6), 8.30 (ddd, 1H, ^{3}J = 9.2 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7), 8.43 (dd, 1H, ^{4}J = 2.5 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.7 (+, $COCH_2\underline{C}H_3$), 17.1 (+, CH_3), 31.3 (-, $CO\underline{C}H_2CH_3$), 109.1 (+, indole), 115.4 (+, indole), 115.8 (+, indole), 118.2 (+, indole), 129.1 (C_{quart}, indole), 139.3 (C_{quart}, indole), 140.6 (C_{quart}, indole), 142.8 (C_{quart}, indole), 174.8 (C_{quart}, $\underline{C}OCH_2CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 232 ([$M^{\bullet+}$], 29), 176 ([M - CH_3CHCO]$^{+}$, 100), 146 ([M - CH_3CHCO - NO]$^{+}$, 10), 130 ([M - CH_3CHCO - NO_2]$^{+}$, 34), 57 ([C_4H_9]$^{+}$, 40)

IR [cm^{-1}]: 3143 (C-H) aromatic, 3093 (C-H) aromatic, 2992 (C-H) aliphatic, 2947 (C-H) aliphatic, 2928 (C-H) aliphatic, 1719 (C=O), 1337 (C-NO_2)

Analysis: $C_{12}H_{12}N_2O_3$ (232.24)

calculated	C: 62.06	H: 5.21	N: 12.06
found	C: 62.10	H: 4.89	N: 12.01

1-Hexanoyl-2-methyl-5-nitro-1*H*-indole (7.13)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (102 mg, 2.55 mmol) and hexanoyl chloride (**7.10**); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.14 g (0.51 mmol, 30 %, yellow solid)

Mp: 76-77.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.89 (t, 3H, ^{3}J = 7.1 Hz, $CO(CH_2)_4C\underline{H}_3$), 1.31-1.41 (m, 4H, $CO(CH_2)_2(C\underline{H}_2)_2CH_3$), 1.66-1.77 (m, 2H, $COCH_2C\underline{H}_2$-), 2.67 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 3.09 (t, 2H, ^{3}J = 7.2 Hz, $COC\underline{H}_2CH_2$-), 6.72 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.7 Hz, H-3), 8.09 (dd, 1H, ^{3}J = 9.2 Hz, ^{4}J = 2.4 Hz, H-6), 8.26 (ddd, 1H, ^{3}J = 9.2 Hz, ^{5}J = 0.7 Hz, ^{5}J = 0.5 Hz, H-7), 8.43 (dd, 1H, ^{4}J = 2.4 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.8 (+, $CO(CH_2)_4\underline{C}H_3$), 17.0 (+, CH_3), 21.9 (-, CH_2), 23.8 (-, CH_2), 30.5 (-, CH_2), 37.7 (-, CH_2), 109.1 (+, indole), 115.4 (+, indole), 115.7 (+, indole), 118.2 (+, indole), 129.1 (C_{quart}, indole), 139.2 (C_{quart}, indole), 140.5 (C_{quart}, indole), 142.8 (C_{quart}, indole), 174.1 (C_{quart}, $\underline{C}O(CH_2)_4CH_3$)

MS (CI-MS, NH$_3$): m/z (%) = 292 ($[M + NH_4]^+$, 100), 275 ($[M + H]^+$, 4), 245 ($[M + H - NO_2]^+$, 19)

Analysis: $C_{15}H_{18}N_2O_3$ (274.32)

calculated	C: 65.68	H: 6.61	N: 10.21
found	C: 65.54	H: 6.43	N: 10.03

1-Decanoyl-2-methyl-5-nitro-1*H*-indole (7.14)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (103 mg, 2.58 mmol) and decanoyl chloride (**7.11**); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.22 g (0.67 mmol, 39 %, pale yellow solid)

Mp: 75-77.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.85 (t, 3H, ^{3}J = 6.7 Hz, $CO(CH_2)_8C\underline{H}_3$), 1.21-1.30 (m, 12H, $CO(CH_2)_2(C\underline{H}_2)_6CH_3$), 1.65-1.76 (m, 2H, $COCH_2C\underline{H}_2$-), 2.67 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 3.10 (t, 2H, ^{3}J = 7.2 Hz, $COC\underline{H}_2CH_2$-), 6.73 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.7 Hz, H-3), 8.10 (dd, 1H, ^{3}J = 9.2 Hz, ^{4}J = 2.5 Hz, H-6), 8.27 (ddd, 1H, ^{3}J = 9.2 Hz, ^{5}J = 0.7 Hz, ^{5}J = 0.5 Hz, H-7), 8.45 (dd, 1H, ^{4}J = 2.5 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, $CO(CH_2)_8\underline{C}H_3$), 17.0 (+, CH_3), 22.0 (-, CH_2), 24.2 (-, CH_2), 28.2 (-, CH_2), 28.5 (-, CH_2), 28.8 (-, $2CH_2$), 31.2 (-, CH_2), 37.8 (-, CH_2), 109.1 (+, indole), 115.4 (+, indole), 115.7 (+, indole), 118.2 (+, indole), 129.1 (C_{quart}, indole), 139.2 (C_{quart}, indole), 140.5 (C_{quart}, indole), 142.8 (C_{quart}, indole), 174.1 (C_{quart}, $\underline{C}O(CH_2)_8CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 330 ([$M^{\bullet+}$], 7), 176 ([M - C_8H_{17} - CHCO]$^+$, 100), 130 ([M - NO_2 - C_8H_{17} - CHCO]$^+$, 9)

Analysis: $C_{19}H_{26}N_2O_3$ (330.42)

calculated	C: 69.06	H: 7.93	N: 8.48
found	C: 68.97	H: 8.14	N: 8.16

2-Methyl-5-nitro-1-(2-phenylethanoyl)-1*H*-indole (7.15)

Method B: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (102 mg, 2.55 mmol) and 1-(2-phenylethanoyl)pyrrolidine-2,5-dione (**7.5**) (0.40 g, 1.71 mmol); purification by flash column chromatography on silica gel, elution with a 4:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.11 g (0.37 mmol, 22 %, yellow solid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.74 (d, 3H, ^4J = 1.0 Hz, CH_3), 4.52 (s, 2H, $COC\underline{H}_2Ph$), 6.77 (dq, 1H, ^4J = 1.0 Hz, ^5J = 0.8 Hz, H-3), 7.23-7.39 (m, 5H, Ar-H), 8.10 (dd, 1H, ^3J = 9.2 Hz, ^4J = 2.5 Hz, H-6), 8.31 (ddd, 1H, ^3J = 9.2 Hz, ^5J = 0.8 Hz, ^5J = 0.5 Hz, H-7), 8.46 (dd, 1H, ^4J = 2.5 Hz, ^5J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 17.1 (+, CH_3), 44.1 (-, $CO\underline{C}H_2Ph$), 109.4 (+, indole), 115.4 (+, indole), 115.9 (+, indole), 118.3 (+, indole), 126.7 (+, Ar-C), 128.1 (+, 2Ar-C), 129.1 (C_{quart}, indole), 129.9 (+, 2Ar-C), 134.1 (C_{quart}, Ar-C), 139.5 (C_{quart}, indole), 140.6 (C_{quart}, indole), 142.9 (C_{quart}, indole), 172.5 (C_{quart}, $\underline{C}OCH_2Ph$)

MS (PI-EIMS, 70 eV): m/z (%) = 294 ([$M^{\bullet+}$], 13), 176 ([M - C_6H_5 - CHCO]$^+$, 54), 118 ([C_6H_5 - CHCO]$^+$, 41), 91 ([C_7H_7]$^+$, 100)

$C_{17}H_{14}N_2O_3$ (294.30)

2-Methyl-5-nitro-1-(3-phenylpropanoyl)-1*H*-indole (7.16)

Method B: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.25 g, 1.42 mmol), sodium hydride (86 mg, 2.15 mmol) and 1-(3-phenylpropanoyl)pyrrolidine-2,5-dione (**7.6**) (0.35 g, 1.42 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.25 g (0.81 mmol, 57 %, yellow solid)

Mp: 130.5-131.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.65 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 3.05 (t, 2H, J = 7.4 Hz, $COCH_2CH_2Ph$), 3.46 (t, 2H, J = 7.4 Hz, $COCH_2CH_2Ph$), 6.73 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.8 Hz, H-3), 7.15-7.34 (m, 5H, Ph-H), 8.10 (dd, 1H, ^{3}J = 9.2 Hz, ^{4}J = 2.4 Hz, H-6), 8.26 (ddd, 1H, ^{3}J = 9.2 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.4 Hz, H-7), 8.44 (dd, 1H, ^{4}J = 2.4 Hz, ^{5}J = 0.4 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 17.0 (+, CH_3), 30.0 (-, CH_2), 39.2 (-, CH_2), 109.2 (+, indole), 115.4 (+, indole), 115.7 (+, indole), 118.3 (+, indole), 126.0 (+, Ar-C), 128.2 (+, 2Ar-C), 128.4 (+, 2Ar-C), 129.1 (C_{quart}, indole), 139.2 (C_{quart}, indole), 140.3 (C_{quart}, Ar-C), 140.5 (C_{quart}, indole), 142.9 (C_{quart}, indole), 173.4 (C_{quart}, $COCH_2CH_2Ph$)

MS (PI-EIMS, 70 eV): m/z (%) = 308 ([$M^{\bullet+}$], 17), 176 ([M - $C_6H_5CH_2$ - CHCO]$^{+}$, 100), 130 ([M - NO_2 - $C_6H_5CH_2$ - CHCO]$^{+}$, 16), 105 ([C_8H_9]$^{+}$, 55), 91 ([C_7H_7]$^{+}$, 79)

Analysis: $C_{18}H_{16}N_2O_3$ (308.33)

calculated	C: 70.12	H: 5.23	N: 9.09
found	C: 70.25	H: 5.34	N: 8.96

2-Methyl-5-nitro-1-(4-phenylbutanoyl)-1*H*-indole (7.17)

Method B: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (102 mg, 2.55 mmol) and 1-(4-phenylbutanoyl)pyrrolidine-2,5-dione (**7.7**) (0.45 g, 1.72 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.32 g (0.99 mmol, 58 %, yellow solid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.95-2.08 (m, 2H, $COCH_2C\underline{H}_2CH_2Ph$), 2.63 (d, 3H, ^4J = 1.1 Hz, CH_3), 2.71 (t, 2H, J = 7.8 Hz, $COC\underline{H}_2CH_2CH_2Ph$), 3.12 (t, 2H, J = 7.2 Hz, $COCH_2CH_2C\underline{H}_2Ph$), 6.72 (dq, 1H, ^4J = 1.1 Hz, ^5J = 0.8 Hz, H-3), 7.13-7.32 (m, 5H, Ph-H), 8.09 (dd, 1H, ^3J = 9.2 Hz, ^4J = 2.4 Hz, H-6), 8.24 (ddd, 1H, ^3J = 9.2 Hz, ^5J = 0.8 Hz, ^5J = 0.5 Hz, H-7), 8.44 (dd, 1H, ^4J = 2.4 Hz, ^5J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 17.0 (+, CH_3), 26.1 (-, CH_2), 34.2 (-, CH_2), 37.3 (-, CH_2), 109.2 (+, indole), 115.4 (+, indole), 115.7 (+, indole), 118.2 (+, indole), 125.8 (+, Ar-C), 128.2 (+, 2Ar-C), 128.3 (+, 2Ar-C), 129.1 (C_{quart}, indole), 139.2 (C_{quart}, indole), 140.5 (C_{quart}, indole), 141.5 (C_{quart}, Ar-C), 142.9 (C_{quart}, indole), 173.8 (C_{quart}, $\underline{C}OCH_2$-)

MS (PI-EIMS, 70 eV): m/z (%) = 322 ([$M^{\bullet+}$], 20), 176 ([M - $C_6H_5CH_2CH_2$ - CHCO]$^+$, 100), 91 ([C_7H_7]$^+$, 56)

$C_{19}H_{18}N_2O_3$ (322.36)

Benzyl 2-methyl-5-nitro-1*H*-indole-1-carboxylate (7.18)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.31 g, 1.76 mmol), sodium hydride (115 mg, 2.88 mmol) and benzyl chloroformate (0.25 ml, 1.78 mmol); purification by recrystallization from methanol.

Yield: 0.49 g (1.58 mmol, 90 %, orange-brown solid)

Mp: 121.5-122.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.56 (d, 3H, ^4J = 1.1 Hz, CH_3), 5.50 (s, 2H, $OC\underline{H}_2Ph$), 6.71 (dq, 1H, ^4J = 1.1 Hz, ^5J = 0.8 Hz, H-3), 7.39-7.48 (m, 3H, Ar-H), 7.54-7.58 (m, 2H, Ar-H), 8.09 (dd, 1H, ^3J = 9.2 Hz, ^4J = 2.4 Hz, H-6), 8.19 (ddd, 1H, ^3J = 9.2 Hz, ^5J = 0.8 Hz, ^5J = 0.5 Hz, H-7), 8.43 (dd, 1H, ^4J = 2.4 Hz, ^5J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 16.4 (+, CH_3), 69.0 (-, CH_2), 108.6 (+, indole), 115.3 (+, indole), 115.6 (+, indole), 118.3 (+, indole), 128.6 (+, 5Ar-C), 129.1 (C_{quart}, indole), 134.8 (C_{quart}, Ar-C), 139.1 (C_{quart}, indole), 141.0 (C_{quart}, indole), 143.1 (C_{quart}, indole), 150.6 (C_{quart}, $\underline{C}OOCH_2Ph$)

MS (PI-EIMS, 70 eV): m/z (%) = 310 ([$M^{\bullet+}$], 14), 266 ([M - CO_2] $^+$, 6), 176 ([M - C_6H_5CH - CO_2] $^+$, 6), 130 ([M - NO_2 - C_6H_5CH - CO_2] $^+$, 4), 91 ([C_7H_7] $^+$, 100)

Analysis: $C_{17}H_{14}N_2O_4$ (310.30)

calculated	C: 65.80	H: 4.55	N: 9.03
found	C: 65.88	H: 4.59	N: 8.96

2-Methyl-5-nitro-1-(2-phenoxyethanoyl)-1*H*-indole (7.19)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (102 mg, 2.55 mmol) and phenoxyacetyl chloride (0.24 ml, 1.74 mmol); purification by column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate and afterwards by column chromatography on silica gel eluting with chloroform.

Yield: 0.18 g (0.58 mmol, 34 %, yellow solid)

Mp: 99.5-100.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.73 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 5.53 (s, 2H, COC$\underline{H}_2$OPh), 6.78 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 6.95-7.07 (m, 3H, Ar-H), 7.26-7.33 (m, 2H, Ar-H), 8.12 (dd, 1H, ^{3}J = 9.2 Hz, ^{4}J = 2.5 Hz, H-6), 8.32 (ddd, 1H, ^{3}J = 9.2 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7), 8.47 (dd, 1H, ^{4}J = 2.5 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 16.4 (+, CH_3), 67.9 (-, CH_2), 109.7 (+, indole), 114.6 (+, 2Ar-C), 115.4 (+, indole), 116.0 (+, indole), 118.6 (+, indole), 121.1 (+, Ar-C), 129.4 (+, 2Ar-C), 129.5 (C_{quart}, indole), 139.5 (C_{quart}, Ar-C), 140.3 (C_{quart}, indole), 143.2 (C_{quart}, indole), 157.5 (C_{quart}, indole), 169.8 (C_{quart}, $\underline{C}OCH_2OPh$)

MS (PI-EIMS, 70 eV): m/z (%) = 310 ([$M^{\bullet+}$], 39), 189 ([M - $C_6H_5OCH_2$ - CH_2] $^+$, 100), 143 ([M - NO_2 - $C_6H_5OCH_2$ - CH_2] $^+$, 22), 107 ([C_6H_5 - NO] $^+$, 37), 77 ([C_6H_5] $^+$, 26)

Analysis: $C_{17}H_{14}N_2O_4$ (310.30)

calculated	C: 65.80	H: 4.55	N: 9.03
found	C: 65.81	H: 4.62	N: 8.95

Synthesis of *N*-alkylated 2-methyl-5-nitro-1*H*-indoles 7.27-7.33

General procedure. Sodium hydride (1.5 eq) was dissolved in DMF (7-10 ml) and cooled with an ice bath under an inert atmosphere. A solution of 2-methyl-5-nitro-1*H*-indole (**7.1**) (1 eq), dissolved in DMF (7-10 ml), was slowly added. After completion of the gas development (about 20 min) the pertinent iodoalkane (1 eq), dissolved in DMF (5 ml), was added with a dropping funnel. The reaction mixture was stirred for further 2 h at 0 °C. Then the solution was diluted with water (50 ml) and extracted three times with ethyl acetate (30 ml). The combined organic layers were dried over magnesium sulfate and concentrated *in vacuo*.

2-Methyl-5-nitro-1-propyl-1*H*-indole (7.27)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (110 mg, 2.75 mmol) and propyl iodide (0.17 ml, 1.74 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.32 g (1.47 mmol, 86 %, yellow solid)

Mp: 46.5-47.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.87 (t, 3H, ^{3}J = 7.4 Hz, $NC_2H_4C\underline{H}_3$), 1.63-1.76 (m, 2H, $NCH_2C\underline{H}_2CH_3$), 2.45 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 4.16 (t, 2H, ^{3}J = 7.3 Hz, $NC\underline{H}_2$-), 6.51 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.62 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.43 (d, 1H, ^{4}J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 10.9 (+, $(CH_2)_2\underline{C}H_3$), 12.4 (+, CH_3), 22.9 (-, CH_2), 44.3 (-, CH_2), 102.1 (+, indole), 109.8 (+, indole), 115.4 (+, indole), 115.9 (+, indole), 126.6 (C_{quart}, indole), 139.4 (C_{quart}, indole), 140.4 (C_{quart}, indole), 140.9 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 218 ([$M^{\bullet+}$], 55), 189 ([M - C_2H_5] $^+$, 100), 143 ([M - C_2H_5 - NO_2] $^+$, 39)

IR [cm^{-1}]: 2969 (C-H) aliphatic, 2873 (C-H) aliphatic, 1317 (C-NO_2)

Analysis: $C_{12}H_{14}N_2O_2$ (218.26)

calculated	C: 66.04	H: 6.47	N: 12.84
found	C: 66.05	H: 6.51	N: 12.60

1-Hexyl-2-methyl-5-nitro-1*H*-indole (7.28)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.3 g, 1.70 mmol), sodium hydride (110 mg, 2.75 mmol) and hexyl iodide (0.26 ml, 1.75 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.41 g (1.57 mmol, 92 %, yellow solid)

Mp: 63-64.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.83 (t, 3H, ^{3}J = 7.0 Hz, N(CH$_2$)$_5$C<u>H</u>$_3$), 1.20-1.32 (m, 6H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_3$CH$_3$), 1.59-1.71 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.45 (d, 3H, ^{4}J = 1.0 Hz, CH$_3$), 4.18 (t, 2H, ^{3}J = 7.4 Hz, NC<u>H</u>$_2$-), 6.51 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.60 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.43 (d, 1H, ^{4}J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.4 (+, CH$_3$), 13.7 (+, (CH$_2$)$_5$<u>C</u>H$_3$), 21.9 (-, CH$_2$), 25.8 (-, CH$_2$), 29.5 (-, CH$_2$), 30.8 (-, CH$_2$), 42.9 (-, CH$_2$), 102.1 (+, indole), 109.7 (+, indole), 115.4 (+, indole), 116.0 (+, indole), 126.6 (C$_{quart}$, indole), 139.3 (C$_{quart}$, indole), 140.4 (C$_{quart}$, indole), 140.8 (C$_{quart}$, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 260 ([M$^{\bullet+}$], 45), 245 ([M - CH$_3$]$^+$, 13), 189 ([M - C$_5$H$_{11}$]$^+$, 100), 143 ([M - C$_5$H$_{11}$ - NO$_2$]$^+$, 32)

Analysis: C$_{15}$H$_{20}$N$_2$O$_2$ (260.34)

calculated	C: 69.20	H: 7.74	N: 10.76
found	C: 69.29	H: 7.91	N: 10.45

2-Methyl-5-nitro-1-octyl-1*H*-indole (7.29)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.2 g, 1.14 mmol), sodium hydride (70 mg, 1.75 mmol) and octyl iodide (0.21 ml, 1.15 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.29 g (1.01 mmol, 89 %, yellow solid)

Mp: 62-63 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.83 (t, 3H, ^{3}J = 6.9 Hz, N(CH$_2$)$_7$C<u>H</u>$_3$), 1.15-1.30 (m, 10H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_5$CH$_3$), 1.59-1.71 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.45 (d, 3H, ^{4}J = 1.0 Hz, CH$_3$), 4.18 (t, 2H, ^{3}J = 7.4 Hz, NC<u>H</u>$_2$-), 6.51 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.59 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.43 (d, 1H, ^{4}J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.4 (+, CH$_3$), 13.8 (+, (CH$_2$)$_7$<u>C</u>H$_3$), 21.9 (-, CH$_2$), 26.1 (-, CH$_2$), 28.5 (-, 2CH$_2$), 29.5 (-, CH$_2$), 31.1 (-, CH$_2$), 42.9 (-, CH$_2$), 102.1 (+, indole), 109.7 (+, indole), 115.4 (+, indole), 116.0 (+, indole), 126.6 (C$_{quart}$, indole), 139.3 (C$_{quart}$, indole), 140.4 (C$_{quart}$, indole), 140.8 (C$_{quart}$, indole)

MS (CI-MS, NH$_3$): m/z (%) = 306 ([M + NH$_4$]$^+$, 75), 289 ([M + H]$^+$, 100), 259 ([M + H - NO]$^+$, 12)

Analysis: C$_{17}$H$_{24}$N$_2$O$_2$ (288.38)

calculated	C: 70.80	H: 8.39	N: 9.71
found	C: 70.93	H: 8.72	N: 9.39

1-Decyl-2-methyl-5-nitro-1*H*-indole (7.30)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.50 g, 2.84 mmol), sodium hydride (0.17 g, 4.26 mmol) and decyl iodide (**7.44**) (0.66 ml, 3.12 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.88 g (2.78 mmol, 98 %, yellow solid)

Mp: 54-55 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.83 (t, 3H, ^{3}J = 6.8 Hz, N(CH$_2$)$_9$C<u>H</u>$_3$), 1.15-1.30 (m, 14H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_7$CH$_3$), 1.59-1.72 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.45 (d, 3H, ^{4}J = 1.0 Hz, CH$_3$), 4.18 (t, 2H, ^{3}J = 7.4 Hz, NC<u>H</u>$_2$-), 6.51 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.59 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8, ^{5}J = 0.5 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.42 (d, 1H, ^{4}J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.4 (+, CH_3), 13.8 (+, $(CH_2)_9\underline{C}H_3$), 22.0 (-, CH_2), 26.1 (-, CH_2), 28.5 (-, $2CH_2$), 28.8 (-, $2CH_2$), 29.5(-, CH_2), 31.1 (-, CH_2), 42.9 (-, CH_2), 102.1 (+, indole), 109.7 (+, indole), 115.4 (+, indole), 116.0 (+, indole), 126.7 (C_{quart}, indole), 139.3 (C_{quart}, indole), 140.4 (C_{quart}, indole), 140.8 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 316 ([$M^{\bullet+}$], 89), 301 ([M - CH_3] $^+$, 49), 287 ([M - C_2H_5] $^+$, 5), 273 ([M - C_3H_7] $^+$, 5), 259 ([M - C_4H_9] $^+$, 5), 245 ([M - C_5H_{11}] $^+$, 5), 231 ([M - C_6H_{13}] $^+$, 10), 217 ([M - C_7H_{15}] $^+$, 15), 203 ([M - C_8H_{17}] $^+$, 15), 189 ([M - C_9H_{19}] $^+$, 100), 143 ([M - C_9H_{19} - NO_2] $^+$, 40)

Analysis: $C_{19}H_{28}N_2O_2$ (316.44)

calculated	C: 72.12	H: 8.92	N: 8.85
found	C: 72.03	H: 8.72	N: 8.74

2-Methyl-5-nitro-1-(3-phenylpropyl)-1*H*-indole (7.31)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.19 g, 1.08 mmol), sodium hydride (70 mg, 1.75 mmol) and 1-iodo-3-phenylpropane (**7.45**) (0.34 g, 1.38 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.11 g (0.37 mmol, 35 %, yellow solid)

Mp: 101-101.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.92-2.05 (m, 2H, $NCH_2C\underline{H}_2CH_2Ph$), 2.42 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 2.64 (t, 2H, ^{3}J = 7.9 Hz, $NCH_2CH_2C\underline{H}_2Ph$), 4.22 (t, 2H, ^{3}J = 7.6 Hz, $NC\underline{H}_2$-), 6.52 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.15-7.32 (m, 5H, Ar-H), 7.56 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8, ^{5}J = 0.5 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.43 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 31.1 (-, CH_2), 32.1 (-, CH_2), 42.6 (-, CH_2), 102.1 (+, indole), 109.6 (+, indole), 115.5 (+, indole), 116.0 (+, indole), 125.9 (+, Ar-C), 126.7 (C_{quart}, indole), 128.1 (+, 2Ar-C), 128.3 (+, 2Ar-C), 139.3 (C_{quart}, indole), 140.4 (C_{quart}, Ar-C), 140.8 (C_{quart}, indole), 140.9 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 294 ([$M^{\bullet+}$], 100), 189 ([M - $C_6H_5C_2H_4$] $^+$, 77), 143 ([M - $C_6H_5C_2H_4$ - NO_2] $^+$, 33), 91 ([C_7H_7] $^+$, 48)

Analysis: $C_{18}H_{18}N_2O_2$ (294.35)

calculated	C: 73.45	H: 6.16	N: 9.52
found	C: 73.36	H: 6.28	N: 9.49

2-Methyl-5-nitro-1-(4-phenylbenzyl)-1*H*-indole (7.32)

Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.20 g, 1.14 mmol), sodium hydride (70 mg, 1.75 mmol) and 4-phenylbenzyl iodide (**7.46**) (0.40 g, 1.36 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.39 g (1.14 mmol, 99 %, yellow solid)

Mp: 122-124.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.43 (d, 3H, ^4J = 1.0 Hz, CH_3), 5.58 (s, 2H, CH_2), 6.64 (dq, 1H, ^4J = 1.0 Hz, ^5J = 0.8 Hz, H-3), 7.06-7.09 (m, 2H, Ar-H), 7.29-7.46 (m, 3H, Ar-H), 7.58-7.63 (m, 4H, Ar-H), 7.64 (ddd, 1H, ^3J = 9.0 Hz, ^5J = 0.8, ^5J = 0.5 Hz, H-7), 7.97 (dd, 1H, ^3J = 9.0 Hz, ^4J = 2.3 Hz, H-6), 8.50 (d, 1H, ^4J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.5 (+, CH_3), 45.8 (-, CH_2), 102.6 (+, indole), 110.0 (+, indole), 115.9 (+, indole), 116.1 (+, indole), 126.5 (+, 2Ar-C), 126.6 (+, 2Ar-C), 126.9 (C_{quart}, indole), 127.0 (+, 2Ar-C), 127.4 (+, Ar-C), 128.8 (+, 2Ar-C), 136.6 (C_{quart}, Ar-C) 139.2 (C_{quart}, Ar-C), 139.5 (C_{quart}, Ar-C), 139.8 (C_{quart}, indole), 140.7 (C_{quart}, indole), 141.1 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 342 ([$M^{\bullet+}$], 29), 312 ([M - NO]$^+$, 6), 167 ([C_6H_5 - C_6H_4 - CH_2]$^+$, 100)

Analysis: $C_{22}H_{18}N_2O_2$ (342.39)

calculated	C: 77.17	H: 5.30	N: 8.18
found	C: 77.58	H: 5.67	N: 7.63

Ethyl 5-(2-methyl-5-nitro-1*H*-indole-1-yl)pentanoate (7.33)

2-Methyl-5-nitro-1*H*-indole (**7.1**) (1.50 g, 8.51 mmol), ethyl 5-bromopentanoate (1.88 ml, 11.88 mmol) and potassium carbonate (2.57 g, 18.59 mmol) were

mixed with acetonitrile (25 ml) under a nitrogen atmosphere and heated under reflux for 48 h. Water (25 ml) was poured into the mixture and it was extracted with ether (3x30 ml). The combined organic phases were washed with water (3x15 ml), dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel eluting with a 95:5 (v/v) mixture of chloroform and methanol.

Yield: 2.38 g (7.82 mmol, 92 %, yellow solid)

Mp: 56-57.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.14 (t, 3H, ^{3}J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.46-1.60 (m, 2H, $N(CH_2)_2C\underline{H}_2$-), 1.61-1.74 (m, 2H, $NCH_2C\underline{H}_2$-), 2.32 (t, 2H, ^{3}J = 7.2 Hz, $N(CH_2)_3C\underline{H}_2$-), 2.45 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 4.02 (q, 2H, ^{3}J = 7.1 Hz, $CO_2C\underline{H}_2$-), 4.21 (t, 2H, ^{3}J = 7.2 Hz, $NC\underline{H}_2$-), 6.51 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 7.61 (ddd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.4 Hz, H-7), 7.95 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.43 (d, 1H, ^{4}J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 14.0 (+, CH_3), 21.6 (-, CH_2), 28.9 (-, CH_2), 32.9 (-, CH_2), 42.5 (-, CH_2), 59.6 (-, CH_2), 102.2 (+, indole), 109.7 (+, indole), 115.4 (+, indole), 116.0 (+, indole), 126.7 (C_{quart}, indole), 139.4 (C_{quart}, indole), 140.4 (C_{quart}, indole), 140.8 (C_{quart}, indole), 172.5 (C_{quart}, $\underline{C}O_2Et$)

MS (PI-EIMS, 70 eV): m/z (%) = 304 ([$M^{\bullet+}$], 78), 289 ([M - CH_3] $^{+}$, 11), 259 ([M - OC_2H_5] $^{+}$, 23), 217 ([M - CH_2CO_2Et] $^{+}$, 22), 189 ([M - $(CH_2)_3CO_2Et$] $^{+}$, 100), 143 ([M - $(CH_2)_3CO_2Et$ - NO_2] $^{+}$, 38)

Analysis: $C_{16}H_{20}N_2O_4$ (304.34)

calculated	C: 63.14	H: 6.62	N: 9.20
found	C: 63.30	H: 6.97	N: 8.91

Reduction of the *N*-alkylated or *N*-acylated 2-methyl-5-nitro-1*H*-indole derivatives (7.20-7.26 and 7.34-7.40)

Method A. Preparation of the catalyst Ni_2B:

The catalyst was prepared just prior to use by dropwise addition of sodium borohydride (10 eq, 1.0 M solution in 0.1 M aqueous sodium hydroxide) to a stirred 0.1 M solution of nickel(II) acetate tetrahydrate (5 eq). After gas evolution

had completely ceased the aqueous solution was decanted, the black granular nickel boride was resuspended in distilled water, then again decanted. After several quick washings with water the catalyst was washed twice with ethanol and used like this for the reduction.

Reduction:
The freshly prepared catalyst, which was obtained as aforementioned from 5 eq of nickel(II) acetate tetrahydrate, was suspended in isopropyl alcohol (10 ml) and the pertinent 5-nitro-1*H*-indole derivative (1 eq), dissolved or suspended in isopropyl alcohol (7-10 ml), was added to form a 0.1 M solution. The reaction mixture was heated to reflux and a solution of hydrazine hydrate (10 eq) in isopropyl alcohol (5 ml) was added dropwise. After addition, reflux was continued for further 30 min. The mixture was allowed to cool down to room temperature before it was filtered through a pad of Celite. Afterwards, the filtrate was concentrated *in vacuo*, the residue was resumed in ethyl acetate (30 ml) and extracted three times with acetic acid (10%, 20 ml). The combined aqueous extracts were basified with concentrated ammonia solution (25%) and extracted three times with ethyl acetate (20 ml). The combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure.

Method B. A mixture of the 5-nitro-1*H*-indole derivative (1 eq), dissolved in ethyl acetate (5 ml), and tin(II) chloride dihydrate (5 eq) was heated at 70 °C under a nitrogen atmosphere and stirred over night. After cooling, the mixture was diluted with ice-cold water (10 ml) and the pH is made slightly basic (pH = 8) by an aqueous solution of sodium bicarbonate (5%). After stirring for 1 h the aqueous phase was extracted three times with ethyl acetate (20 ml). The combined organic layers were washed with brine (15 ml), dried over magnesium sulfate and concentrated under reduced pressure.

1-(5-Amino-2-methyl-1*H*-indol-1-yl)propan-1-one (7.20)

Method B: Reaction of 2-methyl-5-nitro-1-propanoyl-1*H*-indole (**7.12**) (0.33 g, 1.42 mmol) and tin(II) chloride dihydrate (1.60 g, 7.09 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.25 g (1.24 mmol, 87 %, red-brown solid)

Mp: 74.5-76 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.15 (t, 3H, ^{3}J = 7.2 Hz, $CH_2C\underline{H}_3$), 2.55 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 2.97 (q, 2H, ^{3}J = 7.2 Hz, $COC\underline{H}_2CH_3$), 4.84 (br, 2H, NH_2), 6.24 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.8 Hz, H-3), 6.50 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, H-6), 6.58 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4), 7.77 (ddd, 1H, ^{3}J = 8.8 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.9 (+, $COCH_2\underline{C}H_3$), 17.4 (+, CH_3), 30.7 (-, CH_2), 103.1 (+, indole), 109.0 (+, indole), 111.4 (+, indole), 116.0 (+, indole), 128.6 (C_{quart}, indole), 130.3 (C_{quart}, indole), 136.6 (C_{quart}, indole), 144.4 (C_{quart}, indole), 173.4 (C_{quart}, $\underline{C}OCH_2CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 202 ([$M^{\bullet+}$], 40), 146 ([M - CH_3 - CHCO]$^+$, 100)

IR [cm^{-1}]: 3453 (N-H), 3355 (N-H), 2979 (C-H) aliphatic, 2921 (C-H) aliphatic, 1691 (C=O)

Analysis: $C_{12}H_{14}N_2O \cdot 0.1\ H_2O$ (204.05)

calculated	C: 70.64	H: 7.01	N: 13.73
found	C: 70.50	H: 6.88	N: 13.49

1-(5-Amino-2-methyl-1*H*-indol-1-yl)hexan-1-one (7.21)

Method B: Reaction of 1-hexanoyl-2-methyl-5-nitro-1*H*-indole (**7.13**) (0.11 g, 0.40 mmol) and tin(II) chloride dihydrate (0.45 g, 2.00 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.09 g (0.37 mmol, 92 %, brown solid)

Mp: 85-87 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.89 (t, 3H, ^{3}J = 6.9 Hz, $CO(CH_2)_4C\underline{H}_3$), 1.29-1.38 (m, 4H, $CO(CH_2)_2(C\underline{H}_2)_2CH_3$), 1.62-1.73 (m, 2H, $COCH_2C\underline{H}_2$-), 2.54 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 2.94 (t, 2H, ^{3}J = 7.2 Hz, $NCOC\underline{H}_2$-), 4.84 (br, 2H, NH_2), 6.24 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.8 Hz, H-3), 6.50 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, H-

6), 6.58 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4), 7.74 (ddd, 1H, ^{3}J = 8.8 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.8 (+ ,$CO(CH_2)_4C\underline{H}_3$), 17.4 (+, CH_3), 22.0 (-, CH_2), 24.0 (-, CH_2), 30.6 (-, CH_2), 37.3 (-, CH_2), 103.1 (+, indole), 108.9 (+, indole), 111.5 (+, indole), 115.9 (+, indole), 128.6 (C_{quart}, indole), 130.2 (C_{quart}, indole), 136.6 (C_{quart}, indole), 144.4 (C_{quart}, indole), 172.7 (C_{quart}, $\underline{C}O(CH_2)_4CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 244 ([$M^{\bullet+}$], 20), 146 ([M - $C_4H_9CHCO]^+$, 100)

$C_{15}H_{20}N_2O$ (244.33)

1-(5-Amino-2-methyl-1*H*-indol-1-yl)decan-1-one (7.22)

Method B: Reaction of 1-decanoyl-2-methyl-5-nitro-1*H*-indole (**7.14**) (0.13 g, 0.39 mmol) and tin(II) chloride dihydrate (0.45 g, 1.99 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.10 g (0.33 mmol, 85 %, brown solid)

Mp: 86-88 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86 (t, 3H, ^{3}J = 6.7 Hz, $CO(CH_2)_8C\underline{H}_3$), 1.20-1.36 (m, 12H, $CO(CH_2)_2(C\underline{H}_2)_6CH_3$), 1.60-1.71 (m, 2H, $COCH_2C\underline{H}_2$-), 2.54 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 2.94 (t, 2H, ^{3}J = 7.1 Hz, $NCOC\underline{H}_2$-), 4.86 (br, 2H, NH_2), 6.24 (dq, 1H, ^{4}J = 1.0 Hz, ^{5}J = 0.8 Hz, H-3), 6.49 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, H-6), 6.58 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4), 7.73 (ddd, 1H, ^{3}J = 8.8 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 13.9 (+, $CO(CH_2)_8C\underline{H}_3$), 17.4 (+, CH_3), 22.0 (-, CH_2), 24.3 (-, CH_2), 28.4 (-, CH_2), 28.6 (-, CH_2), 28.8 (-, CH_2), 28.9 (-, CH_2), 31.2 (-, CH_2), 37.3 (-, CH_2), 103.1 (+, indole), 108.9 (+, indole), 111.5 (+, indole), 115.9 (+, indole), 128.6 (C_{quart}, indole), 130.2 (C_{quart}, indole), 136.6 (C_{quart}, indole), 144.3 (C_{quart}, indole), 172.7 (C_{quart}, $\underline{C}O(CH_2)_8CH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 300 ([$M^{\bullet+}$], 17), 146 ([M - $C_8H_{17}CHCO]^+$, 100)

$C_{19}H_{28}N_2O$ (300.44)

1-(5-Amino-2-methyl-1*H*-indol-1-yl)-3-phenylpropan-1-one (7.23)

Method B: Reaction of 2-methyl-5-nitro-1-(3-phenylpropanoyl)-1*H*-indole (**7.16**) (0.16 g, 0.52 mmol) and tin(II) chloride dihydrate (0.59 g, 2.61 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.14 g (0.50 mmol, 97 %, brown solid)

Mp: 68-70 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.52 (d, 3H, ^{4}J = 1.1 Hz, CH_3), 3.01 (t, 2H, ^{3}J = 7.4 Hz, $COC\underline{H}_2CH_2Ph$), 3.30 (t, 2H, ^{3}J = 7.4 Hz, $COCH_2C\underline{H}_2Ph$), 4.82 (br, 2H, NH_2), 6.25 (dq, 1H, ^{4}J = 1.1 Hz, ^{5}J = 0.8 Hz, H-3), 6.49 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, H-6), 6.58 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4), 7.15-7.34 (m, 5H, Ar-H), 7.74 (ddd, 1H, ^{3}J = 8.8 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 17.3 (+, CH_3), 30.1 (-, CH_2), 38.9 (-, CH_2), 103.2 (+, indole), 109.0 (+, indole), 111.5 (+, indole), 115.9 (+, indole), 125.9 (+, Ar-C), 128.2 (+, 2Ar-C), 128.4 (+, 2Ar-C), 128.6 (C_{quart}, indole), 130.3 (C_{quart}, indole), 136.5 (C_{quart}, indole), 140.9 (C_{quart}, Ar-C), 144.5 (C_{quart}, indole), 171.9 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 278 ([$M^{\bullet+}$], 43), 146 ([M - $C_6H_5CH_2$ - CHCO]$^+$, 100), 91 ([C_7H_7]$^+$, 14)

$C_{18}H_{18}N_2O$ (278.35)

1-(5-Amino-2-methyl-1*H*-indol-1-yl)-4-phenylbutan-1-one (7.24)

Method B: Reaction of 2-methyl-5-nitro-1-(4-phenylbutanoyl)-1*H*-indole (**7.17**) (0.24 g, 0.74 mmol) and tin(II) chloride dihydrate (0.84 g, 3.72 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.15 g (0.51 mmol, 69 %,brown oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.93-2.04 (m, 2H, $COCH_2C\underline{H}_2CH_2Ph$), 2.51 (d, 3H, ^{4}J = 1.0 Hz, CH_3), 2.70 (t, 2H, ^{3}J = 7.8 Hz, $COC\underline{H}_2CH_2CH_2Ph$), 2.97 (t, 2H, ^{3}J = 7.2 Hz, $COCH_2CH_2C\underline{H}_2Ph$), 4.83 (br, 2H, NH_2), 6.24 (dq, 1H, ^{4}J = 1.0 Hz,

^{5}J = 0.8 Hz, H-3), 6.49 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 2.3 Hz, H-6), 6.58 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4), 7.12-7.33 (m, 5H, Ar-H), 7.70 (ddd, 1H, ^{3}J = 8.8 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 17.3 (+, CH_3), 26.2 (-, CH_2), 34.3 (-, CH_2), 36.8 (-, CH_2), 103.2 (+, indole), 109.0 (+, indole), 111.5 (+, indole), 115.9 (+, indole), 125.7 (+, Ar-C), 128.2 (+, 4Ar-C), 128.5 (C_{quart}, indole), 130.3 (C_{quart}, indole), 136.6 (C_{quart}, indole), 141.7 (C_{quart}, Ar-C), 144.4 (C_{quart}, indole), 172.4 (C_{quart}, CO)

MS (PI-EIMS, 70 eV): m/z (%) = 292 ([$M^{\bullet+}$], 31), 146 ([M - $C_6H_5(CH_2)_2$-CHCO]$^{+}$, 100), 91 ([C_7H_7]$^{+}$, 19)

$C_{19}H_{20}N_2O$ (292.38)

5-Amino-2-methyl-1*H*-indole (7.25)

Method A: Reaction of 2-methyl-5-nitro-1*H*-indole (**7.1**) (0.29 g, 1.65 mmol), sodium borohydride (0.64 g, 16.92 mmol), nickel(II) acetate tetrahydrate (2.12 g, 8.52 mmol) and hydrazine hydrate (0.83 ml, 17.08 mmol); purification by flash column chromatography on silica gel, elution with a 100:1 (v/v) mixture of ethyl acetate and triethylamine.

Yield: 0.17 g (1.16 mmol, 71 %, hazel solid)

Mp: 147.5-148.5 °C (Lit. 158-159 °C[230])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.28 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 4.39 (br, 2H, NH_2), 5.80-5.82 (m, 1H, H-3), 6.36 (dd, 1H, ^{3}J = 8.4 Hz, ^{4}J = 2.1 Hz, H-6), 6.55 (ddd, 1H, ^{4}J = 2.1 Hz, ^{5}J = 0.7 Hz, ^{5}J = 0.6 Hz, H-4), 6.93 (ddd, 1H, ^{3}J = 8.4 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.6 Hz, H-7), 10.36 (br, 1H, indole NH)

MS (PI-EIMS, 70 eV): m/z (%) = 146 ([$M^{\bullet+}$], 100)

$C_9H_{10}N_2$ (146.19)

1-(5-Amino-2-methylindolin-1-yl)propan-1-one (7.26)

2-Methyl-5-nitro-1-propanoyl-1*H*-indole (**7.12**) (146 mg, 0.63 mmol) was dissolved in methanol (10 ml) and THF (2 ml) under an inert atmosphere. After

treatment with palladium (10% Pd) on charcoal, a 0.5 M methanolic solution of ammonium formate (6.5 ml) was added and the reaction was allowed to stir for 1 h at room temperature. Afterwards, the reaction mixture was filtrated through a pad of celite and washed with ethyl acetate. The filtrate was concentrated under reduced pressure and the remaining residue was treated with water (10 ml). After extraction with ethyl acetate (3x15 ml) the combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel eluting with ethyl acetate. The solid was obtained as hydrochloride.

Yield: 0.10 g (0.49 mmol, 78 %, beige solid)

Mp: > 200 °C decomposition

^{1}H-NMR (MeOD): δ [ppm] = 1.21 (t, 3H, ^{3}J = 7.4 Hz, $COCH_2C\underline{H}_3$), 1.28 (d, 2H, ^{3}J = 6.4 Hz, $CHC\underline{H}_3$), 2.48-2.82 (m, 3H, $COC\underline{H}_2CH_3$, $C\underline{H}(CHCH_3)$), 3.46 (dd, 1H, ^{2}J = 17.0 Hz, ^{3}J = 8.2 Hz, $C\underline{H}(CHCH_3)$), 4.66-4.80 (m, 1H, $C\underline{H}CH_3$), 7.20 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 1.6 Hz, H-6), 7.28 (s, 1H, H-4), 8.17 (d, 1H, ^{3}J = 8.5 Hz, H-7)

MS (PI-EIMS, 70 eV): m/z (%) = 204 ([$M^{\bullet+}$], 87), 148 ([M - CH_3CHCO] $^+$, 80), 133 ([M - CH_3CHCO - CH_3] $^+$, 100)

$C_{12}H_{16}N_2O$ (204.27)

5-Amino-2-methyl-1-propyl-1*H*-indole (7.34)

Method A: Reaction of 2-methyl-5-nitro-1-propyl-1*H*-indole (**7.27**) (0.20 g, 0.92 mmol), sodium borohydride (0.35 g, 9.25 mmol), nickel(II) acetate tetrahydrate (1.14 g, 4.58 mmol) and hydrazine hydrate (0.45 ml, 9.26 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.17 g (0.90 mmol, 99 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 7.4 Hz, $N(CH_2)_2C\underline{H}_3$), 1.56-1.69 (m, 2H, $NCH_2C\underline{H}_2CH_3$), 2.31 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 3.93 (t, 2H, ^{3}J = 7.3 Hz, $NC\underline{H}_2$-), 4.45 (br, 2H, NH_2), 5.89 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.8 Hz, H-3), 6.42

(dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.2 Hz, H-6), 6.58 (dd, 1H, ^{4}J = 2.1 Hz, ^{5}J = 0.6 Hz, H-4), 7.04 (ddd, 1H, ^{3}J = 8.5 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.6 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 11.1 (+, $(CH_2)_2\underline{C}H_3$), 12.3 (+, CH_3), 23.0 (-, CH_2), 43.7 (-, CH_2), 98.0 (+, indole), 102.9 (+, indole), 109.3 (+, indole), 110.2 (+, indole), 128.3 (C_{quart}, indole), 130.3 (C_{quart}, indole), 135.7 (C_{quart}, indole), 140.8 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 188 ([$M^{\bullet+}$], 58), 159 ([M - C_2H_5]$^{+}$, 100), 145 ([M - C_3H_7]$^{+}$, 8)

IR [cm^{-1}]: 3413 (N-H), 3340 (N-H), 3017 (C-H) aromatic, 2962 (C-H) aliphatic, 2931 (C-H) aliphatic, 2873 (C-H) aliphatic

Analysis: $C_{12}H_{16}N_2 \cdot 0.1\ H_2O$ (190.07)

calculated	C: 75.83	H: 8.58	N: 14.74
found	C: 75.62	H: 8.19	N: 14.43

5-Amino-1-hexyl -2-methyl-1*H*-indole (7.35)

Method A: Reaction of 1-hexyl-2-methyl-5-nitro-1*H*-indole (**7.28**) (0.20 g, 0.77 mmol), sodium borohydride (0.29 g, 7.67 mmol), nickel(II) acetate tetrahydrate (0.96 g, 3.86 mmol) and hydrazine hydrate (0.37 ml, 7.61 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.09 g (0.39 mmol, 51 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J =6.8 Hz, $N(CH_2)_5C\underline{H}_3$), 1.20-1.30 (m, 6H, $N(CH_2)_2(C\underline{H}_2)_3CH_3$), 1.51-1.65 (m, 2H, $NCH_2C\underline{H}_2$-), 2.30 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 3.95 (t, 2H, ^{3}J = 7.3 Hz, $NC\underline{H}_2$-), 4.43 (br, 2H, NH_2), 5.88 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.8 Hz, H-3), 6.42 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.1 Hz, H-6), 6.56 (dd, 1H, ^{4}J = 2.1 Hz, ^{5}J = 0.6 Hz, H-4), 7.02 (ddd, 1H, ^{3}J = 8.5 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.6 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 13.8 (+, $(CH_2)_5\underline{C}H_3$), 22.0 (-, CH_2), 25.9 (-, CH_2), 29.7 (-, CH_2), 30.9 (-, $2CH_2$), 42.2 (-, CH_2), 98.0 (+, indole), 102.9 (+, indole), 109.2 (+, indole), 110.2 (+, indole), 128.3 (C_{quart}, indole), 130.2 (C_{quart}, indole), 135.6 (C_{quart}, indole), 140.9 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 230 ([$M^{\bullet+}$], 83), 159 ([M - C_5H_{11}] $^+$, 100), 145 ([M - C_6H_{13}] $^+$, 8)

$C_{15}H_{22}N_2$ (230.35)

5-Amino-2-methyl-1-octyl-1*H*-indole (7.36)

Method B: Reaction of 2-methyl-5-nitro-1-octyl-1*H*-indole (**7.29**) (0.14 g, 0.49 mmol) and tin(II) chloride dihydrate (0.55 g, 2.44 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.07 g (0.27 mmol, 56 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 6.7 Hz, N(CH_2)$_7$C$\underline{H}_3$), 1.18-1.31 (m, 10H, N(CH_2)$_2$($C\underline{H}_2$)$_5$$CH_3$), 1.54-1.65 (m, 2H, N$CH_2C\underline{H}_2$-), 2.30 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 3.94 (t, 2H, ^{3}J = 7.3 Hz, N$C\underline{H}_2$-), 4.52 (br, 2H, NH_2), 5.88 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.6 Hz, H-3), 6.42 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.2 Hz, H-6), 6.57 (dd, 1H, ^{4}J = 2.2 Hz, ^{5}J = 0.6 Hz, H-4), 7.02 (ddd, 1H, ^{3}J = 8.5 Hz, ^{5}J = 0.6 Hz, ^{5}J = 0.8 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 13.8 (+, (CH_2)$_7$$\underline{C}H_3$), 22.0 (-, CH_2), 26.2 (-, CH_2), 28.6 (-, 2CH_2), 29.7(-, CH_2), 31.1 (-, CH_2), 42.2 (-, CH_2), 98.0 (+, indole), 102.9 (+, indole), 109.2 (+, indole), 110.2 (+, indole), 128.3 (C_{quart}, indole), 130.2 (C_{quart}, indole), 135.6 (C_{quart}, indole), 140.9 (C_{quart}, indole)

MS (CI-MS, NH_3): m/z (%) = 259 ([M + H] $^+$, 100)

$C_{17}H_{26}N_2$ (258.40)

5-Amino-1-decyl-2-methyl-1*H*-indole (7.37)

Method B: Reaction of 1-decyl-2-methyl-5-nitro-1*H*-indole (**7.30**) (0.88 g, 2.78 mmol) and tin(II) chloride dihydrate (3.14 g, 13.90 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.49 g (1.71 mmol, 62 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.85 (t, 3H, ^{3}J =6.8 Hz, N(CH_2)$_9$C$\underline{H}_3$), 1.18-1.31 (m, 14H, N(CH_2)$_2$($C\underline{H}_2$)$_7$$CH_3$), 1.51-1.65 (m, 2H, N$CH_2C\underline{H}_2$-), 2.30 (d, 3H, ^{4}J =

0.9 Hz, CH_3), 3.94 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2$-), 4.44 (br, 2H, NH_2), 5.88 (dq, 1H, ^4J = 0.9 Hz, ^5J = 0.8 Hz, H-3), 6.42 (dd, 1H, ^3J = 8.5 Hz, ^4J = 2.1 Hz, H-6), 6.56 (dd, 1H, ^4J = 2.1 Hz, ^5J = 0.6 Hz, H-4), 7.01 (ddd, 1H, ^3J = 8.5 Hz, ^5J = 0.8 Hz, ^5J =0.6 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 13.9 (+, $(CH_2)_9\underline{C}H_3$), 22.0 (-, CH_2), 26.2 (-, CH_2), 28.6 (-, CH_2), 28.7 (-, CH_2), 28.8 (-, CH_2), 28.9 (-, CH_2), 29.7 (-, CH_2), 31.2 (-, CH_2), 42.2 (-, CH_2), 98.0 (+, indole), 102.8 (+, indole), 109.2 (+, indole), 110.2 (+, indole), 128.3 (C_{quart}, indole), 130.2 (C_{quart}, indole), 135.6 (C_{quart}, indole), 140.9 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 286 ([$M^{\bullet+}$], 100), 159 ([M - C_9H_{19}]$^+$, 73)

Analysis: $C_{19}H_{30}N_2 \cdot 0.1\ H_2O$ (288.25)

calculated	C: 79.17	H: 10.55	N: 9.72
found	C: 79.15	H: 10.74	N: 9.38

5-Amino-2-methyl-1-(3-phenylpropyl)-1*H*-indole (7.38)

Method B: Reaction of 2-methyl-5-nitro-1-(3-phenylpropyl)-1*H*-indole (**7.31**) (0.05 g, 0.17 mmol) and tin(II) chloride dihydrate (0.19 g, 0.84 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 22 mg (0.08 mmol, 49 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.83-1.96 (m, 2H, $NCH_2C\underline{H}_2CH_2Ph$), 2.27 (d, 3H, ^4J = 0.9 Hz, CH_3), 2.60 (t, 2H, ^3J = 7.8 Hz, $NCH_2CH_2C\underline{H}_2Ph$), 3.99 (t, 2H, ^3J = 7.5 Hz, $NC\underline{H}_2CH_2CH_2Ph$), 4.48 (br, 2H, NH_2), 5.89 (dq, 1H, ^4J = 0.9 Hz, ^5J = 0.8 Hz, H-3), 6.41 (dd, 1H, ^3J = 8.6 Hz, ^4J = 2.2 Hz, H-6), 6.57 (dd, 1H, ^4J = 2.2 Hz, ^5J = 0.6 Hz, H-4), 6.97 (ddd, 1H, ^3J = 8.6 Hz, ^5J = 0.8 Hz, ^5J = 0.6 Hz, H-7), 7.10-7.32 (m, 5H, Ar-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.2 (+, CH_3), 31.3 (-, CH_2), 32.2 (-, CH_2), 41.9 (-, CH_2), 98.1 (+, indole), 102.9 (+, indole), 109.1 (+, indole), 110.2 (+, indole), 125.8 (+, Ar-C), 128.1 (+, 2Ar-C), 128.2 (+, 2Ar-C), 128.3 (C_{quart}, indole), 130.2 (C_{quart}, indole), 135.7 (C_{quart}, indole), 141.0 (C_{quart}, indole), 141.3 (C_{quart}, Ar-C)

MS (CI-MS, NH_3): m/z (%) = 265 ([M + H]$^+$, 100)

$C_{18}H_{20}N_2$ (264.36)

5-Amino-2-methyl-1-(4-phenylbenzyl)-1*H*-indole (7.39)

Method B: Reaction of 2-methyl-5-nitro-1-(4-phenylbenzyl)-1*H*-indole (**7.32**) (0.10 g, 0.29 mmol) and tin(II) chloride dihydrate (0.33 g, 1.46 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.06 g (0.19 mmol, 66 %, brown liquid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.31 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 4.50 (br, 2H, NH_2), 5.31 (s, 2H, NCH_2-), 6.01 (dq, 1H, ^{4}J = 0.9 Hz, H-3), 6.41 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.3 Hz, H-6), 6.62 (dd, 1H, ^{4}J = 2.2 Hz, ^{5}J = 0.6 Hz, H-4), 7.01-7.06 (m, 3H, Ph-H), 7.29-7.37 (m, 1H, Ph-H), 7.40-7.46 (m, 2H, Ph-H), 7.54-7.62 (m, 3H, Ph-H), 7.56 (d, 1H, ^{3}J = 8.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.4 (+, CH_3), 45.2 (-, CH_2), 98.7 (+, indole), 102.9 (+, indole), 109.6 (+, indole), 110.5 (+, indole), 126.5 (+, 2Ph-C), 126.6 (+, 2Ph-C), 126.7 (+, 2Ph-C), 127.3 (+, Ph-C), 128.5 (C_{quart}, indole), 128.8 (+, 2Ph-C), 130.6 (C_{quart}, indole), 136.0 (C_{quart}, indole), 138.0 (C_{quart}, Ph-C), 138.8 (C_{quart}, Ph-C), 139.7 (C_{quart}, Ph-C), 141.4 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 312 ([$M^{\bullet+}$], 73), 167 ([C_6H_5 - C_6H_4 - CH_2]$^{+}$, 100)

$C_{22}H_{20}N_2$ (312.41)

Ethyl 5-(5-amino-2-methyl-1*H*-indol-1-yl)pentanoate (7.40)

Method B: Reaction of ethyl 5-(2-methyl-5-nitro-1*H*-indole-1-yl)pentanoate (**7.33**) (0.20 g, 0.66 mmol) and tin(II) chloride dihydrate (0.74 g, 3.28 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.10 g (0.36 mmol, 55 %, dark red oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.15 (t, 3H, ^{3}J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.45-1.67 (m, 4H, $NCH_2(C\underline{H}_2)_2$-), 2.30 (t, 2H, ^{3}J = 7.2 Hz, $N(CH_2)_3C\underline{H}_2$-), 2.30 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 3.97 (t, 2H, ^{3}J = 7.2 Hz, $NC\underline{H}_2$-), 4.02 (q, 2H, ^{3}J = 7.1 Hz, $CO_2C\underline{H}_2$-), 4.39 (br, 2H, NH_2), 5.89 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.8 Hz, H-3), 6.42 (dd, 1H, ^{3}J = 8.6 Hz, ^{4}J = 2.1 Hz, H-6), 6.56 (dd, 1H, ^{4}J = 2.1 Hz, ^{5}J = 0.6 Hz, H-4), 7.03 (ddd, 1H, ^{3}J = 8.6 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.6 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 14.0 (+, CH_3), 21.8 (-, CH_2), 29.1 (-, CH_2), 33.0 (-, CH_2), 41.9 (-, CH_2), 59.6 (-, CH_2), 98.1 (+, indole), 102.8 (+, indole), 109.2 (+, indole), 110.2 (+, indole), 128.3 (C_{quart}, indole), 130.2 (C_{quart}, indole), 135.6 (C_{quart}, indole), 141.0 (C_{quart}, indole), 172.6 (C_{quart}, $\underline{C}O_2Et$)

MS (CI-MS, NH_3): m/z (%) = 275 ($[M + H]^+$, 100)

$C_{16}H_{22}N_2O_2$ (274.36)

Preparation of the iodides 7.44-7.46 by Finkelstein reaction

General procedure. Sodium iodide (1.3 eq) was suspended with dry acetone (10 ml) and heated under reflux. After complete dissolution of sodium iodide the alkyl halide (1 eq) was slowly added. Subsequently, the reaction mixture was refluxed for further 4 h. Afterwards the cooled mixture was mixed with diethyl ether (20 ml) and filtrated, and the filtrate was concentrated *in vacuo*. The residue was dissolved in diethyl ether (25 ml) and washed successively with a solution of sodium hydroxide (2%, 2x15 ml), water (15 ml) and a saturated solution of sodium chloride (15 ml). The organic phase was dried over magnesium sulfate and the solvent was removed under reduced pressure. The obtained crude product was used for further reactions without purification.

1-Iododecane (7.44)

Reaction of 1-bromodecane (**7.41**) (2.08 ml, 10.00 mmol) and sodium iodide (1.95 g, 13.01 mmol).

Yield: 2.54 g, colorless liquid

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.86 (t, 3H, ^3J = 6.7 Hz, CH_3), 1.20-1.38 (m, 14H, $CH_3(C\underline{H}_2)_7$-), 1.69-1.79 (m, 2H, $ICH_2C\underline{H}_2$-), 3.27 (t, 2H, ^3J = 6.9 Hz, $IC\underline{H}_2$-)

MS (PI-EIMS, 70 eV): m/z (%) = 268 ($[M^{\bullet+}]$, 3), 155 ($[C_2H_4I]^+$, 5), 141 ($[M - I]^+$, 10), 99 ($[C_7H_{15}]^+$, 10), 85 ($[C_6H_{13}]^+$, 57), 71 ($[C_5H_{11}]^+$, 58), 57 ($[C_4H_9]^+$, 100), 43 ($[C_3H_7]^+$, 97)

$C_{10}H_{21}I$ (268.18)

1-Iodo-3-phenylpropane (7.45)

Reaction of 1-chloro-3-phenylpropane (**7.42**) (0.72 ml, 5.02 mmol) and sodium iodide (0.98 g, 6.54 mmol).

Yield: 0.98 g, light brown liquid

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.96-2.06 (m, 2H, $PhCH_2C\underline{H_2}CH_2I$), 2.75 (t, 2H, ^3J = 7.7 Hz, $PhC\underline{H_2}CH_2CH_2I$), 3.61 (t, 2H, ^3J = 6.5 Hz, $PhCH_2CH_2C\underline{H_2}I$), 7.16-7.33 (m, 5H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 246 ($[M^{\bullet+}]$, 20), 119 ($[M - I]^+$, 9), 117 ($[M - I]^+$, 6), 91 ($[C_7H_7]^+$, 100)

$C_9H_{11}I$ (246.09)

4-Phenylbenzyliodide (7.46)

Reaction of 4-phenylbenzyl chloride (**7.43**) (0.40 g, 1.97 mmol) and sodium iodide (0.39 g, 2.60 mmol).

Yield: 0.53 g, beige solid

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 4.70 (s, 2H, CH_2), 7.34-7.78 (m, 9H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 294 ($[M^{\bullet+}]$, 1), 167 ($[M - I]^+$, 100), 165 ($[M - I]^+$, 19)

$C_{13}H_{11}I$ (294.13)

Synthesis of sulfonamide derivatives of 5-amino-1-decyl-2-methyl-1*H*-indole 7.37 (7.47-7.48)

General procedure. 5-Amino-1-decyl-2-methyl-1*H*-indole (**7.37**) (1 eq) was dissolved in pyridine (5 ml) under an inert atmosphere. The pertinent sulfonyl chloride or sulfamoyl chloride, respectively, (1.1 eq) was added dropwise and the mixture was allowed to stir for further 2 h at room temperature. Afterwards, ethyl acetate (20 ml) was added and the mixture was extracted three times with a solution of hydrochloric acid (3 M, 20 ml). The organic phase was washed with water (20 ml), dried over magnesium sulfate and concentrated *in vacuo*.

N-(1-Decyl-2-methyl-1*H*-indol-5-yl)methanesulfonamide (7.47)

Reaction of 5-amino-1-decyl-2-methyl-1*H*-indole (**7.37**) (0.08 g, 0.28 mmol) and methanesulfonyl chloride (0.024 ml, 0.31 mmol); purification by flash column chromatography on silica gel, elution with a 2:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.06 g (0.16 mmol, 59 %, red-pink oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.85 (t, 3H, ^{3}J = 6.7 Hz, N(CH$_2$)$_9$C<u>H</u>$_3$), 1.20-1.29 (m, 14H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_7$CH$_3$), 1.57-1.68 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.38 (d, 3H, ^{4}J = 0.9 Hz, CH$_3$), 2.84 (s, 3H, SO$_2$CH$_3$), 4.06 (t, 2H, ^{3}J = 7.5 Hz, NC<u>H</u>$_2$-), 6.17 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.7 Hz, H-3), 6.94 (dd, 1H, ^{3}J = 8.7 Hz, ^{4}J = 2.1 Hz, H-6), 7.27 (dd, 1H, ^{4}J = 2.1 Hz, ^{5}J = 0.4 Hz, H-4), 7.33 (d, 1H, ^{3}J = 8.7 Hz, H-7), 9.21 (s, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH$_3$), 13.9 (+, CH$_3$), 14.0 (+, CH$_3$), 22.0 (-, CH$_2$), 26.2 (-, CH$_2$), 28.6 (-, 2CH$_2$), 28.8 (-, CH$_2$), 28.9 (-, CH$_2$), 29.7 (-, CH$_2$), 31.2 (-, CH$_2$), 42.4 (-, CH$_2$), 99.3 (+, indole), 109.5 (+, indole), 113.1 (+, indole), 116.1 (+, indole), 127.6 (C$_{quart}$, indole), 129.5 (C$_{quart}$, indole), 134.1 (C$_{quart}$, indole), 137.5 (C$_{quart}$, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 364 ([M$^{\bullet+}$], 22), 285 ([M - SO$_2$CH$_3$]$^+$, 100)

C$_{20}$H$_{32}$N$_2$O$_2$S (364.55)

N'-(1-Decyl-2-methyl-1*H*-indol-5-yl)-*N*,*N*-dimethylsulfamide (7.48)

Reaction of 5-amino-1-decyl-2-methyl-1*H*-indole (**7.37**) (0.08 g, 0.28 mmol) and *N*,*N*-dimethylsulfamoyl chloride (0.033 ml, 0.31 mmol); purification by flash column chromatography on silica gel, elution with a 2:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.05 g (0.13 mmol, 45 %, brown oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 6.6 Hz, N(CH$_2$)$_9$C<u>H</u>$_3$), 1.20-1.29 (m, 14H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_7$CH$_3$), 1.56-1.67 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.37 (d, 3H, ^{4}J = 0.9 Hz, CH$_3$), 2.63 (s, 6H, N(CH$_3$)$_2$), 4.04 (t, 2H, ^{3}J = 7.2 Hz, NC<u>H</u>$_2$-), 6.14 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.5 Hz, H-3), 6.96 (dd, 1H, ^{3}J = 8.6 Hz, ^{4}J = 2.2 Hz, H-6), 7.27 (d, 1H, ^{4}J = 2.2 Hz, H-4), 7.28 (d, 1H, ^{3}J = 8.6 Hz, H-7), 9.42 (s, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 13.9 (+, CH_3), 22.0 (-, CH_2), 26.2 (-, CH_2), 28.5 (-, CH_2), 28.6 (-, CH_2), 28.8 (-, CH_2), 28.9 (-, CH_2), 29.7 (-, CH_2), 31.2 (-, CH_2), 37.8 (+, $N(CH_3)_2$), 42.4 (-, CH_2), 99.3 (+, indole), 109.3 (+, indole), 112.3 (+, indole), 115.5 (+, indole), 127.4 (C_{quart}, indole), 129.7 (C_{quart}, indole), 133.8 (C_{quart}, indole), 137.3 (C_{quart}, indole)

MS (PI-EIMS, 70 eV): m/z (%) = 393 ([$M^{\bullet+}$], 22), 285 ([M - $SO_2N(CH_3)_2$]$^+$, 100)

$C_{21}H_{35}N_3O_2S$ (393.59)

N-Ethyl-*N*'-(1-decyl-2-methyl-1*H*-indol-5-yl)urea (7.49)

5-Amino-1-decyl-2-methyl-1*H*-indole (**7.37**) (0.08 g, 0.28 mmol) was dissolved in acetonitrile (5 ml) under a nitrogen atmosphere. Ethyl isocyanate (0.025 ml, 0.31 mmol) was added dropwise and the reaction mixture was stirred for 1 h at room temperature. The reaction was quenched with water (5 ml) and the mixture was extracted two times with ethyl acetate (10 ml). The combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with ethyl acetate.

Yield: 0.10 g (0.28 mmol, 99 %, beige solid)

Mp: 116-117.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 6.8 Hz, $N(CH_2)_9C\underline{H}_3$), 1.04 (t, 3H, ^{3}J = 7.2 Hz, $NHCH_2C\underline{H}_3$), 1.20-1.29 (m, 14H, $N(CH_2)_2(C\underline{H}_2)_7CH_3$), 1.55-1.65 (m, 2H, $NCH_2C\underline{H}_2$-), 2.35 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 3.10 (dq, 2H, ^{3}J = 7.2 Hz, ^{3}J = 5.7 Hz, $NHC\underline{H}_2CH_3$), 4.02 (t, 2H, ^{3}J = 7.1 Hz, $NC\underline{H}_2$-), 5.91 (t, 1H, ^{3}J = 5.7 Hz, $N\underline{H}CH_2$), 6.06 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.7 Hz, H-3), 6.94 (dd, 1H, ^{3}J = 8.8 Hz, ^{4}J = 1.9 Hz, H-6), 7.19 (d, 1H, ^{3}J = 8.8 Hz, H-7), 7.46 (d, 1H, ^{4}J = 1.9 Hz, H-4), 8.05 (s, 1H, NH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 13.9 (+, CH_3), 15.5 (+, CH_3), 22.0 (-, CH_2), 26.2 (-, CH_2), 28.6 (-, CH_2), 28.7 (-, CH_2), 28.8 (-, CH_2), 28.9 (-, CH_2), 29.7 (-, CH_2), 31.2 (-, CH_2), 33.9 (-, $NH\underline{C}H_2CH_3$), 42.3 (-, CH_2), 99.0 (+, indole), 108.7 (+, indole), 108.9 (+, indole), 113.1 (+, indole), 127.4 (C_{quart}, indole), 132.3 (C_{quart}, 2 indole-C), 136.5 (C_{quart}, indole), 155.6 (C_{quart}, -NHCONH-)

MS (PI-EIMS, 70 eV): m/z (%) = 357 ([$M^{\bullet+}$], 100), 286 ([M - CH_3CH_2NCO] $^+$, 80), 230 ([M - C_9H_{19}] $^+$, 7), 159 ([M - C_9H_{19} - CH_3CH_2NCO] $^+$, 57)

Analysis: $C_{22}H_{35}N_3O \cdot 0.1\ H_2O$ (359.13)

calculated	C: 73.58	H: 9.87	N: 11.70
found	C: 73.42	H: 10.09	N: 11.46

Synthesis of the 3-acylated 1*H*-indoles 7.50-7.53

General procedure. The particular *N*-substituted 2-methyl-5-nitro-1*H*-indole (1 eq) was dissolved in dry dichloromethane (5-10 ml) and cooled in an ice-bath. The corresponding acid chloride (1 eq) and tin(IV) chloride (1 eq) was added consecutively. Then, the ice-bath was removed and the solution was stirred for 4 h at ambient temperature. Ice-cold water (20 ml) was poured into the reaction mixture. After extraction with ethyl acetate (3x15 ml), the organic layers were combined, washed with a solution of sodium hydroxide (0.1 N, 15 ml), dried over magnesium sulfate and evaporated under reduced pressure.

1-(2-Methyl-5-nitro-1-propyl-1*H*-indol-3-yl)-3-phenylpropan-1-one (7.50)

Reaction of 2-methyl-5-nitro-1-propyl-1*H*-indole (**7.27**) (0.13 g, 0.60 mmol), 3-phenylpropanoyl chloride (0.09 ml, 0.60 mmol) and tin(IV) chloride (0.07 ml, 0.60 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.11 g (0.31 mmol, 53 %, yellow solid)

Mp: 106-108 °C

^{1}H-NMR (DMSO-d_6): δ [ppm] = 0.89 (t, 3H, ^3J = 7.4 Hz, $N(CH_2)_2C\underline{H}_3$), 1.64-1.77 (m, 2H, $NCH_2C\underline{H}_2CH_3$), 2.78 (s, 3H, CH_3), 3.00 (t, 2H, ^3J = 7.4 Hz, $COC\underline{H}_2CH_2Ph$), 3.33 (t, 2H, ^3J = 7.4 Hz, $COCH_2C\underline{H}_2Ph$), 4.29 (t, 2H, ^3J = 7.3 Hz, $NC\underline{H}_2$-), 7.15-7.21 (m, 1H, Ph-H), 7.26-7.35 (m, 4H, Ph-H), 7.81 (dd, 1H, ^3J = 9.1 Hz, ^4J = 0.4 Hz, H-7), 8.09 (dd, 1H, ^3J = 9.1 Hz, ^4J = 2.3 Hz, H-6), 8.96 (d, 1H, ^4J = 2.3 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 10.8 (+, CH_3), 12.9 (+, CH_3), 22.5 (-, CH_2), 29.4 (-, CH_2), 43.6 (-, CH_2), 44.4 (-, CH_2), 111.0 (+, indole), 114.6 (C_{quart}, indole-C3), 117.0 (+, indole), 117.2 (+, indole), 125.1 (C_{quart}, indole), 125.7 (+, Ph-C), 128.2 (+, 2Ph-C), 128.4 (+, 2Ph-C), 138.8 (C_{quart}, indole), 141.5 (C_{quart}, indole), 142.4 (C_{quart}, indole), 147. 8 (C_{quart}, Ph-C), 194.7 (C_{quart}, $\underline{C}O(CH_2)_2$-)

MS (PI-EIMS, 70 eV): m/z (%) = 350 ([$M^{\bullet+}$], 49), 245 ([M - $C_6H_5CH_2CH_2$] $^+$, 100), 218 ([M - $C_6H_5CH_2CHCO$] $^+$, 55)

Analysis: $C_{21}H_{22}N_2O_3$ (350.41)

calculated	C: 71.98	H: 6.33	N: 7.99
found	C: 71.60	H: 6.25	N: 7.79

Ethyl 5-(3-*m*-chlorobenzoyl-2-methyl-5-nitro-1*H*-indol-1-yl)pentanoate (7.51)

Reaction of Ethyl 5-(2-methyl-5-nitro-1*H*-indole-1-yl)pentanoate (**7.33**) (0.30 g, 0.99 mmol), *m*-chlorobenzoyl chloride (0.13 ml, 1.01 mmol) and tin(IV) chloride (0.12 ml, 1.02 mmol); purification by flash column chromatography on silica gel, successive elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate and then with dichloromethane.

Yield: 0.18 g (0.41 mmol, 41 %, yellow solid)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.14 (t, 3H, ^{3}J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.52-1.66 (m, 2H, $N(CH_2)_2(C\underline{H}_2)$-), 1.68-1.80 (m, 2H, $NCH_2C\underline{H}_2$-), 2.35 (t, 2H, ^{3}J = 7.3 Hz, $N(CH_2)_3C\underline{H}_2$-), 2.46 (s, 3H, CH_3), 4.03 (q, 2H, ^{3}J = 7.1 Hz, $CO_2C\underline{H}_2$-), 4.34 (t, 2H, ^{3}J = 7.3 Hz, $NC\underline{H}_2$-), 7.53-7.64 (m, 2H, Ph-H), 7.67-7.76 (m, 2H, Ph-H), 7.86 (dd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.5 Hz, H-7), 8.10 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.29 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.5 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.7 (+, CH_3), 14.0 (+, CH_3), 21.5 (-, CH_2), 28.3 (-, CH_2), 32.8 (-, CH_2), 43.0 (-, CH_2), 59.7 (-, CH_2), 111.1 (+, indole), 113.6 (C_{quart}, indole-C3), 116.2 (+, indole), 117.4 (+, indole), 125.8 (C_{quart}, indole-C3a), 127.1 (+, Ph-C), 127.9 (+, Ph-C), 130.5 (+, Ph-C), 131.6 (+, Ph-C), 133.4 (C_{quart}, Ph-C), 138.9 (C_{quart}, Ph-C), 142.2 (C_{quart}, indole), 142.3 (C_{quart}, indole), 148.4 (C_{quart}, indole), 172.5 (C_{quart}, $\underline{C}O_2Et$), 189.5 (C_{quart}, $\underline{C}OPh(3\text{-}Cl)$)

MS (PI-EIMS, 70 eV): m/z (%) = 442 ([$M^{\bullet+}$], 30), 397 ([M - OC_2H_5] $^+$, 28), 355 ([M - $CH_2CO_2C_2H_5$] $^+$, 16), 327 ([M - $(CH_2)_3CO_2Et$] $^+$, 35), 313 ([M - $(CH_2)_4CO_2Et$] $^+$, 71), 139 ([ClC_6H_4CO] $^+$, 100)

$C_{23}H_{23}ClN_2O_5$ (442.89)

Ethyl 5-(2-methyl-5-nitro-3-propanoyl-1*H*-indol-1-yl)pentanoate (7.52)

Reaction of Ethyl 5-(2-methyl-5-nitro-1*H*-indole-1-yl)pentanoate (**7.33**) (0.30 g, 0.99 mmol), propionyl chloride (0.09 ml, 1.03 mmol) and tin(IV) chloride (0.12 ml, 1.02 mmol); purification by flash column chromatography on silica gel, elution with a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.29 g (0.80 mmol, 82 %, yellow solid)

Mp: 83-84 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.13 (t, 3H, ^3J = 7.2 Hz, $COCH_2C\underline{H}_3$), 1.14 (t, 3H, ^3J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.51-1.62 (m, 2H, $N(CH_2)_2(C\underline{H}_2)$-), 1.64-1.75 (m, 2H, $NCH_2C\underline{H}_2$-), 2.34 (t, 2H, ^3J = 7.2 Hz, $N(CH_2)_3C\underline{H}_2$-), 2.79 (s, 3H, CH_3), 3.02 (q, 2H, ^3J = 7.2 Hz, $COC\underline{H}_2CH_3$), 4.02 (q, 2H, ^3J = 7.1 Hz, $CO_2C\underline{H}_2$-), 4.34 (t, 2H, ^3J = 7.2 Hz, $NC\underline{H}_2$-), 7.81 (dd, 1H, ^3J = 9.1 Hz, ^5J = 0.4 Hz, H-7), 8.10 (dd, 1H, ^3J = 9.1 Hz, ^4J = 2.4 Hz, H-6), 8.97 (dd, 1H, ^4J = 2.4 Hz, ^5J = 0.4 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.0 (+, CH_3), 12.8 (+, CH_3), 14.0 (+, CH_3), 21.5 (-, CH_2), 28.5 (-, CH_2), 32.8 (-, CH_2), 35. 3 (-, CH_2), 42.6 (-, CH_2), 59.7 (-, CH_2), 110.9 (+, indole), 114.6 (C_{quart}, indole-C3), 117.1 (+, 2 indole-CH), 125.2 (C_{quart}, indole), 138.7 (C_{quart}, indole), 142.4 (C_{quart}, indole), 147.5 (C_{quart}, indole), 172.5 (C_{quart}, $\underline{C}O_2Et$), 196.1 (C_{quart}, $\underline{C}OEt$)

MS (PI-EIMS, 70 eV): m/z (%) = 360 ([$M^{\bullet+}$], 31), 331 ([M - C_2H_5] $^+$, 100), 315 ([M - OC_2H_5] $^+$, 11)

Analysis: $C_{19}H_{24}N_2O_5$ (360.40)

calculated	C: 63.32	H: 6.71	N: 7.77
found	C: 63.58	H: 7.02	N: 7.52

1-(1-Hexyl-2-methyl-5-nitro-1*H*-indol-3-yl)propan-1-one (7.53)

Reaction of 1-hexyl-2-methyl-5-nitro-1*H*-indole (**7.28**) (0.14 g, 0.54 mmol), propionyl chloride (0.05 ml, 0.57 mmol) and tin(IV) chloride (0.07 ml, 0.60 mmol); purification by flash column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.16 g (0.51 mmol, 94 %, pale yellow solid)

Mp: 112.5-113 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 7.1 Hz, N(CH$_2$)$_5$C<u>H</u>$_3$), 1.13 (t, 3H, ^{3}J = 7.2 Hz, COCH$_2$C<u>H</u>$_3$), 1.21-1.36 (m, 6H, N(CH$_2$)$_2$(C<u>H</u>$_2$)$_3$CH$_3$), 1.60-1.72 (m, 2H, NCH$_2$C<u>H</u>$_2$-), 2.79 (s, 3H, CH$_3$), 3.01 (q, 2H, ^{3}J = 7.1 Hz, COC<u>H</u>$_2$-), 4.31 (t, 2H, ^{3}J = 7.5 Hz, NC<u>H</u>$_2$-), 7.79 (dd, 1H, ^{3}J = 9.1 Hz, ^{5}J = 0.4 Hz, H-7), 8.09 (dd, 1H, ^{3}J = 9.1 Hz, ^{4}J = 2.3 Hz, H-6), 8.97 (dd, 1H, ^{4}J = 2.3 Hz, ^{5}J = 0.4 Hz, H-4)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.0 (+, CH$_3$), 12.8 (+, CH$_3$), 13.8 (+, CH$_3$), 21.9 (-, CH$_2$), 25.7 (-, CH$_2$), 29.2 (-, CH$_2$), 30.8 (-, CH$_2$), 35.2 (-, CH$_2$), 43.0 (-, CH$_2$), 110.9 (+, indole), 114.6 (C$_{quart}$, indole-C3), 117.1 (+, 2 indole-CH), 125.2 (C$_{quart}$, indole), 138.7 (C$_{quart}$, indole), 142.3 (C$_{quart}$, indole), 147.5 (C$_{quart}$, indole), 196.1 (C$_{quart}$, <u>C</u>OEt)

MS (PI-EIMS, 70 eV): m/z (%) = 316 ([M$^{\bullet+}$], 19), 301 ([M - CH$_3$]$^+$, 8), 287 ([M - C$_2$H$_5$]$^+$, 100)

$C_{18}H_{24}N_2O_3$ (316.39)

Reduction of the 3-acylated 1*H*-indoles 7.54-7.56

General procedure. A mixture of the *N*-substituted 3-acylated-2-methyl-5-nitro-1*H*-indole derivative (1 eq), dissolved in ethyl acetate (5-7 ml), and tin(II) chloride dihydrate (5 eq) was heated at 70 °C and stirred under a nitrogen atmosphere overnight. After cooling, the mixture was diluted with ice-cold water (10-20 ml) and the pH is made slightly basic (pH = 8) by an aqueous solution of sodium bicarbonate (5 %). After stirring for 1 h the aqueous phase was extracted three times with ethyl acetate (20 ml). The combined organic layers were

washed with brine (15 ml), dried over magnesium sulfate and concentrated under reduced pressure.

Ethyl 5-(5-amino-2-methyl-3-*m*-chlorobenzoyl-1*H*-indol-1-yl)pentanoate (7.54)

Reaction of ethyl 5-(3-*m*-chlorobenzoyl-2-methyl-5-nitro-1*H*-indol-1-yl)pentanoate (**7.51**) (0.11 g, 0.25 mmol) and tin(II) chloride dihydrate (0.28 g, 1.24 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.08 g (0.19 mmol, 78 %, red-brown oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.15 (t, 3H, ^{3}J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.50-1.77 (m, 4H, $NCH_2(C\underline{H}_2)_2$-), 2.33 (s, 3H, CH_3), 2.34 (t, 2H, ^{3}J = 7.2 Hz, $N(CH_2)_3C\underline{H}_2$-), 4.03 (q, 2H, ^{3}J = 7.1 Hz, $CO_2C\underline{H}_2$-), 4.12 (t, 2H, ^{3}J = 7.2 Hz, $NC\underline{H}_2$-), 4.74 (br, 2H, NH_2), 6.53 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 2.2 Hz, H-6), 6.58 (d, 1H, ^{4}J = 2.2 Hz, H-4), 7.23 (d, 1H, ^{3}J = 8.5 Hz, H-7), 7.49-7.53 (m, 2H, Ph-H), 7.55-7.58 (m, 1H, Ph-H), 7.61-7.67 (m, 1H, Ph-H)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 12.3 (+, CH_3), 14.0 (+, CH_3), 21.7 (-, CH_2), 28.4 (-, CH_2), 32.9 (-, CH_2), 42.2 (-, CH_2), 59.6 (-, CH_2), 103.5 (+, indole), 110.4 (+, indole), 111.3 (C_{quart}, indole-C3), 111.5 (+, indole), 126.8 (+, Ph-C), 127.6 (+, Ph-C), 127.6 (C_{quart}, indole), 129.0 (C_{quart}, indole), 130.3 (+, Ph-C), 130.3 (C_{quart}, Ph-C), 130.6 (+, Ph-C), 133.1 (C_{quart}, Ph-C), 143.7 (C_{quart}, indole), 143.8 (C_{quart}, indole), 172.6 (C_{quart}, $\underline{C}O_2Et$), 189.4 (C_{quart}, COPh(3-Cl))

MS (CI-MS, NH_3): m/z (%) = 413 ($[M + H]^+$, 100)

$C_{23}H_{25}ClN_2O_3$ (412.91)

Ethyl 5-(5-amino-2-methyl-3-propanoyl-1*H*-indol-1-yl)pentanoate (7.55)

Reaction of ethyl 5-(2-methyl-5-nitro-3-propanoyl-1*H*-indol-1-yl)pentanoate (**7.52**) (0.21 g, 0.58 mmol) and tin(II) chloride dihydrate (0.66 g, 2.93 mmol);

purification by flash column chromatography on silica gel, elution with a 100:1 (v/v) mixture of ethyl acetate and triethylamine.

Yield: 0.16 g (0.48 mmol, 83 %, light brown solid)

Mp: 88-91 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 1.10 (t, 3H, ^{3}J = 7.2 Hz, $COCH_2C\underline{H}_3$), 1.14 (t, 3H, ^{3}J = 7.1 Hz, $CO_2CH_2C\underline{H}_3$), 1.47-1.69 (m, 4H, $NCH_2(C\underline{H}_2)_2$-), 2.32 (t, 2H, ^{3}J = 7.1 Hz, $N(CH_2)_3C\underline{H}_2$-), 2.65 (s, 3H, CH_3), 2.85 (q, 2H, ^{3}J = 7.2 Hz, $COC\underline{H}_2CH_3$), 4.03 (q, 2H, ^{3}J = 7.1 Hz, $CO_2C\underline{H}_2CH_3$), 4.10 (t, 2H, ^{3}J = 7.3 Hz, NCH_2-), 4.86 (br, 2H, NH_2), 6.54 (dd, 1H, ^{3}J = 8.5 Hz, ^{4}J = 1.9 Hz, H-6), 7.15 (d, 1H, ^{4}J = 1.9 Hz, H-4), 7.21 (d, 1H, ^{3}J = 8.5 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.4 (+, CH_3), 12.4 (+, CH_3), 14.0 (+, CH_3), 21.6 (-, CH_2), 28.5 (-, CH_2), 32.9 (-, CH_2), 35.2 (-, CH_2), 41.8 (-, CH_2), 59.6 (-, CH_2), 104.3 (+, indole), 110.4 (+, indole), 111.0 (+, indole), 112.1 (C_{quart}, indole-C3), 126.7 (C_{quart}, indole), 129.0 (C_{quart}, indole), 143.1 (C_{quart}, indole), 143.6 (C_{quart}, indole), 172.6 (C_{quart}, $\underline{C}O_2Et$), 195.6 (C_{quart}, $\underline{C}OEt$)

MS (PI-EIMS, 70 eV): m/z (%) = 330 ([$M^{\bullet+}$], 74), 301 ([M - C_2H_5]$^{+}$, 100)

$C_{19}H_{26}N_2O_3$ (330.42)

1-(5-Amino-1-hexyl-2-methyl-1*H*-indol-3-yl)propan-1-one (7.56)

Reaction of 1-(1-hexyl-2-methyl-5-nitro-1*H*-indol-3-yl)propan-1-one (**7.53**) (0.13 g, 0.41 mmol) and tin(II) chloride dihydrate (0.47 g, 2.08 mmol); purification by flash column chromatography on silica gel, elution with a 50:50:1 (v/v) mixture of petroleum ether (60-80 °C), ethyl acetate and triethylamine.

Yield: 0.09 g (0.31 mmol, 76 %, red-brown oil)

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 0.84 (t, 3H, ^{3}J = 7.0 Hz, $N(CH_2)_5C\underline{H}_3$), 1.10 (t, 3H, ^{3}J = 7.3 Hz, $COCH_2C\underline{H}_3$), 1.21-1.33 (m, 6H, $N(CH_2)_2(C\underline{H}_2)_3CH_3$), 1.54-1.67 (m, 2H, $NCH_2C\underline{H}_2$-), 2.65 (s, 3H, CH_3), 2.84 (q, 2H, ^{3}J = 7.3 Hz, $COC\underline{H}_2CH_3$), 4.08 (t, 2H, ^{3}J = 7.4 Hz, $NC\underline{H}_2$-), 4.87 (br, 2H, NH_2), 6.54 (dd, 1H, ^{3}J = 8.6 Hz, ^{4}J = 2.0 Hz, H-6), 7.16 (d, 1H, ^{4}J = 2.0 Hz, H-4), 7.19 (d, 1H, ^{3}J = 8.6 Hz, H-7)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 8.4 (+, CH_3), 12.4 (+, CH_3), 13.8 (+, CH_3), 21.9 (-, CH_2), 25.8 (-, CH_2), 29.2 (-, CH_2), 30.8 (-, CH_2), 35.2 (-, CH_2), 42.1 (-, CH_2), 104.3 (+, indole), 110.4 (+, indole), 111.0 (+, indole), 112.0 (C_{quart}, indole-C3), 126.7 (C_{quart}, indole), 129.0 (C_{quart}, indole), 143.1 (C_{quart}, indole), 143.5 (C_{quart}, indole), 195.6 (C_{quart}, <u>C</u>OEt)

MS (PI-EIMS, 70 eV): m/z (%) = 286 ([$M^{\bullet+}$], 49), 257 ([M - C_2H_5]$^+$, 100)

$C_{18}H_{26}N_2O$ (286.41)

Method for the *N*-acylation of the 1*H*-indole derivatives 7.60-7.62

The corresponding 1*H*-indole derivative (1 eq) and TEBAC (0.1 eq) was dissolved in dry dichloromethane (5-15 ml) (if necessary small amounts of THF were added to dissolve the remaining starting material). After addition of sodium hydroxide (2.5 eq) and 3-phenylpropanoyl chloride (1.5 eq), dissolved in 10 ml anhydrous dichloromethane, the mixture was allowed to stir for 30 min at room temperature. Subsequently, the reaction mixture was filtrated and washed with dichloromethane (25 ml). The filtrate was concentrated and purified by column chromatography on silica gel.

1-(1*H*-Indol-1-yl)-3-phenylpropan-1-one (7.60)

Reaction of 1*H*-indole (**7.57**) (0.50 g, 4.27 mmol), TEBAC (126 mg, 0.43 mmol), sodium hydroxide (0.40 g, 10.00 mmol) and 3-phenylpropanoyl chloride (0.95 ml, 6.39 mmol); purification by column chromatography on silica gel, elution with a 1:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.50 g (2.01 mmol, 47 %, white solid)

Mp: 76-77.5 °C (Lit. 78.5-79.5 °C[214])

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 3.03 (t, 2H, ^{3}J = 7.6 Hz, C<u>H$_2$</u>CH$_2$Ph), 3.40 (t, 2H, ^{3}J = 7.6 Hz, CH$_2$C<u>H$_2$</u>Ph), 6.73 (dd, 1H, ^{3}J = 3.8 Hz, ^{5}J = 0.8 Hz, H-3), 7.16-7.36 (m, 7H, Ar-H), 7.59-7.63 (m, 1H, Ar-H), 7.95 (d, 1H, ^{3}J = 3.8 Hz, H-2), 8.34-8.39 (m, 1H, Ar-H)

MS (PI-EIMS, 70 eV): m/z (%) = 249 ([$M^{\bullet+}$], 31), 117 ([M - $C_6H_5CH_2$ - CHCO]$^+$, 100)

$C_{17}H_{15}NO$ (249.31)

1-(3-Acetyl-1*H*-indol-yl)-3-phenylpropan-1-one (7.61)

Reaction of 3-acetyl-1*H*-indole (**7.58**) (0.50 g, 3.14 mmol), TEBAC (93 mg, 0.31 mmol), sodium hydroxide (0.32 g, 7.85 mmol) and 3-phenylpropanoyl chloride (0.70 ml, 4.71 mmol); purification by column chromatography on silica gel, elution with a 3:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate.

Yield: 0.71 g (2.44 mmol, 77 %, white solid)

Mp: 115-115.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 2.55 (s, 3H, CH_3), 3.06 (t, 2H, ^3J = 7.8 Hz, $C\underline{H}_2CH_2Ph$), 3.54 (t, 2H, ^3J = 7.8 Hz, $CH_2C\underline{H}_2Ph$), 7.18-7.44 (m, 7H, Ar-H), 8.21-8.25 (m, 1H, Ar-H), 8.37-8.41 (m, 1H, Ar-H), 8.85 (s, 1H, H-2)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 27.6 (+, CH_3), 29.5 (-, CH_2), 36.2 (-, CH_2), 115.8 (+, Ar-C), 120.0 (C_{quart}, C-3), 121.6 (+, Ar-C), 124.6 (+, Ar-C), 125.6 (+, Ar-C), 126.0 (+, Ar-C), 126.7 (C_{quart}, Ar-C), 128.2 (+, 2Ar-C), 128.5 (+, 2Ar-C),134.3 (+, Ar-C), 135.4 (C_{quart}, Ar-C), 140.4 (C_{quart}, Ar-C), 172.1 (C_{quart}, $\underline{C}O(CH_2)_2Ph$), 194.1 (C_{quart}, $\underline{C}OCH_3$)

MS (PI-EIMS, 70 eV): m/z (%) = 291 ([$M^{\bullet+}$], 51), 159 ([M - $C_6H_5CH_2$ - CHCO]$^+$, 94), 144 ([M - $C_6H_5CH_2$ – CHCO - CH_3]$^+$, 100), 91 ([$C_6H_5CH_2$]$^+$, 45)

Analysis: $C_{19}H_{17}NO_2$ (291.34)

calculated	C: 78.33	H: 5.88	N: 4.81
found	C: 78.13	H: 5.79	N: 4.59

1-(3-Phenylpropanoyl)-1*H*-indole-5-carboxylic acid (7.62)

Reaction of 1*H*-indole-5-carboxylic acid (**7.59**) (0.15 g, 0.93 mmol), TEBAC (28 mg, 0.09 mmol), sodium hydroxide (0.11 g, 2.79 mmol) and 3-phenylpropanoyl chloride (0.21 ml, 1.41 mmol); purification by column chromatography on silica gel, elution with a 2:1 (v/v) mixture of petroleum ether (60-80 °C) and ethyl acetate; analytical pure product was obtained by recrystallization from methanol.

Yield: 0.16 g (0.55 mmol, 59 %, pink solid)

Mp: 188.5-189.5 °C

^{1}H-NMR (DMSO-*d*$_6$): δ [ppm] = 3.04 (t, 2H, ^{3}J = 7.6 Hz, COC<u>H$_2$</u>CH$_2$Ph), 3.43 (t, 2H, ^{3}J = 7.6 Hz, COCH$_2$C<u>H$_2$</u>Ph), 6.85 (dd, 1H, ^{3}J = 3.8 Hz, ^{5}J = 0.8 Hz, H-3), 7.16.7.37 (m, 5H, Ar-H), 7.92 (dd, 1H, ^{3}J = 8.7 Hz, ^{4}J = 1.7 Hz, H-6), 8.06 (d, 1H, ^{3}J = 3.8 Hz, H-2), 8.24 (dd, 1H, ^{4}J = 1.7 Hz, ^{5}J = 0.7 Hz, H-4), 8.42 (ddd, 1H, ^{3}J = 8.7 Hz, ^{5}J = 0.8 Hz, ^{5}J = 0.7 Hz, H-7), 12.83 (br, 1H, COOH)

^{13}C-NMR (DMSO-*d*$_6$): δ [ppm] = 29.6 (-, CH$_2$), 36.2 (-, CH$_2$), 108.5 (+, Ar-C), 115.5 (+, Ar-C), 122.6 (+, Ar-C), 125.6 (+, Ar-C), 126.0 (+, Ar-C), 127.9 (+, Ar-C), 128.2 (+, 2Ar-C), 128.4 (+, 2Ar-C), 130.0 (C_{quart}, Ar-C), 134.43 (C_{quart}, Ar-C), 137.2 (C_{quart}, Ar-C), 140.4 (C_{quart}, Ar-C), 167.4 (C_{quart}, COOH), 171.6 (C_{quart}, <u>C</u>OCH$_2$-)

MS (PI-EIMS, 70 eV): m/z (%) = 293 ([M$^{\bullet+}$], 21), 161 ([M - $C_6H_5CH_2CHCO$] $^{+}$, 100), 144 ([M - $C_6H_5CH_2CHCO$ - OH] $^{+}$, 13), 116 ([M - $C_6H_5CH_2CHCO$ - CO_2H] $^{+}$, 7), 91 ([C_7H_7] $^{+}$, 49)

Analysis: $C_{18}H_{15}NO_3$ (293.32)

calculated	C: 73.71	H: 5.15	N: 4.78
found	C: 73.46	H: 5.09	N: 4.80

2-Methyl-1*H*-indole-5-sulfonic acid (7.65)

A suspension of 4-hydrazinobenzenesulfonic acid hemihydrate (**7.63**) (4.00 g, 20.28 mmol), acetone (2 ml, 27.20 mmol) and glacial acid (40 ml) was heated at 110 °C for 3 h. After evaporation of the solvent the resulting hydrazone **7.64** was treated with concentrated sulfuric acid (21.9 ml) and heated at 130 °C for 3 h. The annealed solution was made alkaline by addition of sodium carbonate. The solvent was removed under reduced pressure. Then again water was added and the solution was adjusted to slight acidity with hydrochloric acid (2N). The solvent was removed *in vacuo*. The resulting solid was elutriated with methanol, filtered off and washed thoroughly with methanol. Evaporation of the solvent yielded a pale brown solid.

Yield: 3.89 g (18.42 mmol, 91 %, pale brown solid)

Mp: >270 °C

^{1}H-NMR (DMSO-*d$_6$*): δ [ppm] = 2.37 (d, 3H, ^{4}J = 0.9 Hz, CH_3), 6.12 (dq, 1H, ^{4}J = 0.9 Hz, ^{5}J = 0.9 Hz, H-3), 7.16 (ddd, 1H, ^{3}J = 8.4 Hz, ^{5}J = 0.5 Hz, ^{5}J = 0.9 Hz, H-7), 7.29 (dd, 1H, ^{3}J = 8.4 Hz, ^{4}J = 1.6 Hz, H-6), 7.67 (dd, 1H, ^{4}J = 1.6 Hz, ^{5}J = 0.7 Hz, H-4)

MS (ESI, H_2O/MeOH + 10 mM NH_4Ac): m/z (%) = 234 ([M + Na]$^+$, 34), 229 ([M + NH_4]$^+$, 100), 212 ([M + H]$^+$, 19)

$C_9H_9NO_3S$ (211.24)

7.5.3 Pharmacological methods

All synthesized indole derivatives were investigated for their inhibitory activity on the enzymatic activities of both bovine testicular hyaluronidase and hyaluronate lyase of *S.agalactiae* in a turbidimetric assay, which was developed in our laboratory based on the method of Di Ferrante[145] as described in section 5.5.3.

Chapter 8

Summary

Hyaluronan and hyaluronidases have been used in several medical fields for many years. For instance, sodium hyaluronate is frequently applied in the treatment of arthritic joints, whereas preparations of bovine testicular hyaluronidase are beneficial in the therapy of different diseases in internal medicine, ophthalmology or orthopedics. It has been reported that some hyaluronidases are implicated in biological processes like meningitis, septicemia, arthroses, fertilization and cancer. Therefore, potent and selective inhibitors are required as pharmacological tool to prove and substantiate the physiological and pathophysiological role of hyaluronidases and their preferred substrate, hyaluronic acid. Moreover, such inhibitors might be of therapeutic value, *e.g*. in the treatment of arthritis, as new anti-fertility agents or as antimicrobial agent against penicillin-resistant bacteria. Inspired by the lack of such compounds, the main goal of this thesis was the synthesis and identification of lead-like compounds and their structural optimization as inhibitors of bovine testicular hyaluronidase and hyaluronate lyase from *S. agalactiae* ($hylB_{4755}$). The inhibitory activities on both hyaluronidases were determined by means of a turbidimetric assay and the structure-activity relationships were elaborated. Based on the crystal structure of a hyaluronate lyase in complex with inhibitors the binding mode of structurally related compounds was suggested and used as a model for the design of new inhibitors.

L-Ascorbic acid, a purported inhibitor of bovine testicular hyaluronidase, was found to weakly inhibit hyaluronate lyase of *S. pneumoniae* (hylSpn). X-ray analysis of the vitamin C-hylSpn complex confirmed that vitamin C binds to the active site of the bacterial enzyme suggesting that additional hydrophobic interactions might increase the inhibitory effect. Therefore, as a part of this doctoral

project a series of L-ascorbic acid derivatives with additional lipophilic partial structures was synthesized and investigated for inhibitory activity against bovine testicular hyaluronidase and hyaluronate lyase of *S. agalactiae* strain 4755 ($hylB_{4755}$). Four 6-*O*-acyl derivatives of L-ascorbic acid were identified as moderate to strong inhibitors of hyaluronidases with preference for the bacterial enzyme. At optimum pH (pH 5) the IC_{50} values for inhibition of $hylB_{4755}$ range from 0.9 µM to 475 µM with highest activity for the esters of the long chain alkanoic acids supporting the hypothesis that additional hydrophobic interactions in the active site contribute to the high affinity. Among the studied vitamin C derivatives, L-ascorbic acid-6-octadecanoate proved to be the most potent inhibitor of $hylB_{4755}$ and BTH with IC_{50} values of 0.9 µM and 39 µM at pH 5, respectively. The octadecanoate is up to 6500 times more potent than the parent compound vitamin C and is the most potent inhibitor of bacterial and bovine testicular hyaluronidase described to date.

The binding mode of L-ascorbic acid-6-hexadecanoate at hyaluronate lyase from *S. pneumoniae* was determined by X-ray analysis[‡‡]. The inhibitor binds within the catalytic site of the enzyme whereby hydrophobic interactions with Trp291, Phe343, His399 and Thr400 and a hydrogen bond between the carboxylic group of the inhibitor and the hydroxyl side chain of Tyr408 are involved. This insight into enzyme-inhibitor interactions at molecular level delivered new information for further development of more potent and selective inhibitors of hyaluronidases.

Starting from a *de novo* substrate-based inhibitor design 1,3-diacetylbenzimidazole-2-thione was identified as lead compound for hyaluronate lyase of *S. agalactiae* (IC_{50} values of 5 µM and 160 µM at physiological pH and optimum pH, respectively). The investigation of structural diverse benzimidazoles derived from the lead revealed that *N*-monoacylated and *N*,*N'*-diacylated benzimidazole-2-thiones inhibit the bacterial enzyme $hylB_{4755}$ but not the bovine testicular hyaluronidase (BTH). For *N*-monoacylated derivatives IC_{50} values in the low µM range were determined at physiological pH whereas the *N*,*N'*-diacylated compounds showed strong inhibition of $hylB_{4755}$ at both pH optimum and physiologi-

[‡‡] These studies were accomplished in cooperation with *M. J. Jedrzejas* (Children's Hospital Oakland Research Institute, Oakland, California 94609, USA). The X-ray structure of the complex was solved by *D. Rigden* (National Centre of Genetic Resources and Biotechnology, Cenargen/Embrapa, Brasília, D.F. 70770-900, Brazil).

cal pH. Nevertheless, only the *N*,*N'*-diacylated compound displayed IC_{50} values in the same range as the lead. Regardless of the high inhibitory potency of *N*,*N'*-diacylated benzimidazoles the development of this class of compounds was discontinued due to insufficient stability against moisture.

Owing to their structural similarity with the aforementioned benzimidazoles corresponding benzoxazoles and indoles were also considered promising scaffolds for the preparation of hyaluronidase inhibitors. The structure-activity relationships of recently described 2-phenylindole derivatives were in accordance with a model based on the X-ray structure of hylSpn co-crystallized with 1-decyl-2-(4-sulfamoyloxyphenyl)-1*H*-indol-6-yl-sulfamate. This 3D structure was also used to suggest the putative binding mode of benzoxazole-2-thiones. To prove this model a series of benzoxazole derivatives was synthesized and investigated for hyaluronidase inhibition. All tested compounds were highly selective for the hyaluronate lyase of *S.agalactiae* strain 4755 *vs*. the bovine testicular hyaluronidase. 3-Acylated benzoxazole-2-thiones inhibited $hylB_{4755}$ with IC_{50} values in the µM range. Interestingly, the thiones were much more potent than the corresponding oxo analogues. Within this series, 3-phenyl-1-(2-thioxo-benzo[*d*]oxazol-3(2*H*)-yl)propan-1-one was the strongest inhibitor of $hylB_{4755}$ with an IC_{50} value of 15 µM at optimum pH. The 3-phenylpropanoyl proved to be the optimal substituent at the benzoxazole-N. This result is in good agreement with the suggestions from molecular modeling studies. Because of the sensitivity of 3-phenylpropanoyl-benzoxazole-2-thione against moisture (hydrolysis of the labile amide bond) various structural modifications were made. But replacing the alkanoyl with a sulfonyl or an alkyl group or using a benzofurane instead of the benzoxazole moiety resulted in a marked decrease in inhibitory activity on $hylB_{4755}$. In summary, the 3-acylated benzoxazole-2-thione derivatives were successfully introduced as a new class of bacterial hyaluronidase inhibitors. The applicability of the *de-novo* design strategy starting from the 3D structure of hylSpn was confirmed by the structure-activity relationships of benzoxazole-2-thiones, especially, by the identification of the 3-phenylpropanoyl derivative as a potent hyaluronate lyase inhibitor.

Based on the analysis of the binding mode of the aforementioned 2-phenylindoles and stimulated by the successful design of benzimidazole- and benzoxazole-type hyaluronidase inhibitors a set of additional indole derivatives was pre-

pared and screened for activity on $hylB_{4755}$ and BTH. Generally, the investigated indoles showed a preference for the bacterial hyaluronidase with highest activity residing in three *N*-alkylated 5-amino-2-methyl-indoles (inhibitory activities on $hylB_{4755}$ in the micromolar range at optimum pH). Different functional groups such as nitro, amino, urea, sulfonamide, carboxylic acid and sulfonic acid were introduced in position 5 of the indole scaffold. As a result only the amino group and the acidic groups seem to fulfill the structural requirements for potent inhibitors derived from indole moiety. Ionic interactions as well as hydrogen bonds may play an important role in this enzyme-inhibitor interaction. Surprisingly, the *N*-acylated 5-amino-2-methylindoles had only minor inhibitory activities. The synthesized 1,3-disubstituted 5-amino-2-methyl-indoles weakly inhibited $hylB_{4755}$. Compared to the benzoxazole-2-thione the indole structure proved to be a less promising scaffold for the synthesis of hyaluronidase inhibitors.

In summary, ligand-based and structure-based approaches led to the identification of hyaluronidase inhibitors with micromolar and submicromolar activities. Particularly, the molecular modeling based approach for the design of novel hyaluronate lyase inhibitors was very successful. Such hyaluronidase inhibitors may be valuable pharmacological tools for the investigation of the role of the enzyme and its substrate, hyaluronan, in many biological processes.

Chapter 9 Appendix

List of abbreviations

2D	two-dimensional
3D	three-dimensional
Ac	acetyl
AIDS	acquired immunodeficiency syndrome
Bn	benzyl
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
BTH	bovine testicular hyaluronidase
BVH	bee venom hyaluronidase
CADD	Computer-Aided Drug Design
$CDCl_3$	chloroform
CDI	*N*,*N*'-carbonyldiimidazole
CTAB	cetyltrimethylammonium bromide
Cy	cyclohexyl
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DMAP	*p*-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DSCG	disodium cromoglycate
DTT	dithiothreitol
EC	enzyme commission
GAG	glycosaminoglycan
GBS	group B streptococci
GPI	glycosylphosphatidylinositol
h	hour(s)

HA	hyaluronan
HAc	acetic acid
HPLC	high-pressure liquid chromatography
HR-MS	high resolution mass spectroscopy
HSAB	Hard Soft Acid Base
$hylB_{3502}$	hyaluronate lyase of *S. agalactiae* strain 3502
$hylB_{4755}$	hyaluronate lyase of *S. agalactiae* strain 4755
hylSpn	*S. pneumoniae* hyaluronidase
IC_{50}	concentration of an inhibitor required to give 50% inhibition of enzyme activity
IR	infrared spectroscopy
IU	international unit
kDa	kilo Dalton
Mp	melting point
MS	mass spectrometry
MsCl	methanesulfonyl chloride
n.d.	not determined
NMR	nuclear magnetic resonance
PAD	proton acceptance and donation
PG	protecting group
pH	negative logarithm of the hydrogen ion concentration
Ph	phenyl
ppm	parts per million
rms	root mean square
RT	room temperature
SAR	structure-activity relationships
SEM	standard error of the mean
TCDI	*N*,*N*'-thiocarbonyldiimidazole
TEBAC	tetrabutylammonium chloride
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSCl	trimethylsilyl chloride
UV	ultra violet

List of publications and abstracts

Botzki, A.; Rigden, D.J.; Braun, S.; Nukui, M.; Salmen, S.; Hoechstetter, J.; Bernhardt, G.; Dove, S.; Jedrzejas, M.J.; Buschauer, A.: L-ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor. X-ray structure and molecular modeling of enzyme-inhibitor complexes*. J. Biol. Chem.* **2004**, 279(44), 45990-7.

Braun, S., Botzki, A., Jedrzejas, M., Bernhardt, G., Dove, S., Buschauer, A.: Vitamin C derivatives as inhibitors of bacterial hyaluronidase*.* Jahrestagung der DPhG, Germany, 9.-12. October 2002, *Arch. Pharm. Pharm. Med. Chem.* 335, Suppl.1, **2002**.

Spickenreither M., Braun S., Botzki A., Jedrzejas M., Bernhardt G., Dove S., von Angerer E., Buschauer A., Design, *Synthesis and structure-activity relationships of acylated indoles and benzoxazoles as inhibitors of bacterial hyaluronidase*. Poster, 6th Carbohydrate Bioengineering Meeting, Barcelona, Spain, 3-6 April **2005**.

Spickenreither M., Braun S., Botzki A., Jedrzejas M., Bernhardt G., Dove S., von Angerer E., Buschauer A., *Acylated indoles and benzoxazoles as potential hyaluronidase inhibitors: Design, Synthesis and structure-activity relationships*, Poster, Frontiers in Medicinal Chemistry, Leipzig, Germany, 13-16 March **2005**.

Braun S., Botzki A., Schneider L., Bernhardt G., Buschauer A., *Structure-activity relationships of benzoxazole-2-thiones as inhibitors of bacterial hyaluronidase*, Poster, Jahrestagung der DPhG und Joint Meeting, Regensburg, Germany, 6-9 October **2004**.

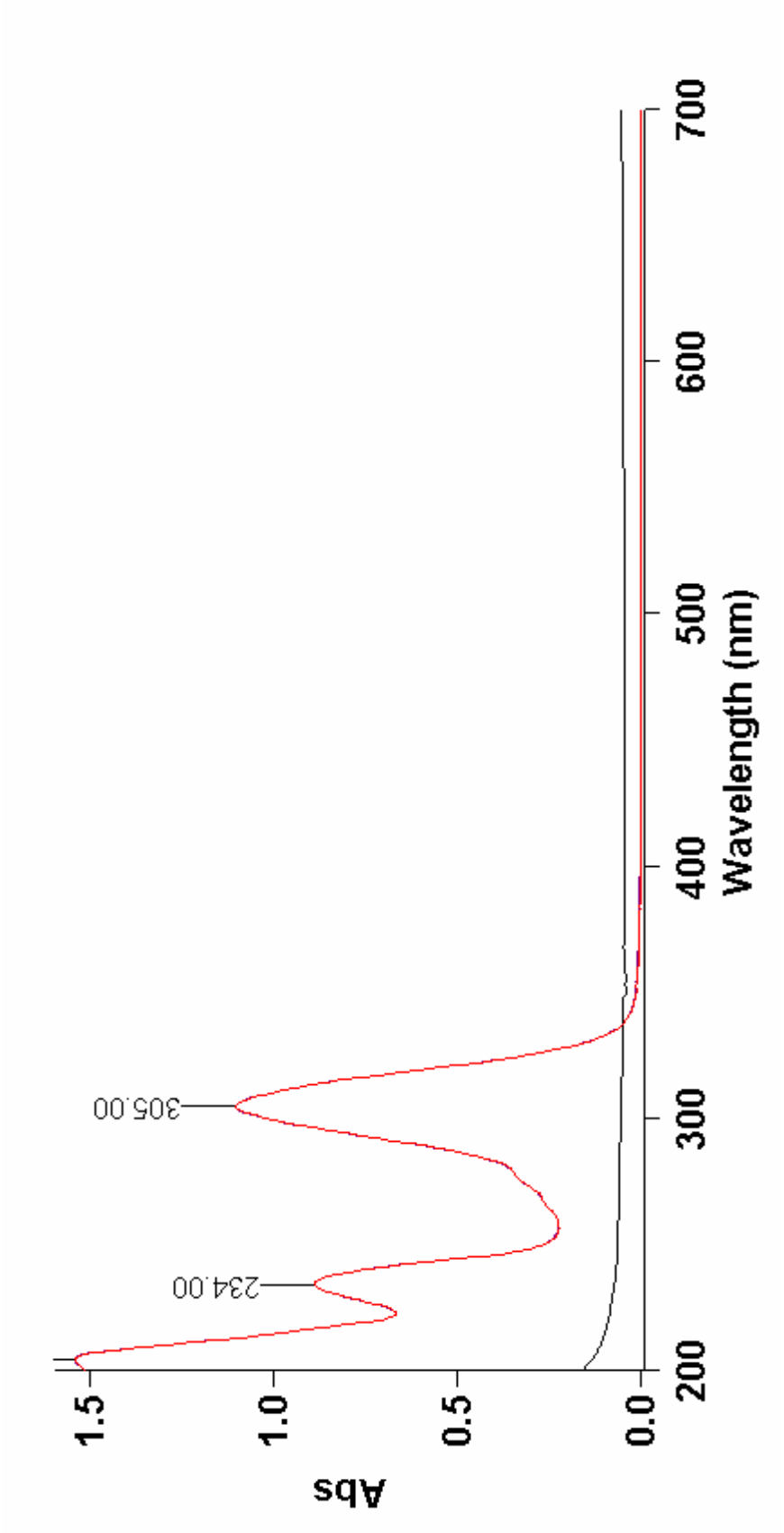
Braun S., Botzki A., Schneider L., Bernhardt G., Buschauer A., *Benzoxazole-2-thiones - potent inhibitors of bacterial hyaluronidase*, Poster, Frontiers in Medicinal Chemistry, Erlangen, Germany, 15-17 March **2004**.

Botzki A., Braun S., Ridgen D.J., Nukui M., Bernhardt G., Dove S., Jedrzejas M.J., Buschauer A., *L-ascorbic acid-6-hexadecanoate: a potent inhibitor of hyaluronidases*, Poster, Jahrestagung der DPhG, Würzburg, Germany, 8 - 11 October **2003**.

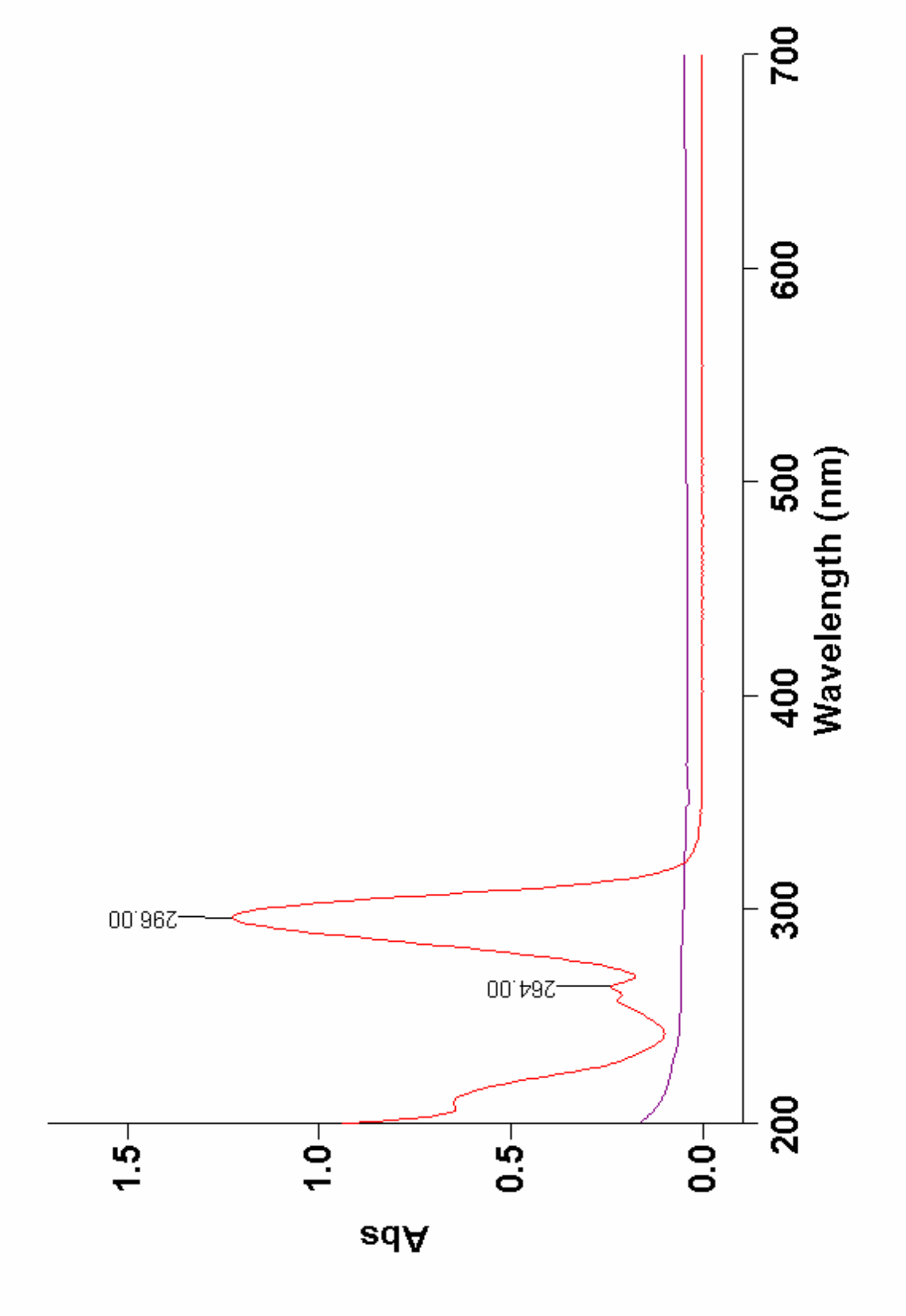
Braun S., Botzki A., Jedrzejas M.J., Bernhardt G., Dove S., Buschauer A., *Acylated ascorbic acids as inhibitors of hyaluronate lyase*, Poster, Summer School "Medicinal Chemistry" in Regensburg, Germany, 15 - 18 September **2002**.

Braun S., Botzki A., Jedrzejas M.J., Bernhardt G., Dove S., Buschauer A., *Vitamin C derivatives as hyaluronidase inhibitors with selectivity for bacterial lyases*, Poster, 17th International Symposium on Medicinal Chemistry, Barcelona, Spain, 1 - 5 September **2002**.

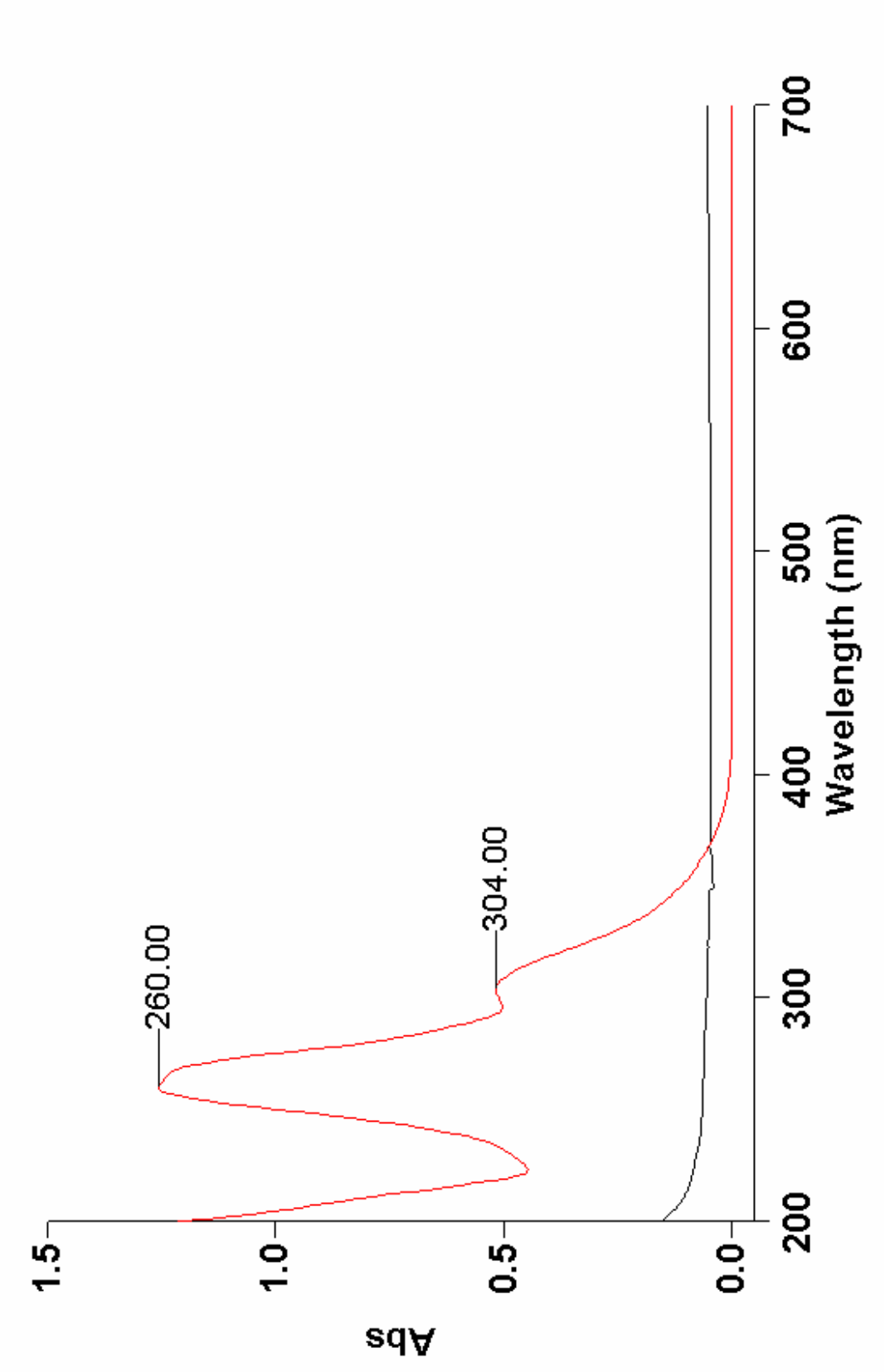
UV/VIS-spectra of **6.11** (50 µM in MeCN)



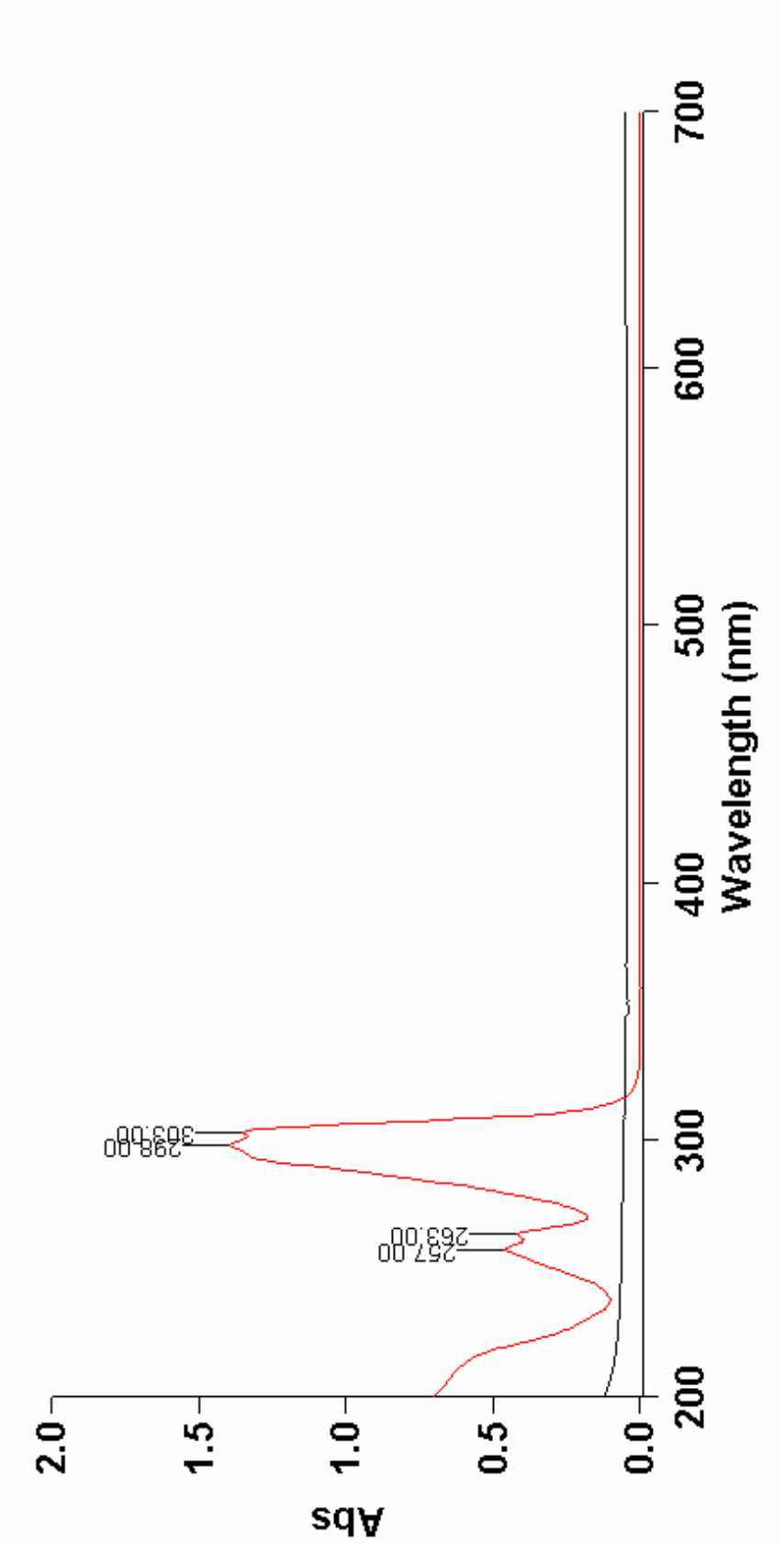
UV/VIS-spectra of **6.19** (50 µM in MeCN)



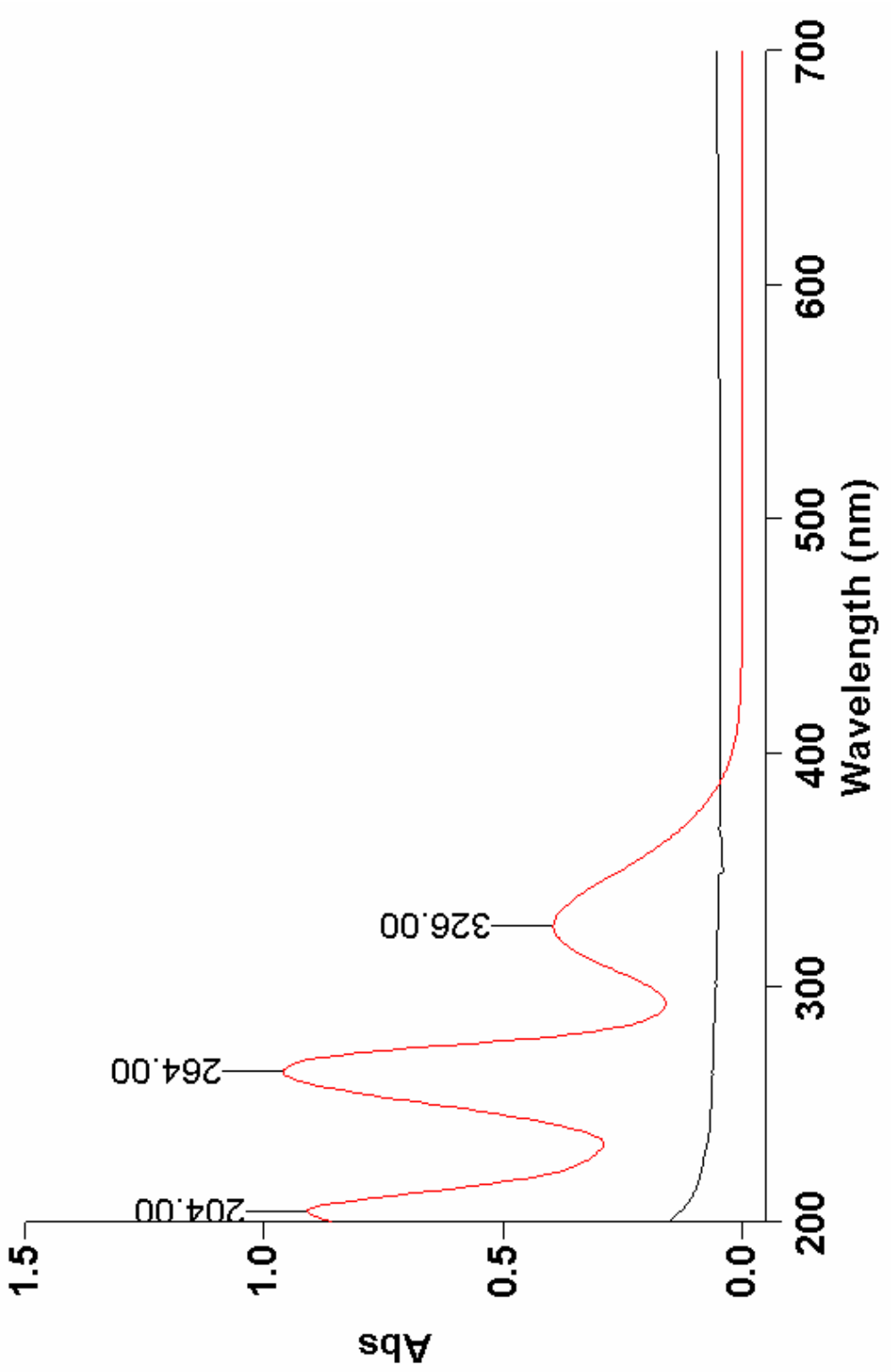
UV/VIS-spectra of **7.16** (50 µM in MeCN)



UV/VIS-spectra of **6.1** (50 µM in MeCN)



UV/VIS-spectra of **7.1** (50 µM in MeCN)



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