# Preparation, properties and pharmacological testing of sulfonic acid nitrophenyl esters:

## Towards the development of sulfonic acid prodrugs of P1 and P2 receptor antagonists with potential peroral bioavailability

**Dissertation**

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn


vorgelegt von

**Luo Yan**

aus

Jiangxi, China



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Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

| | |
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*With Love and Affection for*

*My husband and parents*

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# Chapter 1

# Introduction

## 1.1 History of purine and pyrimidine receptors

Physiological actions of adenosine were first described by Drury and Szent-Györgyi in 1929.[1] They found that adenosine and adenosine 5'-monophosphate (AMP) exert a large number of biological effects upon the mammalian heart, including lowering of arterial pressure, slowing of the rate of heart beating and impairing of the conduction from auricle to ventricle. In 1970, Sattin and Rall[2] reported that adenosine mediates an increase of cAMP level in slices of guinea pig brain, and the effect of adenosine could be blocked by methylxanthines. These results strongly suggested that adenosine receptors exist in the central nervous system (CNS).

Van Calker et al.[3] proposed the first subclassification of adenosine receptors. According to their results, adenosine could regulate the cAMP level in the brain cells of rats through two different receptors: $A_1$ and $A_2$ receptors. The $A_1$ receptor subtype mediates the **i**nhibition of the accumulation of cAMP, and the $A_2$ receptor subtype mediates the **s**timulation of the accumulation of cAMP. At the same time, Londos et al. similarly proposed two receptor subtypes as $R_i$- and $R_a$-receptors.[4] In this case, R stands for ribose since a virtually intact ribose moiety was needed for the adenylate cyclase activity. The *"i"* stands for the **i**nhibition of adenylate cyclase activity, while *"a"* stands for the **a**ctivation of adenylate cyclase activity.

The $A_2$ receptor was further divided into two receptor subtypes by Daly et al. in 1983.[5] Based on the adenylate cyclase (AC) assay, they postulated that there was a high-affinity $A_2$ receptor (localized in striatal membranes) and a low-affinity $A_2$ receptor (overall) for adenosine in rat brain. Almost at the same time, Elfmann et al.[6] reported that a high-affinity $A_2$ receptor in cultured neuroblastoma cells and a low-affinity $A_2$ receptor in glioma cells was found. Later on, the existence of these receptors was proven

*via* the cloning and sequencing from various species including rat, mouse and human. In 1986, Bruns et al. proposed the terms $A_{2A}$- and $A_{2B}$-receptors based on labelling experiments with [$^3$H]NECA of $A_{2A}$ receptors in rat striatal membranes.[7]

Unlike the $A_1$, $A_{2A}$ and $A_{2B}$ receptors, which were first discovered and classified by studying of agonist pharmacology, followed by confirming their existence and classification by cloning, sequencing and expression, the $A_3$ receptor was first discovered by molecular biology studies in 1992 from rat striatal cells by Zhou et al.[8] Like the $A_1$ receptor, the $A_3$ receptor is also coupled to adenylate cyclase in an inhibitory manner.[9]

In 1992, Cornfield et al.[10] proposed an $A_4$ receptor subtype based on the pharmacological results of agonist binding profiles. But there was also evidence disputing this proposal.[11] So the existence of a new adenosine receptor subtype is still unclear, and needs to be further examined.

Simultaneously with the discovery of adenosine receptors, Gillespie[12] gave the first indication of different actions of ATP in 1934 after research on the structure-activity relationships of adenine compounds. He concluded that ATP causes the increase in blood pressure in rabbit and cat, which was rarely or never observed with AMP or adenosine.

Holton[13] reported in 1959 that ATP was released to produce vasodilatation of rabbit ear arteries during antidromic stimulation of sensory nerves. This result gave the first hint that ATP might be a neurotransmitter. Two years later Gaarder et al.[14] identified ADP as an active component in red blood cell extract, which gave the first indication of the presence of receptors for ADP.

In 1972 Burnstock[15] postulated that ATP was released as the principal neurotransmitter from some non-adrenergic, non-cholinergic (NANC) nerves, and these nerves were tentatively termed "purinergic". In 1978 he made an important suggestion[16] that there are two classes of purinergic receptors in membranes of peripheral cells: namely P1-purinoceptors, which are selective for adenosine, act through adenylate cyclase, and are antagonized by lower concentrations of methylxanthines than those which produce phosphodiesterase inhibition; P2-purinoceptors, which are selective for adenine nucleotides. Pyrimidine receptors (UTP, UDP) were later found in 1993 by Lin et al in NG108-15 cells.[17]

P2-purinoceptors were suggested to be further divided into P2X and P2Y subtypes by Burnstock and Kennedy in 1985,[19] based on the rank order of agonist potency of structural analogues of ATP and also on the activity of antagonists at the P2-purinoceptors.

The detailed classification of P1 and P2 receptors will be discussed in the following chapter.

## 1.2 Overview of purine and pyrimidine receptors

Membrane receptors for physiological purine and pyrimidine derivatives are subdivided into two separate families:[18] purine P1 receptors or adenosine receptors (AR), which are the only extracellular nucleoside membrane receptors described so far; purine and pyrimidine P2 receptors, a big family of nucleotide receptors with ATP, ADP, UDP and/or UTP or other nucleotides as physiological agonists.

P1 receptors were further divided into four subtypes according to their genetic and pharmacological character. They are $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ adenosine receptors (ARs).

P2 receptors were subdivided into two families based on the signal transduction mechanisms and molecular structure:[20],[21] a P2X receptor family consisting of ligand-gated cation channels; and a P2Y receptor family consisting of G-protein coupled receptors. Up to now, seven mammalian P2X receptor subunits $P2X_{1-7}$, and eight P2Y receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ have been cloned, pharmacologically characterized and accepted as valid members of the P2 receptor family. Table 1.1 shows the overview of the purine and pyrimidine receptors.

**Table 1.1:** *Purine and pyrimidine receptors*[22],[23]

| | P1 receptors (Nucleoside Receptors) | P2 receptors (Nucleotide Receptors) | |
|---|---|---|---|
| Natural Agonists | Adenosine | ATP, ADP, UTP, UDP and diadenine nucleotides | |
| Subgroups | | **P2X** | **P2Y** |
| Type | G-protein coupled | Ion channel | G-protein coupled |
| Subtype | $\mathbf{A_1}, \mathbf{A_{2A}}, \mathbf{A_{2B}}, \mathbf{A_3}$ | $\mathbf{P2X_{1-7}}$ | $\mathbf{P2Y_1}, \mathbf{P2Y_2}, \mathbf{P2Y_4}$ $\mathbf{P2Y_6}, \mathbf{P2Y_{11}}, \mathbf{P2Y_{12}}$ $\mathbf{P2Y_{13}}, \mathbf{P2Y_{14}}$ |

## 1.3 P1 (adenosine) receptors

Adenosine (P1) receptors belong to the superfamily of G-protein coupled receptors.[18] Up to now, four subtypes $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ have been identified. All subtypes are coupled with adenylate cyclase (AC) through G-proteins. $A_1$ and $A_3$ ARs preferably inhibit adenylate cyclase (AC) *via* $G_{i/o}$ proteins, whereas $A_{2A}$ and $A_{2B}$ ARs activate adenylate cyclase (AC) *via* $G_s$ proteins.[24],[25] Table 1.2 gives a summary of the subtypes of ARs.

**Table 1.2:** *Subtypes of adenosine receptors (P1)*[26]–[30]

| | $A_1$ | $A_{2A}$ | $A_{2B}$ | $A_3$ |
|---|---|---|---|---|
| **Selective agonists** | CHA , CPA | CGS21680, APEC | / | PENECA, AB-MECA |
| **Selective antagonists** | DPCPX, KFM-19, XAC, KW-3902 | CSC, MSX-2, ZM-241385 | / | PSB-11, MRS1067, MRS-1097 |
| **G-Protein coupling** | $G_{i/o}$ | $G_s$ | $G_s$ | $G_i$, $G_q$ |
| Effectors | ↓ cAMP | ↑ cAMP | ↑cAMP | ↓cAMP |
| Phospholipase C: | ↑ $IP_3$ | | ↑ $IP_3$ | ↑ $IP_3$ |
| Ion channels: | ↑ $K^+$, ↓ $Ca^{2+}$ | | | |
| **Tissue distribution in humans** | | | | |
| High levels | Brain (hippocampus, cortex) | Brain (striatum, nucleus accumbens), olfactory tubercle | Caecum, large intestine, urinary bladder | Liver, lung |
| Low levels | Heart, kidney, lung, testis, fat cells | Liver, lung, heart, kidney | Brain, fibroblasts, heart, lung | Brain, heart, testis, kidney |
| **Potential therapeutic application** | | | | |
| Therapeutic application for agonists | Pain, antiepileptic, neuroprotective, | Vasodilator, antihypertensive, antiinflammatory | Antiinflammatory, septic shock | Cardioprotective, antiinflammatory, asthma |
| Therapeutic application for antagonists | Alzheimer's disease, antihypertensive, asthma | Morbus Parkinson, hypotension | Asthma, type II diabetes, Morbus Alzheimer | Stroke, glaucoma, asthma |

Adenosine receptors show ubiquitous distribution and are involved in a variety of biological processes. The effects of activation of ARs by endogenous ligands can be modulated by agonists and antagonists.

All adenosine receptor agonists are derivatives of the nucleoside adenosine (Figure 1.1), three positions in the molecule may be modified to increase affinity to specific receptor subtypes without destroying the agonistic activity: these are the 2- and $N^6$-positions of the purine and the 5'-position of the ribose. The agonistic effects of different substitutions at different positions were reviewed recently by experts.[26], [31]

In contrast to agonists, adenosine receptor antagonists have different structures. Normally they are divided into xanthine and non-xanthine derivatives. The first reported xanthine antagonists were the naturally occurring caffeine and theophylline.[32] Until now

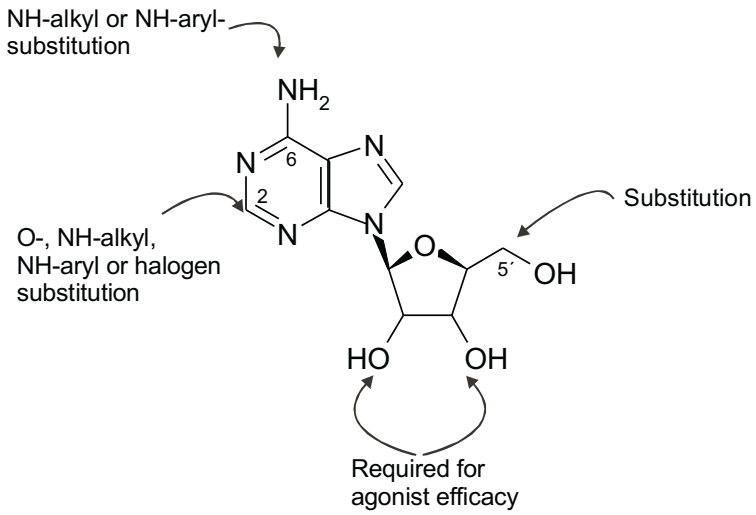



**Figure 1.1:** *Structure of adenosine, showing the effects of structural modifications at various sites on receptor binding*

a large number of xanthines has been synthesized in the quest of potent and selective ligands, among which selective antagonists for $A_{2B}$- and $A_3$-ARs are still sparse, since $A_{2B}$ is a low affinity receptor and $A_3$ is quite a new subtype. Antagonists and agonists for both of these two AR subtypes are to be further studied. Experts have published a lot of reviews about the antagonists of each subtype of ARs.[27], [33]–[37]

J. W. Daly[38] gave the first prospective review about the potential use of adenosine receptors as drug targets in 1982. After that, different reviews about the therapeutic aspects of the agonists and antagonists of adenosine receptors have been published.[39]–[46]

Despite great success in finding new antagonists, only a few xanthine antagonists have been approved as drugs, including the well-known caffeine and theophylline, useful for their CNS-stimulating, diuretic and bronchodilating effects, respectively. Their effects are mainly mediated by $A_{2A}$ and $A_1$ receptor antagonism, perhaps also by $A_{2B}$ antagonism. DPCPX is in clinical trials as an orphan drug for the treatment of cystic fibrosis.[28], [29] Further selective adenosine receptor ligands are currently in clinical development.

## 1.4 P2 receptors

P2 receptors are subdivided into two main classes: P2X and P2Y subtypes. The P2X receptors belong to the ligand-gated cation channel family, whereas the P2Y receptors are members of the seven transmembrane G-protein coupled receptor (Table 1.3).[47]

Almost all current synthetic P2 agonists are variations of the physiological nucleotides

ATP or UTP (Figure 1.2) with modifications at one or more positions in the purine or pyrimidine ring system, the ribose moiety, or the triphosphate chain. Aim of these modifications is usually to improve the ligand's potency, enzymatic stability and/or receptor subtype selectivity.[48]

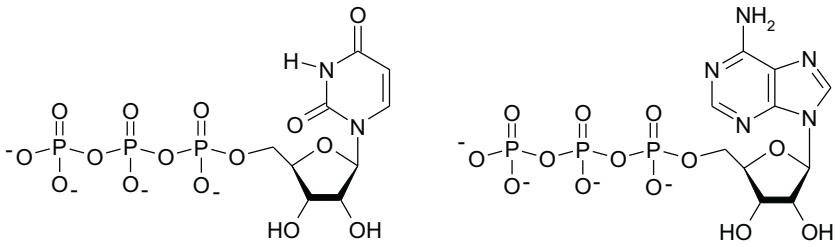



**Figure 1.2:** *Structures of UTP and ATP*

Antagonists at these receptors are structurally more diverse, ranging from polyaromatic polysulfonated suramin, NF023, PPADS etc. to various 2'- or 3'-deoxy adenosine bisphosphate derivatives, e.g. MRS 2179 (Figure 1.3). Most of these compounds exhibit low potency, and only marginal selectivity for one type of P2 receptor over another. Besides this, they may interact with other nucleotide (ATP) binding sites and modulate the activity of other receptors, thus their usefulness is limited.[49]

In comparison with the adenosine receptors, much less is known about the specific effects of P2 receptors. Studies of P2 receptors are hindered by several factors, e.g. general lack of selective and effective agonists and antagonists, the coexistence of different P2 receptors, fast enzymatic nucleotide degradation and interconversion,[48] and the lack of reliable binding assays, although functional fluorescent imaging (FLIPR) in cell lines transfected with rat or human P2 receptors has proven to be useful.[50]

A lot of experts published reviews about P2 receptors, including agonists, antagonists, and their potential therapeutical applications.[48], [49], [51]–[55]

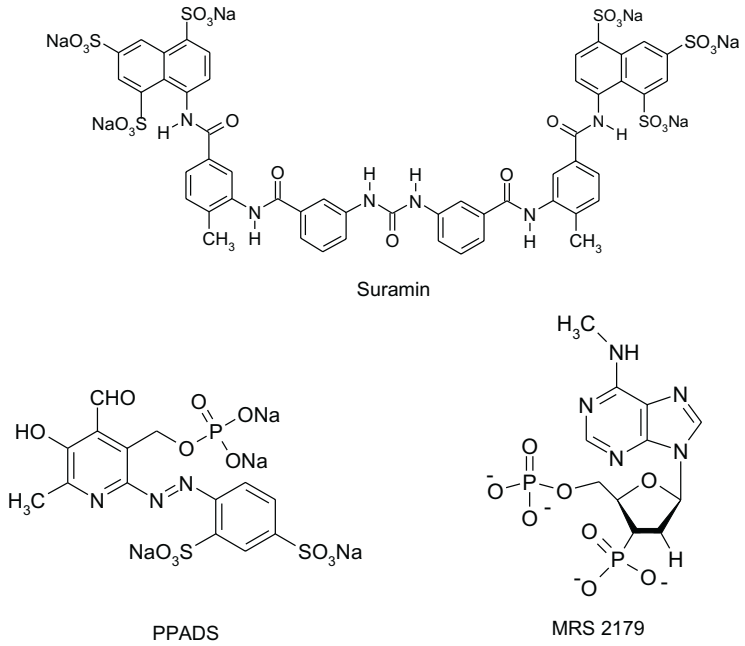



**Figure 1.3:** *Structures of P2 antagonists (selected)*

**Table 1.3:** *Subtypes of P2 receptors*[25]

| | | **P2X** | **P2Y** |
|---|---|---|---|
| **Receptor type** | | Ion channel | G-Protein coupled |
| **Signaling pathway** | | / | PLC, AC, $PLA_2$, $K^+$ channels, PLD, PKC, MAPK |
| **Effectors** | | $Ca^{2+} \gg Na^+ \gg K^+$ | $\uparrow IP_3$, $\uparrow Ca^{2+}$, ↑ DAG, ↓cAMP |
| **Agonists(selected)** | **Nonselective** | ATP, ATPγS, 2 MESATP, $Ap_4A$ | ATP, ATPγS, 2 MESATP, $Ap_4A$ |
| | **P2X/P2Y-selective** | $\alpha$, $\beta$-meATP, $\beta$,$\gamma$-meATP, BzATP | ADP, UTP, UDP, 2Cl-ADP, 2MeSADP, ADP$\beta$S |
| **Antagonists (selected)** | **Nonselective** | Suramin, PPADS, Reactive Blue 2 | Suramin, PPADS, Reactive Blue 2 |
| | **P2X/P2Y-selective** | NF023, NF279, KN-62 | ARL67085, MRS2179, 2-cyclohexylthio-ATP |

# Chapter 2

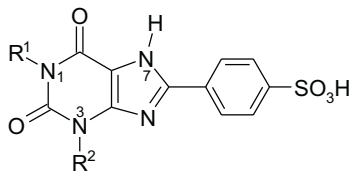
# Introduction of the project

## 2.1 Introduction of P1 antagonists containing sulfonic acid groups

Up to now, a series of mono- and di-substituted 8-phenylxanthine derivatives were synthesized and their potency and selectivity as antagonists at $A_1$-, $A_{2A}$-, $A_{2B}$- and $A_3$-adenosine receptors were evaluated. One obstacle that inhibits the progress of these antagonists in drug application is their low bioavailablity due to their hydrophobicity and low water-solubility, and attempts to generate hydrophilic antagonists usually results in a substantial loss of affinity.[29]

Daly et al.[56] had the idea of introducing polar substituents in the 8-phenyl position of xanthines. 8-*p*-Sulfophenyltheophylline has a very good water solubility which reaches more than 20 mM. So far, different 1,3-substituted derivatives of 8-*p*-sulfophenylxanthine have been synthesized.[57]–[59],[63] Figure 2.1 gives some examples of 1-monosubstituted and 1,3-disubstituted 8-*p*-sulfophenylxanthine which have been reported up to now.

Comparing the $K_i$ values of these 1-monosubstituted and 1,3-disubstituted 8-*p*-sulfophenylxanthines with those of the corresponding 8-phenylxanthine derivatives (Table 2.1), it is obvious that the introduction of a *p*-sulfo substituent in the 8-phenyl xanthine decreases the $A_1$ and $A_{2A}$ affinity heavily, and the $A_{2B}$-affinity was also decreased. For example, if we compare 1-propyl-8-phenylxanthine with 1-propyl-8-*p*-sulfophenylxanthine[63] (PSB-1115), the introduction of a *p*-sulfo acid group results in a loss of affinity at the receptors at $A_1$: 71-fold, $A_{2A}$: 13-fold and $A_{2B}$: 11-fold. So attempts to generate these hydrophilic antagonists usually result in a substantial loss of receptor affinity. But the $A_1/A_{2B}$ receptor selectivity increases from 7-fold to 41-fold, and the $A_{2A}/A_{2B}$ selectivity increases from 97-fold to 453-fold. Therefore the *p*-sulfo substitution in the




| $R^1$ | $R^2$ | Name |
|---|---|---|
| Methyl | Methyl | 1,3-Dimethyl-8-(*p*-sulfophenyl)xanthine |
| Ethyl | Ethyl | 1,3-Diethyl-8-*p*-(sulfophenyl)xanthine |
| Propyl | Propyl | 1,3-Dipropyl-8-*p*-(sulfophenyl)xanthine |
| Propyl | H | 1-Propyl-8-(*p*-sulfophenyl)xanthine |
| Butyl | H | 1-Butyl-8-(*p*-sulfophenyl)xanthine |
| H | Methyl | 3-Methyl-8-(*p*-sulfophenyl)xanthine |
| H | Propyl | 3-Propyl-8-(*p*-sulfophenyl)xanthine |

**Figure 2.1:** *Examples of 1,3-substituted 8-p-sulfophenylxanthines*

**Table 2.1:** *Comparison of $K_i$ values of 1-monosubstituted or 1,3-disubsustituted 8-phenylxanthines and 1-monosubstituted or 1,3-disubsustituted 8-p-sulfophenylxanthines*

| **Compound** | **$K_i$[nM] or % inhibition of radioligand binding at 10 $\mu$M** | | | |
|---|---|---|---|---|
| | $A_1$ | $A_{2A}$ | $A_{2B}$ | $A_3$ |
| 1,3-Dimethyl-8-phenylxanthine | 89[65]a(86[60]a) | 830[65]a(850[60]a) | n.d. | n.d. |
| 1,3-Dimethyl-8-(*p*-sulfophenyl)xanthine | 14000[60]a | 14000[60]a | 1200[59]b | 11000[59] |
| 1,3-Dipropyl-8-phenylxanthine | 10[60]a | 180[60]a | n.d. | n.d. |
| 1,3-Dipropyl-8-(*p*-sulfophenyl)xanthine | 210[60]a | 1400[60]a | 250[61]c | 183[62]d (90100[66]e) |
| 1-Propyl-8-phenylxanthine | 67[64]a(31[63]f) | 1900[64]a(458[63]f) | 4.7[63]f | n.d. |
| 1-Propyl-8-(*p*-sulfophenyl)xanthine | 2200[64]a | 24000[64]a | 53.4[63]f | 14 %[63]f |
| 1-Butyl-8-phenylxanthine | 40[63]f | 642[63]f | 11.8[63]f | n.d |
| 1-Butyl-8-(*p*-sulfophenyl)xanthine | 475[63]f | 8070[63]f | 70[63]f | 39 %[63]f |

n.d. not determined

[a] [$^3$H]PIA was used as $A_1$-radioligand and [$^3$H]NECA as $A_{2A}$-radioligand, at rat cortical and striatal brain membrane preparations respectively.

[b] Inhibition of adenosine-induced stimulation of adenylate cyclase in human-CHO cell membranes.

[c] Inhibition of NECA-induced stimulation of adenylate cyclase in human fibroblast cells.

[d] Sheep $A_3$ receptors, [$^{125}$I]ABA as $A_3$-radioligand.

[e] [$^{125}$I]ABA-MECA as the $A_3$-radioligand, rat-CHO cell membranes.

[f] [$^3$H]CCPA was used as $A_1$-radioligand, [$^3$H]MSX-2 as $A_{2A}$-radioligand, [$^3$H]ZM241385 as $A_{2B}$-radioligand and [$^3$H]PSB-11 as $A_3$-radioligand; $A_1$and $A_{2A}$: rat cortical and striatal membrane preparations respectively, $A_{2B}$and $A_3$: human-CHO cell membranes.

8-phenylxanthine decreases the affinity at ARs, but the selectivity for $A_{2B}$ is greatly enhanced. PSB-1115 is one of the most selective $A_{2B}$ antagonists described to date. The sulfonic acid group appears to contribute to the high selectivity of the compound versus the other AR subtypes, especially *versus* $A_1$ ARs.

Compared with other xanthines which are badly water-soluble, these 8-*p*-sulfophenylxanthine derivatives have much better parenteral bioavailability because of their excellent water solubility, thus they play a role as very important pharmacological tools. Sulfophenylxanthine derivatives are deprotonated under physiological condition due to the low $pK_a$ ($pK_a < 1$[67]) value of free sulfonic acid groups, which means that they do not penetrate into the central nervous system (CNS) and are only peripherally active, thus the central stimulant properties of theophylline and caffeine can be avoided.[68], [70] And because of their polarity, they will not penetrate into cells and hence will not affect phosphodiesterases or calcium-release channels.[71] Due to their polar character they might probably only locally exert their activity in the intestine if perorally applied. For systemic effects they are only parenterally applicable and can not be applied as peroral drugs.

8-*p*-Sulfonamidophenylxanthines (Figure 2.2) were first synthesized by Hamilton et al.[57]They concluded that sulfonamidophenylxanthines exhibit higher affinity for adenosine receptors in the rat brain than free 8-*p*-sulfophenylxanthines. These sulfonamide derivatives are expected to be soluble across a wide pH range and have a potential to be well absorbed because of their amphoteric nature.

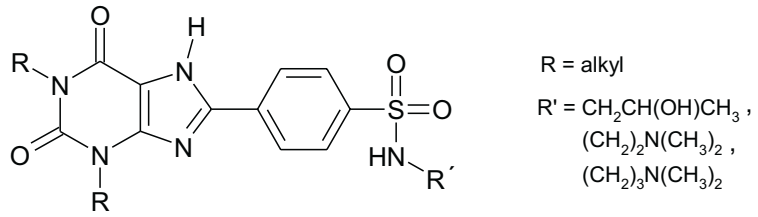



**Figure 2.2:** *1,3-Disubstituted-8-p-sulfoamidophenylxanthine derivatives*

After that publication,[57] no further research on these *p*-sulfonamidophenylxanthines has been reported, especially no research on the 1-monosubstituted sulfonamidophenylxanthine derivatives. However, 1,8-disubstituted xanthine derivatives have now become of particular interest due to their high $A_{2B}$ adenosine receptor affinity and selectivity.[63]

## 2.2 Introduction of P2 antagonists containing sulfonic acid groups

So far, seven P2X ($P2X_{1-7}$) and eight P2Y receptors ($PY_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been cloned and expressed, they are distinct molecular entities that elicit functional responses. Pharmacological characterization of P2 receptors has generally been based on the rank order of their activation by agonists related to ATP and UTP since the majority of available P2 receptor antagonists are relatively weak and only marginally selective for one P2 receptor subtype over another.[55] These antagonists also interact with other ATP recognition sites and with other receptor classes and signal transduction systems.[48]

Compared with the successful development of P1 antagonists, there has been a lacking of highly selective, competitive P2-purinoceptor antagonists. Novel P2 antagonists have been synthesized recently, belonging to different structural classes. If we compare the structure of these antagonists, we can see that most of them contain one or several anionic sulfonate groups from the oldest suramin up to the recent developments, such as PPADS, NF023 etc. (Figure 2.3).

Suramin (8-(3-benzamido-4-methylbenzamido)naphthalene-1,3,5-trisulfonic acid) is the most widely used P2 antagonist ever since its introduction by Dunn and Blakeley as a reversible P2 purinoceptor antagonist in the mouse vas deferens.[72] Actually it is a nonselective antagonist, since it has potency as antagonist for both P2X and P2Y receptors,[25] although its sensitivity at subtypes of P2X or P2Y is different.[73]–[76] Furthermore, suramin inhibits ecto-nucleotidase[77],[78] and neutral ecto-diadenosine polyphosphate hydrolase activity.[79] Besides, suramin is currently investigated in clinical trials against AIDS and cancer, due to its anti-angiogenic activity, thus it shows interesting pharmacological properites.[80]

NF023 (8,8'-carbonyl-bis-imino-3,1-phenylene-bis-1,3,5-naphtalene trisulfonic acid) is moderately selective as an antagonist of P2X receptors of the rat mesenteric arterial bed, rat bladder, rat and rabbit vas deferens, rat saphenous artery and aorta as well as heterologously expressed P2X receptors.[81]–[85] Like the parent compound suramin, NF023 inhibits ecto-nucleotidase activity.[77]

Reactive blue 2 is a non-competitive P2 receptor antagonist which does not discriminate adequately between P2X and P2Y subtypes.[25] It has micromolar affinity and some selectivity for endothelial $P2Y_1$ and smooth muscle $P2Y_1$-like receptors versus other vascular P2X and P2Y receptors.[86]

XAMR 0721 (3',5'-dinitro-phenylenecarbonylimino-1,3,5-naphthalene trisulfonic acid) inhibited the binding of ADP$\beta^{35}$S to turkey erythrocytes with relatively high potency ($K_i$

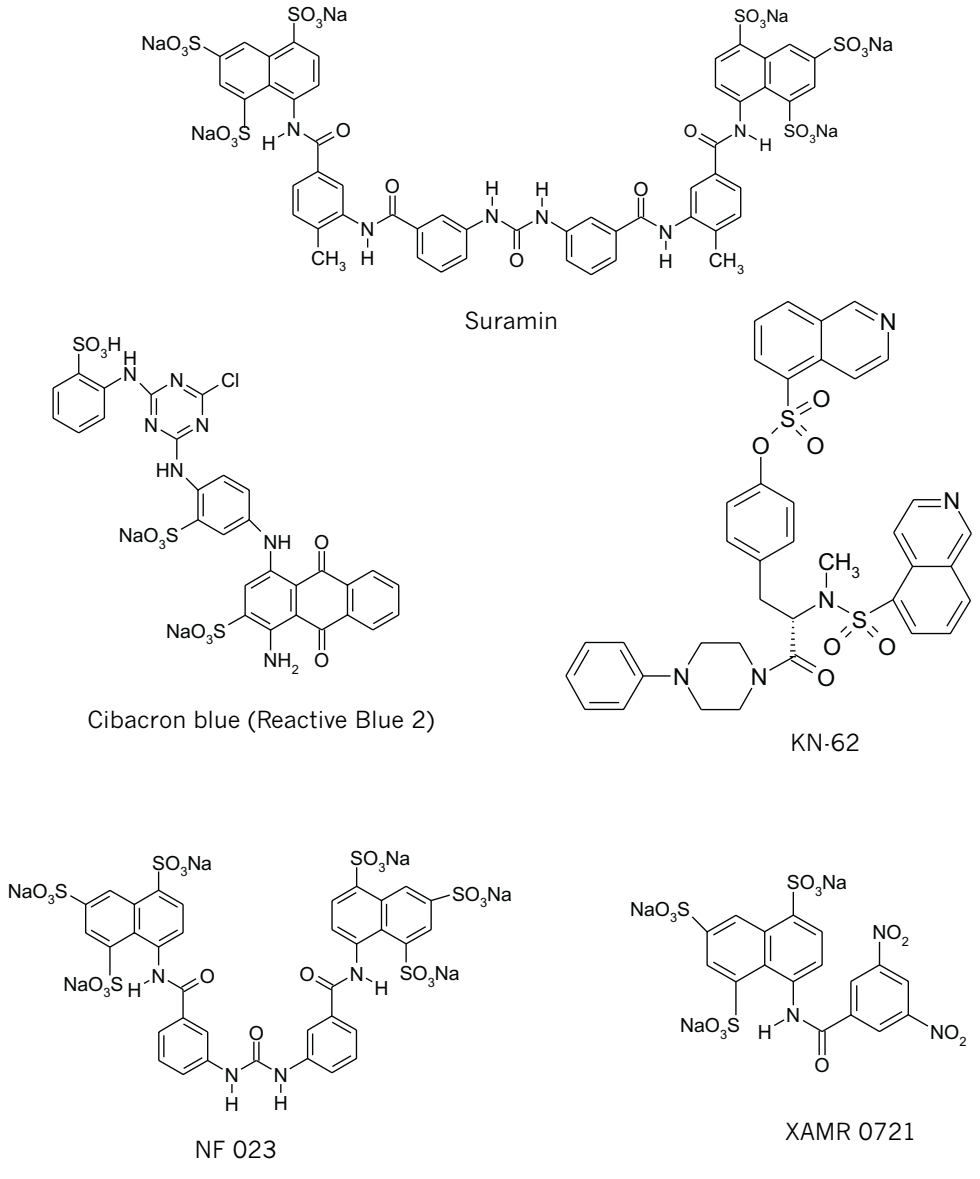



**Figure 2.3:** *Selected structures of P2 receptor antagonists*

19 $\mu M$).[87] But in the taenia coli, it antagonized ADP$\beta$S-induced, i.e. P2Y-receptor-mediated, relaxation only at 1000 $\mu M$.[88] The compound has very little affinity for the P2X receptor of rat vas deferens. It was also found that it does not inhibit ectonucleotidases.[77]

PPADS (pyridoxal-5'-phosphate-6-phenylazo-2,4-disulfonic acid) was originally put forward as a P2X-selective antagonists,[89]–[92] but unfortunately it has now to be accepted that it is in fact a non-selective (but non-universal) P2 receptor antagonist.[25]

KN62 (1-N,O-bis-1,5-isoquinolinesulfonyl-N-methyl-L-tyrosly-4-phenylpiperazine) is a calcium-calmodulin-dependent protein kinase-II (Camk-II) inhibitor,[93] it inhibits the ability of $P2Y_7$ receptors to activate phospholipase D in THP-1 monocytes.[94] It is also a potent antagonist of the $P2X_7$ receptor on human lymphocytes.[95], [96] Unlike suramin and analogues, which contain free sulfonic acid groups, it features two isoquinoline-5-sulfonyl moieties which act as an "ATP mimic",[97] and it seemed that this sulfonyl moiety plays an important role in its antagonistic activity.

## 2.3 Aim of the project

As discussed above, many pharmacologically active compounds contain polar sulfonate groups, which are in many cases essential for their pharmacological activities and/or selectivities. These compounds belong to the P1 and P2 receptor antagonists. Therefore the whole project is divided into two parts: P1 antagonists and P2 antagonists.

In the P1 field, 8-*p*-sulfophenylxanthine derivatives have favorable pharmacological properties, e.g. increased selectivity for $A_{2B}$ adenosine receptors, and exhibit high water-solubility, but on the other hand, they cannot be applied as peroral drugs, and are only parenterally applicable. Therefore we plan to make some structural changes on the sulfonic acid group, that is, introduce a prodrug concept for these sulfophenylxanthine derivatives. Prodrugs of these 8-*p*-sulfophenylxanthine derivatives should be applicable as peroral drugs. They should have the following characteristics: 1.) be more lipophilic than their parent drugs, thus they can better cross the cell membranes; 2.) since these prodrugs are designed as oral drugs, they should be able to resist the gastric acid media in the stomach and keep intact as prodrugs before they are absorbed and reach the target cell; 3.) they should be capable to release the parent active drugs *in vivo*, preferably by an enzymatic mechanism.

Sulfonamidophenylxanthine derivatives were also set as a synthesis aim of the P1 antagonists, since we want to investigate if differently substituted sulfonamidophenylxanthines possess different AR antagonistic activities. Thus we can have a wide overview about the antagonist properties of xanthines which contain sulfur, from free sulfonic acid,

sulfonate esters to sulfonamides.

In the P2 field, several novel P2 antagonists which contain anionic sulfonate groups have been reported, and it is proven that they are more or less selective at P2X or P2Y receptors. But because of the polarity of sulfonic acid, they are not able to cross the cell membrane, thus are only parenterally applicable. Therefore the design and synthesis of the prodrugs of these P2 antagonists was planned, in order to 1.) improve the *in vivo* applicability of these drugs; 2.) investigate whether the antagonistic activity at P2 receptors will be changed if the sulfonic acid is esterified to yield sulfonate esters.

# Chapter 3

# Stability tests of model sulfonate esters

## 3.1 Results and discussion

Two kinds of sulfonic acid derivatives are conceivable as potential prodrugs: sulfonamides or sulfonate esters. However, sulfonamides are generally very stable *in vivo*, the sulfonamide bond cannot be cleaved under physiological conditions. Compared with sulfonamides, sulfonate esters are relatively unstable at least *in vivo*, their instability greatly depends on the substitution pattern, therefore stable sulfonate esters might be obtained by suitable substitution.

As discussed above in chapter 2, one of the aims of the project can be described as follows (Figure 3.1). Esters of P1 and P2 antagonists bearing a sulfonate group are expected to be potential prodrug and/or antagonists with improved properties.

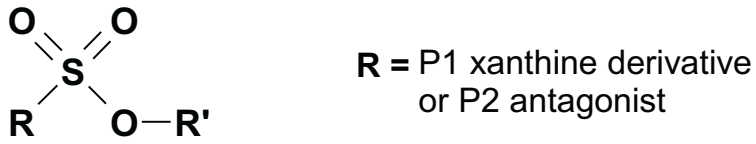



**Figure 3.1:** *Simplified target structure*

An important factor is the choice of the R' group. Before working on the aimed P1 and P2 antagonists, some model compounds were designed, that is, instead of the xanthine derivatives or P2 antagonists, which are normally big molecules, the simple structure of R = tosylate was chosen as a model compound.

Hydrolysis and stability tests of different sulfonates have been reported before.[98]–[103] What we can clearly see from these articles is that the nitrophenyl sulfonate ester is one of the most stable esters among differently substituted sulfonates. But there is no further

report about the comparison of the hydrolysis constants of *o*-, *m*- and *p*-nitrophenyl sulfonates under different pH values. Therefore the synthesis and stability tests of these model compounds, i.e. *o*-, *m*- and *p*-nitrophenyltosylate were first performed.

*o*-, *m*- and *p*-Nitrophenyltosylates were synthesized by reacting toluene sulfonyl chloride with *o*-, *m*- and *p*-nitrophenol in dichloromethane in the presence of triethylamine (TEA) as described,[104] and their stabilities were investigated.

Since these esters are not water-soluble, acetonitrile was used as a co-solute for the stability test. The ratio of acetonitrile to buffer was 10 : 90 and 5 : 95 respectively, in order to see if the co-solute acetonitrile has any significant effect on the hydrolysis rate. Five different pH values were tested, namely pH 1, 6, 7, 8 and 9.8, reaching from acidic to alkaline media. Reaction rates (K) were measured spectro-photometrically by following the appearance of the absorption due to released *o*-, *m*- and *p*-nitrophenolate. It was proven that all the hydrolysis were first-order reactions. Plots of log ($A_t$ -$A_0$) versus time were linear for three half-lives, the slopes yielding the rate constants. Figure 3.2 gives the rate constants (K) of the three sulfonate esters at 5 different pH values in acetonitrile : buffer (5 : 95) and acetonitrile : buffer (10 : 90).

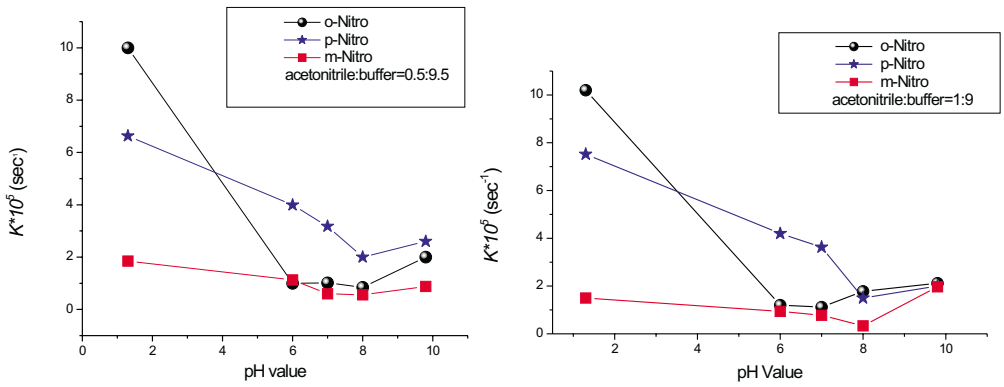



**Figure 3.2:** *Hydrolysis rate of o-, m- and p-substituted nitrophenyl tosylates in different media as a function of pH*

It was proven that the co-solute didn't significantly affect the hydrolysis of the sulfonate esters, since the K values were almost the same under the two conditions. In Figure 3.2 we can see that the *m*-nitrophenyl tosylate is the most stable ester among these three differently substituted sulfonate esters. Therefore the *m*-nitrophenol was used as the relevant phenol for the synthesis of the xanthine sulfonate prodrugs.

## 3.2 Experimental part

| | |
|---|---|
| Apparatus: | HP 8452A Diode-Array spectrophotometer |
| Buffers: | KCl-HCl buffer, 50 mM, pH 1 |
| | $KH_2PO_4$ buffer, 50 mM, pH 6, pH 7, pH 8 |
| | $H_3BO_3$-KCl, 50 mM, pH 9.8 |
| Wavelength: | 400 nm: *p*-nitrophenolate |
| | 376 nm: *m*-nitrophenolate |
| | 400 nm: *o*-nitrophenolate |

Esters were dissolved in a certain amount of acetonitrile, then diluted with different buffers in a ratio of 10 : 90 or 5 : 95, the final concentration reached $5 \times 10^{-5}$ M. Hydrolysis rate constants were measured by following the appearance of the hydrolysis product continuously at fixed wavelength using a HP 8452A Diode-Array spectrophotometer. All reactions were carried out under pseudo-first-order conditions while the buffer concentration was maintained in large excess over that of the esters. Rate constants were calculated as the slope of plots of log ($A_t$ -$A_o$) *vs.* time.

# Chapter 4

# Syntheses, stabilities and pharmacological tests of sulfonate esters of P1 (adenosine) receptor antagonists

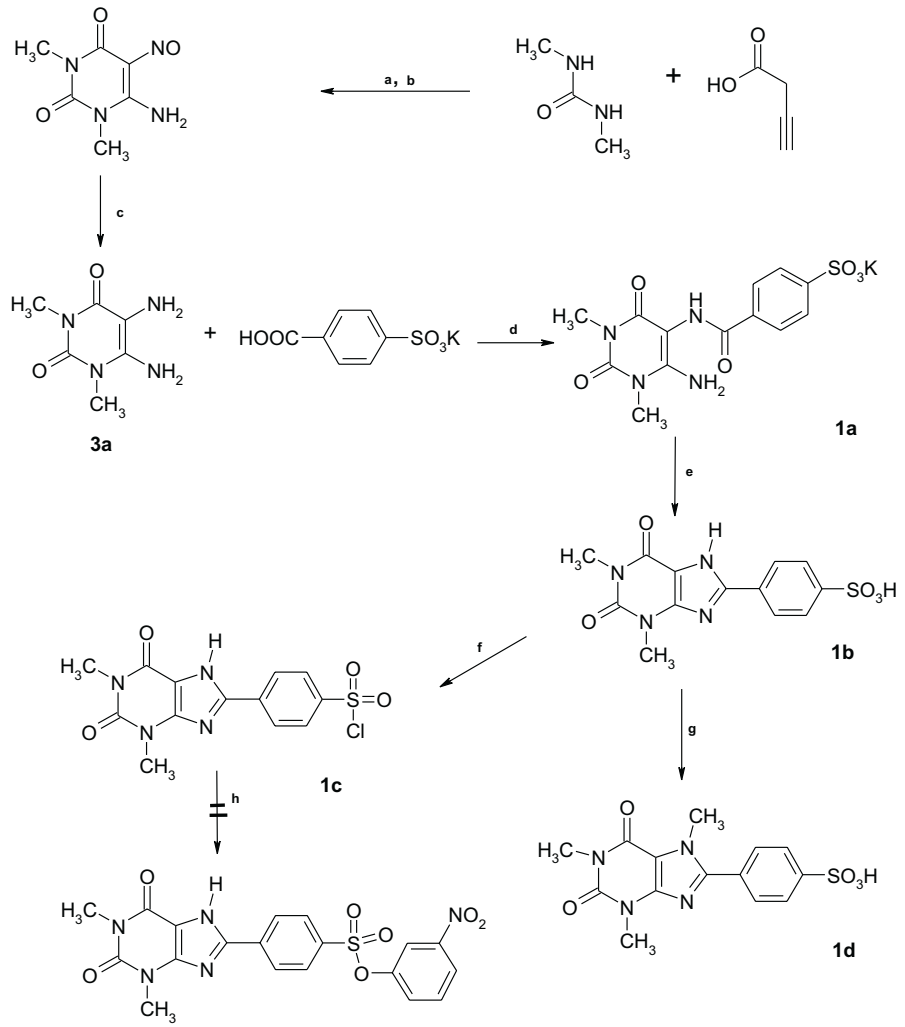
## 4.1 Syntheses of *m*-nitrophenylsulfonate esters of xanthine derivatives

The preparation of 5,6-diamino-1,3-dimethyluracil **3a** was carried out according to the classical synthetic route,[105],[106] i.e. condensation of 1,3-dimethylurea with cyanoacetic acid, ring closure by means of sodium hydroxide, nitrosation using sodium nitrite in acetic acid, followed by reduction with sodium dithionite in aqueous ammonia.

6-Amino-1,3-dimethyl-5-(*p*-sulfobenzamido)uracil **1a** was obtained when 5,6-diamino-1,3-dimethyluracil was reacted with the potassium salt of *p*-sulfobenzoic acid by using water-soluble N-dimethylaminopropyl-N'-ethylcarbodiimide-HCl (EDC) as the condensing agent. The uracil **1a** was cyclized to 1,3-dimethyl-8-*p*-sulfophenylxanthine **1b** by using 2.5 M NaOH solution.[56],[57]

Esterification was supposed to be performed by converting the 1,3-dimethyl-8-*p*-sulfophenylxanthine **1b** to chlorosulfonylphenylxanthine **1c**, followed by esterification with phenol or phenoxide under basic condition. In fact, chlorination of the sulfophenylxanthine was easy to be performed by refluxing it with thionyl chloride, which can be confirmed by the mass spectrum of the product. But subsequent esterification of the chlorosulfonylphenylxanthine **1c** with an excess of *m*-nitrophenol or sodium *m*-nitrophenoxide

could not be achieved even though different reaction conditions, e.g. triethylamine or pyridine as base catalysts, at different temperatures ranging from -30 °C to 30 °C, were tried (Figure 4.1).



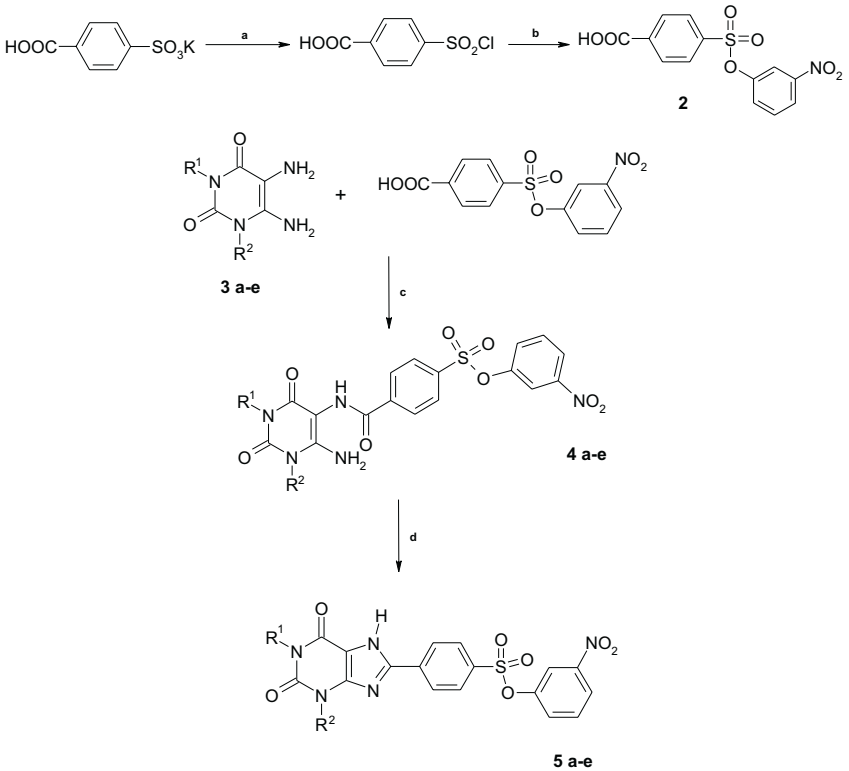

(a) anhydrous acetic acid, 80 °C, 2 h; (b) 1.) 10 % NaOH, 50 % EtOH, 85 °C, 2 hr; 2.) $NaNO_2$, 50 % acetic acid; (c) $Na_2S_2O_4$, 25 % $NH_3 \cdot H_2O$, 30 min; (d) EDC, water, 1 hr, 67 % yield; (e) 2.5 N NaOH, 10 min, 65 % yield; (f) $SOCl_2$, 2 h; (g) MeI, DMF, $K_2CO_3$, overnight, 35 % yield; (h) *m*-nitrophenol or sodium *m*-nitrophenoxide

**Figure 4.1:** *Attempted synthesis of xanthine m-nitrophenylsulfonate ester*

It was reported[107] that methylation of xanthine substituted in the 8-position with a naphthalene carboxylic acid using methyl iodide in the presence of potassium carbonate in DMF gave not only a methylation in the 7-position, but also a methyl ester of the carboxylic acid group. So methylation of **1b** by means of methyl iodide was tried as well, to see if the same reaction will happen when applied to a *p*-sulfophenylxanthine. But in

fact, only the 7-position of the xanthine was methylated, the para-position of the 8-phenyl substituent was still occupied by the free sulfonic acid group, 1,3,7-trimethyl-8-*p*-sulfophenylxanthine **1d** was obtained (Figure 4.1).

Since the normal way of esterification of sulfonic acids was not successful, an alternative synthesis was planned (Figure 4.2).




| Compd. | $R^1$ | $R^2$ | Yield (%) |
|---|---|---|---|
| **5a** | Methyl | Methyl | 40 |
| **5b** | Propyl | Propyl | 36 |
| **5c** | Methyl | H | 65 |
| **5d** | Propyl | H | 50 |
| **5e** | Butyl | H | 88 |

(a) 1.) $ClSO_2H$, overnight; 2.) chipped ice; (b) *m*-nitrophenol, THF, pH 8-9, 4 h; (c) EDC, MeOH, 2-3 h; (d) PPSE, 1-1.5 h.

**Figure 4.2:** *Synthesis of xanthine m-nitrophenylsulfonate esters*

First, *p*-sulfobenzoic acid potassium salt was chlorinated using chlorosulfonic acid under low temperature.[108],[109] Then this sulfonyl chloride was converted to 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2**.[110]

5,6-Diamino-1,3-disubstituted uracils **3a**, **3b** were synthesized from corresponding 1,3-

disubstituted urea with cyanoacetic acid according to the Figure 4.1. 5,6-Diamino-3-substituted uracils **3c** - **3e** were synthesized *via* alkylation of 6-aminouracil according to the reported methods.[111], [112]

Compound **2** was used to form the benzamido derivatives **4a** - **4e** with different 5,6-diamino-1,3-substituted uracil derivatives **3a** - **3e** utilizing EDC as the condensing agent.

Many different methods have been reported for the ring closure to get xanthines, e.g. with NaOH in MeOH,[56] with HMDS,[63] with PPSE,[63] or with triethyloxonium tetrafluoroborate (Meerwein's reagent).[113] At last it was found that refluxing of **4a** - **4e** in PPSE at 160 °C - 180 °C for 1 - 1.5 h, the desired xanthine sulfonate esters **5a** - **5e** could be obtained without destroying the sulfonate ester, since PPSE is a powerful, but mild condensing agent and has been used successfully in the closure of imidazole rings.[114]–[116]

## 4.2 Novel strategy for the synthesis of 1-substituted 8-*p*-sulfophenyl xanthine derivatives

The synthesis of 1-substituted 8-*p*-sulfophenylxanthine derivatives, such as 1-propyl and 1-butyl-8-*p*-sulfophenylxanthines has already been reported by using different methods.[63], [64] But actually it is difficult to practically handle the synthesis, since 1-substituted, 3-unsubstituted carboxamidouracils have a relatively low reactivity. It is difficult to perform the ring closure to get 1-substituted 8-*p*-sulfophenylxanthine derivatives, therefore the reaction conditions which have been reported were very hard, e.g. reflux with HMDS for more than 50 h. Besides, if the PPSE method which has been reported, was applied, it is difficult to isolate the desired product since PPSE and *p*-sulfophenylxanthine are both well soluble in water and methanol. The reproducibility of the reaction is not as good as expected. The 1-substituted 8-*p*-sulfophenylxanthines have a good pharmaceutical potential because of their high water-solubility and high selectivity at $A_{2B}$ ARs.[63] Thus gram amounts of the compounds were needed for further pharmacological investigation including animal experiments. But the required large amounts were difficult to be prepared if the reported methods were applied.

In order to find a convenient way to synthesize 1-substituted-8-*p*-sulfophenylxanthine derivatives, hydrolysis of the 1-substituted-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine was applied (Figure 4.3). Simultaneously the stability of these sulfonate esters towards aqueous alkaline solution was also investigated.

1-Methyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5c** and 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** were subjected to the hydrolysis. If the hydrolysis

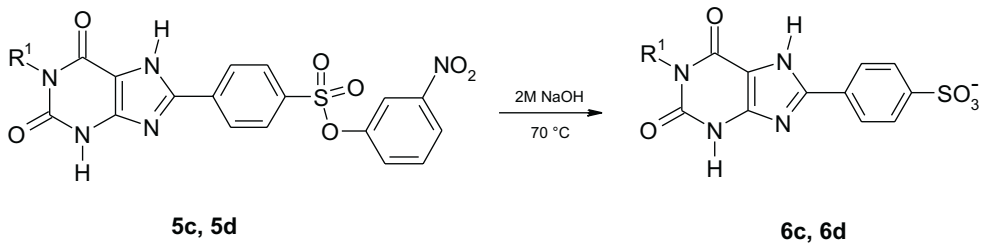



**Figure 4.3:** *Hydrolysis of 1-substituted xanthine m-nitrophenyl sulfonate esters to 1-substituted-8-p-sulfophenylxanthine derivatives.*

was allowed to proceed for 10 min in 2 M NaOH at 70 °C, the xanthine sulfonate esters still could be identified by TLC. Only when the hydrolysis time was prolonged to 20 min, the reaction was completely performed and no sulfonate ester could be identified by TLC. Yield of the isolated hydrolysis product reached ca. 80 %.

Besides the recrystallization from $H_2O$ for the purification of the 1-substituted 8-*p*-sulfophenylxanthines which has been reported,[63],[64] preparative HPLC was also used for the purification. Figure 4.4 shows the chromatogram of the purification of 1-propyl-8-*p*-sulfophenylxanthine **6d** with prepative HPLC.

At the same time, capillary electrophoresis was also used for the purity determination of 1,3-substituted-8-*p*-sulfophenylxanthines. Figure 4.5 shows the CE spectrum of the synthesized 1-propyl-8-*p*-sulfophenylxanthine **6d**. From this figure we can see that the purity of the synthesized sulfophenylxanthine derivative reaches 100 %.

Thus, hydrolysis of esters is a convenient practical method to get large gram amount of 1-substituted-8-*p*-sulfophenylxanthine derivatives. On the other side, we realized that these sulfonate esters are relatively stable, since the complete hydrolysis lasted as long as 20 min at 70 °C in 2 M NaOH.

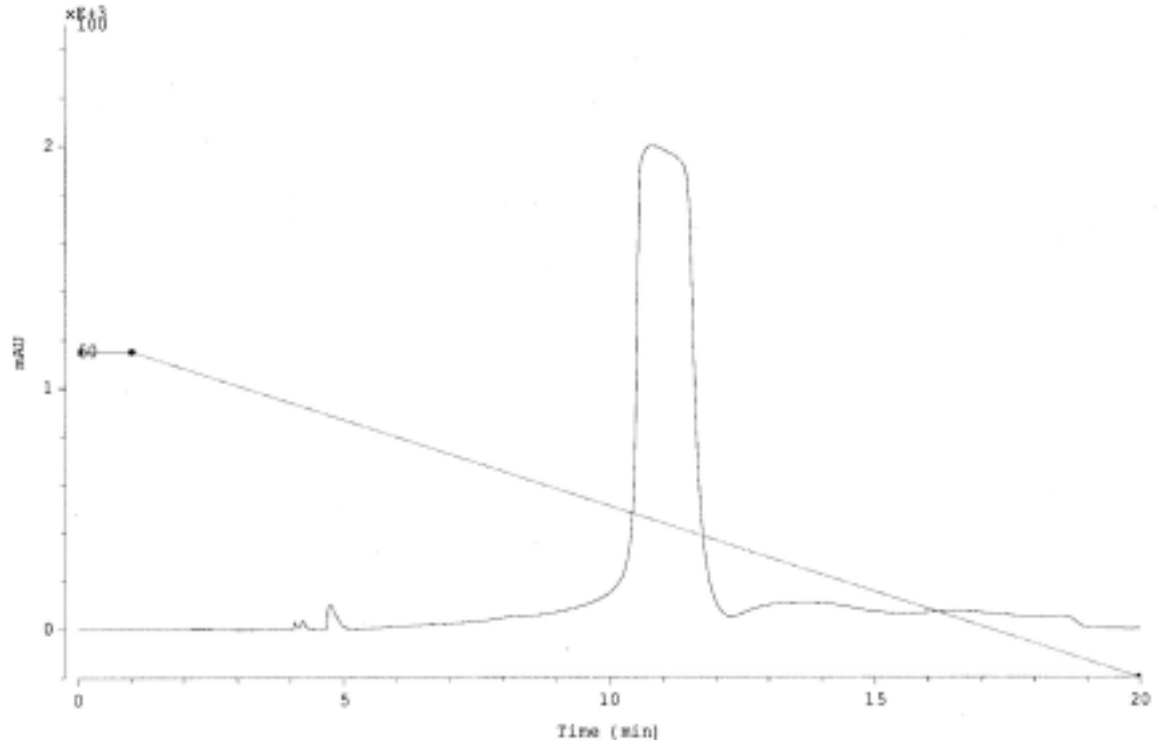



**Figure 4.4:** *HPLC chromatogram of compound* ***6d*** *(preparative HPLC)*

| | | |
|---|---|---|
| Condition: | column: | Eurospher 100, C18, 10 $\mu$m, 250 $\times$ 20 mm ID |
| | precolumn: | Eurospher 100, C18, 5 $\mu$m, 30 $\times$ 20 mm ID |
| | injection speed: | 10 ml/min |
| | mobile phase A: | 35 % methanol + 65 % $H_2O$ |
| | mobile phase B: | $H_2O$ |
| | gradient: | 0 min: A: 50 % B: 50 %(equal:17.5 % methanol) |
| | | 20 min: A: 100 % B: 0 % |
| | elution speed: | 20 ml/min |
| Retention time: | 11 min | |

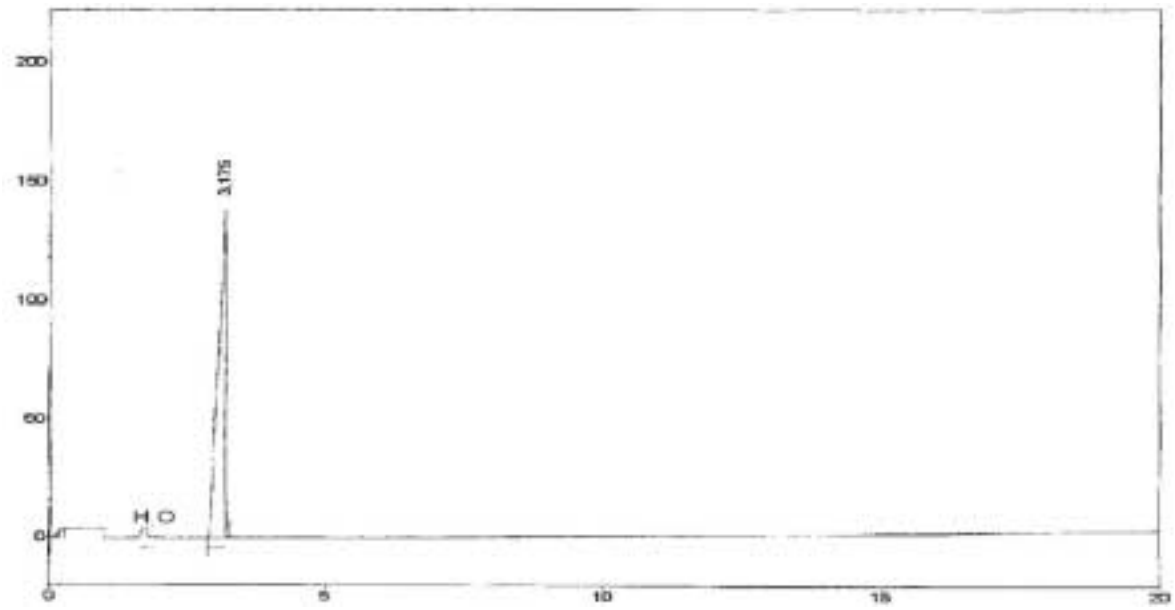



**Figure 4.5:** *CE spectrum for the purity determination of compound* ***6d***

| | | |
|---|---|---|
| Condition: | buffer | phosphate buffer 20 mM, pH 7.4 |
| | voltage: | 10 kV |
| Migration time: | 3.175 min | |
| Purity: | 100 % | |

## 4.3 Synthesis of xanthine *p*-nitrophenylsulfonate esters and 8-*p*-sulfonamidophenylxanthine derivatives

8-*p*-Sulfonamidophenylxanthines were first introduced by Hamilton et al. because of their amphoteric nature. These sulfonamidophenylxanthine derivatives were expected to be soluble across a wide pH range and have a potential to be well absorbed.[57]

Normally, preparation of sulfonamides is performed by converting the sulfonic acids to the corresponding chlorosulfonyl derivatives with thionyl chloride or phosphorus pentachloride, then these chlorosulfonyl derivatives are reacted with ammonia or amine to obtain the desired sulfonamides,[57],[117]–[125] These methods gave good yields of sulfonamides at ambient temperature[118],[123],[125] or with reflux.[124],[126] But when these methods were applied to xanthine derivatives, most of the syntheses did not work. N-Benzyl-4-(2,6-dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-benzenesulfonamide (Figure 4.6) was the only substance which could be synthesized by applying the standard method, but hard reaction condition had to be used, i.e. stirring of the reactants in pyridine for 4

days at r.t. followed by reflux for 3 h, nevertheless the yields reached only 14 %, and the purification was very difficult even by column chromatography.[127]

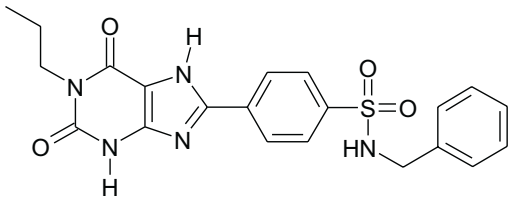


**Figure 4.6:** *Structure of N-benzyl-4-(2,6-dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonamide*

In order to improve the synthesis of the desired 8-*p*-sulfonamidophenylxanthine derivatives, aminolysis of xanthine sulfonate esters to xanthine sulfonamides was tried, in order to investigate whether the nitrophenylester was a good leaving group for the aminolysis. First, 1,3-dimethyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5a** and 1-methyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5c** were used for the aminolysis (Figure 4.7). But unfortunately both compounds did not react, even though different alkaline catalysts were tried. No expected sulfonamide was obtained, only some unidentified compounds could be seen in the NMR spectrum. This means that the *m*-nitrophenyl is not a suitable leaving group for the aminolysis.

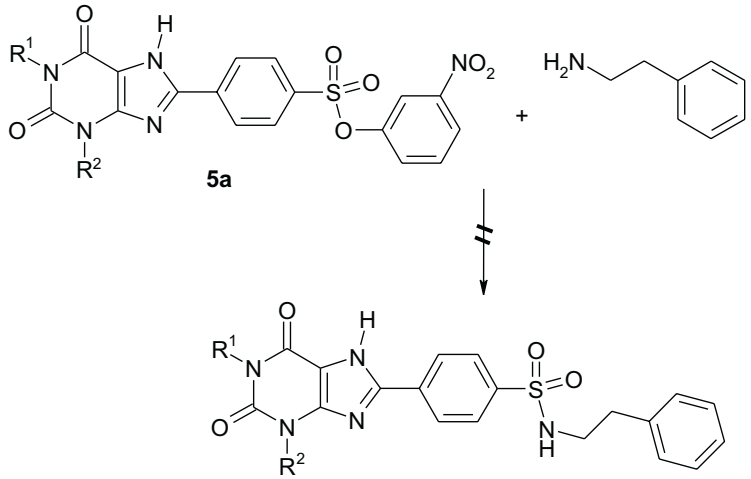
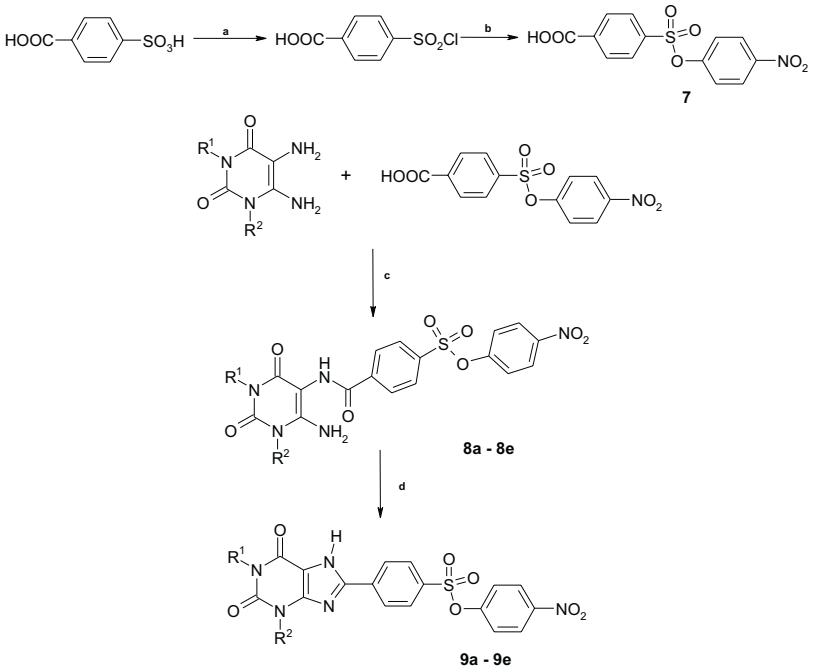


**Figure 4.7:** *Attempted aminolysis by using m-nitrophenyl as a leaving group*

Therefore another suitable leaving group had to be found for the aminolysis reaction. According to the stability test results of the model compounds in chapter 3, *p*-nitrophenyl sulfonate esters are less stable than the *m*-nitrophenyl sulfonate esters, so




| Compd. | $R^1$ | $R^2$ | Yield (%) |
|---|---|---|---|
| **9a** | Methyl | Methyl | 38 |
| **9b** | Propyl | Propyl | 31 |
| **9c** | Methyl | H | 83 |
| **9d** | Propyl | H | 65 |
| **9e** | Butyl | H | 76 |

(a) 1.) $ClSO_2H$, overnight; 2.) chipped ice; (b) *p*-nitrophenol, THF, pH 8 - 9, ca 4 h; (c) EDC, MeOH; (d) PPSE, 1.5 - 2 h.

**Figure 4.8:** *Synthesis of xanthine p-nitrophenyl sulfonate esters*

the *p*-nitrophenyl group may be used as an effective leaving group. The synthetic route of xanthine *p*-nitrophenyl sulfonate esters is almost the same as for the xanthine *m*-nitrophenyl sulfonate esters (Figure 4.8). Through the synthesis of these compounds, it was also proven that the synthetic method for the sulfonate esters is very convenient and applicable for this kind of xanthine sulfonate esters.

Altogether five differently substituted xanthine *p*-nitrophenyl sulfonate esters were synthesized. They are less stable than xanthine *m*-nitrophenyl sulfonate esters according to the stability results of chapter 3, so *p*-nitrophenyl should be a better leaving group for the aminolysis to prepare the expected sulfonamides.

First, 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9a** was used for the aminolysis (Figure 4.9). Stirring of the ester with the appropriate amine in DMSO, first

at r.t. for 30 min, then heating at 150 °C for 3 h, yielded the corresponding sulfonamide which were purified by flash chromatography (dichloromethane : methanol = 50 : 1 or 20 : 1).

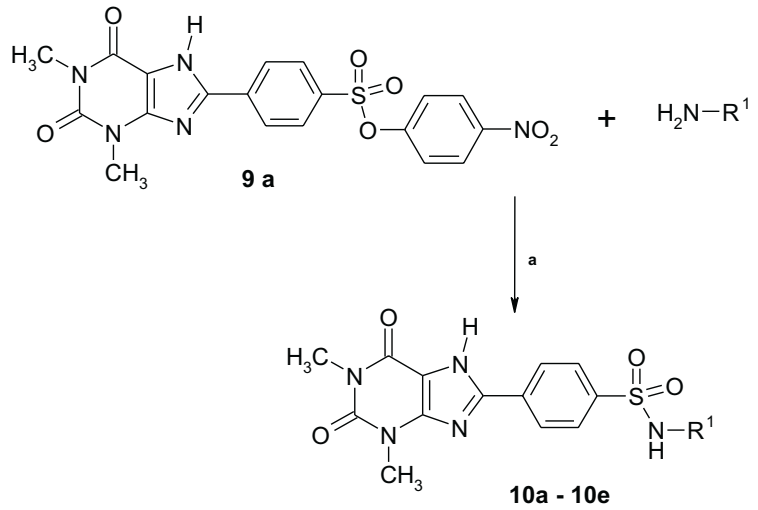
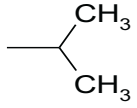
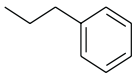
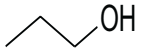
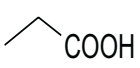



**Figure 4.9:** *Synthesis of 1,3-dimethyl-substituted xanthine sulfonamide derivatives*

Table 4.1 gives an overview of the synthesized 1,3-dimethyl-substituted xanthine sulfonamide derivatives. Compounds **10d** and **10e** are especially interesting, because they have polar -OH and -COOH substituents, which increase the hydrophilicity of the compounds. Therefore they may exert favorable pharmacokinetic properties.

**Table 4.1:** *Synthesized 1,3-dimethyl-substituted xanthine sulfonamide derivatives*

| Compd. | $R^1$-$NH_2$ | Yield (%) |
|---|---|---|
| **10a** |  | 61 |
| **10b** |  | 44 |
| **10c** |  | 44 |
| **10d** |  | 51 |
| **10e** |  | 28 |

It has been reported that 1,8-disubstituted xanthines generally possess high affinity

to $A_{2B}$ adenosine receptors.[63] So it is necessary to perform the aminolysis also at the 1-substituted xanthines. Normally 1-substituted xanthines are less reactive than 1,3-disubstituted xanthines, so harder reaction condition should be applied to achieve this aminolysis. It was proven that, after the heating time was prolonged to 5 h at 150 °C in DMSO, or ca. 72 h at r.t. in DMSO under the protection of argon, 1-substituted xanthine sulfonamide derivatives could be successfully obtained and purified by flash chromatography (dichloromethane : methanol = 40 : 1 or 20 : 1) (Figure 4.10).

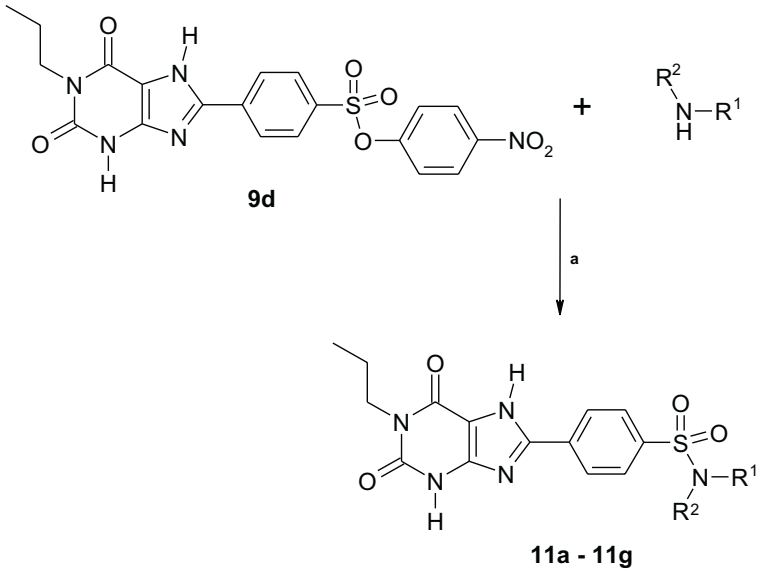
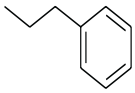
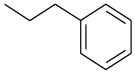
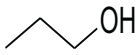
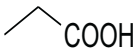
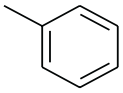
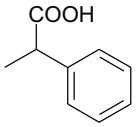



**Figure 4.10:** *Synthesis of 1-propyl-substituted xanthine sulfonamide derivatives*

In table 4.2 the synthesized 1-propyl-substituted xanthine sulfonamide derivatives are collected.

From tables 4.2 and 4.1 it can be clearly concluded that using *p*-nitrophenyl sulfonate as a leaving group is a very efficient way for synthesizing sulfonamides, since the yields generally reach more than 30 %, in some cases even 88 % (**11b**). Not only primary amines can easily be reacted but also the secondary amines, which have lower reactivity, can also be easily employed and the yield reaches 33 % (compound **11e**).

**Table 4.2:** *Synthesized 1-propyl-substituted xanthine sulfonamide derivatives*

| Compd. | $R^1$ | $R^2$ | Yield (%) |
|---|---|---|---|
| **11a** |  | H | 65 |
| **11b** |  | H | 88 |
| **11c** |  | H | 53[a], 28[b] |
| **11d** |  | H | 34 |
| **11e** |  |  | 33 |
| **11f** |  | H | 40 |
| **11g** |  | H | 44 |

[a] r.t. under argon
[b] 150 °C, 5h

## 4.4 Stability tests of *m*-nitrophenylsulfonate esters of xanthine derivatives

For a designed, effective oral prodrug, it must be: 1.) resistant enough to any hydrolysis that might occur before it reaches the bloodstream; 2.) lipophilic enough to cross the gastrointestinal wall; 3.) capable to release the active parent drug in the body through enzymatic biotransformation. So, studying the chemical and the biological *in vitro* stabilities of the prodrug plays an important role in the whole project.

The most popular instruments for stability tests is HPLC. But because the synthesized sulfonate esters are very lipophilic, they are insoluble in most of the common solvents for HPLC, e.g. methanol, acetonitrile, water etc. Therefore it is impossible to use HPLC for the stability tests. So capillary electrophoresis (CE) was chosen as an alternative for the stability test.

### 4.4.1 Short introduction to capillary electrophoresis (CE)

Electrophoresis has been a mainstay of biochemical separation methods since the mid-1930s when it was first described by Tiselius.[128] Electrophoresis refers to the migration of charged electrical species when dissolved, or suspended, in an electrolyte through which an electric current is passed. Cations migrate toward the negatively charged electrode (cathode) and anions are attracted toward the positively charged electrode (anode). Neutral solutes are not attracted to either electrode.

The advantages of conducting electrophoresis in capillaries was highlighted in the early 1980s by the work of Jorgenson and Lukacs[129], [130]who popularized the use of CE. Compared with other analytic techniques, capillary electrophoresis has advantages of speed, versatility, low running costs and high separation efficiency. The application field of CE is not only in the separation of proteins, DNA fragments, carbohydrates, but also in various drugs and drug metabolism studies. In stability tests, CE has its advantage such as not requiring any sample pretreatment so that the incubation solution can directly be injected e.g. without centrifugation to remove proteins.

Briefly, the principle of CE are the following: separations are carried out in a capillary tube, normally the length of this capillary is in the range of 20 to 50 cm, the diameter of it is from 20 $\mu m$ to 80 $\mu m$. The capillary is filled with running buffer and the sample is introduced by dipping one end of the capillary into the sample solution and applying an electric field (electrokinetic injection) or by applying gas pressure (pressure injection). Migration through the capillary is driven, directly or indirectly, by an electric field. Analytes are detected as they pass the window at the far end. Detection is normally by UV absorbance or fluorescence (Figure 4.11).

CE can be further divided into several subgroups according to their different separation modes. These include: capillary zone electrophoresis (CZE), free solution CE (FSCE), whose separation is based on size and charge differences between analytes; micellar electrokinetic capillary chromatography (MECC or MEKC), which separates neutral compounds using surfactant micelles; capillary gel electrophoresis (GCE), which sieves solutes through a gel network; and capillary isoelectric focusing (CIEF), whose separation of zwitterionic solutes is performed within a pH gradient.[132]

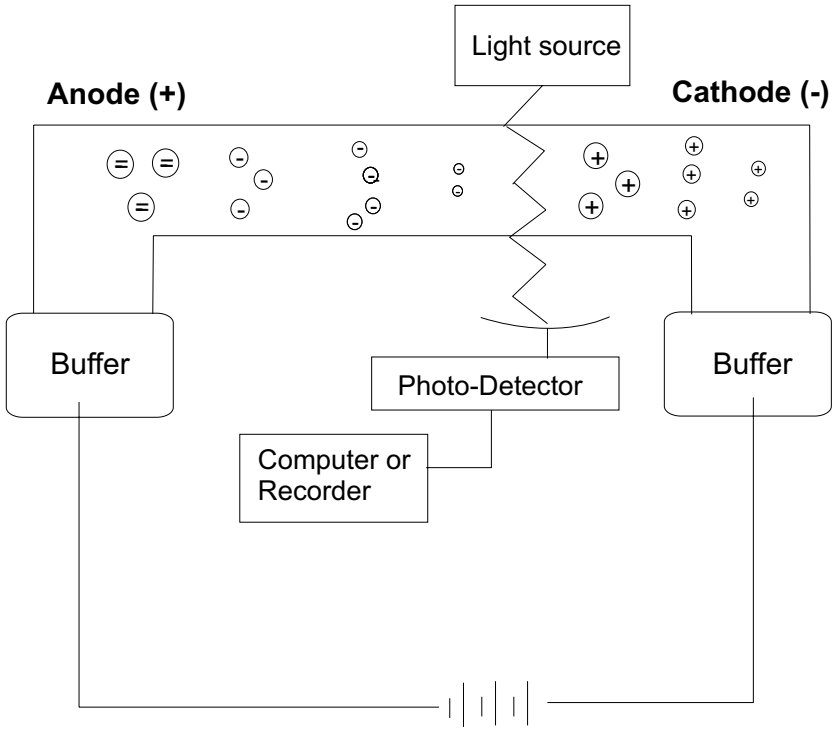



**Figure 4.11:** *Simple principle of Capillary Electrophoresis (CE)*[131]

### 4.4.2 Chemical stability tests

At first, chemical stability of nitrophenyl sulfonic acid esters was investigated. The decomposition pathways and kinetic data of compounds **5a** - **5e** were studied at 37 °C in 0.001 M NaOH in order to determine their chemical stability.

A 2 mM DMSO stock solution was applied since the prodrugs are very well soluble in DMSO. In order to eliminate the influence of DMSO, stock solutions were then diluted with different aqueous buffer solutions at a ratio of 1 : 100. However, if this dilution ratio was applied, esters were precipitated again at a concentration of 0.02 mM.

Therefore different conditions had to be applied. The xanthine sulfonate esters are soluble in alkaline aqueous media because the 7-N position of the xanthines can be deprotonated. So 0.001 M NaOH (pH 11) was used for the incubation. It is known that esters are less stable in alkaline media than in neutral media. It is likely that, if they are stable in alkaline medium, they will also be stable in neutral media.

Hydrolysis products of these sulfonate esters are the corresponding 8-*p*-sulfophenylxanthine and *m*-nitrophenol (Figure 4.12). This can be confirmed from the CE spectrum, since the free xanthine sulfonic acid and *m*-nitrophenol appear in the spectrum after several hours of incubation (Figure 4.13).

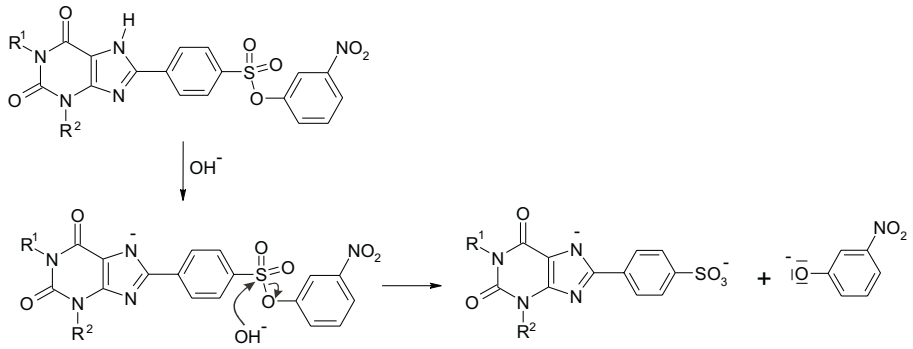


**Figure 4.12:** *Chemical hydrolysis of xanthine m-nitrophenyl sulfonate esters*

We found that the hydrolysis reaction of the esters is a first order reaction. This means:

$$t_{1/2} = ln2/K \qquad (K: \ slope\ of\ the\ hydrolysis\ curve) \tag{4.1}$$

Two kinds of buffers were chosen for the separation. One was 20 mM phosphate buffer (pH 7.4) plus 100 mM sodium dodecylsulfate (SDS), the other one was 100 mM borate buffer (pH 8) plus 50 mM SDS. It was proven that borate buffer was better suitable for the separation, because the migration time of the esters was about 9 min by using borate buffer, compared to about 25 to 30 min when using phosphate buffer. The applied voltage for the separation was 10 kV (Figure 4.13).

Measurement of the hydrolysis kinetics revealed an exponential decay of first order for all esters. These are documented by the diagrams in Figure 4.14 - 4.18 in which $\ln(C/C_0)$ (C = concentration of compound, $C_0$ = concentration at time zeros) plotted over the time (t); the diagrams show linear relationships for all esters under the condition of 0.001 M NaOH. Table 4.3 gives the half-lives ($t_{1/2}$) of each ester, it can be concluded that the synthesized sulfonate esters are very stable, since the half-lives of all esters reach about 20 hours.

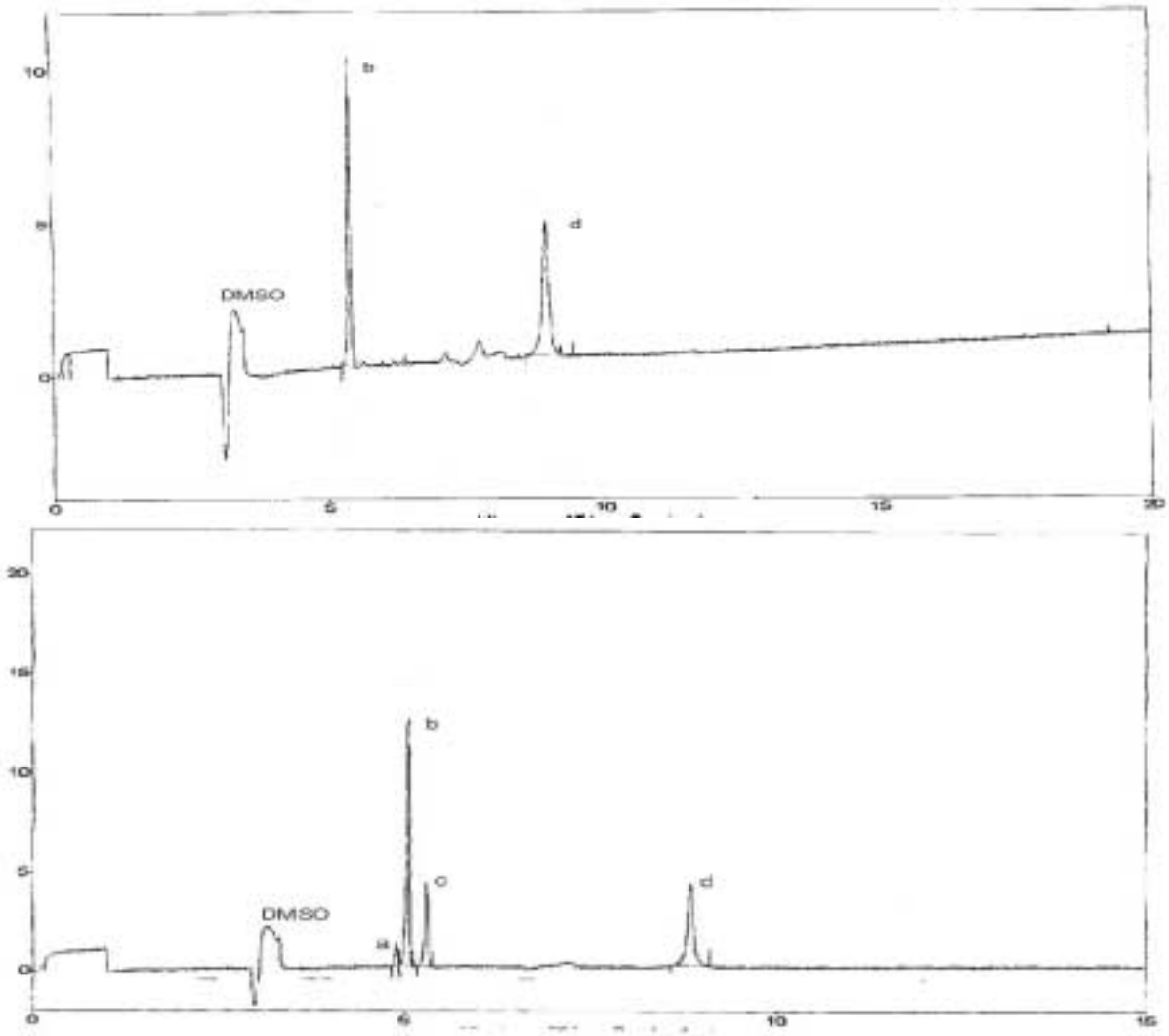
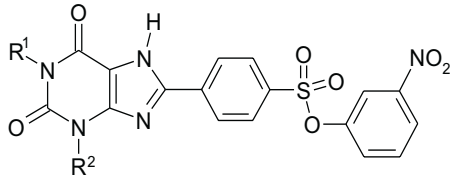



**Figure 4.13:** *CE spectrum of compound 1-propyl-8-[4'-[[m-nitrophenoxy]sulfonyl]-phenyl]xanthine (**5d**) before incubation and after 26 hours of incubation in 0.001 M NaOH at 37 °C*

| | | |
|---|---|---|
| Condition: | buffer | Borate buffer 100 mM + SDS 50 mM, pH 8 |
| | voltage: | 10 kV |
| Migration: | a: | *m*-Nitrophenol, 4.9 min |
| | b: | Internal standard, 5.1 min |
| | c: | 1-Propyl-8-*p*-sulfophenylxanthine, 5.3 min |
| | d: | Compound 5d, 8.8 min |

**Table 4.3:** *Stability of compounds **5a** - **5e** towards 0.001 M aqueous NaOH solution at 37 °C*




| compd. | $R^1$ | $R^2$ | $t_{1/2}$ (h) | $K$ |
|---|---|---|---|---|
| **5a** | Methyl | Methyl | 20.8 | $9.24 \times 10^{-6}$ |
| **5b** | Propyl | Propyl | 21.1 | $9.1 \times 10^{-6}$ |
| **5c** | Methyl | H | 20.1 | $9.6 \times 10^{-6}$ |
| **5d** | Propyl | H | 23.8 | $8.1 \times 10^{-6}$ |
| **5e** | Butyl | H | 19.1 | $10.1 \times 10^{-6}$ |

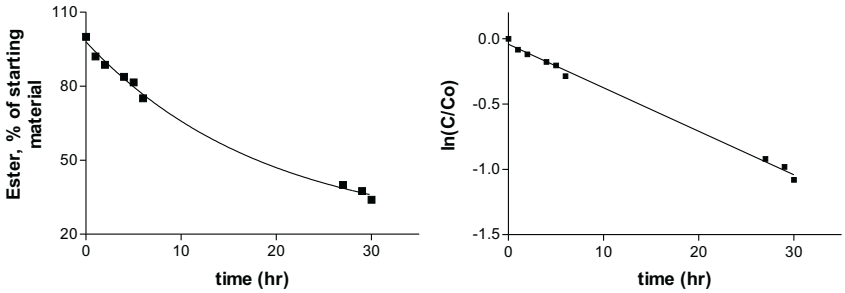



**Figure 4.14:** *Hydrolysis rate of 1,3-dimethyl-8-[4'-[[m-nitrophenoxy]sulfonyl]phenyl]xanthine (**5a**). Slope of the curve, -0.03328; $t_{1/2}$ = 20.8 h; $r^2$ = 0.9956*

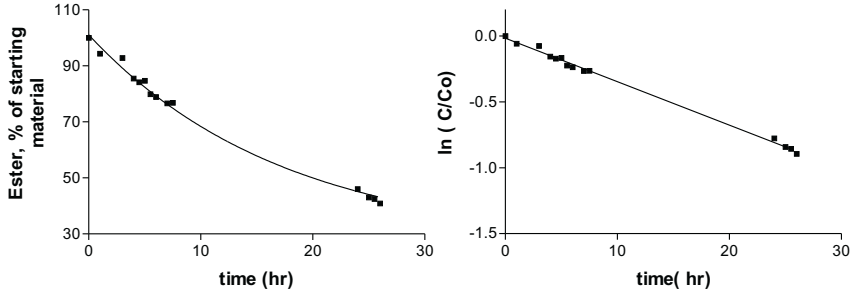



**Figure 4.15:** *Hydrolysis rate of 1,3-dipropyl-8-[4'-[[m-nitrophenoxy]sulfonyl]phenyl]xanthine (**5b**). Slope of the curve, -0.03296; $t_{1/2}$ = 21.1 h; $r^2$ = 0.9963*

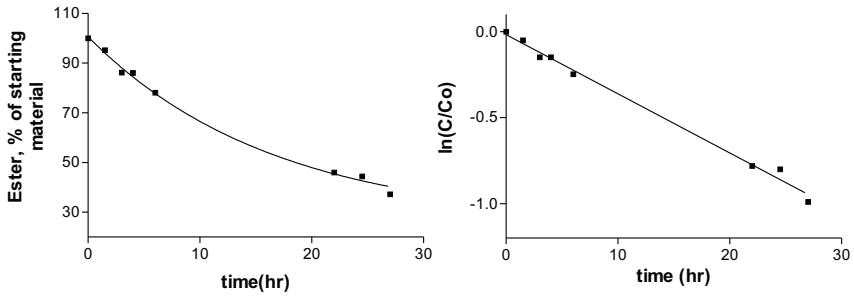



**Figure 4.16:** *Hydrolysis rate of 1-methyl-8-[4'-[[m-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5c**). Slope of the curve, -0.03441; $t_{1/2}$= 20.1 h; $r^2$ = 0.993*

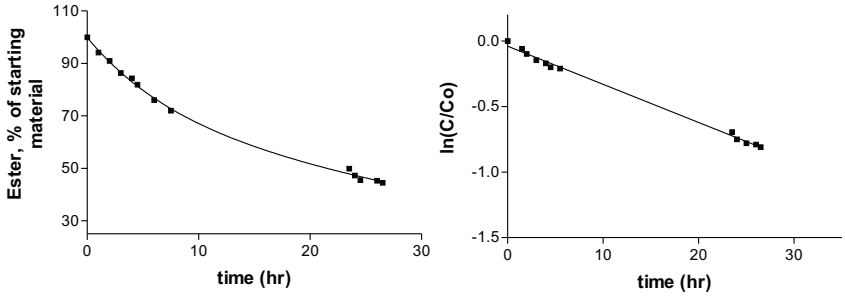



**Figure 4.17:** *Hydrolysis rate of 1-propyl-8-[4'-[[m-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5d**). Slope of the curve, -0.02911; $t_{1/2}$ = 23.8 h; $r^2$ = 0.996*

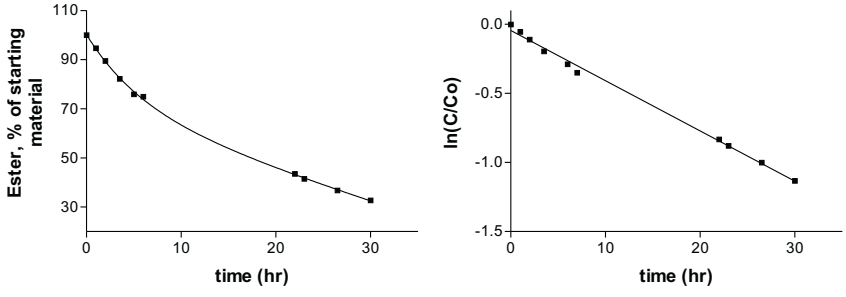



**Figure 4.18:** *Hydrolysis rate of 1-butyl-8-[4'-[[m-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5e**). Slope of the curve, -0.03634; $t_{1/2}$ = 19.1 hr; $r^2$ = 0.996*

### 4.4.3 Biological *in vitro* stability tests

Since the chemical structure of these esters is similar, i.e. *m*-nitrophenyl sulfonate ester, their stability properties should be almost the same. This was confirmed in the chemical stability tests, as there was no great difference between the half-lives of these esters. Therefore only one compound was selected for the further biological *in vitro* stability tests.

1-Propyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** was chosen as an example to study its biological stability at 37 °C in: i.) fetal calf serum; ii.) simulated gastric acid; iii.) rat liver homogenate. These various media are thought to be valid biological *in vitro* models for the degradation that may affect the prodrugs during oral application.

#### 4.4.3.1 Stability in fetal calf serum

To eliminate interference of the proteins from the fetal calf serum, different CE separation conditions were used than in the chemical stability test.

Firstly, the buffer used was 100 mM borate buffer (pH 8) plus 100 mM SDS instead of 50 mM SDS used in the chemical stability tests, thus the migration times of the ester and hydrolyzed product could be clearly distinguished from that of the proteins in fetal calf serum.

Secondly, 320 nm wavelength was chosen for the detection instead of 254 nm used in the chemical stability test, since the UV absorbance of the protein in fetal calf serum reaches 300 nm maximally, therefore at a wavelength of 320 nm one can eliminate the interference of the protein absorption during the investigation.

Thirdly, *p*-aminosalicylate sodium salt was used as the internal standard instead of diclofenac sodium salt, which was used in the chemical stability test, because the latter one doesn't have any absorbance at a wavelength of 320 nm.

Table 4.4 shows the migration times of the ester and the hydrolysis products. We can see that there is no interference with the ester and the internal standard under the CE test conditions.

**Table 4.4:** *Migration times of prodrug, parent drug and internal standard*

| | prodrug | free sulfonic acid | *m*-nitrophenol | internal standard (*p*-aminosalicylate Na) |
|---|---|---|---|---|
| migration time (min) | 12.89 | 5.85 | 6.304 | 5.00 |

However no hydrolyzed products could be detected in the CE spectrum after the hydrolysis in fetal calf serum (Figure 4.19). The only change that could be detected was

the decrease of the peak area of the ester with the prolongation of the incubation time. Figure 4.19 gives the CE spectrum of ester **5d** in fetal calf serum before incubation and after 24 h of incubation at 37 °C.

The reason why no hydrolyzed product could be detected by CE analysis is not clear. Two possible explanations are reasonable: one is that the pH value of the fetal calf serum is ca. 6.9 - 7.6, so the hydrolysis products of the free xanthine sulfonic acid and the *m*-nitrophenol are not charged and stay as neutral molecules in the fetal calf serum, therefore they are not detectable by CE; the other possibility is that both hydrolyzed products interacted with the proteins in the fetal calf serum, forming neutral insoluble compounds, thus they are not detectable anymore by CE either. It seemed that the second explanation is more reasonable, because, if the free sulfonic acid of xanthine and *m*-nitrophenol were mixed with fetal calf serum in a separate experiments, precipitation could be observed after some time.

The hydrolysis is documented by the diagram in figure 4.20 in which $C/C_0$ (C = concentration of compound, $C_0$ = concentration at time zero) is plotted over the time (t); the diagram shows linear relationships for **5d** under the conditions in fetal calf serum, i.e. the hydrolysis of **5d** in fetal calf serum is a zero-order reaction. Therefore the half-life is as follows:

$$t_{1/2} = 1/2K \quad (K: \ slope\ of\ the\ hydrolysis\ curve) \tag{4.2}$$

From figure 4.20 we can get the slope = 0.03058, therefore the half-life time of compound **5d** in fetal calf serum is ca. 16.4 hours.

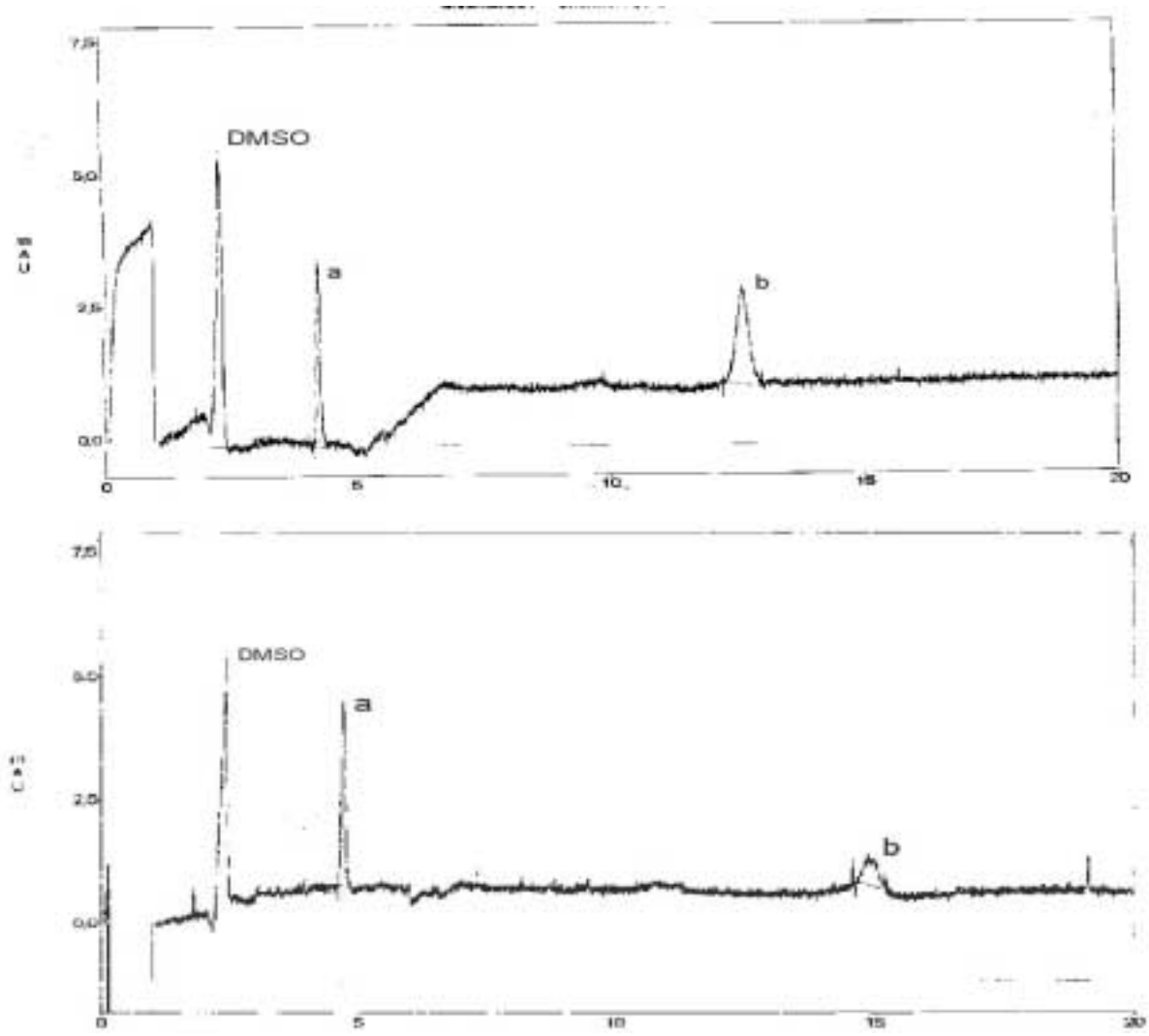



**Figure 4.19:** *CE spectrum of 1-propyl-8-[4'-[[[m-nitrophenoxy]sulfonyl]phenyl]-xanthine* ***5d*** *before incubation and after 24 hours of incubation in fetal calf serum at 37 °C*

| | | |
|---|---|---|
| Condition: | buffer | Borate buffer 100 mM + SDS 100 mM, pH 8 |
| | voltage: | 15 kV |
| Migration: | a: | internal standard, 4.9 min |
| | b: | compound 5d, 15 min |

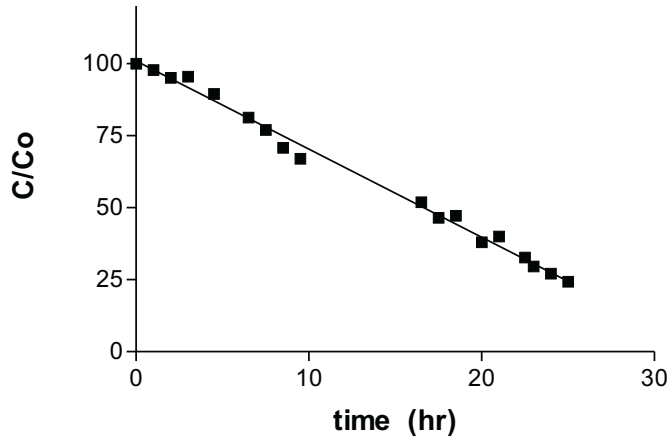



**Figure 4.20:** *Hydrolysis rate of 1-propyl-8-[4'-[[[m-nitrophenoxy]sulfonyl]phenyl]-xanthine* ***5d*** *in fetal calf serum at 37 °C*
*Slope of the curve, -0.03058 ;* $t_{1/2}$ *= 16.4 h;* $r^2$ *= 0.995*

#### 4.4.3.2 Stability in simulated gastric acid

When simulated gastric acid was applied as the biological medium, only 4 hours of incubation was done. Drugs will generally remain in the stomach for less than 4 hours, so 4 hours of incubation will be enough for the stability tests of the prodrug in simulated gastric acid. The simulated gastric acid consisted of hydrochloride acid, sodium chloride, pepsin dissolved in water.[133] Stability of the sulfonic acid ester **5d** was determined by CE. It was ovserved that migration time of the prodrug was delayed for about 2 - 3 min after each test, so after several measurements the migration time of the prodrug was prolonged to more than 20 min and the peak became wider. However it is difficult to exactly determine the peak area if the peak is too wide. The longer the migration time is, the wider the peak is, and the more difficult is the determination.

The delay of the migration time can be possibly explained by a jam in the capillary. An alkaline borate buffer (pH 8) was applied as the balance buffer, but pepsin in simulated gastric acid is not stable anymore at pH > 6. When the denatured pepsin precipitates in the capillary, it induces a change of the capillary's surface. Thus, the migration time of the compound will be delayed after each test.

It was proven that after 4 hours of incubation at 37 °C in simulated gastric acid, there are still 94 % of **5d** remaining in the solution. Therefore, it can be concluded that the *m*-nitrophenylsulfonate esters are likely to be very stable in the stomach.

#### 4.4.3.3 Stability in rat liver homogenate

Fresh rat liver was collected directly after slaughtering, homogenized in DPBS (pH 7.2), centrifuged at 4 °C at 9000 × g for 30 min to obtain the rat liver homogenate containing 32 % of homogenized liver. The final protein concentration was 16 mg/ml as determined by the Bradford method.[137]

Compared with the tests in fetal calf serum and simulated gastric acid, different CE separation conditions were used to eliminate interference of the protein in rat liver homogenate. Borate buffer (100 mM) plus 150 mM SDS (pH 8) was used instead of 50 mM or 100 mM SDS in the previous studies. In place of 15 kV voltage for the separation in fetal calf serum and simulated gastric acid, 12 kV voltage was used in the rat liver homogenate test in order to eliminate the influence of proteins in rat liver homogenate.

It was proven that the hydrolysis in rat liver homogenate is a first order reaction (Figure 4.21), the half-life ($t_{1/2}$) of compound **5d** being about 41 min. Like the test in fetal calf serum, no decomposition products could be seen in the CE spectrum, only the decrease of the peak area of **5d** with the prolongation of time was detected. Again no exact reason was found, only the supposition described in Chapter 4.4.3.1.

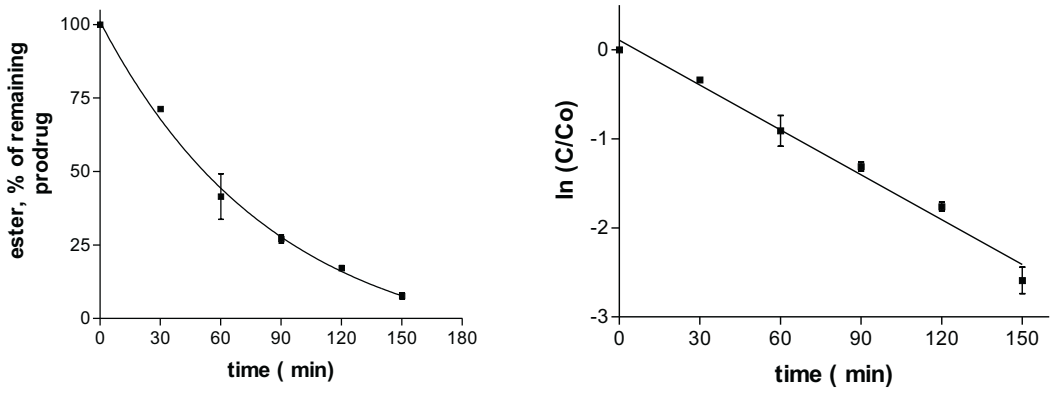



**Figure 4.21:** *Hydrolysis of compound **5d** in rat liver homogenate at 37 °C. Slope of the curve, -0.0168; $t_{1/2}$ = 41 min; $r^2$ = 0.96*

Since no hydrolyzed *p*-sulfophenylxanthine and *m*-nitrophenol could be detected from the CE spectra during the stability test in rat liver homogenate, in order to prove whether the ester can be hydrolyzed to the parent drugs through enzymatic hydrolysis, TLC analysis of the hydrolysis products was performed. It was proven that after ca. 2 hours of incubation in rat liver homogenate at 37 °C, the *p*-sulfophenylxanthine could be detected by TLC using dicloromethane : methanol = 4 : 1 as mobile phase. This means that the

ester is cleaved to the active parent drug through enzymatic biotransformation.

### 4.4.4 Conclusions

The synthesized xanthine *m*-nitrophenyl sulfonate esters are relatively stable, since half-life of these esters in 0.001 M NaOH at 37 °C reaches 20 h on average. The 1-propyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** showed high stability in artificial gastric acid and in serum as well. However, the ester was cleaved by incubation with liver homogenate indicating that nitrophenyl esters have a potential as peroral prodrugs of sulfonic acid drugs.

# 4.5 Pharmacological tests

## 4.5.1 Results and discussion

The synthesized xanthines were tested in radioligand binding assays for their affinity to $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ adenosine receptors in rat cortical membrane, rat striatal membrane and CHO-cell preparations which recombinantly express the human $A_{2B}$ and $A_3$ adenosine receptors, respectively.

The $A_1$ AR radioligand binding assay was performed in rat cortical membranes, with a protein content of about 70 $\mu g/mL$. [$^3$H]2-Chloro-N$^6$-cyclopentyladenosine (CCPA, conc: 0.5 nM) was used as selective $A_1$ radioligand.

The $A_{2A}$ AR affinity test was performed using the $A_{2A}$-selective radioligand [$^3$H]3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargyl xanthine (MSX-2, conc: 1 nM) at rat striatal membranes, which are used with a protein content of 70 $\mu g/mL$.

The $A_{2B}$ affinity test was performed using the $A_{2B}$-selective radioligand [$^3$H]8-((4-(2-hydroxyethylamino)-2-oxo-ethoxy)phenyl)-1-propyl xanthine (PSB-298, conc: 5 nM), membranes of human CHO-cells transfected with human $A_{2B}$-adenosine receptor were used with a protein content of 100 $\mu g/mL$.

The $A_3$ affinity test was performed using the $A_3$-selective radioligand [$^3$H]2-(2',3',5'-trichlorophenyl)-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidzo[2,1-*i*]purin-5-one (P SB-11, conc: 0.5 nM), membranes of CHO-cells transfected with human $A_3$-adenosine receptor were used with a protein content of 50 $\mu g/mL$.

Inhibition of receptor radioligand binding was determined by 7 concentrations of the compounds in triplicate in at least three separate experiments. The Cheng-Prusoff equation and $K_D$ values of 0.2 nM for the [$^3$H]CCPA,[136] 8 nM for [$^3$H]MSX-2,[138] 56 nM for [$^3$H]PSB-298[139] and 4.9 nM for [$^3$H]PSB-11[140] were used to calculate the $K_i$ values from $IC_{50}$ values, determined by the nonlinear curve fitting program GraphPad Prism$^{TM}$, version 2.0 (GraphPad, San Diego, California, USA).

### 4.5.1.1 Results of xanthine nitrophenylsulfonate esters

The determined affinities of synthesized 1,3-substituted-8-[4'-[[*m*-nitrophenoxy]sulfonyl]-phenyl]xanthines **5a** - **5e** are collected in table 4.5. Figures 4.22 - 4.23 show the inhibition curves of **5a** - **5e** at $A_1$ and $A_{2A}$ ARs.

The determined affinities of the synthesized 1,3-substituted-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthines **9a** - **9e** are collected in table 4.6. Figures 4.24 - 4.25 give the inhibition curves of **9a** - **9e** at $A_1$ and $A_{2A}$ ARs.

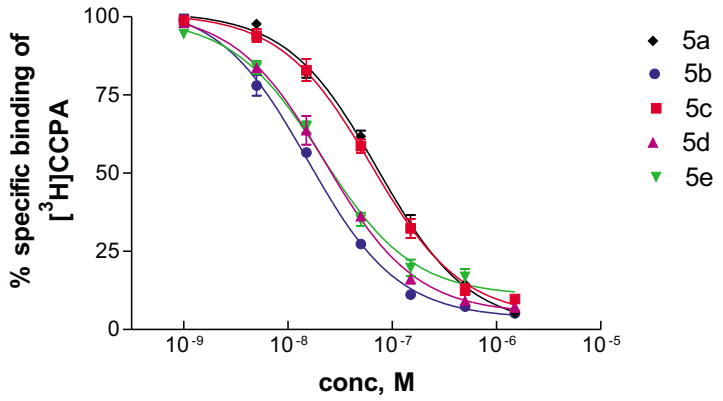



**Figure 4.22:** *Binding of compounds* ***5a*** *-* ***5e*** *to the* $A_1$ *adenosine receptor of rat brain cortical membranes*

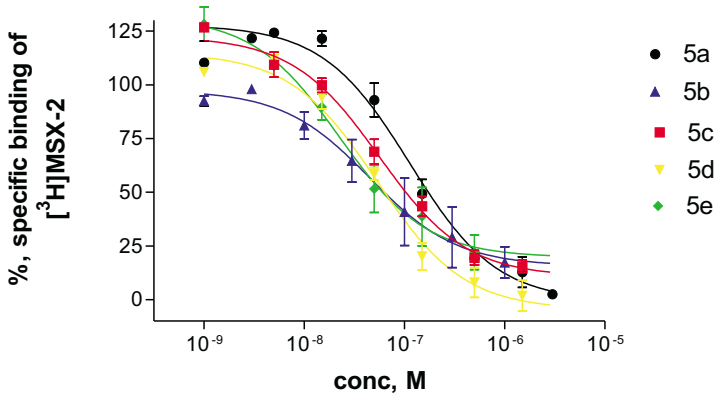



**Figure 4.23:** *Binding of compounds* ***5a*** *-* ***5e*** *to the* $A_{2A}$ *adenosine receptor of rat brain striatal membranes*

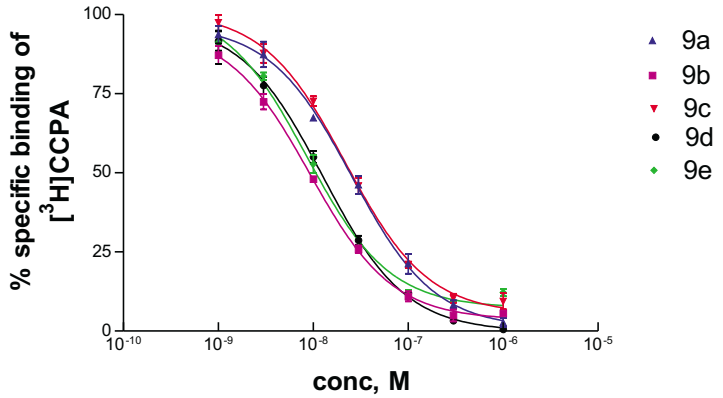



**Figure 4.24:** *Binding of compounds* ***9a*** *-* ***9e*** *to the* $A_1$ *adenosine receptor of rat brain cortical membranes*

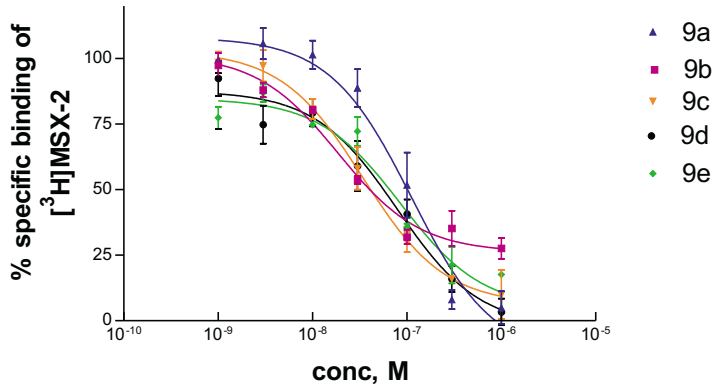
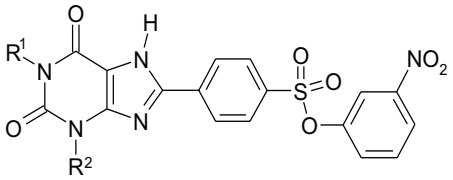



**Figure 4.25:** *Binding of compounds* ***9a*** *-* ***9e*** *to the* $A_{2A}$ *adenosine receptor of rat brain striatal membranes*

**Table 4.5:** *Affinity of compounds* ***5a*** *-* ***5e*** *towards* $A_1$, $A_{2A}$ *and* $A_3$ *ARs*



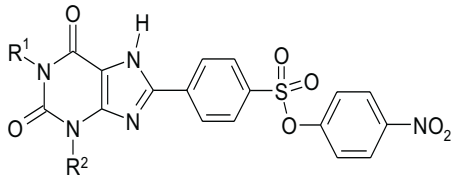

| | | | $K_i \pm$ SEM [nM] or % inhibition of radioligand binding at 10 $\mu M$ | | |
|---|---|---|---|---|---|
| **compd.** | **$R^1$** | **$R^2$** | **$A_1$-Affinity** Rat [$^3$H]CCPA (n = 3) | **$A_{2A}$-Affinity** Rat [$^3$H]MSX-2 (n = 3) | **$A_3$-Affinity** Human [$^3$H]PSB-11 (n = 2) |
| **5a** | Methyl | Methyl | 21 ± 5 | 117 ± 22 | 25 % ± 1.2 |
| **5b** | Propyl | Propyl | 4.4 ± 0.3 | 28 ± 29 | 66 % ± 6.6 |
| **5c** | Methyl | H | 18 ± 2 | 63 ± 29 | 11 % ± 4.3 |
| **5d** | Propyl | H | 6.5 ± 0.9 | 54 ± 5 | 14 % ± 3.1 |
| **5e** | Butyl | H | 5.7 ± 0.1 | 21 ± 3 | 21 % ± 1.3 |

Comparing the $K_i$ values of the xanthine *m*-nitrophenylsulfonates **5a** - **5e** and the xanthine *p*-nitrophenylsulfonates **9a** - **9e**, we can see that *p*-nitrophenyl sulfonate xanthines have at least two fold higher potency at $A_1$ than *m*-nitrophenylsulfonate xanthines. Especially *p*-nitrophenylsulfonate xanthines have a very high affinity for $A_1$ ARs roughly from 2.7 nM (**9b**) to 7.7 nM (**9a**). 1,3-Disubstituted and 1-methyl-substituted xanthine *p*-nitrophenylsulfonate esters (**9a**, **9b**, **9c**)have higher affinity at $A_{2A}$ ARs than the corresponding xanthine *m*-nitrophenylsulfonate esters (**5a**, **5b**, **5c**) , but the 1-propyl and 1-butyl-substituted xanthine *p*-nitrophenylsulfonate esters (**9d**, **9e**) have a less affinity at $A_{2A}$ AR than 1-propyl and 1-butyl-substituted xanthine *m*-nitrophenylsulfonate esters (**5d**, **5e**). Except for the 1-propyl-substituted xanthine derivative, all xanthine *m*-nitrophenylsulfonate esters are more potent than xanthine *p*-nitrophenylsulfonate esters at $A_3$ AR.

Both of these two kinds of esters have a higher affinity at $A_1$ ARs than at $A_{2A}$ ARs, but the selectivity between the two receptors is not great, $A_1/A_{2A}$ selectivity ranging from 3-fold (**5e**) to 30 fold (**9e**). So these two types of xanthine sulfonate esters are potent but not selective $A_1$ AR antagonists.

The 1,3-dipropyl substituted compound (**5b**) has almost the highest $A_1$, $A_{2A}$ and $A_3$ affinity among the five *m*-nitrophenylsulfonate esters, which reaches $K_i$ values of 4.4 nM

**Table 4.6:** *Affinity of compounds **9a** - **9e** towards $A_1$, $A_{2A}$ and $A_3$ ARs*




| | | | **$K_i$ ± SEM [nM] or % inhibition of radioligand binding at 10 $\mu M$** | | |
|---|---|---|---|---|---|
| **compd.** | **$R^1$** | **$R^2$** | **$A_1$-Affinity** Rat [$^3$H]CCPA (n = 3) | **$A_{2A}$-Affinity** Rat [$^3$H]MSX-2 (n = 3) | **$A_3$-Affinity** Human [$^3$H]PSB-11 (n = 2) |
| **9a** | Methyl | Methyl | 7.7 ± 0.6 | 99 ± 21 | 16 % ± 9 |
| **9b** | Propyl | Propyl | 2.7 ± 0.1 | 16 ± 10 | 44 % ± 2.4 |
| **9c** | Methyl | H | 6.3 ± 7 | 34 ± 12 | 5 % ± 3 |
| **9d** | Propyl | H | 3.6 ± 0.1 | 74 ± 2 | 51 % ± 9 |
| **9e** | Butyl | H | 2.6 ± 0.3 | 78 ± 18 | 9 % ± 8.6 |

at $A_1$ ARs and 28 nM at $A_{2A}$ ARs, and the inhibition of $A_3$ AR at the concentration of 10 $\mu M$ reaches 66 %. So does compound **9b**, it has also similarly high $A_1$, and $A_{2A}$ affinity, which is 2.7 nM affinity at $A_1$ ARs and 16 nM at $A_{2A}$ ARs. The $A_3$ AR affinity of **9b** is 44 % inhibition at 10 $\mu M$, a little less than for the 1-propyl substituted compound **9d** with 51 % inhibition at 10 $\mu M$ concentration. The 1,3-dimethyl substituted compound **5a** has the lowest $A_1$ and $A_{2A}$ affinity among the five *m*-nitrophenylsulfonate esters, which reaches a $K_i$ value of 21 nM at $A_1$ and 117 nM at $A_{2A}$ AR, and so does the compound **9a**, which has the lowest $A_1$ and $A_{2A}$ ARs affinity among the five *p*-nitrophenylsulfonate esters, with a $K_i$ of 7.7 nM at $A_1$ AR and 99 nM at $A_{2A}$ AR. The lowest affinity at $A_3$ ARs exhibit the 1-methyl substituted compounds **5c**, **9c**, which show only 11 %, 5 % inhibition respectively of radioligand binding at 10 $\mu M$ concentration. So the introduction of a 3-propyl substituent increases the affinity to $A_1$, $A_{2A}$ and $A_3$ subtypes, but the introduction of a 3-methyl substituent decreases the affinity to the $A_1$ and $A_{2A}$ AR subtypes, but not the $A_3$ AR affinity.

One thing needs to be mentioned, 1-methyl substituted xanthine *p*-nitrophenylsulfonate ester (**9c**) has two times or three times lower $A_1$ affinity than 1-propyl (**9d**) and 1-butyl (**9e**) substituted xanthines, but it has a two times higher $A_{2A}$ affinity than the other two 1-substituted compounds. But the $A_1$ and $A_{2A}$ AR affinity of *m*-nitrophenylsulfonate

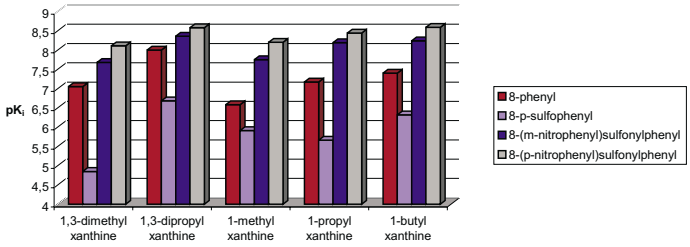
ester **5c** is reversed with respect to **9c**.

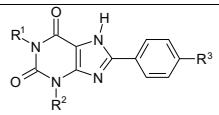
As we see, there is no great difference between 1-propyl- and 1-butyl-substituted xanthines **5d**, **5e** at $A_1$ ARs (6.5 nM, 5.7 nM) and **9d**, **9e** at $A_1$ (3.6 nM, 2.6 nM), but the 1-propyl *m*-nitrophenyl xanthine **5d** has a lower $A_{2A}$ AR affinity (54 nM) than 1-butyl *m*-nitrophenyl xanthine **5e** (21 nM). On the contrary, there is no difference between **9d** and **9e** with respect to the $A_{2A}$ affinity.

The introduction of an 8-phenyl residue in xanthines leads to a large increase in receptor affinity and $A_1$ selectivity of the compounds, and further introduction of a *p*-sulfophenyl moiety in that 8-position of xanthines results in a decrease in activity and selectivity at $A_1$ receptors. In our studies, the substitution on the 8-position with a big nitrophenylsulfonylphenyl ester molecule leads to an even higher increase in $A_1$ AR affinity, on the average all of these esters reach $A_1$ and $A_{2A}$ ARs affinities of $< 10$ nM. Figure 4.26 gives the comparison of $pK_i$ values at $A_1$ ARs of differently 8-substituted 1,3-substituted xanthines.

Compared with 8-phenylxanthine, the biggest affinity increase at $A_1$ AR is observed with 1-methyl-8-(*p*-nitrophenylsulfonyl)xanthine **9c**, which reaches 40 times, and compared with 8-sulfophenylxanthine, the greatest affinity increase at $A_1$ AR exhibits compound **9a**, which reaches ca. 1820 times.

The introduction of a polar sulfonate substitution at 1,3-substituted 8-phenyl xanthines has almost the same effect for $A_{2A}$ ARs as for $A_1$ ARs, that is the potency greatly decreased (from 7 fold to 65 fold). After the sulfonate substitution was substituted by big nitrophenyl residues, the $A_{2A}$ affinity greatly increased, from 30-fold (**9d**) to 350-fold (**9c**)(See figure 4.27).

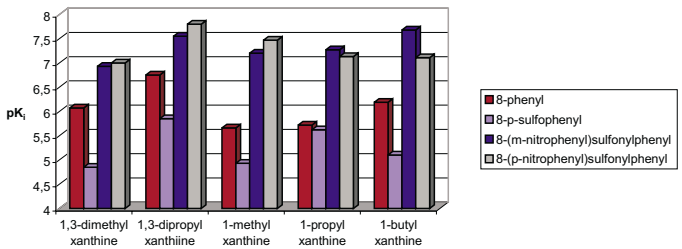


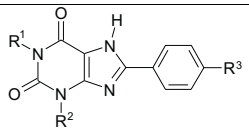



| | | | Ki [nM] | | |
|---|---|---|---|---|---|
| $R^1$ | $R^2$ | $R^3$ = H | $R^3$=$SO_3H$ | $R^3$ = *m*-nitrophenylsulfonyl | $R_3$= *p*-nitrophenylsulfonyl |
| Methyl | Methyl | 89 [a,65] | 14000 [a,60] | 21 [b] | 7.7 [b] |
| Propyl | Propyl | 10 [a,60] | 210 [a,60] | 4.4 [b] | 2.7 [b] |
| Methyl | H | 260 [a,65] | 1238 [b] | 18 [b] | 6.3 [b] |
| Propyl | H | 67 [a,64] | 2200 [a,64] | 6.5 [b] | 3.6 [b] |
| Butyl | H | 40 [b,63] | 475 [b,63] | 5.7 [b] | 2.6 [b] |

[a] [$^3$H]PIA was used as $A_1$-radioligand. [b] [$^3$H]CCPA was used as $A_1$-radioligand.

**Figure 4.26:** *Comparison of differently 1,3,8-substituted xanthines at rat $A_1$ ARs*



| | | | Ki [nM] | | |
|---|---|---|---|---|---|
| R1 | R2 | $R^3$ = H | $R^3$=$SO_3H$ | $R^3$ = *m*-nitrophenylsulfonyl | $R_3$ = *p*-nitrophenylsulfonyl |
| Methyl | Methyl | 850 [a,60] | 14000 [a,60] | 117 [b] | 99 [b] |
| Propyl | Propyl | 190 [a,50] | 1400 [a,60] | 28 [b] | 16 [b] |
| Methyl | H | 2200 [a,64] | 11800 [b] | 62.7 [b] | 34 [b] |
| Propyl | H | 1900 [a,64] | 24000 [a,64] | 54.3 [b] | 74 [b] |
| Butyl | H | 642 [b,63] | 8070 [b,63] | 21 [b] | 78 [b] |

[a] [$^3$H]NECA was used as $A_{2A}$-radioligand. [b] [$^3$H]MSX-2 was used as $A_1$-radioligand.

**Figure 4.27:** *Comparison of differently 1,3,8-substituted xanthines at rat $A_{2A}$ ARs*

#### 4.5.1.2 Results of sulfonamidophenylxanthines

Determined $A_1$, $A_{2A}$ and $A_3$ affinities of synthesized sulfonamidophenyl xanthines **10a** - **10e** and **11b** - **11f** are collected in table 4.7. Figures 4.28 - 4.29 show the $A_1$ and $A_{2A}$ affinity curves of compounds **10a** - **10e**.

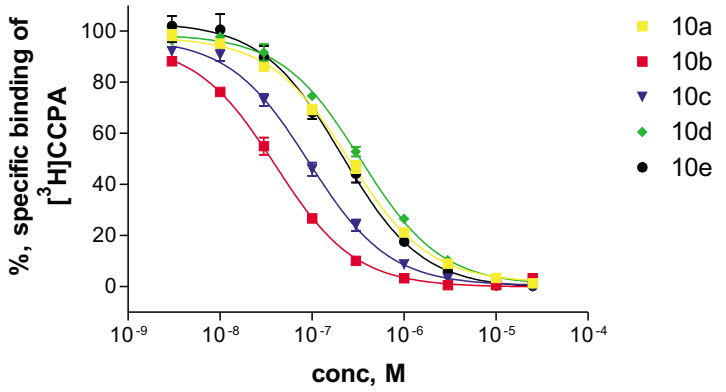



**Figure 4.28:** *Binding of 1,3-dimethylsulfonamidophenylxanthines* ***10a*** *-* ***10e*** *to* $A_1$ *adenosine receptors of rat brain cortical membranes*

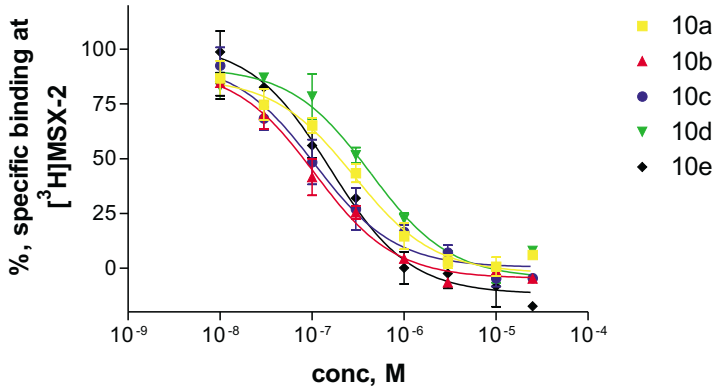
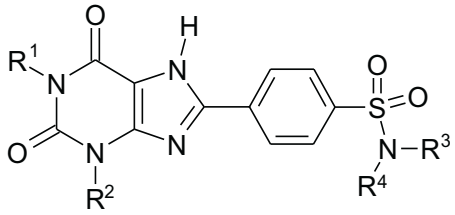
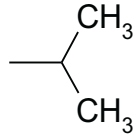
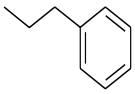
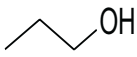
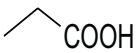
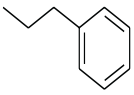
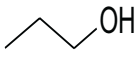
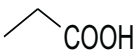
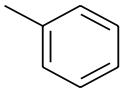



**Figure 4.29:** *Binding of 1,3-dimethylsulfonamidophenylxanthines* ***10a-10e*** *to* $A_{2A}$ *adenosine receptors of rat brain striatal membranes*

**Table 4.7:** Affinity of compounds **10a - 10e** and **11b - 11f** towards $A_1$, $A_{2A}$ and $A_3$ ARs



| | | | | | $K_i \pm$ SEM [nM] or % inhibition of radioligand binding at 10 $\mu M$ | | |
|---|---|---|---|---|---|---|---|
| **co.** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^4$** | **$A_1$-Affinity** Rat [$^3$H]CCPA (n = 3) | **$A_{2A}$-Affinity** Rat [$^3$H]MSX-2 (n = 3) | **$A_3$-Affinity** Human [$^3$H]PSB-11 (n = 2) |
| **10a** | Methyl | Methyl |  | H | 75 ± 9 | 283 ± 13 | 27 % ± 0.6 |
| **10b** | Methyl | Methyl |  | H | 12 ± 1 | 113 ± 9 | 66 % ± 5 |
| **10c** | Methyl | Methyl |  | H | 27 ± 2 | 165 ± 69 | 43 % ± 9 |
| **10d** | Methyl | Methyl |  | H | 98 ± 2 | 347 ± 37 | 49 % ± 3 |
| **10e** | Methyl | Methyl |  | H | 64 ± 4 | 182 ± 65 | 41 % ± 0.3 |
| **11b** | Propyl | H |  | H | 10 ± 2 | 297 ± 81 | 68 % ± 8 |
| **11c** | Propyl | H |  | H | 45 ± 7 | 287 ± 15 | 36 % ± 22 |
| **11d** | Propyl | H |  | H | 100 ± 15 | 320 ± 32 | 25 % ± 13 |
| **11e** | Propyl | H |  |  | 5.5 ± 2 | 42 ± 3 | not determined |
| **11f** | Propyl | H |  | H | 1.8 ± 1 | 23 ± 13 | not determined |

Comparing the $A_1$ affinity of differently N-substituted 1,3-dimethyl sulfonamidophenyl xanthines **10a** - **10e** (Figure 4.28), it can be seen that N-phenylethyl-substituted sulfonamide xanthine **10b** has the highest $A_1$ AR affinity (12.3 nM), N-2-hydroxyethyl-substituted sulfonamide **10d** has the lowest $A_1$ affinity (98 nM), N-carboxymethyl substituted sulfonamide **10e** has a 64 nM affinity at $A_1$ AR. The difference between **10b**, **10d**, **10e** is only 5-fold to 8-fold. This means that the introduction of a polar -OH or -COOH to the sulfonamide does not decrease the $A_1$ AR affinity greatly, comparing the introduction of a polar -COOH or -$SO_3$H in 8-phenylxanthine which induced a large decrease in $A_1$ affinity.

Comparing the affinity of 1,3-dimethylsulfonamidophenylxanthines **10a** - **10e** at rat striatal $A_{2A}$ AR (Figure 4.29) no great difference between these five sulfonamidophenylxanthines can be observed. The $K_i$ values reach from 113 nM (**10b**) to 347 nM (**10d**), so the different substitutions at the nitrogen have little effect on the $A_{2A}$ affinity. Like at $A_1$ AR, the N-phenylethyl substituted sulfonamide **10b** has the highest affinity among these five xanthines.

Comparing the $A_3$ AR affinity of 1,3-dimethyl substituted sulfonamidophenylxanthines, the N-phenylethyl sulfonamide **10b** has the highest affinity with a 65 % inhibition at a concentration of 10 $\mu M$. No great difference between the other N-substituted sulfonamides can be seen.

Like the xanthine sulfonate esters described before, there is also no selectivity of these 1,3-dimethylsulfonamidophenylxanthine for $A_1$ or $A_{2A}$ ARs. The $A_1/A_{2A}$ selectivity ranges from 3-fold (**10e**) to 10-fold (**10b**).

Figure 4.30 - 4.31 give the $A_1$ and $A_{2A}$ affinity curves of the different N-substituted 1-propyl-*p*-sulfonamidoxanthines. From these curves it can be concluded that compound **11f** has the highest $A_1$ affinity among all of the 1-propyl-*p*-sulfonamidoxanthines which reaches 1.8 nM, and the $A_{2A}$ affinity is also high reaching 23 nM. N-Disubstituted compound **11e** also has very high $A_1$ and $A_{2A}$ affinity among these five 1-propyl substituted *p*-sulfonamidoxanthine derivatives, reaching 5.6 nM for $A_1$ and 42 nM for $A_{2A}$. But the $A_1/A_{2A}$ selectivity of these 1-propyl-*p*-sulfoamidoxanthine derivatives is very low, ranging from 30-fold (**11b**) to 3-fold (**11d**). The introduction of a polar -OH or -COOH decreases the affinity at $A_1$ affinity and to a low extent at $A_{2A}$ AR.

So the introduction of an N-phenyl (**11g**) or N-dipropyl subsituted amine (**11f**) in the sulfonamidoxanthine greatly increases the $A_1$ and $A_{2A}$ affinity. Besides this, there is no great difference between the other N-mono-substituted sulfonamidoxanthines concerning the $A_1$, $A_{2A}$ and $A_3$ AR affinity. That means that a polar substitution does not affect the ARs affinity greatly.

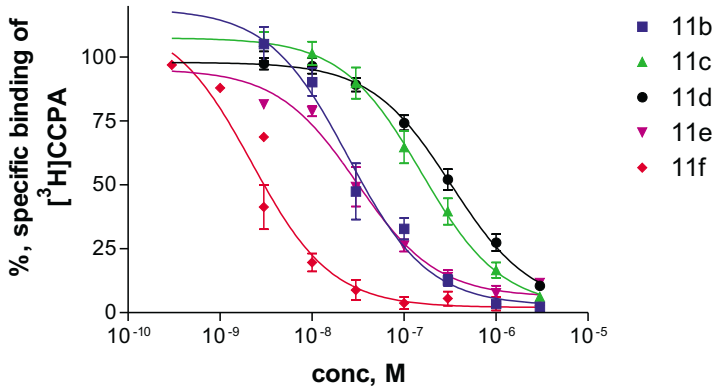



**Figure 4.30:** *Binding of 1-propyl-p-sulfonamidophenylxanthines* ***11b*** *-* ***11f*** *to* $A_1$ *adenosine receptors of rat brain cortical membranes*

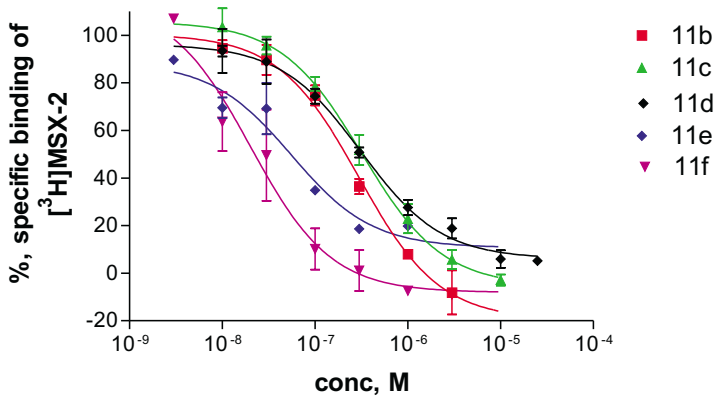



**Figure 4.31:** *Binding of 1-propyl-p-sulfonamidophenylxanthines* ***11b*** *-* ***11f*** *to* $A_{2A}$ *adenosine receptors of rat brain striatal membranes*

#### 4.5.1.3 Results of nitrophenylsulfonate esters at human CHO-$A_{2B}$ ARs

Compounds **5d** and **9d** were selected for the human CHO-$A_{2B}$ binding tests. Figure 4.32 give the inhibition curves of the two compounds at human CHO-$A_{2B}$ ARs.

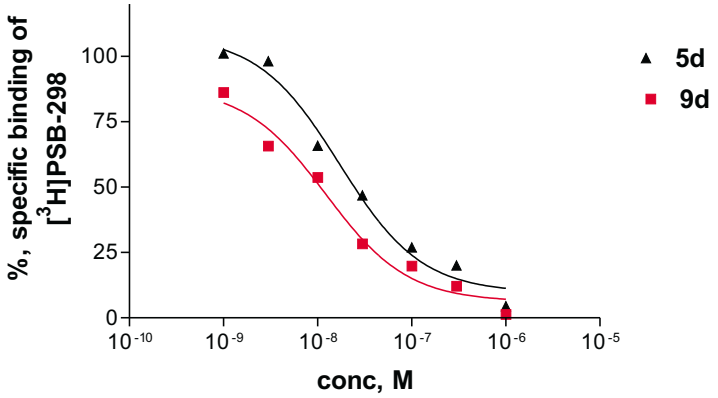



**Figure 4.32:** *Binding of compound **5d** and **9d** to to human CHO-$A_{2B}$ adenosine receptors*

From the curves we can see that both compounds have a relative high affinity at $A_{2B}$ ARs. The $K_i$ value of compound **9d** reaches 5.43 ± 4.3 $nM$, and compound **5d** has a 13.3 ± 3.6 $nM$ $K_i$ value at human CHO-$A_{2B}$ ARs.

### 4.5.2 Conclusions

The synthesized sulfonate esters and sulfoamidoxanthine derivatives were tested in radioligand binding assays for their affinity to $A_1$, $A_{2A}$ and $A_3$ adenosine receptors, two sulfonate esters (**5d**, **9d**) were selected to be tested for $A_{2B}$ receptors as well. It was concluded that all the sulfonate esters and sulfonamide derivatives are very potent $A_1$ and $A_{2A}$ AR antagonists, but the $A_1/A_{2A}$ selectivity is not high, ranging from 3-fold (**5e**) to 30-fold (**9e**). And the two compounds which tested for the $A_{2B}$ showed high potency as well, which have a $K_i$ value of 5.43 $nM$ and 13.3 $nM$ respectively. Most of the compounds don't have activity at $A_3$ receptors.

# Chapter 5

# Syntheses of sulfonate esters of P2 receptor antagonists

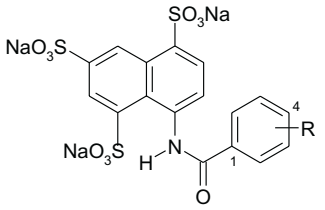
This project was focused on developing a prodrug concept for phenylsulfonic acids and related pharmacologically active sulfonic acid derivatives. Sulfonate groups appear to be essential for receptor affinity and/or selectivity of many P2 purine and pyrimidine receptor antagonists. Therefore, after the work on the sulfonate esters of P1 receptor antagonists which described in Chapter 4, efforts were made to synthesize sulfonate esters of P2 receptor antagonists as well.

As previously discussed in Chapter 1 and 2, P2 antagonists have been developed including suramin, NF023, PPADS and XAMR analogues etc., which contain one or several anionic sulfonate groups. But no further research was reported about the esters of these sulfonates which may be applied as effective sulfonate prodrugs.

Two kinds of P2 antagonists were chosen for the synthesis of the sulfonate esters, these are XAMR and NF023 analogues. Because of the lack of naphthalene trisulfonic acid monosodium salt as starting materials, analogues e.g. sulfanilic acid sodium salt and 2-amino-1,4-benzenedisulfonic acid monosodium salt were applied for the syntheses.

## 5.1 Synthesis of sulfonate esters of XAMR analogues

XAMR analogues (Figure 5.1) were first selected because they belong to the simplest P2 receptor antagonists which contain sulfonic acids. Such analogues with different substituents at the phenyl residue had been synthesized and tested at different P2Y-purinoceptors.[87] Some of them have a good prospect as competitive antagonists for P2 purinoceptor, e.g. XAMR 0721 shows a high affinity for the P2Y purinoceptors ($K_i$ value




| Name | R |
|---|---|
| XAMR0716 | H |
| XAMR0710 | 3-CN |
| XAMR0722 | 4-Cl |
| XAMR0708 | 4-$NO_2$ |
| XAMR0714 | 4-CN |
| XAMR0709 | 3-$NO_2$, 4-$CH_3$ |
| XAMR0721 | 3-$NO_2$, 5-$NO_2$ |

**Figure 5.1:** *Examples of XAMR analogues*

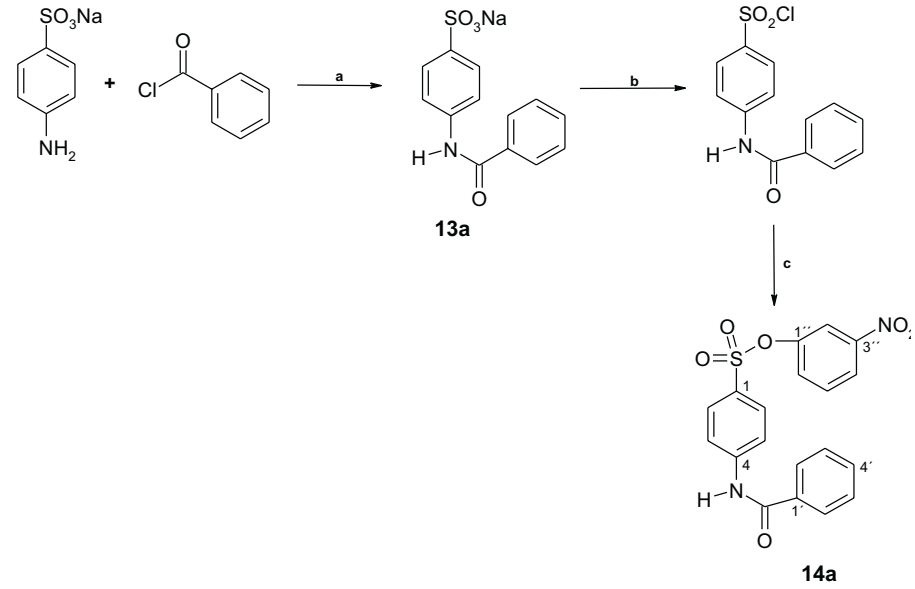
of 19 $\mu M$) inhibiting the binding of the radioligand ADP$\beta^{35}$S to P2Y purinoceptors on turkey erythrocyte membranes. XAMR 0716 is inactive ($> 1000\ \mu M$) at P2Y purinoceptor on the same membranes.[87] XAMR 0721 is weak at the P2Y-receptor of the guinea pig taenia coli, with a $K_d$ value of 785.5 $\mu M$ against ADP$\beta^{35}$S.[88] But XAMR 0721 has good selectivity on P2 purinoceptors and is inactive in inhibiting ecto-nucleotidase activity.[85] Therefore it still plays an important role in the research of P2 purinoceptor-mediated effects.

In this project, sulfonate esters of two XAMR analogues are synthesized. One is the ester of XAMR 0721 which has the highest P2 antagonist affinity among XAMR analogues, the other is the ester of XAMR 0716.

### 5.1.1 Synthesis of 4-benzoylamino-1-benzenesulfonic acid *m*-nitrophenyl ester (14a)

An aqueous solution of sulfanilic acid was treated at pH 3 with a solution of benzoyl chloride in toluene to obtain benzamides **13a** (Figure 5.2). It was reported[144] that the hydrolyzed benzoic acid, which formed as a by-product, could be removed completely by extracting the solution with diethyl ether at pH 3. But due to the lack of a special diethyl ether extracting apparatus, extracting of the water phase with enough toluene was applied, until no more benzoic acid could be detected by TLC in the water phase.

The synthesis of the desired sulfonate ester was accomplished in the usual way: chlorination of the free sulfonic acid was followed by esterification of the resulting sulfonyl--




(a) $H_2O$/toluene, pH 3; (b) 1.) pyridine, acetic anhydride, r.t. overnight; 2.) $PCl_5$, 60 °C, 2 h;
(c) sodium *m*-nitrophenoxide, TEA, $CH_2Cl_2$.

**Figure 5.2:** *Synthesis of 4-benzoylamino-1-benzenesulfonic acid m-nitrophenyl ester* ***14a***

chloride (Figure 5.2). The sodium sulfonate **13a** was converted to the corresponding sulfonyl chloride by first converting the sodium sulfonate to the pyridinium sulfonate, then chlorination by heating it with phosphorus pentachloride.[145],[146] Then the sulfonyl chloride was reacted with sodium *m*-nitrophenoxide to yield the expected sulfonate ester **14a**.

*Confirmation of the structure*

The $^1$H-NMR of compound **14a** is shown in Figure 5.3. Integration of the protons displays the signals of fourteen protons. The amide NHCO signal is at 10.65 ppm as one proton. The four protons of the sulfonate ester can be assigned according to the previous P1 sulfonate esters which are described in Chapter 4. The four protons of the main phenyl ring are an AA'BB'-system, the chemically but not magnetically equivalent protons H2 and H6 form the AA'part, and because of the effect from sulfur (S), they appear at lower field than H3 and H5, the signal of H2 and H6 being at 8.09 ppm (the $J_{AB}$ or $J_{A'B'}$ coupling is 9.14 Hz). The signal of H3 and H5 is at 7.9 ppm (the $J_{BA}$ or $J_{B'A'}$ coupling is 8.51 Hz). The five protons of the benzamide residue are an ABXB'A'system, the H4' couples with the other two ortho protons, therefore the coupling constant reaches 8.35 Hz, and the signal appears at 7.63 ppm. H3' and H5' have the same chemical shifts, and they are also coupled with the ortho position protons, therefore they are at 7.55 ppm.

H2'and H6' as the AA' have also the same chemical shifts, the signal is at 7.96 ppm.

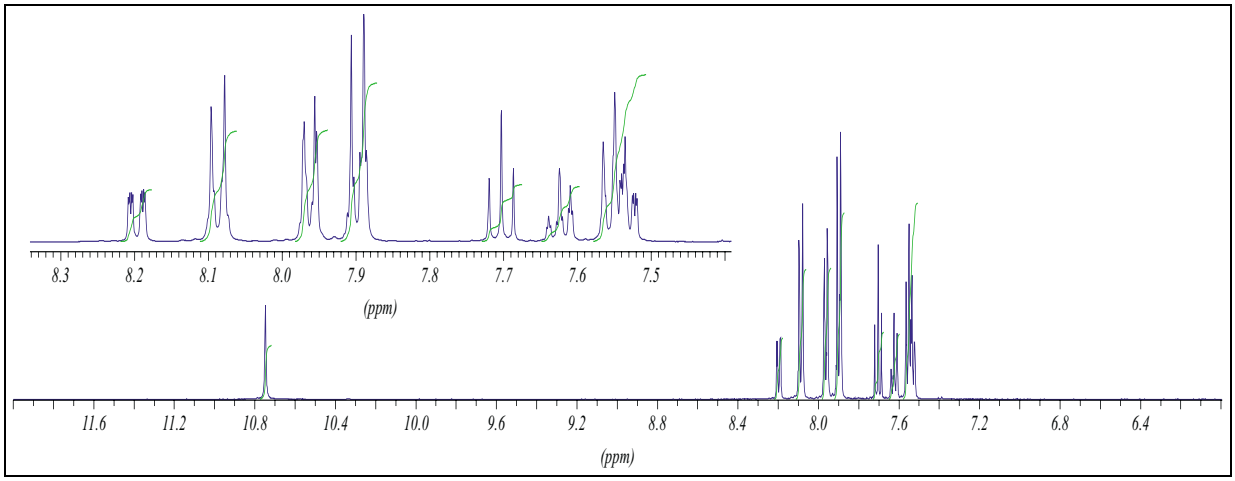



**Figure 5.3:** *Part of the* $^{1}$*H-NMR spectrum of* ***14a*** *in DMSO-*$d_6$

Figure 5.4 - 5.5 show the $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra of **14a**. The $^{13}$C-NMR spectra displayed signals of fifteen carbon atoms, and DEPT $^{13}$C-NMR subspectra indicated that there are nine methyl and six quaternary carbon atoms. At 166.5 ppm the signal of CONH appears clearly. Figure 5.6 gives the assignment of proton and carbon signals for compound **14a**.

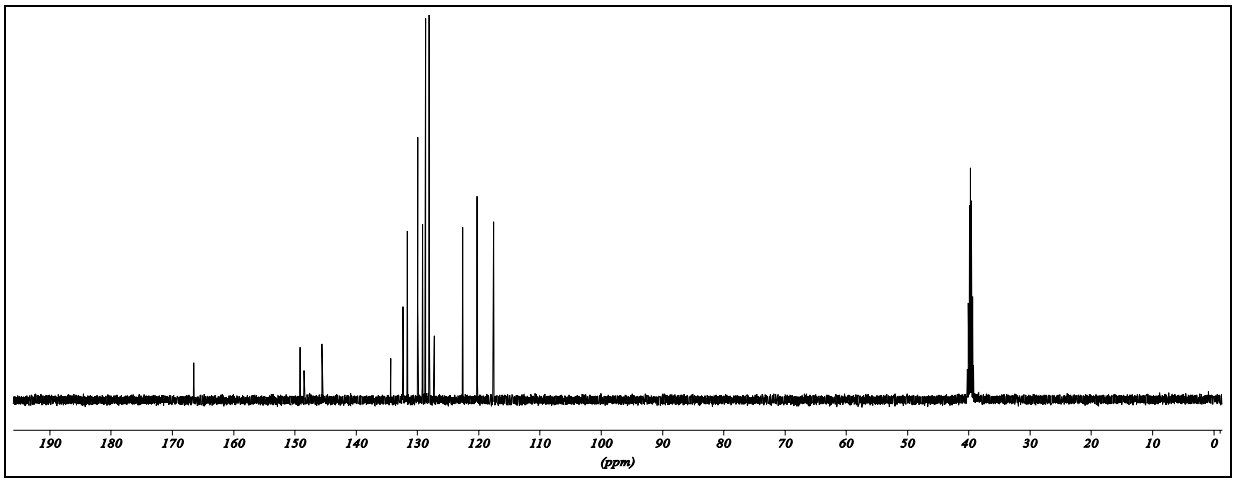



**Figure 5.4:** *Part of the* $^{13}$*C-NMR spectrum of* ***14a*** *in DMSO-*$d_6$

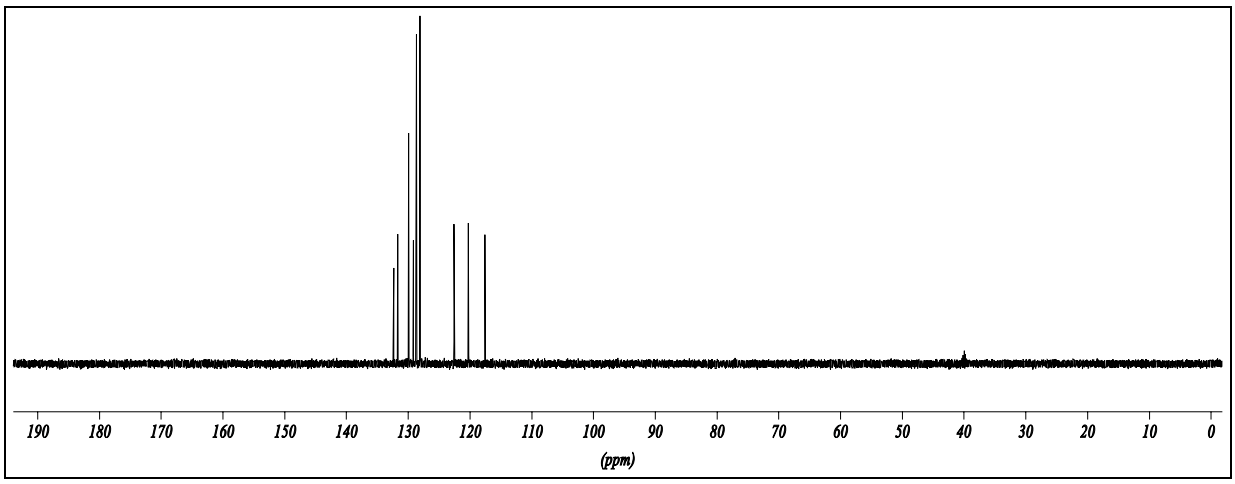



**Figure 5.5:** *Part of the DEPT* $^{13}$*C-NMR spectrum of* ***14a*** *in DMSO-*$d_6$

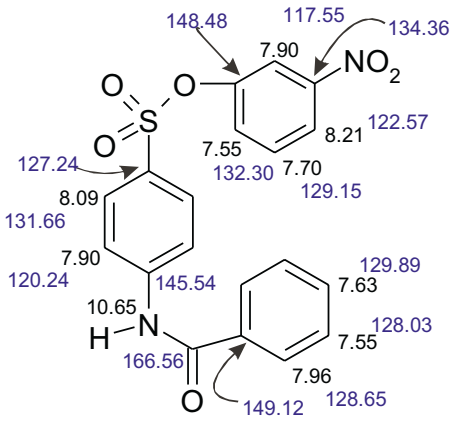
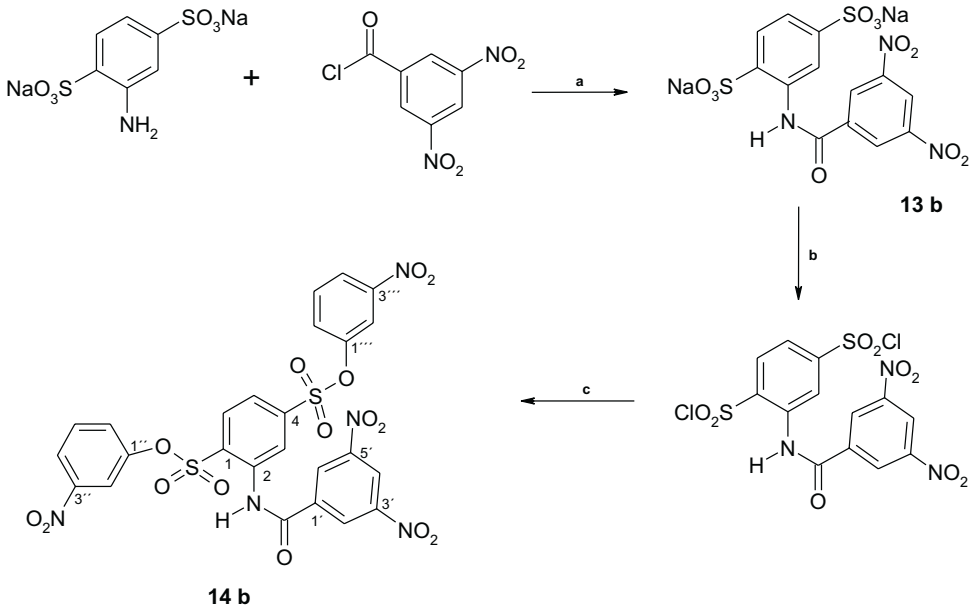



**Figure 5.6:** *Assignment of proton and carbon signals for compound* ***14a***

### 5.1.2 Synthesis of 2-(3',5'-dinitrobenzoylamino)benzene-1,4-disulfonic acid bis-*m*-nitrophenyl ester (14b)

An aqueous solution of the 2-amino-1,4-benzenedisulfonic acid monosodium salt was treated at pH 4.5 with a solution of 3,5-dinitrobenzoyl chloride in toluene to obtain the benzamide **13b**. The disodium sulfonate **13b** was converted to the corresponding bis-sulfonyl chloride by heating it directly with phosphorus pentachloride, then pour it was carefully poured onto chipped ice. The bis-sulfonyl chloride was subsequently reacted with the sodium *m*-nitrophenoxide to get the desired sulfonate ester **14b** (Figure 5.7).




(a) $H_2O$/toluene, pH 4.5; (b) 1.) $PCl_5$, 150 °C, 2hr; 2.) chipped ice; (c) sodium *m*-nitrophenoxide, TEA, $CH_2Cl_2$

**Figure 5.7:** *Synthesis of 2-(3',5'-dinitro-benzoylamino)benzene-1,4-disulfonic acid bis-m-nitrophenyl ester* ***14b***

*Confirmation of the structure:*

Figures 5.8 - 5.9 show the HH-COSY and HMQC spectra of compound **14b**. Integration of the protons displays fourteen protons, there are altogether twenty-three carbon signals in the $^{13}$C-NMR spectrum. The NHCO signal cannot be seen in the $^{1}$H-NMR spectrum, but it appears in the $^{13}$C-NMR at 157.09 ppm. Since the two sulfonate esters are in different position of the main body, they have quite different chemical shifts for protons and also for carbons. The benzamide signals can easily be distinguished, because they moved to lower field with the influence of the $NO_2$ group.

With the help of the $^{1}$H-NMR, $^{13}$C-NMR, DEPT $^{13}$C-NMR, HHCOSY and HMQC spectra, all chemical shifts of each proton and carbon can be assigned (Figure 5.10).

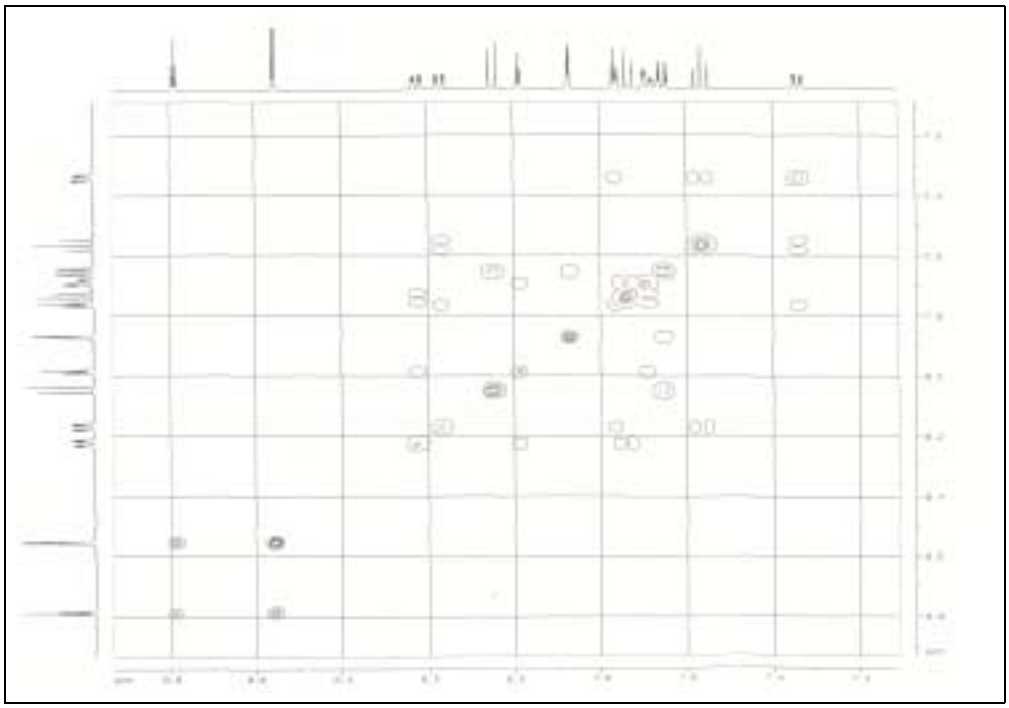


**Figure 5.8:** *Part of the HH-COSY spectrum of* ***14b*** *in DMSO-*$d_6$

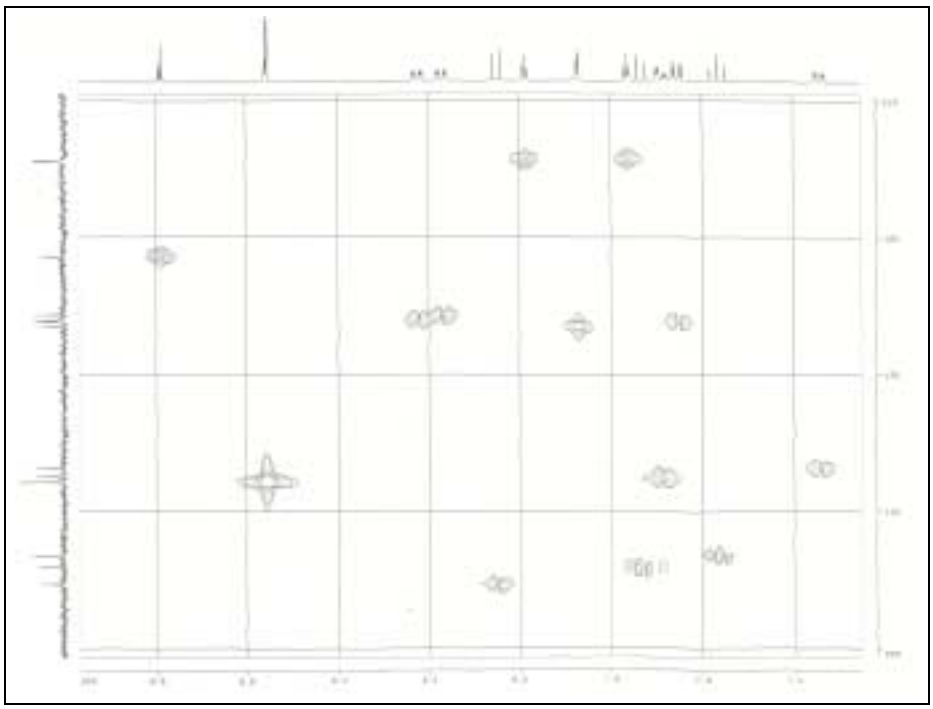


**Figure 5.9:** *Part of the HMQC spectrum of* ***14b*** *in DMSO-*$d_6$

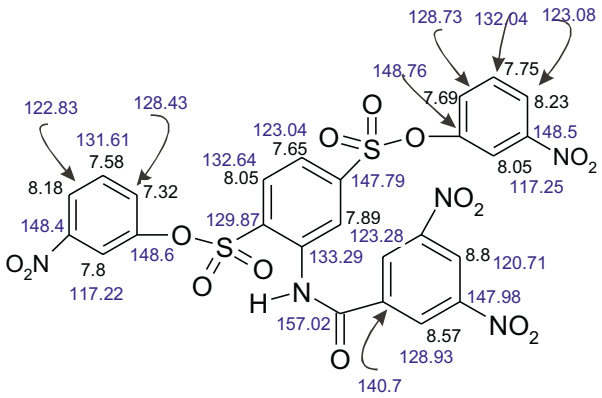



**Figure 5.10:** *Assignment of proton and carbon signals for compound* ***14b***

## 5.2 Synthesis of sulfonate esters of NF023 analogues

As we known, most P2 antagonists are symmetrical polysulfonate compound, e.g. suramine, NF023 etc. But there is no report about the isophthaloylamido-bisnaphthalen trisulfonic acid, although it is one of the simplest symmetrical compounds (Figure 5.11). Sulfanilic acid sodium salt and 2-amino-1,4-benzenedisulfonic acid monosodium salt were used as the starting materials for the synthesis instead of naphthalene trisulfonic acid, because of the lack of 1,3,5-naphtalene trisulfonic acid as the raw material.

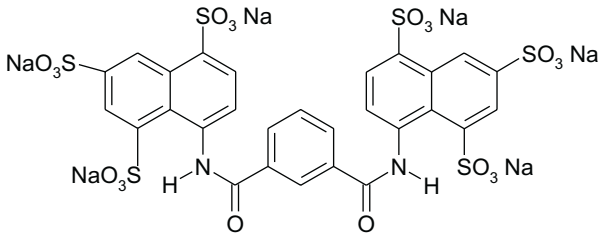
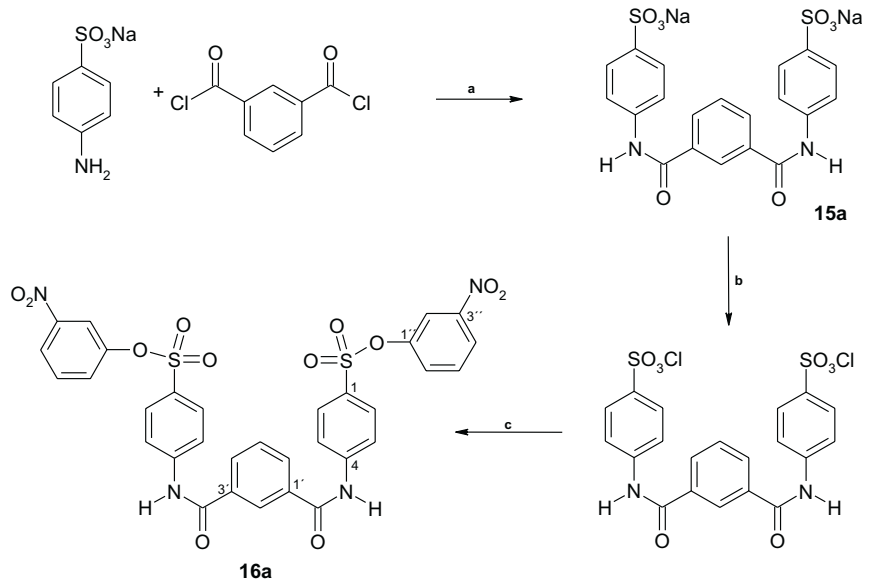



**Figure 5.11:** *Structure of isophthaloyl bisnaphthalene trisulfonic acid*

### 5.2.1 Synthesis of isophthaloyl-N,N-bis-(1-benenesulfonic acid)-*m*-nitrophenyl ester (16a)

The synthetic route is shown in Figure 5.12. Sulfanilic acid reacted with isophthaloyl dichloride at pH 3 yielding the free sulfonic acid sodium salt **15a**. Then this sulfonate sodium salt was chlorinated to sulfonyl chloride with $PCl_5$, and the produced sulfonyl chloride reacted with sodium *m*-nitrophenoxide using TEA as a base catalyst to get the

desired ester **16a**.




(a) $H_2O$/Toluene, pH 3; (b) 1.) $PCl_5$, 150°C 2 h; 2.) chipped ice; (c) sodium *m*-nitrophenoxide, TEA, $CH_2Cl_2$.

**Figure 5.12:** *Synthesis of isophthaloyl-N,N-bis-(1-benenesulfonicacid)-m-nitrophenyl ester* ***16a***

*Confirmation of the structure:*

Figures 5.13 - 5.15 show the $^1$H-NMR, $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra of compound **16a**. The compound is symmetrical, therefore integration of the protons displayed signals of twenty protons, and fifteen carbon signals. The amide NHCO signal is at 10.65 ppm as one proton, the *m*-nitrophenyl ester residue can be assigned after comparison with the other sulfonate esters. The main body is an AA'BB'system, see chapter 5.1, the coupling constants of $J_{AB}$ or $J_{A'B'}$ is 9.14 Hz. Because of the influence of the carboxamido group, C2'H is shifted downfield to 8.55 ppm, coupled with H4' and H6' as 1.3 Hz, with H5' as 1.57 Hz.

The $^{13}$C-NMR showed signals of fifteen carbon atoms, and the DEPT $^{13}$C-NMR indicated nine methyl, and six quaternary carbon atoms. The NHCO signal appears at 165.86 ppm. The rest can be assigned after comparison with the previous described sulfonate ester analogues **14a**.

After comparison of the $^1$H-NMR, $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra, the chemical shifts of protons and carbons of compound **16a** can be assigned (Figure 5.16).

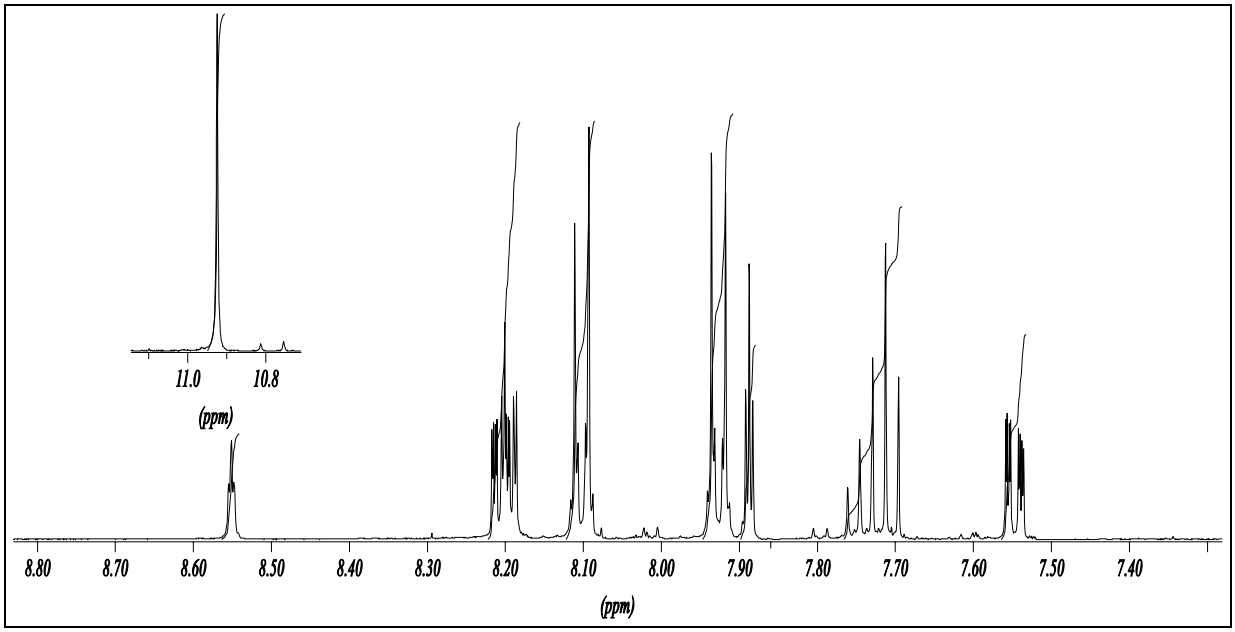



**Figure 5.13:** *Part of the* $^{1}$*H-NMR spectrum of compound* ***16a*** *in DMSO-*$d_6$

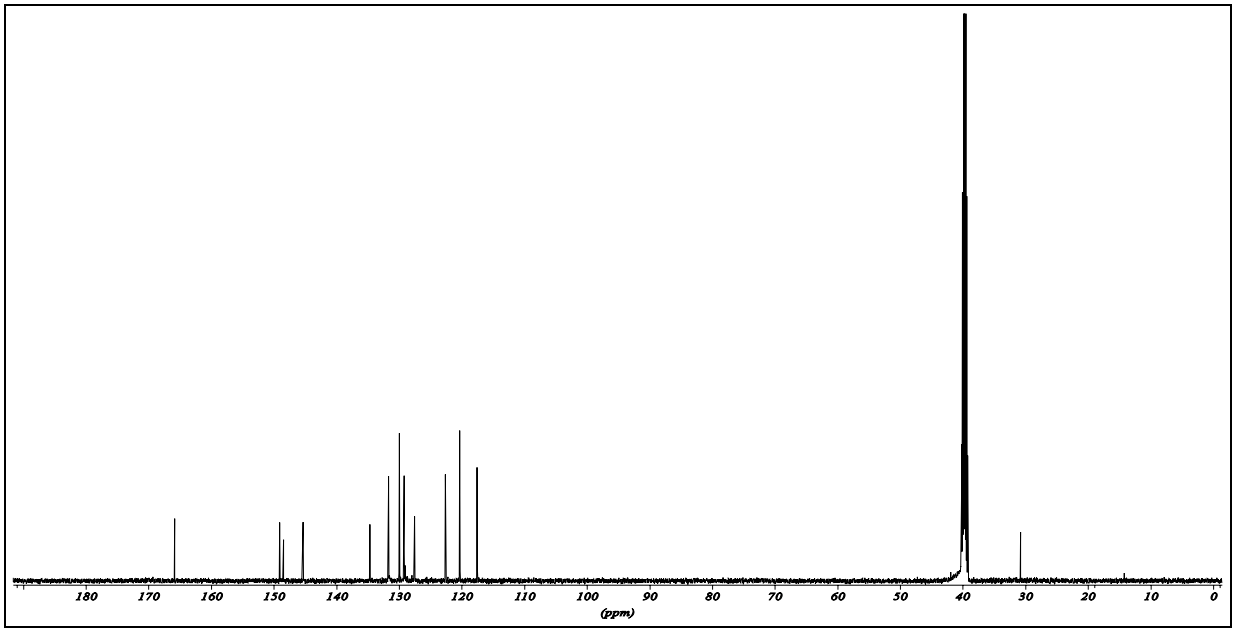



**Figure 5.14:** *Part of the* $^{13}$*C NMR spectrum of compound* ***16a*** *in DMSO-*$d_6$

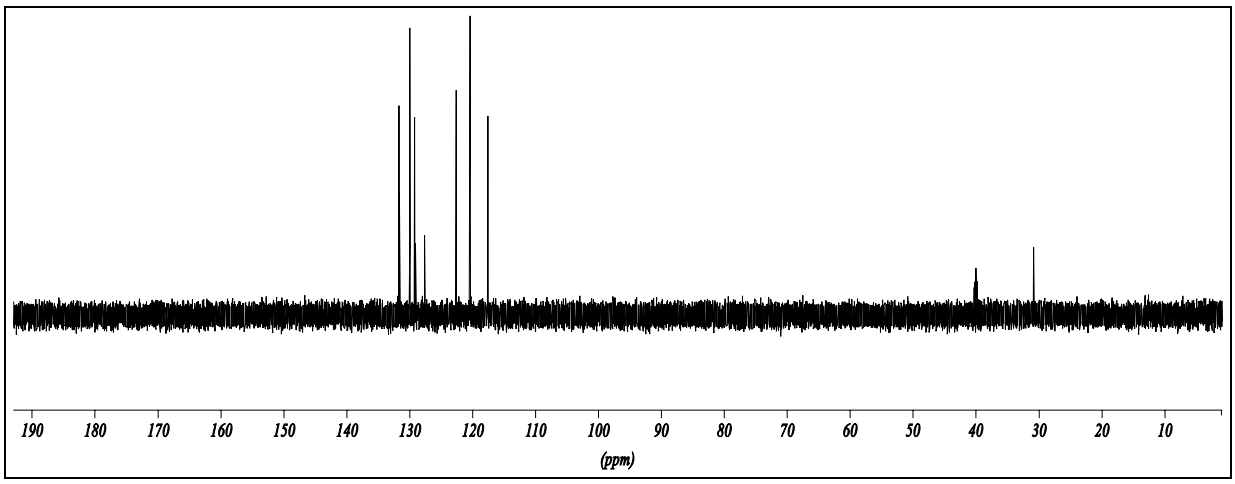



**Figure 5.15:** *Part of DEPT* $^{13}$*C-NMR spectrum of compound* ***16a*** *in DMSO-*$d_6$

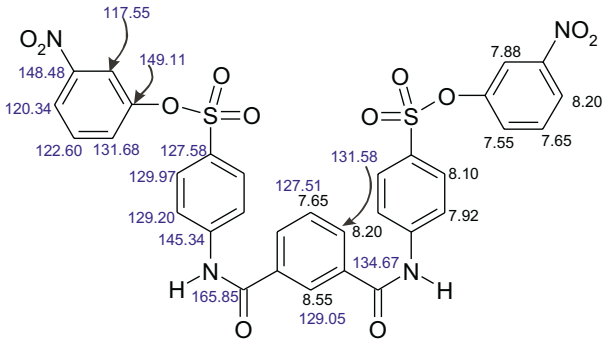
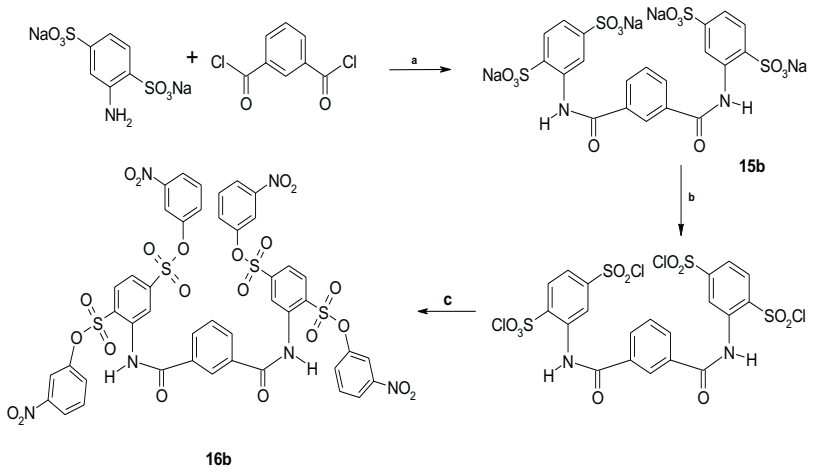



**Figure 5.16:** *Assignment of proton and carbon signals for compound* ***16a***

### 5.2.2 Synthesis of isophthaloyl-N,N-bis-(benzene-1,4-disulfonic acid)-*m*-nitrophenyl ester (16b)

The synthetic route is shown in Figure 5.17. 2-Amino-1,4-benzene disulfonic acid monosodium salt reacted with isophthaloyl dichloride at pH 3 to yield the free sulfonic acid sodium salt **15b**. Then this sodium sulfonate was chlorinated to sulfonyl chloride by means of $PCl_5$, and subsequently reacted with the sodium m-nitrophenoxide to yield the ester **16b**. Unfortunately no clean **16b** was obtained even though flash chromatography was done several times, but the NMR spectra confirmed that the esterification had happened. The problem in this procedure is the isolation and purification of this product.




(a) pH 3, $H_2O$/toluene. (b) $PCl_5$, 150°C 2hr. (c) sodium m-nitrophenoxide, TEA,$CH_2Cl_2$.

**Figure 5.17:** *Synthesis of isophthaloylamino-N,N-bis-benzene-1,4-disulfonic acid m-nitrophenyl ester* ***16b***

## 5.3 Synthesis of NF023 analogues

NF023 (8,8'-(carbonyl-bis-(imino-4-methyl-3,1-phenylene)carbonylimino)-bis-(1,3,5-naphthalenetrisulfonic acid)) was first introduced by Nickel et al. in 1986.[144] Instead of 1,3,5-naphthalenetrisulfonic acid, sulfanilic acid and 2-amino-1,4-benzenedisulfonic acid monosodium salt were used as raw materials for the synthesis. The esterification of these NF023 analogues was not done up because macromolecular compounds were not the target compounds of our research, so only these NF023 analogues were synthesized.

### 5.3.1 Synthesis of 4,4-(carbonylbis-(imino-1',3'-phenylene)carbonylimino)bis-1-sulfonic acid sodium salt (19a)

The synthetic route of the aimed compound is showed in Figure 5.18. An aqueous solution of sulfanilic acid was treated at pH 3 with a solution of 3-nitrobenzoyl chloride in toluene to get benzamide **17a**. 4-(3'-Amino-benzoylamino)-benzenesulfonic acid sodium salt **18a** was obtained by hydrogenation of 4-(3'-nitro-benzoylamino)-benzenesulfonic acid sodium salt **17a** in water at pH 8 using palladium/charcoal as the catalyst. Later the urea was prepared by treating the amides with phosgene.[148] During the whole phosgene reaction, pH 3 was strictly controlled by automatic addition of 2 M $Na_2CO_3$. When the reaction happened, the reaction color turned from yellow to white, and large amounts of precipitate were formed from the solution. Unfortunately it was impossible to push the reaction to

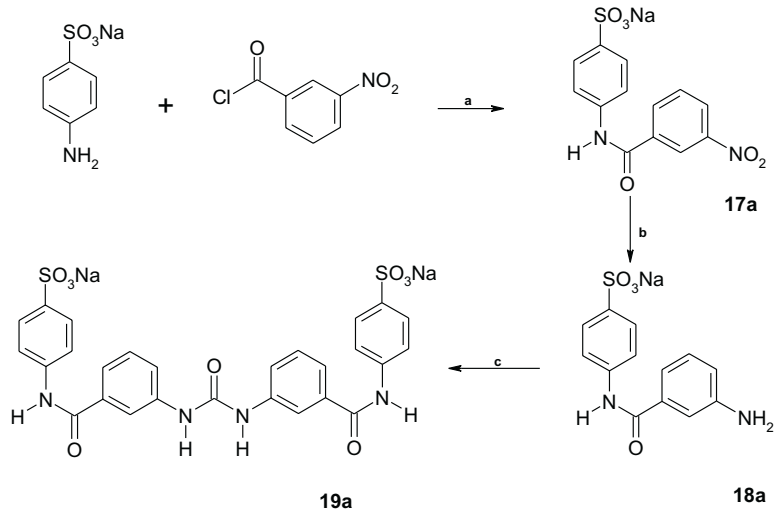



**Figure 5.18:** *Synthesis of 4,4-(carbonylbis-(imino-1',3'-phenylene)carbonylimino)-bis-1-sulfonic acid sodium salt* ***19a***

completion, even when a very large excess of phosgene was used (30 moles per mole of **18a**), the starting material could still be detected on TLC, but all these starting materials stay in solution, and the precipitated substance at this pH value was only the desired urea which produced.

*Confirmation of the structure:*

Figure 5.19 - 5.21 show the $^{1}$H-NMR, $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra of compound **19a**. The compound is symmetrical, therefore integration of the protons displayed signals of ten protons. There are twelve carbon signals in the $^{13}$C-NMR spectrum, and six methyl carbon, and six quaterary carbon from the DEPT $^{13}$C-NMR spectrum. The NHCONH signal appears at 9.1 ppm in $^{1}$H-NMR and at 152.79 ppm in $^{13}$C-NMR. The CONH signal is seen at 10.3 ppm in $^{1}$H-NMR and at 165.86 ppm in the $^{13}$C-NMR spectraum.

The chemical shifts of protons and carbons of the compound **19a** can be assigned according to the $^{1}$H-NMR and $^{13}$C-NMR spectra (see Figure 5.27).

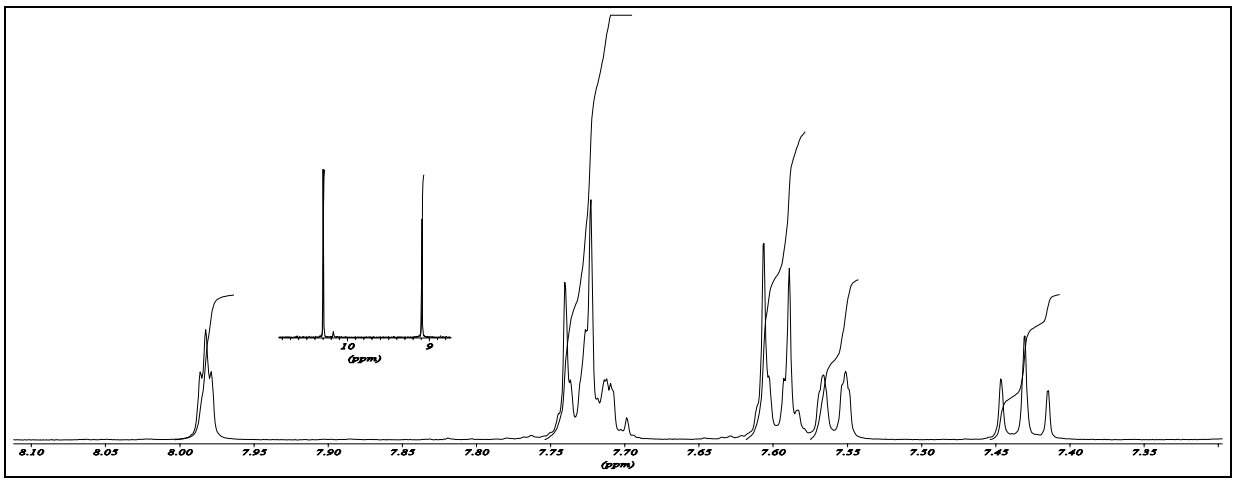



**Figure 5.19:** *Part of the* $^{1}$*H-NMR spectrum of compound* ***19a*** *in DMSO-*$d_6$

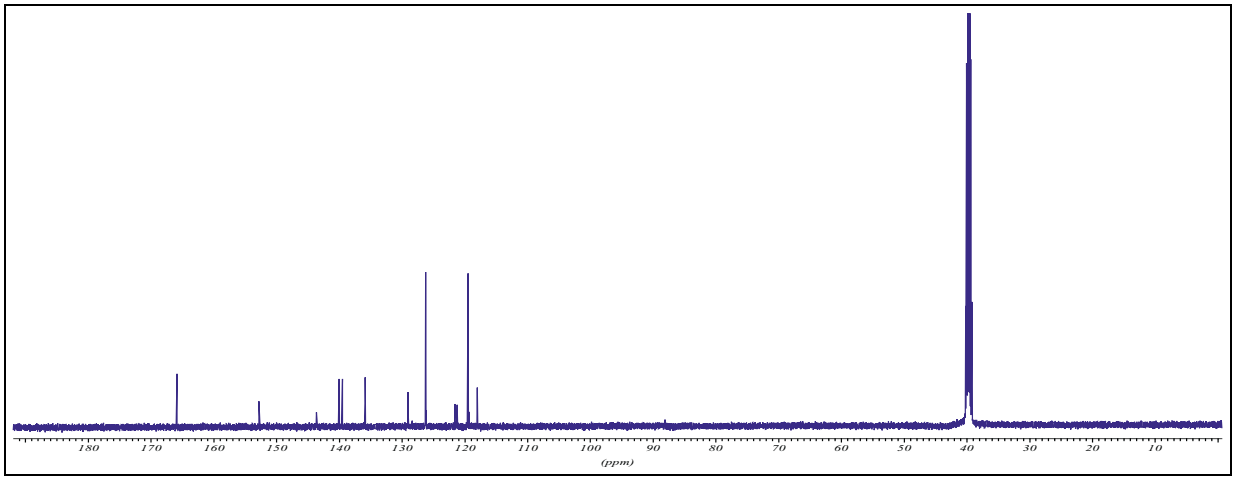



**Figure 5.20:** *Part of* $^{13}$*C NMR spectrum of compound* ***19a*** *in DMSO-*$d_6$

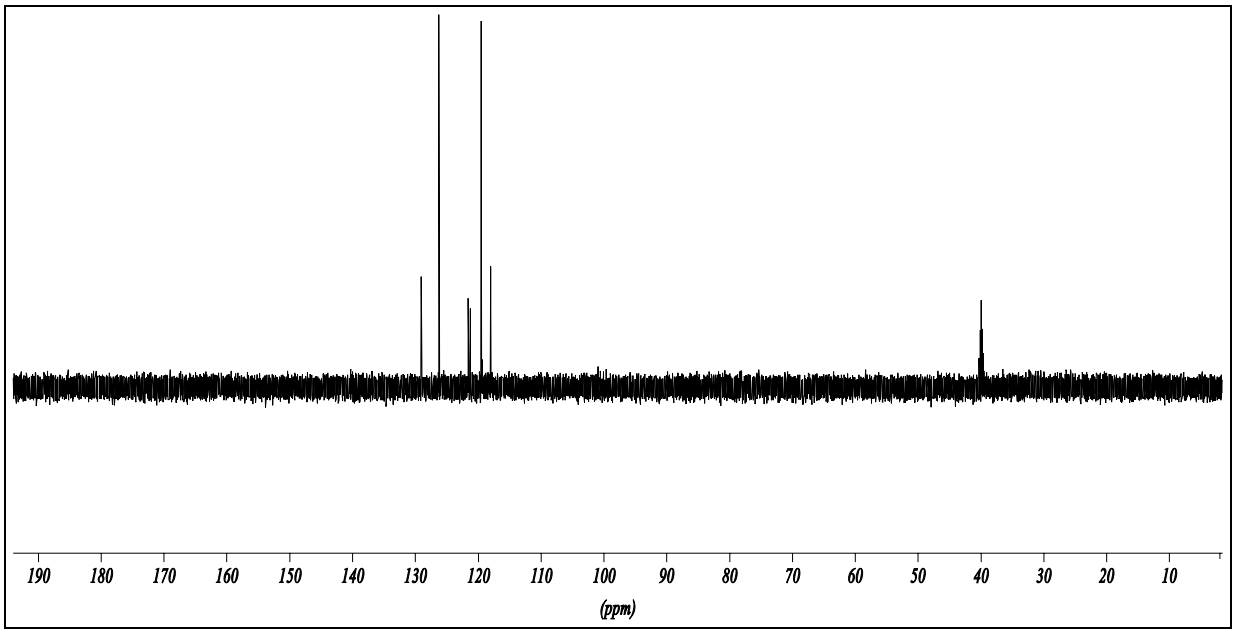



**Figure 5.21:** *DEPT* $^{13}$*C-NMR spectrum of compound* ***19a*** *in DMSO-*$d_6$

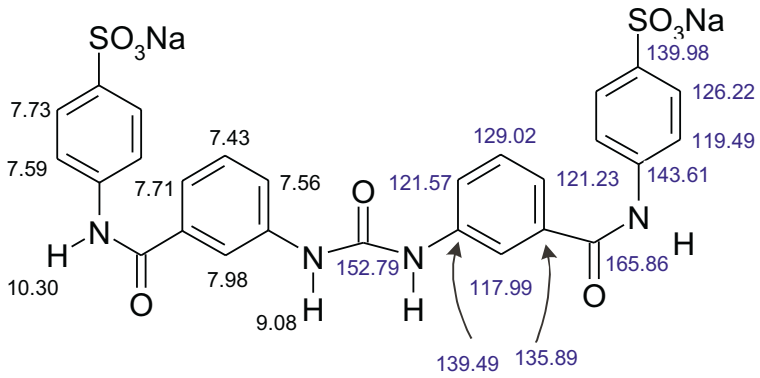



**Figure 5.22:** *Assignment of proton and carbon signals of* ***19a***

### 5.3.2 Synthesis of 2,2-(carbonylbis-(imino-1',3'-phenylene)carbonylimino)-bis-1,4-disulfonic acid monosodium salt (19b)

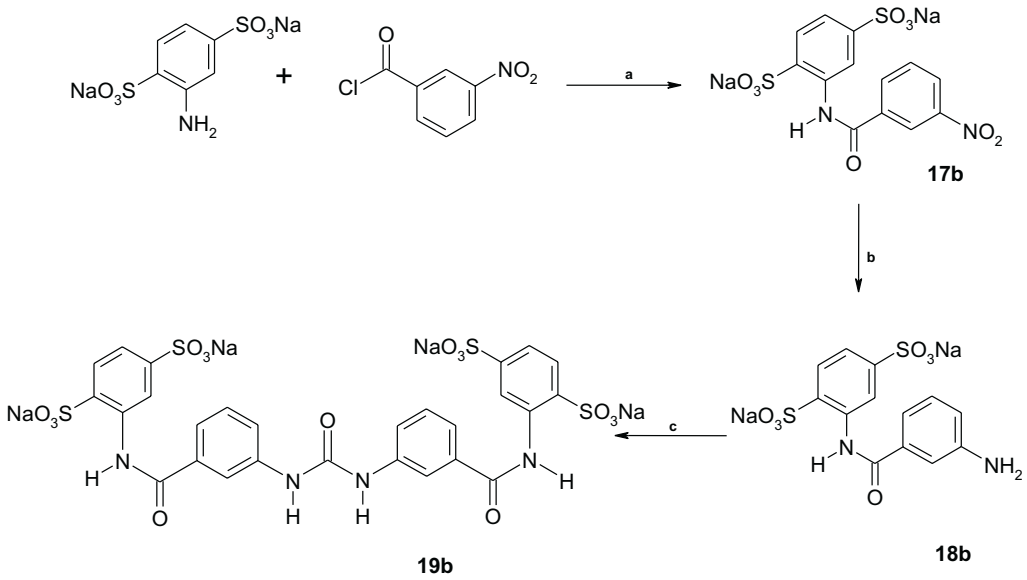



**Figure 5.23:** *Synthesis of 2,2-(carbonylbis-(imino-1',3'-phenylene)carbonylimino)-bis-1,4-disulfonic acid monosodium salt* ***19b***

The synthetic route of the desired compound is shown in Figure 5.23. The synthetic procedure is almost the same as for compound **19a**. The only difference is the reaction with phosgene, since the 2-(3'-amino-benzoylamino)benzene-1,4-disulfonic acid sodium salt **18b** has a better water-solubility than 4-(3'-amino-benzoylamino)-benzenesulfonic acid sodium salt **18a**, therefore all the reactants are well soluble in water, thus the reaction proceeds to completion.

*Comfirmation of the structure:*

Figure 5.24 - 5.26 show the $^{1}$H-NMR, $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra of compound **19b**. The compound is symmetrical, therefore integration of the proton displayed signals of nine protons. There are thirteen carbon signals in $^{13}$C-NMR spectrum, and seven methyl carbons, and six quaterary carbons in the DEPT $^{13}$C-NMR spectrum. The NHCONH signal is at 9.66 ppm in the $^{1}$H-NMR and at 152.84 ppm in the $^{13}$C-NMR, the CONH signal is at 11.33 ppm in the $^{1}$H-NMR and at 164.02 ppm in the $^{13}$C-NMR spectrum.

The chemical shifts of protons and carbons of compound **19b** can be assigned according to the $^{1}$H-NMR, $^{13}$C-NMR and DEPT $^{13}$C-NMR spectra (see Figure 5.27).

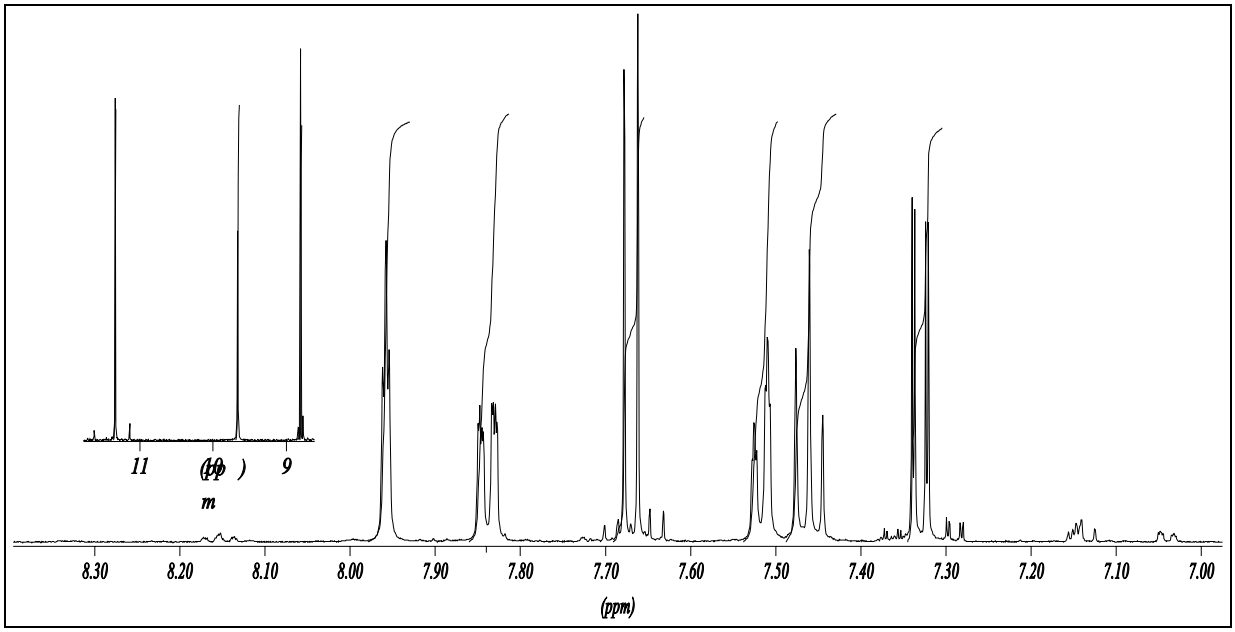



**Figure 5.24:** *Part of* $^{1}$*H-NMR spectrum of compound* ***19b*** *in DMSO-*$d_6$

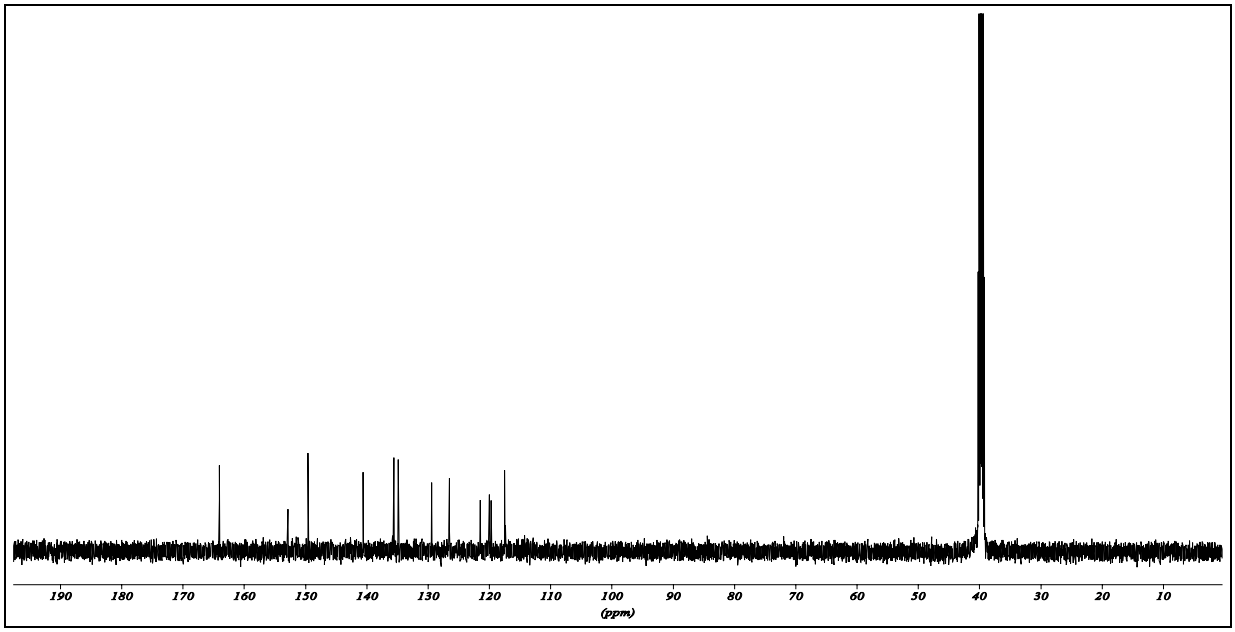



**Figure 5.25:** *Part of* $^{13}$*C-NMR spectrum of compound* ***19b*** *in DMSO-*$d_6$

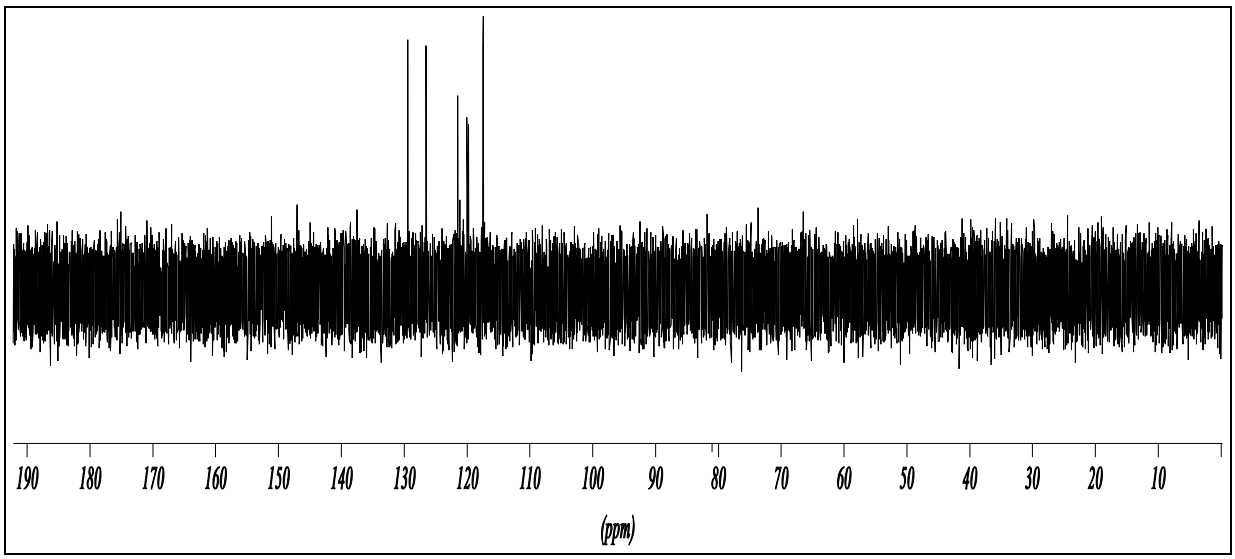



**Figure 5.26:** *Part of DEPT* $^{13}$*C-DEPT spectrum of compound* ***19b*** *in DMSO-*$d_6$

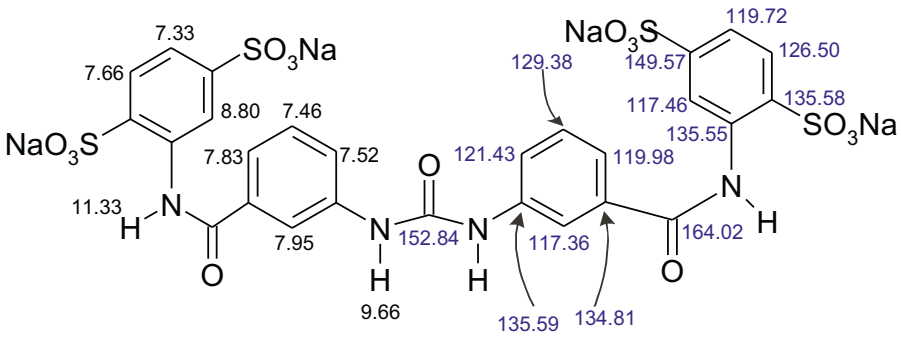
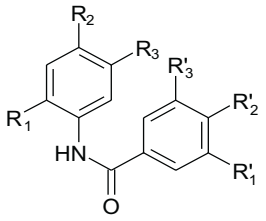


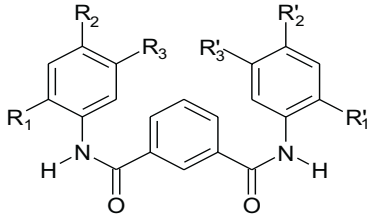

**Figure 5.27:** *Assignment of proton and carbon signals of* ***19b***

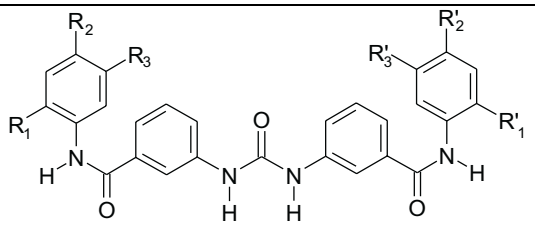
## 5.4 Pharmacological results and discussion

Some of the synthesized compounds were tested at the $P2Y_2$ receptor of NG108-15 (mouse neuroblastoma x rat glioma) hybrid cells in our group by Andrea Hunder and Marko Kaulich. Table 5.1 gives the percentage of inhibition of UTP-stimulated increase in intracellular calcium release mediated by $P2Y_2$ receptors. The compounds were investigated at a concentration of 100 $\mu M$.

**Table 5.1:** *Antagonist activity of compounds **13a** - **19b** towards $P2Y_2$ receptors (n = 2).*




| compd. | $R_1$ | $R_2$ | $R_3$ | $R'_1$ | $R'_2$ | $R'_3$ | % inhibition ± SEM |
|---|---|---|---|---|---|---|---|
| **13a** | H | $SO_3Na$ | H | H | H | H | 19 ± 12 |
| **14a** | H | *m*-NPS[a] | H | H | H | H | 100 ± 5 |
| **17a** | H | $SO_3Na$ | H | $NO_2$ | H | H | 8.2 ± 2 |
| **13b** | $SO_3Na$ | H | $SO_3Na$ | $NO_2$ | H | $NO_2$ | 7.9 ± 1.3 |
| **14b** | *m*-NPS[a] | H | *m*-NPS[a] | $NO_2$ | H | $NO_2$ | 34 ± 7.8 |
| **17b** | $SO_3Na$ | H | $SO_3Na$ | $NO_2$ | H | H | 18 ± 5.4 |
| | | |  | | | | |
| **15a** | H | $SO_3Na$ | H | H | $SO_3Na$ | H | 11.6 ± 7.6 |
| **16a** | H | *m*-NPS[a] | H | H | *m*-NPS[a] | H | 49 ± 10 |
| **15b** | $SO_3Na$ | H | $SO_3Na$ | $SO_3Na$ | H | $SO_3Na$ | 4.8 ± 1.4 |




| compd. | $R_1$ | $R_2$ | $R_3$ | $R'_1$ | $R'_2$ | $R'_3$ | % inhibition ± SEM |
|---|---|---|---|---|---|---|---|
| **19a** | H | $SO_3Na$ | H | H | $SO_3Na$ | H | 27.3 ± 3.8 |
| **19b** | $SO_3Na$ | H | $SO_3Na$ | $SO_3Na$ | H | $SO_3Na$ | 4.8 ± 0.7 |

[a] *m*-NPS: m-nitrophenylsulfonate

It was shown that most of these sulfonic acids and sulfonate esters are inactive at $P2Y_2$ receptors except compound **14a** which shows 100 % inhibition at the concentration of 100 $\mu M$. The inhibition of the other compounds are less than 50 % at 100 $\mu M$ concentration.

The $IC_{50}$ of compound **14a** was determined by stimulation of intracellular calcium release with 3 $\mu M$ UTP. It was shown that the $IC_{50}$ of **14a** reaches 8.6 ± 1.0 $\mu M$. Further antagonist activity tests towards other P2 receptor subtypes are still in progress.

## 5.5 Conclusions

In the P2 receptor antagonist field, new XAMR analogues and NF023 analogues using sulfanilic acid and 2-amino-1,4-benzenedisulfonic acid monosodium salt as starting material were synthesized.

Synthesis of these sulfonic acid analogues could be easily performed according to the reported method with some modification, i.e the pH value during the synthesis of compounds **13a, 13b, 15a** and **16b** had not need to be strictly controlled, manual addition of 2 M $Na_2CO_3$ was sufficient. For the synthesis of urea compounds **19a** and **19b** by using phosgene, a pH value of 3 had to be strictly controlled by automatical addition of 2 M $Na_2CO_3$, otherwise no products could be obtained. During the synthesis of compounds **13a, 13b, 15a** and **16b**, by-product of hydrolyzed benzoic acid could be removed by extracting the solution with toluene several times without the specific ether extraction apparatus which was described in the literature.[144]

The *m*-nitrophenyl sulfonate esters of the XAMR analogues and some of the NF023 analogues **14a, 14b** and **16a** were synthesized by chlorination and esterification of the sulfonic acid derivatives **13a, 13b** and **15a**.

The inhibitory potency of some of the synthesized potential P2 receptor antagonists was tested at $P2Y_2$ receptors of NG108-15 cells. It is concluded that the sulfanilic acid and 2-amino-1,4-benzene disulfonic acid monosodium salts of XAMR and NF023 analogues are inactive at $P2Y_2$ receptors. The sulfonate esters of XAMR and NF023 derivatives are inactive at the $P2Y_2$ receptor as well except compound **14a** which has an $IC_{50}$ of 8.6 $\mu M$ (stimulation with 3 $\mu M$ UTP).

Comparing the inhibition by the free sulfonic acid derivatives and the sulfonate ester derivatives ( **13a, 14a** have 19 % and 100 % inhibition respectively; **13b, 14b** have 7.9 % and 34 % inhibition respectively; **15a, 16a** have 11.6 % and 48 % inhibition respectively at the concentration of 100 $\mu M$), we can conclude that sulfonate esters have somewhat higher antagonistic activity than the free sulfonic acids at $P2Y_2$ receptors.

# Chapter 6

# Experimental Part

## 6.1 Stability - experimental part

### 6.1.1 Materials and instruments

#### 6.1.1.1 Materials

The xanthine derivatives were synthesized according to chapter 4.1. Stock solutions of the xanthines were prepared in DMSO at concentrations of 2 mM, 20 mM and 50 mM respectively and stored at 4 °C. For the chemical stability test, diclofenac-Na was used as internal standard at a concentration of 1 mg/mL. *p*-Aminosalicylate sodium was used as internal standard at a concentration of 1 mg/mL for fetal calf serum, and at a concentration of 0.1 mg/mL for simulated gastric acid and rat liver homogenate.

Fetal calf serum was bought from Sigma (F 7524), and stored at -20 °C before use.

Simulated gastric acid was prepared as described.[133] To 3.20 g pepsin, 2.0 g NaCl and 80 mL HCl (1 mol/L), distilled water was added to get a volume of 1000 mL. The solution had a pH value of 2. This solution was stored at 4 °C before use.

Rat liver homogenate was prepared as described with small changes.[134],[135] Fresh rat liver (6.5 g) was homogenized in DPBS buffer, and centrifuged at 9000 × g for 30 min at 4 °C. The supernant was carefully decanted to get the rat liver homogenate containing 32 % of homogenized liver. The protein concentration was 16 mg/mL measured by methods of Bradford.[137] Rat liver homogenate was kept at -80 °C before use.

#### 6.1.1. 2 Buffer

DPBS buffer (Dulbecco's Phosphate Buffered Saline):
$CaCl_2 \cdot 2\ H_2O$: 132.5 mg, $MgCl_2 \cdot 6\ H_2O$: 100 mg, KCl: 200 mg, $KH_2PO_4$: 200 mg, NaCl: 8000 mg, $Na_2HPO_4$: 1500 mg filled with $H_2O$ to 1000 mL.

#### 6.1.1. 3 Instruments

| | |
|---|---|
| Centrifuge | Beckman Avanti$^{TM}$ J-201 |
| Homogenizer | IKA Labortechnic RW 16 |
| pH-meter | WTW pH 197 |
| Pipettes | Eppendorf (10 - 100 $\mu$L, 100 - 1000 $\mu$L) |
| Waterbath | Memmert WB/OB 7-45 WBO 45 |
| Software | Prism, version 3.0, Graph Pad, San Diego, California, USA |
| Thermomixer | Thermoxixer comfort, Eppendorf |
| Vortexer | IKA Labortechnic MS1 minishaker |

### 6.1.2 Capillary electrophoresis conditions

| | |
|---|---|
| Apparatus: | Beckmann P/ACE 5000 station |
| Capillary: | Fused silica capillary; length: 30/37 cm; diameter: 75 $\mu m$ |
| Detector: | Diode-Array Detector (DAD) |
| Voltage: | 10 kV (chemical stability) |
| | 12 kV (rat liver homogenate stability) |
| | 15 kV (fetal calf serum and simulated gastric acid stability) |
| Buffer: | a) Chemical stability |
| | 20 mM phosphate buffer + 100 mM SDS (pH 7.4) |
| | 100 mM borate buffer + 50 mM SDS (pH 8) |
| | b) Fetal calf serum and simulated gastric acid stability |
| | 100 mM borate buffer + 100 mM SDS (pH 8) |
| | c) Rat liver homogenate stability |
| | 100 mM borate buffer + 150 mM SDS (pH 8) |

### 6.1.3 Stability tests

#### 6.1.3. 1 Chemical stability test

DMSO stock solution of compound **5d** (2 mM, 1 mL) was incubated with 99 mL of 0.001 M NaOH at 37 °C in a water bath. At certain time intervals, 4.8 mL of incubation solution was withdrawn and mixed with 0.1 mL of internal standard for CE analysis. The final concentration of the test solution was $1.96 \times 10^{-5}$ M.

#### 6.1.3. 2 Fetal calf serum stability test

DMSO stock solution of compound **5d** (50 mM, 30 $\mu$L) was added to 1470 $\mu$L of fetal calf serum, and incubated at 37 °C with a rotation speed of 900 rpm. At certain time intervals, 100 $\mu$L of incubation solution was withdrawn and diluted with 4.7 mL of $H_2O$, and 50 $\mu$L of internal standard. After vortexing, it was analyzed by CE. The final drug concentration was $2.1 \times 10^{-5}$ M.

#### 6.1.3. 3 Simulated gastric acid stability test

DMSO stock solution of compound **5d** (2 mM, 150 $\mu$L) was added to 9850 $\mu$L of simulated gastric acid and incubated at 37 °C with a rotation speed of 900 rpm. At certain time intervals, 400 $\mu$L of incubation solution was withdrawn and mixed with 100 $\mu$L of internal standard. After vortexing, it was analyzed by CE. The final drug concentration was 2.4 $\times 10^{-5}$ M.

#### 6.1.3. 4 Rat liver homogenate stability test

DMSO stock solution of compound **5d** (20 mM, 10 $\mu$L) was added to 990 $\mu$L of rat liver homogenate, and incubated at 37 °C with a rotation speed of 900 rpm. At certain time intervals, 50 $\mu$L of incubation solution was withdrawn, and 100 $\mu$L of 0.1 % TFA to precipitated the denaturate proteins, 250 $\mu$L of DPBS buffer and 100 $\mu$L of internal standard were added. The quenched sample was shortly vortexed and directly analyzed by CE. The final drug concentration was $2 \times 10^{-5}$ M.

## 6.2 Radioligand binding assays - experimental part

### 6.2.1 Materials and instruments

| | |
|---|---|
| Cell-harvester | Brandell MP-48, Gaithersburg, Maryland, USA |
| Centrifuge | Beckmann Avanti$^{TM}$ J-201 |
| Filter | Whatman GF/B, 57 × 303 m |
| Glasfaserfilter | Whatman GF/B, fired, FDP-148, Brandell, Geithersburg, MD USA |
| Harvester | Brandell M24, Gaithersburg, MD, USA |
| | Brandell M48, Gaithersburg, MD, USA |
| Homogenizer | IKA Labortechnik RW16 basic |
| Incubator | Jouan IG 850 |
| LSC | Tri-Carb Model 2100 TR, Canberra Packard |
| LSC Cocktail | Ultima Gold TM, Canberra Packard |
| Multipipette | Eppendorf Multipipette plus |
| PE-vials | Polyethylene vials 4 mL |
| Photometer | Beckmann DU 530 |
| pH-meter | WTW pH 197 |
| Pipette | Eppendorf research (10 - 100 $\mu$L, 100 - 1000 $\mu$L) |
| Pipettepoint | Plastibrand, Greiner |
| Software | Prism, version 3.0, Graph Pad, San Diego, California, USA |
| Ultraturray | IKA Labortechnic T25 basic |
| Vortex | IKA Labortechnic MS1 minishaker |
| Waterbath | GFL 1083 |

| | |
|---|---|
| ADA | Sigma, Typ IV from calf intestinal mucosa, 2000 IU/1.4 mL |
| [$^3H$]CCPA | NEN life science (54.9 C$_i$/mmol) |
| DMSO | Sigma |
| DMEM F12 | D-6421, Sigma |
| Fetal Calf Serum | F-7524, Sigma |
| HCl 37 % | Merck |
| [$^3H$]MSX-2 | Amersham (85 C$_i$/mmol) |
| NECA | Sigma |
| Penicillin | |
| Streptomycin | Sigma, P-0781 |
| [$^3H$]PSB-11 | Amersham (53 C$_i$/mmol) |
| Trizma (Tri-salt) | Sigma |
| Typsin EDTA | T-3924, Sigma |

TRIS-HCl buffer (50 mM, pH 7.4):
TRIS-base 6.05 g is dissolved in 1 L $H_2O$, the pH is adjusted to 7.4 by 37 % HCl.

PBS-free buffer (pH 7.2):
NaCl: 8 g, KCl: 0.2 g, $Na_2HPO_4$: 1.4 g, $KH_2PO_4$: 0.2 g filled with $H_2O$ to 1000 mL, pH adjusted to 7.2 by 1 M HCl.

## 6.2.2 Membrane preparations

### 6.2.2.1 Preparation of cortical membranes of rat brain for $A_1$-binding assays[141]

Rat brains (unstripped, code 56004-2, Lot 32220, 25ea, Pel Freez Biological, Rogers, Arkansas, USA) were thawed slowly at 0 - 4 °C, then they were put into ice-cold 0.32 M sucrose solution.

The cerebral cortex of each brain was carefully scraped off and stored in an ice-cold sucrose solution, then the cortex and sucrose solution were homogenized for 2 min at full speed. The homogenate was centrifuged for 10 min at 1,000 g at 4 °C. The supernatant was centrifuged again at 37,000 g for 1 h at 4 °C. Membrane pellets were resuspended in 50 mM TRIS-buffer (pH 7.4) and shortly homogenized. These procedures: centrifuging at 37,000 g, pellet suspension, homogenization were repeated twice. In the end the membrane preparation was resuspended in 50 mM TRIS-buffer (pH 7.4), and stored at -80 °C before use.

### 6.2.2.2 Preparation of striatal membranes of rat brain for $A_{2A}$-binding assays[142]

The rat striata are separated from the same rat brains and stored in 50 mM TRIS buffer (pH 7.4), homogenized at stage 3 for 10 sec. The homogenate was centrifuged at 37,000 g for 15 min at 4 °C, the membrane pellets were resuspended in small amounts of TRIS-buffer, and homogenized again for 2 sec at stage 3. This procedure: centrifugation, resuspension, was repeated once. At last the membrane pellet was suspended in 50 mM TRIS buffer (pH 7.4), and stored at -80 °C before use.

### 6.2.2.3 Membranes from CHO cells for $A_{2B}$-binding assays[143]

Chinese Hamster Ovary (CHO) cells stably transfected with the human $A_{2B}$-adenosine receptors were grown and maintained at 37 °C in 5 % $CO_2$ and 95 % air on petri dishes. Then cells were washed with PBS free buffer and frozen at -80 °C till preparation of

membrane. For the preparation of membranes for $A_{2B}$ receptor binding, the frozen cells were thawed and scraped off in ice-cold Tris buffer (50 mM Tris-HCl, pH 7.4), The cell suspension was homogenized (Ultra-Turrax, 2 × 15 s, at full speed) and the homogenate was spun for 10 min at 1,000 g. The supernatant was then centrifuged for 45 min at 45,000 g. The membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4, frozen in liquid nitrogen at a protein concentration of 1 - 3 mg/mL and stored at -80 °C.

#### 6.2.2.4 Membranes from CHO cells for $A_3$-binding assays[143]

Chinese Hamster Ovary (CHO) cells stably transfected with the human $A_3$-adenosine receptors were grown and maintained at 37 °C in 5 % $CO_2$ and 95 % air on petri dishes. Then cells were washed with PBS free buffer and frozen at -80 °C till preparation of membrane. For the preparation of membranes for $A_3$-receptor binding, the frozen cells were thawed and scraped off in ice-cold hypotonic buffer (5 mM TRIS-HCl, 2 mM EDTA, pH 7.4), The cell suspension was homogenized (Ultra-Turrax, 2 × 15 s, at full speed) and the homogenate was spun for 10 min at 1,000 g. The supernatant was then centrifuged for 40 min at 50,000 g. The membrane pellet was resuspended in 50 mM TRIS-HCl, pH 8.25, containing 1 mM EDTA and 10 mM $MgCl_2$, frozen in liquid nitrogen at a protein concentration of 1 - 3 mg/mL and stored at -80 °C.

### 6.2.3 Binding assays

#### 6.2.3.1 Radioligand binding assay at $A_1$-adenosine receptors with $[^3H]$CCPA using cortical membranes of rat brain

| | |
|---|---|
| 25 $\mu L$ | DMSO, CADO (25 $\mu M$), or diluted test substance |
| 800 $\mu L$ | 50 mM TRIS buffer (pH 7.4) |
| 75 $\mu L$ | $[^3H]$CCPA (end concentration 0.5 nM, $K_D$ 0.2 nM) |
| 100 $\mu L$ | Rat brain cortical membrane (protein concentration: 70 $\mu g$/mL) in TRIS-buffer, 15 min preincubation with 0.12 IU ADA |
| 1000 $\mu L$ | End volume |

*Dilution scheme:*

Compounds **5a** - **5e**

1 mM Stock solution

| | solution($\mu L$) | DMSO ($\mu L$) | conc. in assay ($\mu M$) |
|---|---|---|---|
| 1 | 60 | 940 | 1.5 |
| 2 | 300 | 600 | 0.5 |
| 3 | 300 | 700 | 0.15 |
| 4 | 300 | 600 | 0.05 |
| 5 | 300 | 700 | 0.015 |
| 6 | 300 | 700 | 0.005 |
| 7 | 200 | 800 | 0.001 |

Compounds **9a** - **9e**

0.1 mM Stock solution

| | solution($\mu L$) | DMSO ($\mu L$) | conc. in assay($\mu M$) |
|---|---|---|---|
| 1 | 200 | 300 | 1 |
| 2 | 300 | 700 | 0.3 |
| 3 | 300 | 600 | 0.1 |
| 4 | 300 | 700 | 0.03 |
| 5 | 300 | 600 | 0.01 |
| 6 | 300 | 700 | 0.003 |
| 7 | 300 | 600 | 0.001 |

Compounds **10a** - **10e**, **11b-11f**

0.4 mM Stock solution

| | solution($\mu L$) | DMSO ($\mu L$) | conc. in assay($\mu M$) |
|---|---|---|---|
| 1 | 300 | 700 | 3 |
| 2 | 300 | 600 | 1 |
| 3 | 300 | 700 | 0.3 |
| 4 | 300 | 600 | 0.1 |
| 5 | 300 | 700 | 0.03 |
| 6 | 300 | 600 | 0.01 |
| 7 | 300 | 700 | 0.003 |

The test compounds were dissolved in DMSO, and diluted according to the above dilution schemes. Rat brain cortex membrane was suspended in 50 mM TRIS-buffer (pH

7.4) and preincubated for 20 min with 0.12 IU/mL ADA in order to remove endogenous adenosine, which would otherwise compete for the binding sites.[7] DMSO (to determine the total binding), CADO (to determine the unspecific binding) and the test compound solutions were pipetted in triplicate into test tubes. 50 mM TRIS-HCl buffer (pH 7.4), radioligand and protein suspension were added and the mixture was mixed. The suspension was filtered through glass fiber filters (GFB) using the harvester after incubation at r.t. for 90 min. The filters were washed with ice-cold ca. 10 mL 50 mM TRIS-buffer (pH 7.4) twice. The filter papers were punched out and transferred to scintillation vials and 2 mL scintillation fluid was added. After 6 hours of incubation, radioactivity was counted with a liquid scintillation counter.

#### 6.2.3. 2 Radioligand binding assay at $A_{2A}$-adenosine receptors with $[^3H]$MSX-2 using striatal membranes of rat brain

| | |
|---|---|
| 25 $\mu L$ | DMSO, NECA (50 $\mu M$), or diluted test substance |
| 800 $\mu L$ | 50 mM TRIS buffer (pH 7.4) |
| 75 $\mu L$ | $[^3H]$MSX-2 (end concentration 1 nM, $K_D$ 8 nM) |
| 100 $\mu L$ | Rat brain cortical membrane (protein concentration: 70 $\mu g$/mL) in TRIS-buffer 15 min incubation with 0.12 IU ADA |
| 1000 $\mu L$ | End volumn |

*Dilution scheme:*

Compounds **5a** - **5e** and Compounds **9a** - **9e** were the same as the $A_1$ assay

Compounds **10a** - **10e**, **11b**-**11f**

1 mM Stock solution

| | solution($\mu L$) | DMSO ($\mu L$) | conc. in assay($\mu M$) |
|---|---|---|---|
| 1 | 200 | 300 | 10 |
| 2 | 300 | 700 | 3 |
| 3 | 300 | 600 | 1 |
| 4 | 300 | 700 | 0.3 |
| 5 | 300 | 600 | 0.1 |
| 6 | 300 | 700 | 0.03 |
| 7 | 300 | 600 | 0.01 |

This assay was performed in the same manner as the $A_1$ radioligand binding assay, except that the incubation time at r.t was shortened to 30 min, the filter paper was immersed in 0.3 % aqueous PEI solution for 1 hour before filtering in order to reduce non-specific binding.

#### 6.2.3. 3 Radioligand binding assay at $A_{2B}$-adenosine receptors with $[^3H]$PSB-298 using CHO-$A_{2B}$ membranes

| | |
|---|---|
| 10 $\mu L$ | DMSO, NECA (50 $\mu M$), or diluted test substance |
| 70 $\mu L$ | 50 mM TRIS buffer (pH 7.4) |
| 50 $\mu L$ | $[^3H]$PSB-298 (end concentration 5 nM, $K_D$ 56 nM) |
| 70 $\mu L$ | CHO-$A_{2B}$ (protein concentration: 100 $\mu g$/mL) in TRIS-buffer 15 min incubation with 0.22 IU ADA |
| 200 $\mu L$ | End volumn |

*Dilution scheme:*

Compounds **5d**, **9d**

0.02 mM 20 % DMSO stock solution

| | solution($\mu L$) | 20 % DMSO ($\mu L$) | conc. in assay($\mu M$) |
|---|---|---|---|
| 1 | 300 | 700 | 0.3 |
| 2 | 300 | 600 | 0.1 |
| 3 | 300 | 700 | 0.03 |
| 4 | 300 | 600 | 0.01 |
| 5 | 300 | 700 | 0.003 |
| 6 | 300 | 600 | 0.001 |

This assay was performed in the same manner as the $A_1$ radioligand binding assay. The dilution solution using 20 % DMSO, the end concentration of radioligand is 5 nM, CHO-$A_{2B}$ was preincubated for 30 min with 0.22 IU/mL ADA in order to remove endogenous adenosine. The suspension was filtered through glass fiber filter (GFB) using the harvester after 90 min incubation at r.t.

# 6.3 Chemistry - experimental part

## 6.3.1 Instruments, materials and methods

### 6.3.1.1 $^{1}H$-NMR and $^{13}C$-NMR spectra

Apparatus: Bruker Advance 500 MHz spectrometer

The chemical shifts $\delta$ were given in ppm. The coupling constants J are given in Hz. Chemical shifts of the deuterated solvents served as internal standards for spectra recorded in DMSO-$d_6$: $\delta$ $^{1}$H 2.51 ppm, $\delta$ $^{13}$C 39.7 ppm. Tetramethylsilane was used as internal standard for spectra recorded in $D_2O$ and $CD_3COCD_3$.

### 6.3.1.2 Melting points

Melting points were taken on a Buechi 530 melting point apparatus and were uncorrected.

### 6.3.1.3 Thin layer chromatography

| | | |
|---|---|---|
| TLC-Aluminum Plates: | | Silica gel 60 $F_{254}$<br>Merck, Art No. 1.05554 |
| Mobile phase: | S 1: | Dichloromethane : methanol = 4 : 1 |
| | S 2: | Dichloromethane : methanol = 8 : 1 |
| | S 3: | Dichloromethane : methanol = 20 : 1 |
| | S 4: | Dichloromethane : methanol = 40 : 1 |
| | S 5: | Dichloromethane : methanol = 50 : 1 |
| | S 5: | 2-Propanol : $NH_3$ 25 % : methanol = 5 : 2 : 1 |
| | S 7: | 2-Propanol : $NH_3$ 25 % = 5 : 2 |
| | S 8: | 2-Propanol : $NH_3$ 25 % = 6 : 1 |
| | S 9: | Aceton : petroleum ether = 1 : 3.5 |
| | S 10: | Aceton : petroleum ether = 1 : 2 |
| Detection: | -UV light at 254 nm | |
| | -UV light at 366 nm | |
| Color reagent | Ehrlichs-reagent<br>(1 g 4-dimethylaminobenzaldehyde, 25 mL 37 % HCl,<br>75 mL methanol) | |

### 6.3.1.4 Elemental analysis

Apparatus: Vario EL from ,,Elementar Analysensysteme GmbH"

The elemental analysis were carried out at the Pharmaceutical Institute, Endenich, University of Bonn.

#### 6.3.1.5 Mass spectra

Apparatus: MS-50 A.E.I

The mass spectra were determined at the Institute of Organic Chemistry, University of Bonn.

#### 6.3.1.6 Capillary electrophoresis (CE) analysis

| | |
|---|---|
| Apparatus: | Beckmann P/ACE 5000 station |
| Capillary: | Fused silica capillary; length: 30/37 cm; diameter 75 $\mu m$ |
| Detector: | Diode-Array Detector (DAD) |
| Voltage: | 10 kV |
| Buffer: | a) 20 mM phosphate buffer (pH = 7.4) |
| | b) 20 mM phosphate buffer (pH = 7.4) + 100 mM SDS |
| | c) 50 mM phosphate buffer (pH = 7.4) |

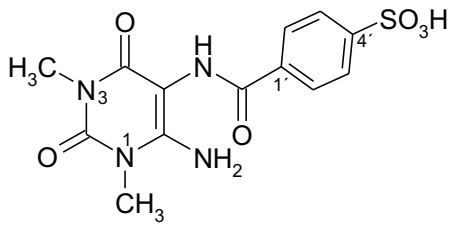
#### 6.3.1.7 Preparative HPLC

| | |
|---|---|
| Pump: | Preparative HPLC pump, Knauer GmbH |
| Precolumn: | Eurospher 100, $C_{18}$, 5 $\mu m$, 30× 20 mm ID |
| Column: | Eurospher 100, $C_{18}$, 10 $\mu m$, 250 × 20 mm ID |
| Detector: | Spectrophotometer K-2600 |
| Software: | Eurochrom Version 3.05 preparative software |
| Mobile phase A: | 35 % methanol + 65 % $H_2O$ |
| Mobile phase B: | $H_2O$ |
| Gradient: | 0 min: A: 50 % B: 50 % (equal: 17.5 % methanol) |
| | 20 min: A: 100 % B: 0 % (equal: 35 % methanol) |
| Injection speed: | 10 ml/min |
| Elute speed: | 20 ml/min |

1,3-Disubstituted-5,6-diaminouracils (**3a**, **3b**) were prepared from 1,3-dimethylurea or 1,3-dipropylurea with cyanoacetic acid followed by ring closure, nitrosation and reduction as described.[105],[106] 3-Substituted-5,6-diaminouracils **3c** - **3e** were prepared from 6-aminouracil *via* regioselective alkylation with the appropriate alkyl iodide followed by nitrosation and reduction as described.[111],[112]

## 6.3.2 Monographs

### 6.3.2. 1 6-Amino-1,3-dimethyl-5-(*p*-sulfobenzamido)uracil (1a)



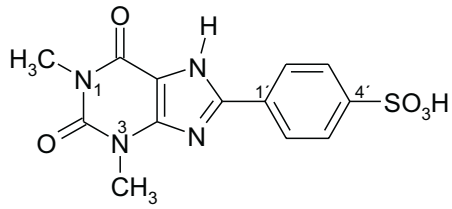

To a solution of 1.3 g (5.4 mmol) *p*-sulfobenzoic acid potassium salt in 20 mL water, 0.9 g (5.3 mmol) of 5,6-diamino-1,3-dimethyluracil and 1 g (5.2 mmol) of EDC were added while stirring. The solution was stirred at room temperature for 1 h. Then most of the solvent was removed *in vacuo*. 6-Amino-1,3-dimethyl-5-(*p*-sulfobenzamido)uracil **1a** was precipitated from the solution by addition of methanol, collected by filtration and washed with methanol to obtain the pure product.

| | | |
|---|---|---|
| Yield: | 1.8 g | (67 %) |
| m.p.: | > 300 °C | (Lit > 300 °C[56]) |
| $R_f$: | 0.24 | (S 1) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.13 (s, 3H, N3-$CH_3$); 3.33 (s, 3H; N1-$CH_3$); 6.68 (s, 2H, $NH_2$); 7.66 (d, 2H, J = 8.2 Hz, C3'H, C5'H ); 7.91 (d, 2H, J = 8.2 Hz, C2'H, C6'H); 8.89 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.67 (N3-$CH_3$); 30.15 (N1-$CH_3$); 87.66 (C5); 125.19 (C3', C5'); 127.67 (C2', C6'); 134.61 (C1'); 150.8, 151.02 (C2, C6); 152.41 (C4'); 159.39 (C4); 166.51 (NHCO).

### 6.3.2. 2 1,3-Dimethyl-8-*p*-sulfophenylxanthine (1b)




6-Amino-1,3-dimethyl-5-(*p*-sulfobenzamido)uracil **1a** (0.91 g, 0.26 mmol) was dissolved in 25 mL of 2.5 N NaOH and heated at 70 °C for 15 min. After cooling down

to 0 °C, the solution was adjusted to pH 6 with conc. HCl. The formed precipitate was washed with cold 1 M HCl and recrystallized by NaOH/HCl treatment.

| | | |
|---|---|---|
| Yield: | 0.61 g | (65 %) |
| m.p.: | > 300 °C | (Lit >300 °C[56]) |
| $R_f$: | 0.53 | (S 1) |

C,H,N analysis: $C_{13}H_{12}N_4O_5S \cdot 2\ H_2O$ [372.37 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 41.89 | 4.30 | 15.05 |
| Found: | 41.63 | 4.24 | 14.85 |

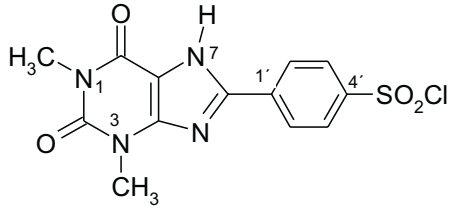
$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.25 (s, 3H, N1-$CH_3$); 3.5 (s, 3H; N3-$CH_3$); 7.7 (d, 2H, J = 8.51 Hz, C3'H, C5'H); 8.07 (d, 2H, J = 8.51 Hz, C2'H, C6'H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.96 (N1-$CH_3$); 29.95 (N3-$CH_3$); 108.01 (C5); 126.11 (C3', C5'); 126.26 (C2', C6'); 128.72 (C1'); 148.63 (C4), 149.47 (C8); 149.95 (C4'); 151.37 (C2); 154.38 (C6).

Determination of purity by CE:

| | |
|---|---|
| Buffer: | Phosphate 20 mM, pH 7.4 |
| Currency: | 90 $\mu A$ |
| Retention time: | 4.41 min |
| Purity: | 100 % |

### 6.3.2. 3 1,3-Dimethyl-8-*p*-chlorosulfophenylxanthine (1c)




1,3-Dimethyl-8-*p*-sulfophenylxanthine **1b** (56 mg, 0.16 mmol) was dissolved in 10 mL of $SOCl_2$. The mixture was refluxed for 2 h. The remaining $SOCl_2$ was removed under

reduced pressure to yield a white solid residue. Since it is unstable, only NMR and MS were used to confirm the structure.

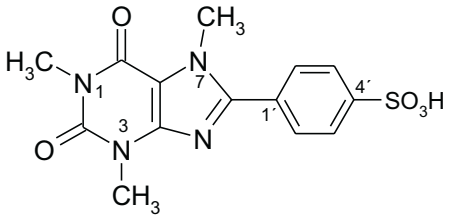
Calcd. Mass: 354.77 g/mol ($C_{13}H_{11}ClN_4O_4S$).

EI-MS (70 eV): m/z (%): 354 ($M^+$; 100); 255 ([M-$SO_2Cl$]$^+$, 25).

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.25 (s, 3H, N1-$CH_3$); 3.5 (s, 3H; N3-$CH_3$); 7.7 (d, 2H, C3'H, C5'H); 8.07 (d, 2H, C2'H, C6'H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.91 (N1-$CH_3$); 29.94 (N3-$CH_3$); 107.02 (C5); 126.08 (C3', C5'); 126.26 (C2', C6'); 128.76 (C1'); 148.65, 149.48, 149.88 (C4, C8, C4'); 151.34 (C2); 154.33 (C6).

#### 6.3.2. 4 1,3,7-Trimethyl-8-*p*-sulfophenylxanthine (1d)




To a solution of 100 mg (0.3 mmol) 1,3-dimethyl-8-*p*-sulfophenylxanthine **1b** in 10 mL of DMF 82.8 mg (0.6 mmol) of $K_2CO_3$ and 0.37 mL (6 mmol) of $CH_3I$ were added, and the mixture was stirred overnight at room temperature. The formed precipitate was filtered off and washed with DMF. After the addition of diethyl ether to the filtrate, a white precipitate was obtained which was collected by filtration, and washed with diethyl ether.

| | | |
|---|---|---|
| Yield: | 0.37 g | (35 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.31 | (S 1) |

C,H,N analysis: $C_{14}H_{14}N_4O_5S \cdot 2.5\ H_2O$ [395.41 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 42.49 | 4.81 | 14.16 |
| Found: | 42.07 | 4.30 | 14.18 |

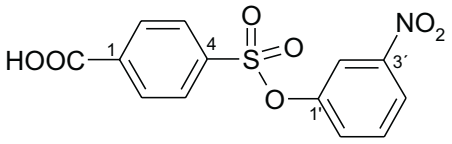
$^{1}$H-NMR ($D_2O$) $\delta$ [ppm]:
3.34 (s, 3H, N1-$CH_3$); 3.53 (s, 3H, N3-$CH_3$); 3.97 (s, 3H, N7-$CH_3$); 7.83 (d, 2H, J = 8.35 Hz, C3'H, C5'H); 7.99 (d, 2H, J = 8.35 Hz, C2'H, C6'H).

$^{13}$C-NMR ($D_2O$) $\delta$ [ppm]:
30.92 (N1-$CH_3$); 32.78 (N3-$CH_3$); 36.53 (N7-$CH_3$); 111.91 (C5); 128.87 (C3', C5'); 132.86 (C2', C6'); 132.74 (C1'); 147.34 (C4); 150.85 (C8); 154.76 (C4'); 155.37 (C2); 159.2 (C6).

Determination of purity by CE:

| | |
|---|---|
| Buffer: | Phosphate 20 mM, pH 7.4 |
| Currency: | 90 $\mu A$ |
| Retention time: | 3.64 min |
| Purity: | 100 % |

#### 6.3.2.5 4-[[*m*-Nitrophenoxy]sulfonyl]benzoic acid (2)




Chlorosulfonic acid (50 mL) was slowly added to 5 g (21 mmol) of *p*-sulfobenzoic acid potassium salt, while the temperature was kept below 30 °C, then the mixture was stirred at r.t. overnight. The clear solution was carefully poured onto chipped ice, and the granular white solid was collected by filtration and washed with cold water. 4-Sulfochlorobenzoic acid was obtained and dried.

*m*-Nitrophenol (1.47 g, 11 mmol) was dissolved in 50 mL of THF and 50 mL of TRIS-buffer (50 mM, pH 9), 4-sulfochlorobenzoic acid (2.5 g, 11 mmol) in 50 mL of THF was added drop by drop into the above solution. The pH value was kept between 9 - 10 by addition of 2.5 M NaOH, while the reaction mixture was stirred for about 4 h at r.t.. Then the solution was neutralized with 1 M HCl to pH 7. THF was removed *in vacuo*, and the aqueous solution was further acidified to pH 1 with 1 M HCl. The formed precipitate was filtered and recrystallized from acetone : cyclohexane (1 : 2).

| | | |
|---|---|---|
| Yield: | 3.18 g | (45 % calcd. from *p*-sulfobenzoic acid) |
| m.p.: | 213.5 °C | |
| $R_f$: | 0.32 | (S 2) |

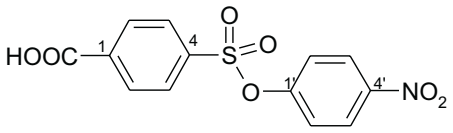
C,H,N analysis: $C_{13}H_9NO_7S$ [323.28 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 48.30 | 2.81 | 4.33 |
| Found: | 48.22 | 2.69 | 4.42 |

$^1$H-NMR ($CD_3COCD_3$) $\delta$ [ppm]:
7.56 (m, 1H, C6'H); 7.74 (t, 1H; J = 8.35 Hz, C5'H); 7.93 (t, 1H, J = 2.2 Hz, C2'H); 8.09 (d, 2H, J = 8.51 Hz, C3H, C5H); 8.25 (m, 1H, C4'H); 8.3 (d, 2H, J = 8.51 Hz, C2H, C6H).

$^{13}$C-NMR ($CD_3COCD_3$) $\delta$ [ppm]:
118.57 (C2'); 123.32 (C4'); 129.7 (C5'); 129.76 (C3, C5); 131.67 (C2, C6); 132.19 (C6'); 137.27 (C1, C4); 139.38 (C3'); 150.38 (C1'); 165.99 (COOH).

#### 6.3.2.6 4-[[*p*-Nitrophenoxy]sulfonyl]benzoic acid (7)




Compound **7** was prepared as described for compound **2**, from 5 g (21 mmol) of *p*-sulfobenzoic acid potassium salt and 1.5 g (11 mmol) of *p*-nitrophenol.

| | | |
|---|---|---|
| Yield: | 2.9 g | (41 % calcd. from *p*-sulfobenzoic acid) |
| m.p.: | 218 - 219 °C | |
| $R_f$: | 0.37 | (S 2) |

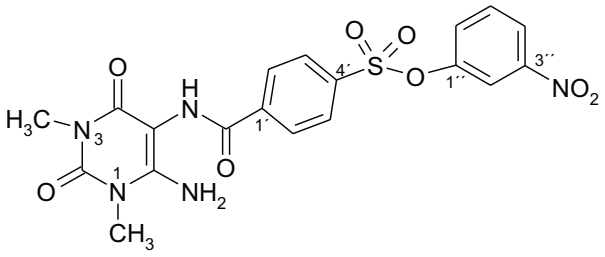
C,H,N analysis: $C_{13}H_9NO_7S$ [323.28 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 48.30 | 2.81 | 4.33 |
| Found: | 48.15 | 2.87 | 4.47 |

$^1$H-NMR (DMSO-$d_6$) $\delta$[ppm]:
7.36 (d, J = 9.14 Hz, 2H, C3'H, C5'H); 8.02 (d, J = 8.82 Hz, 2H, C3H, C5H); 8.18 (d, 2H, J = 8.82 Hz, C2H, C6H); 8.26 (d, J = 9.14 Hz, 2H, C2'H, C6'H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$[ppm]:
123.81 (C3', C5'); 126.26 (C2', C6'); 129.06 (C3, C5); 131.03 (C2, C6); 137.12, 137.54 (C4, C1); 146.62 (C4'); 153.3 (C1'); 166.11 (COOH).

### 6.3.2. 7 6-Amino-1,3-dimethyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (4a)




To a solution of 262 mg (1.54 mmol) 1,3-dimethyluracil **3a** in 60 mL of methanol, 500 mg (1.54 mmol) of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2** and 250 mg (1.31 mmol) of EDC were added. After 3 h of stirring, a white precipitate was obtained by filtration. The solid was washed with cold methanol. Recrystallization from DMF/$H_2O$ afforded pure **4a**.

| | | |
|---|---|---|
| Yield: | 442 mg | (60 %) |
| m.p.: | 239 °C | |
| $R_f$: | 0.55 | (S 2) |

C,H,N analysis: $C_{19}H_{17}N_5O_8S \cdot 0.5\ H_2O$ [484.45 g/mol]

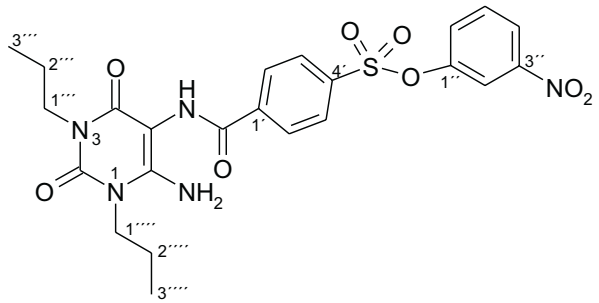
| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 47.06 | 3.72 | 14.45 |
| Found: | 47.52 | 3.61 | 14.55 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.14 (s, 3H, N3-$CH_3$); 3.35 (s, 3H, N1-$CH_3$); 6.28 (s, 2H, $NH_2$); 7.55 (m, 1H, C6"H); 7.71 (t, 1H, J = 8.35 Hz, C5"H); 7.99 (t, 1H, J = 2.2 Hz, C2"H); 8.05 (d, 2H, J = 8.51 Hz, C3'H, C5'H); 8.2 (d, 2H, J = 8.51 Hz, C2'H, C6'H); 8.28 (m, 1H, C4"H); 9.24 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.68 (N3-$CH_3$); 30.16 (N1-$CH_3$); 87.06 (C5); 117.5 (C2"); 122.78 (C4"); 128.35 (C3',

C5'); 129.61 (C5"); 128.91 (C2', C6'); 131.78 (C6"); 135.72 (C1'); 140.86 (C3"); 148.57, 148.94 (C2, C6); 150.74 (C1"); 152.43 (C4'); 159.29 (C4); 165.23 (NHCO).

#### 6.3.2. 8 6-Amino-1,3-dipropyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (4b)



Compound **4b** was obtained as described above for **4a**, from 546 mg (2.42 mmol) of 5,6-diamino-1,3-dipropyluracil **3b**, 780 mg (2.42 mmol) of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2** and 461 mg (2.41 mmol) of EDC.

| | | |
|---|---|---|
| Yield: | 700 mg | (55 %) |
| m.p.: | 245 °C | |
| $R_f$: | 0.62 | (S 2) |

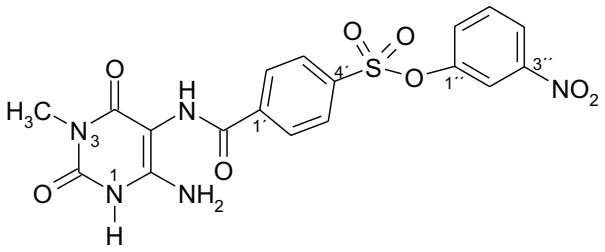
C,H,N analysis: $C_{23}H_{25}N_5O_8S \cdot 0.5\ H_2O$ [540.57 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 51.06 | 4.81 | 12.95 |
| Found: | 51.55 | 4.65 | 12.45 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.83 (m, 3H, C3"'H); 0.88 (m, 3H, C3""H); 1.52 (m, 2H, C2"'H); 1.57 (m, 2H, C2""H); 3.7 (m, 2H, C1"'H); 3.84 (m, 2H, C1""H); 6.79 (s, 2H, $NH_2$); 7.54 (m, 1H, C6"H); 7.69 (t, 1H, C5"H); 7.99 (t, 1H, C2"H); 8.05 (d, 2H, C3'H, C5'H); 8.20 (d, 2H, C2'H, C6'H); 8.22 (m, 1H, C4"H); 9.21 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
10.85 (C3"'); 11.31 (C3""); 20.96 (C2"'); 20.99 (C2""); 41.99 (C1"'); 43.84 (C1""); 86.99 (C5); 117.51 (C2"); 122.78 (C4"); 128.35 (C3', C5'); 128.91 (C5"); 129.58 (C2', C6'); 131.78 (C6"); 135.72 (C1'); 140.83 (C3"); 148.56, 148.93 (C6, C2); 150.47 (C1"); 151.79 (C4'); 159.08 (C4); 165.17 (NHCO).

### 6.3.2. 9 6-Amino-3-methyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (4c)



Compound **4c** was obtained as described above for **4a**, from 1.12 g (7.2 mmol) of 5,6-diamino-3-methyluracil **3c**, 2.35 g (7.2 mmol) of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2** and 3 g (6.8 mmol) of EDC.

| | | |
|---|---|---|
| Yield: | 1.89 g | (55 %) |
| m.p.: | 268 °C | |
| $R_f$: | 0.59 | (S 2) |

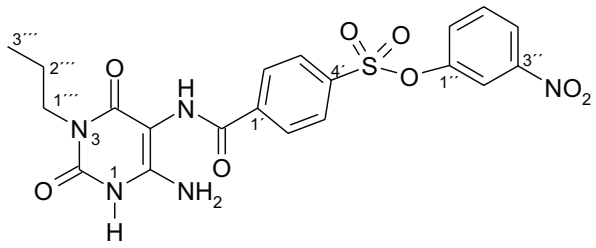
C,H,N analysis: $C_{18}H_{15}N_5O_8S \cdot 0.5\ H_2O$ [470.42 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 45.92 | 3.40 | 14.88 |
| Found: | 45.92 | 3.31 | 14.92 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.07 (s, 3H, N3-$CH_3$); 6.2 (s, 2H, $NH_2$); 7.54 (m, 1H, C6"H); 7.7 (t, 1H, J = 8.2 Hz, C5"H); 7.99 (t, 1H, J = 2.2 Hz, C2"H); 8.05 (d, 2H, J = 8.51 Hz, C3'H, C5'H); 8.19 (d, 2H, J = 8.51Hz, C2'H, C6'H); 8.2 (m, 1H, C4"H); 9.2 (s, 1H, NHCO); 10.55 (s,1H, N1-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
26.64 (N3-$CH_3$); 86.54 (C5); 117.53 (C2"); 122.8 (C4"); 128.42 (C3', C5'); 128.93 (C5"); 129.63 (C2', C6'); 131.79 (C6"); 135.74 (C1'); 140.82 (C3"); 148.57, 148.94 (C6, C2); 150.32 (C1"); 152.76 (C4'); 160.84 (C4); 165.07 (NHCO).

### 6.3.2. 10 6-Amino-3-propyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (4d)




Compound **4d** was obtained as described above for **4a**, from 1.3 g (7.2 mmol) of 5,6-diamino-3-propyluracil **3d**, 2.35 g (7.2 mmol) of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2** and 3 g (6.8 mmol) of EDC.

| | | |
|---|---|---|
| Yield: | 2.01 g | (57 %) |
| m.p.: | 252 °C | |
| $R_f$: | 0.44 | (S 2) |

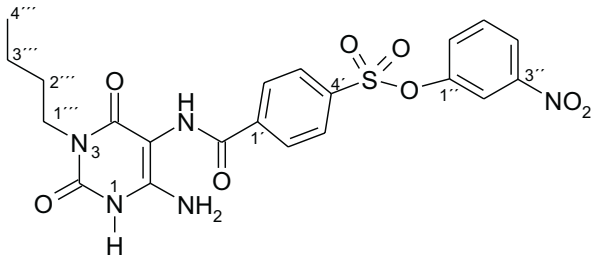
C,H,N analysis: $C_{20}H_{19}N_5O_8S$ [489.47 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 49.08 | 3.91 | 14.31 |
| Found: | 48.88 | 3.93 | 14.24 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.82 (t, 3H, C3"'H); 1.5 (m, 2H, C2"'H); 3.65 (s, 3H, C1"'H); 6.19 (s, 2H, $NH_2$); 7.53 (m, 1H, C6"H); 7.7 (t, 1H, C5"H); 7.98 (t, 1H, C2"H); 8.05 (d, 2H, C3'H, C5'H); 8.2 (d, 2H, C2'H, C6'H); 8.2 (m, 1H, C4"H); 9.2 (s, 1H, NHCO); 10.48 (s, 1H, N1-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.32 (C3"'); 21.09 (C2"'); 26.64 (C1"'); 86.54 (C5); 117.52 (C2"); 122.78 (C4"); 128.4 (C3', C5'); 128.92 (C5"); 129.51 (C2', C6'); 131.77 (C6"); 135.72 (C1'); 140.79 (C3"); 148.56, 148.93 ((C6, C2); 150.01 (C1"); 150.62 (C4'); 160.64 (C4); 165.02 (NHCO).

### 6.3.2. 11 6-Amino-3-butyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (4e)



Compound **4e** was obtained as described above for **4a**, from 1.4 g (7.2 mmol) of 5,6-diamino-3-butyluracil **3e**, 2.35 g (7.2 mmol) of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **2** and 3 g (6.8 mmol) of EDC.

| | | |
|---|---|---|
| Yield: | 1.53 g | (42 %) |
| m.p.: | 253 °C | |
| $R_f$: | 0.60 | (S 2) |

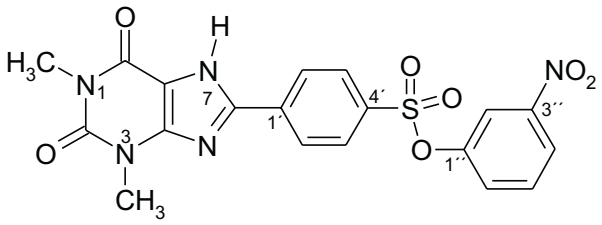
C,H,N analysis: $C_{21}H_{21}N_5O_8S \cdot 0.5\ H_2O$ [512.5 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 49.17 | 4.29 | 13.66 |
| Found: | 49.51 | 4.18 | 13.62 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.87 (t, 3H, $CH_3$); 1.25 (m, 2H, C3"'H); 1.46 (m, 2H, C2"'H); 3.7 (s, 3H, C1"'H); 6.19 (s, 2H, $NH_2$); 7.54 (m, 1H, C6"H); 7.71 (t, 1H, C5"H); 7.98 (t, 1H, C2"H); 8.05 (d, 2H, C3'H, C5'H); 8.2 (d, 2H, C2'H, C6'H); 8.17 (m, 1H, C4"H); 9.2 (s, 1H, NHCO); 10.48 ( s, 1H, N1-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
13.85 ($CH_3$); 19.75 (C3"'); 30 (C2"'); 39.24 (C1"'H); 86.57 (C5); 117.52 (C2"); 122.79 (C4"); 128.4 (C3', C5'); 128.92 (C5"); 129.51 (C2', C6'); 131.78 (C6"); 135.72 (C1'); 140.79 (C3"); 148.56, 148.93 ((C6, C2); 150.01 (C1"); 150.62 (C4'); 160.64 (C4); 165.02 (NHCO).

### 6.3.2. 12 1,3-Dimethyl-8-[4'-[[*m*-nitrophenoxy]sulfony]phenyl]xanthine (5a)



To 300 mg (0.32 mmol) of 6-amino-1,3-dimethyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil **4a**, ca. 4 g of PPSE was added. First the mixture was heated to 120 °C for 10 min, then the heating temperature was increased to 170 °C for 1.5 h. After cooling down to r.t., the residue was treated with 20 mL of methanol and the formed precipitate was filtered off and recrystallized from DMF/$H_2O$.

Yield: 117 mg (40 %)
m.p.: 296 °C

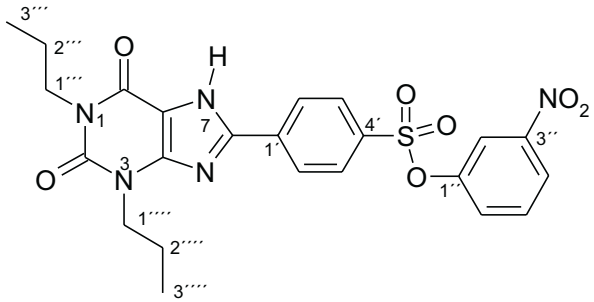
C,H,N analysis: $C_{19}H_{15}N_5O_7S$ [457.42 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 49.89 | 3.31 | 15.31 |
| Found: | 49.75 | 3.33 | 15.23 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.26 (s, 3H, N1-$CH_3$); 3.5 (s, 3H, N3-$CH_3$); 7.56 (m, 1H, C6"H); 7.71 (t, 1H, C5"H); 7.92 (t, 1H, C2"H); 8.05 (d, 2H, C3'H, C5'H); 8.2 (m, 1H, C4"H); 8.37 (d, 2H, C2'H, C6'H); 14.27 (s, 1H, NH).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
28 (N3-$CH_3$); 29.95 (N1-$CH_3$); 106.77 (C5); 117.6 (C2"); 122.78 (C4"); 127.44 (C3', C5'); 129.13 (C5"); 129.36 (C2', C6'); 131.75 (C6"); 134.15 (C1'); 134.87 (C3"); 147.14, 147.13 (C1", C4'); 148.51 (C8); 148.87 (C4); 151.26 (C2); 154.52 (C6).

### 6.3.2. 13 1,3-Dipropyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine (5b)



Compound **5b** was prepared from 324 mg (0.61 mmol) of 6-amino-1,3-dipropyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil **4b** and ca. 4 g of PPSE as described above for **5a**.

Yield: 112 mg (36 %)
m.p.: 315 °C

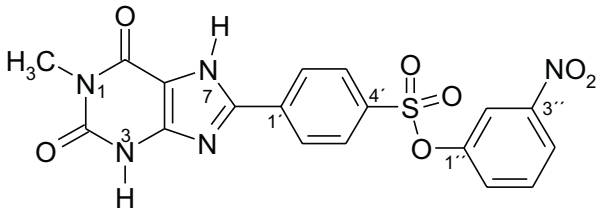
C,H,N analysis: $C_{23}H_{23}N_5O_7S \cdot 0.5\ H_2O$ [457.42 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 53.82 | 4.59 | 13.40 |
| Found: | 53.51 | 4.52 | 13.46 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.86 (t, 3H, C3"'H); 0.88 (t, 3H, C3""H); 1.57 (m, 2H, C2"'H); 1.73 (m, 2H, C2""H); 3.86 (t, 2H, C1"'H); 4.01 (t, 2H, C1""H); 7.55 (m, 1H, C6"H); 7.71 (t, 1H, C5"H); 7.92 (t, 1H, C2"H); 8.1 (d, 2H, C3'H, C5'H); 8.2 (m, 1H, C4"H); 8.36 (d, 2H, C2'H, C6'H); 14.28 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.16 (C3"'); 11.29 (C3""); 20.95 (C2"'); 20.96 (C2""); 42.4 (C1"'); 44.66 (C1""); 109.12 (C5); 117.62 (C2"); 122.78 (C4"); 127.78 (C3', C5'); 129.13 (C5"); 129.38 (C2', C6'); 131.77 (C6"); 134.24 (C1'); 134.86 (C3"); 147.37, 148.36 (C1", C4'); 148.53 (C8); 148.9 (C4); 150.75 (C2); 154.34 (C6).

### 6.3.2.14 1-Methyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine (5c)



Compound **5c** was prepared from 92 mg (0.2 mmol) of 6-amino-3-methyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil **4c** and ca. 2 g of PPSE as described above for **5a**.

Yield: 58 mg (65 %)
m.p.: 349 °C

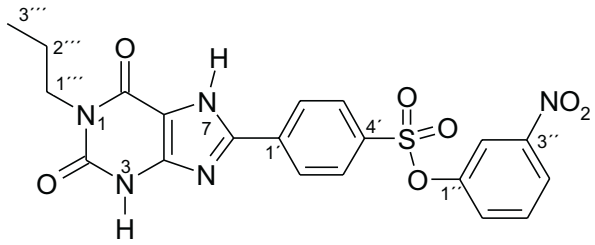
C,H,N analysis: $C_{18}H_{13}N_5O_7S \cdot 0.4\ H_2O$ [450.61 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 47.94 | 3.06 | 15.53 |
| Found: | 48.35 | 3.19 | 15.19 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.2 (s, 3H, N1-$CH_3$); 7.55 (m, 1H, C6"H); 7.7(t, 1H, C5"H); 7.92 (t, 1H, C2"H); 8.05 (d, 2H, C3'H, C5'H); 8.2 (m, 1H, C4"H); 8.34 (d, 2H, C2'H, C6'H); 12.0 (s, 1H, N3-H); 14.14 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.27 (N1-$CH_3$); 108.93 (C5); 117.63 (C2"); 122.77 (C4"); 127.43 (C3', C5'); 129.13 (C5"); 129.36 (C2', C6'); 131.75 (C6"); 134.15 (C1'); 134.87 (C3"); 147.14, 147.13 (C1", C4'); 148.51 (C8); 148.87 (C4); 151.26 (C2); 154.52 (C6).

### 6.3.2. 15 1-Propyl-8-[4'-[[*m*-nitrophenyoxy]sulfonyl]phenyl]xanthine (5d)




Compound **5d** was prepared from 100 mg (0.2 mmol) of 6-amino-3-propyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil **4d** and ca. 2 g of PPSE as described above for **5a**.

Yield: 50 mg (50 %)
m.p.: 309 °C

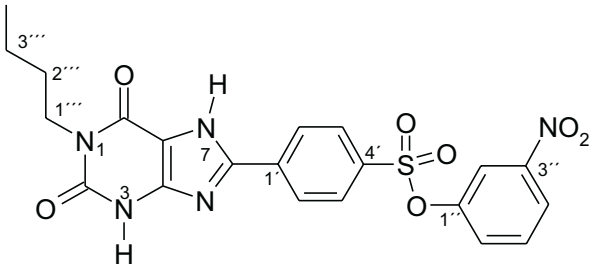
C,H,N analysis: $C_{20}H_{17}N_5O_7S \cdot 0.5\ H_2O$ [480.46 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 49.95 | 3.75 | 14.57 |
| Found: | 50.08 | 3.67 | 14.60 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.87 (s, 3H, C3"'H); 1.57 (m, 2H, C2"'H); 3.81 (t, 2H, C1"'H); 7.56 (m, 1H, C6"H); 7.71 (t, 1H, C5"H); 7.92 (t, 1H, C2"H); 8.1 (d, 2H, C3'H, C5'H); 8.2 (m, 1H, C4"H); 8.34 (d, 2H, C2'H, C6'H); 11.96 (s, 1H, N3-H); 14.12 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm] :
11.3 (C3"'); 20.98 (C2"'); 41.65 (C1"'); 108.96 (C5); 117.62 (C2"); 122.77 (C4"); 127.44 (C3', C5'); 129.13 (C5"); 129.35 (C2', C6'); 131.75 (C6"); 134.14 (C1'); 135 (C3"); 147.46, 147.72 (C1", C4'); 148.51 (C8); 148.87 (C4); 151.06 (C2); 155.06 (C6).

### 6.3.2. 16 1-Butyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine (5e)




Compound **5e** was prepared from 150 mg (0.3 mmol) of 6-amino-3-butyl-5-[4'-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil **4e** and ca. 2 g of PPSE as described above for **5a**.

Yield: 122 mg (88 %)
m.p.: 313 °C

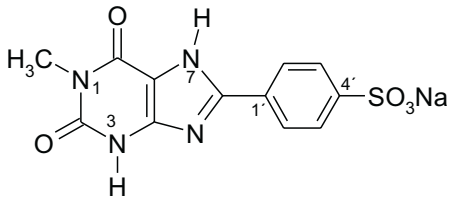
C,H,N analysis: $C_{21}H_{19}N_5O_7S$ [485.48 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 51.96 | 3.94 | 14.43 |
| Found: | 52.57 | 4.05 | 14.25 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.88 (t, 3H, C4"'H); 1.23 (m, 2H, C3"'H); 1.56 (m, 2H, C2"'H); 3.91 (t, 2H, C1"'H); 7.56 (m, 1H, C6"H); 7.71 (t, 1H, C5"H); 7.96 (t, 1H, C2"H); 8.07 (d, 2H, C3'H, C5'H); 8.2 (m, 1H, C4"H); 8.35 (d, 2H, C2'H, C6'H); 11.96 (s, 1H, N3-H); 14.12 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO- $d_6$) $\delta$ [ppm]:
13.85 (C4"'); 19.77 (C3"'); 29.85 (C2"'); 39 (C1"'); 117.62 (C2"); 122.77 (C4"); 127.43 (C3', C5'); 129.13 (C5"); 129.35 (C2', C6'); 131.75 (C6"); 134.12 (C1'); 135.02 (C3"); 147.45, 147.71 (C1", C4'); 148.51 (C8); 148.87 (C4); 151.04 (C2); 155.03 (C6).

### 6.3.2. 17 1-Methyl-8-*p*-sulfophenylxanthine (6c)




A solution of 3 g (6 mmol) of 1-methyl-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5c** in 60 mL of 2 M NaOH was heated at 70 °C for 30 min. After cooling down to r.t., the pH of the mixture was carefully adjusted to 7 by conc. HCl, then the solution was extracted with dichloromethane twice. The product precipitated when the aqueous solution was carefully acidified to pH 4 with 1 M HCl. It was filtered off and recrystallized by treatment with NaOH/HCl.

| | | |
|---|---|---|
| Yield: | 1.92 g | (83 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.55 | (S 1) |

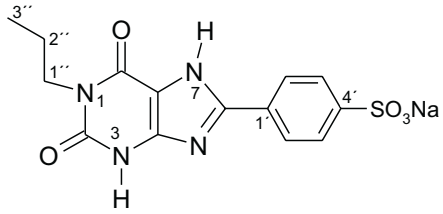
C,H,N analysis: $C_{12}H_9NaN_4O_2S \cdot 2.5\ H_2O$ [389.33 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 36.98 | 3.59 | 14.38 |
| Found: | 36.44 | 2.91 | 14.03 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.2 (s, 3H, N1-$CH_3$); 7.69 (d, 2H, J = 8.51 Hz, C3'H, C5'H); 8.03 (d, 2H, J = 8.2 Hz, C2'H, C6'H); 11.89 (s, 1H, N3-H); 13.68 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.20 (N1-$CH_3$); 107.83 (C5); 126.01 (C3', C5'); 126.21 (C2', C6'); 128.9 (C1'); 147.79 (C4'); 149.73, 149.89 (C8, C4); 151.37 (C2); 155.11 (C6).

### 6.3.2. 18 1-Propyl-8-*p*-sulfophenylxanthine (6d)




Compound **6d** was prepared from 3 g (6 mmol) of 1-propyl-8-[4'-[[*m*-nitrophenoxy]-sulfonyl]phenyl]xanthine **5d** as described above for **6c**.

| | | |
|---|---|---|
| Yield: | 1.85 g | (83 %) |
| m.p.: | >300 °C | (Lit >300 °C[63]) |
| $R_f$: | 0.53 | (S 1) |

C,H,N analysis: $C_{14}H_{13}NaN_4O_2S \cdot 2.5\ H_2O$ [417.39 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 40.25 | 4.31 | 13.42 |
| Found: | 40.16 | 4.33 | 13.27 |

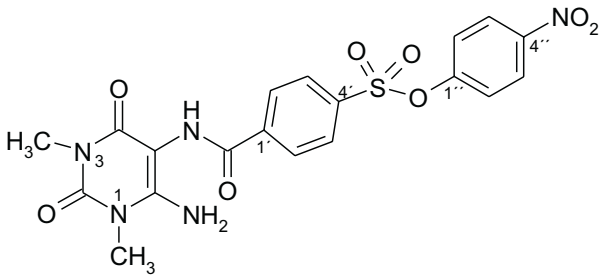
$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.87 (t, J = 7.56 Hz, 3H, C3"'H); 1.57 (m, 2H, C2"'H); 3.81 (t, J = 7.57 Hz, 2H, C1"'H); 7.68 (d, 2H, J = 8.51 Hz, C3'H, C5'H); 8.03 (d, 2H, J = 9.1 Hz, C2'H, C6'H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.32 (C3"'); 21.03 (C2"'); 41.54 (C1"'); 109.76 (C5); 126.04 (C3', C5'); 126.21 (C2', C6'); 128.96 (C1'); 147.78 (C4'); 148.88, 149.78 (C8, C4); 151.14 (C2); 154.95 (C6).

Determination of purity by CE:

| | |
|---|---|
| Buffer: | Phosphate 20 mM, pH 7.4 |
| Voltage: | 10 kV |
| Retention time: | 3.48 min |
| Purity: | 100 % |

### 6.3.2. 19 6-Amino-1,3-dimethyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (8a)




To a solution of 450 mg (2.94 mmol) of 5,6-diamino-1,3-dimethyluracil **3a** in 20 mL of methanol, 997 mg (2.94 mmol) of 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7** and 840 mg (4.4 mmol) of EDC were added. The reaction mixture was stirred until no more starting material could be detected by TLC. The mixture was treated with a large amount of water and the formed precipitate was filtered off and washed with methanol.

Yield: 1.03 g (71 %)
m.p.: >300 °C

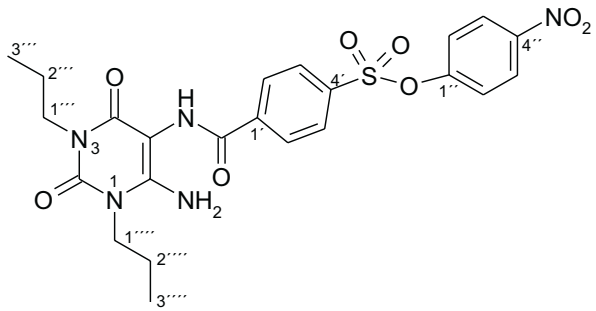
C,H,N analysis: $C_{19}H_{17}N_5O_8S$ [475.44 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 48.00 | 3.60 | 14.73 |
| Found: | 47.76 | 3.76 | 14.31 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.13 (s, 3H, N3-$CH_3$); 3.34 (s, 3H, N1-$CH_3$); 6.79 (s, 2H, $NH_2$); 7.39 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.04 (d, J = 8,82 Hz, 2H, C3'H, C5'H); 8.21 (d, J = 8.51 Hz, 2H, C2'H, C6'H); 8.27 (d, J = 9.45 Hz, 2H, C3"H, C5"H); 9.24 (s, 1H, $NH_2$).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.68 (N3-$CH_3$); 30.16 (N1-$CH_3$); 87.04 (C5); 123.45 (C3", C5"); 126.06 (C2", C6"); 128.28 (C3', C5'); 129.63 (C2', C6'); 135.8 (C1'); 140.86 (C4'); 146.34 (C4"); 150.74 (C6); 152.43 (C2); 153.22 (C1"); 159.29 (C4); 165.21 (NHCO).

### 6.3.2. 20 6-Amino-1,3-dipropyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (8b)



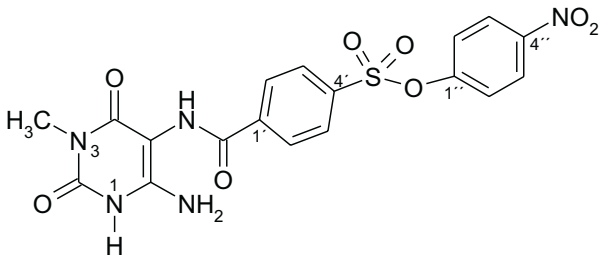

Compound **8b** was obtained as described above for **8a**, from 400 mg (1.77 mmol) of 5,6-diamino-1,3-dipropyluracil **3b**, 600 mg (1.77 mmol) of 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7** and 380 mg (1.99 mmol) of EDC.

Yield: 656 mg (68 %)
m.p.: >300 °C

$^{1}$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.83 (t, J = 7.41 Hz, 3H, C3"'H); 0.90 (t, J =7.25 Hz, 3H, C3""H); 1.50 (m, 2H, C2"'H); 1.56 (m, 2H, C2""H); 3.71 (t, J = 7.25 Hz, 2H, C1"'H); 3.84 (t, J = 7.56 Hz, 2H, C1""H); 6.25 (s, 2H, $NH_2$); 7.39 (d, J = 9.46 Hz, 2H, C2"H, C5"H); 8.03 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.20 (d, J = 8.82 Hz, 2H, C2'H, C6'H); 8.27 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 9.21 (s, 1H, $NH_2$).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
10.85 (C3"'); 11.31 (C3""); 20.96 (C2"'); 20.99 (C2""); 41.99 (C1"'); 43.85 (C1""); 86.99 (C5); 123.45 (C3", C5"); 126.06 (C2", C6");128.29 (C2', C5'); 129.61 (C2', C6'); 135.81 (C1'); 140.85 (C4'); 146.34 (C4"); 150.48 (C6); 151.81 (C2); 153.22 (C1"); 159.09 (C4); 165.16 (NHCO).

### 6.3.2. 21 6-Amino-3-methyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (8c)

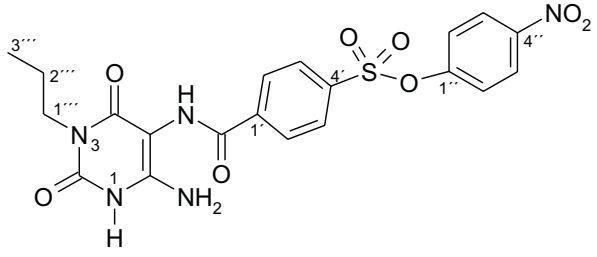


Compound **8c** was obtained as described above for **8a**, from 500 mg (3.21 mmol) of 5,6-diamino-3-methyluracil **3c**, 1.09 g (3.22 mmol) of 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7** and 612 mg (3.2 mmol) of EDC.

Yield: 892 mg (60 %)
m.p.: >300 °C

$^1$H-NMR (DMSO-d$_6$) $\delta$ [ppm]:
3.06 ( s, 3H, N3-CH$_3$); 6.25 (s, 2H, NH$_2$); 7.39 (d, J = 9.45 Hz, 2H, C2"H, C5"H); 8.04 (d, J = 8.51 Hz, 2H, C3'H, C5'H); 8.2 (d, J = 8.51 Hz, 2H, C2'H, C6'H); 8.27 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 9.24 (s, 1H, NH$_2$).

### 6.3.2. 22 6-Amino-3-propyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (8d)



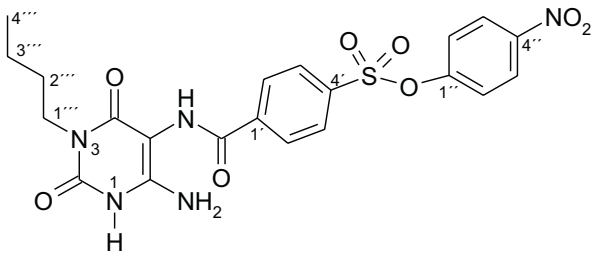
Compound **8d** was obtained as described above for **8a**, from 1.0 g (5.44 mmol) of 5,6-diamino-3-propyluracil **3c**, 1.84 g (5.4 mmol) of 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7** and 1.78 g (9.32 mmol ) of EDC.

Yield: 1.54 g (57 %)
m.p.: >300 °C

$^{1}$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.83 (m, 3H, C3"'H); 1.50 (m, 2H, C2"'H); 3.65 (m, 2H, C1"'H); 6.25 (s, 2H, $NH_2$); 7.38 (d, 2H, C2"H, C5"H); 8.03 (d, 2H, C3'H, C5'H); 8.19 (d, 2H, C2'H, C6'H); 8.27 (d, 2H, C3"H, C5"H); 9.20 (s, 1H, $NH_2$).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.27 (C3"'); 30.92 (C2"'); 35.91 (C1"'); 123.62 (C3", C5"); 126.04 (C2", C6"); 127.42 (C3', C5'); 129.25 (C2', C6'); 135.21 (C1'); 146.34 (C4"); 151.32 (C2); 153.20 (C1"); 155.35 (C4); 162.44 (NHCO).

### 6.3.2. 23 6-Amino-3-butyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (8e)

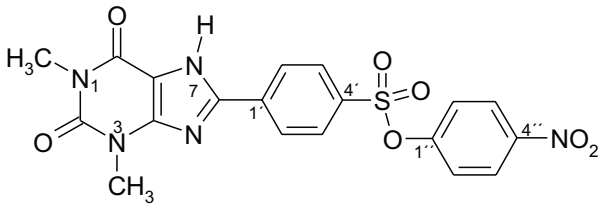



Compound **8e** was obtained as described above for **8a**, from 500 mg (2.53 mmol) of 5,6-diamino-3-butyluracil **3e**, 865 mg (5.55 mmol) of 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7** and 600 mg (3.14 mmol) of EDC.

| | | |
|---|---|---|
| Yield: | 870 mg | (66 %) |
| m.p.: | >300 °C | |

$^{1}$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.89 (t, J = 7.25 Hz, 3H, C4"'H); 1.25 (m, 2H, C3"'H); 1.46 (m, 2H, C2"'H); 3.67 (t, J = 7.41 Hz, 2H, C1"'H); 6.25 (s, 2H, $NH_2$); 7.39 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.04 (d, J = 8.81 Hz, 2H, C3'H, C5'H); 8.2 (d, J = 8.82 Hz, 2H, C2'H, C6'H); 8.27 (d, J = 9.45 Hz, 2H, C3"H, C5"H); 9.24 (s, 1H, NHCO); 10.4 (s, 1H, N1-H).

### 6.3.2. 24 1,3-Dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (9a)




To 800 mg (1.63 mmol) of 1,3-dimethyl-6-amino-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil **8a**, ca. 6.5 g of PPSE was added, and the mixture was heated at 170 °C for 1.5 h. After cooling down to r.t., the reaction mixture was treated with 20 mL of methanol and the formed precipitate was filtered off and recrystallized from DMF/$H_2O$.

Yield: 292 mg (38 %)
m.p.: >300 °C

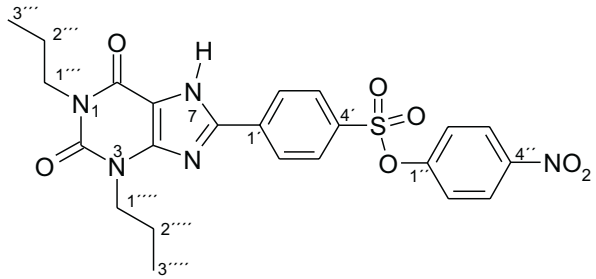
C,H,N analysis: $C_{19}H_{15}N_5O_7S \cdot H_2O$ [475.44 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 48.00 | 3.58 | 14.72 |
| Found: | 48.05 | 3.39 | 14.40 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.26 (s, 3H, N3-$CH_3$); 3.5 (s, 3H, N1-$CH_3$); 7.39 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.04 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.27 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 8.36 (d, J = 8.51 Hz, 2H, C2'H, C5'H); 14.25 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
29.94 (N1-C); 30.80 (N3-C); 109.04 (C5); 123.62 (C3", C5"); 126.03 (C2", C6"); 127.52 (C3', C5'); 129.28 (C2', C6' ); 134.34 (C1'); 134.84 (C4'); 146.34 (C4"); 147.15 (C4); 148.55 (C8); 151.26 (C2); 153.19 (C1"); 154.49 (C6).

### 6.3.2. 25 1,3-Dipropyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (9b)



Compound **9b** was prepared from 500 mg (0.92 mmol) of 6-amino-1,3-dipropyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil **8b** and ca. 5 g of PPSE as described above for **9a**.

Yield: 150 mg (31 %)
m.p.: >300 °C

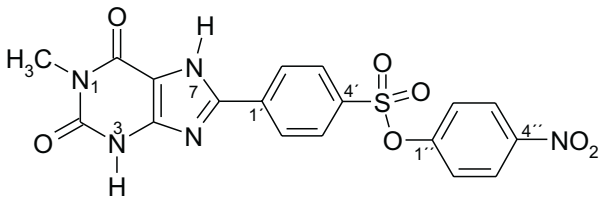
C,H,N analysis: $C_{23}H_{23}N_5O_7S \cdot 0.5\ H_2O$ [522.54 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 52.82 | 4.59 | 13.40 |
| Found: | 53.01 | 4.52 | 13.39 |

$^1$H-NMR (DMSO-d$_6$) $\delta$ [ppm]:
0.92 (t, J = 7.57 Hz, 3H, C3"'H); 0.96 (t, J = 7.56 Hz, 3H, C3""H); 1.65 (m, 2H, C2"'H); 1.82 (m, 2H, C2""H); 3.95 (t, J = 7.40 Hz, 2H, C1"'H); 4.10 (t, J = 7.25 Hz, 2H, C1""H); 7.41 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.02 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.29 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 8.49 (d, J = 8.82 Hz, 2H, C2'H, C5'H); 14.09 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-d$_6$) $\delta$ [ppm]:
11.17 (C3"'); 11.30 (C3""); 20.96 (C2"', C2""); 42.40 (C1"'); 44.67 (C1""); 123.62 (C3", C5"); 126.05 (C2", C6"); 127.56 (C3', C5'); 129.29 (C2', C6'); 134.28 (C1'); 146.34 (C4"); 150.76 (C2); 153.21 (C1"); 154.42 (C6).

### 6.3.2. 26 1-Methyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (9c)



Compound **9c** was prepared from 650 mg (1.36 mmol) of 6-amino-3-methyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil **8c** and ca. 4.5 g of PPSE as described above for **9a**.

Yield: 446 mg (93 %)
m.p.: >300 °C

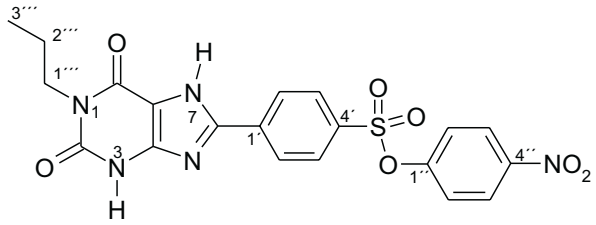
C,H,N analysis: $C_{18}H_{13}N_5O_7S \cdot 1.5\ H_2O$ [470.43 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 45.92 | 3.40 | 14.88 |
| Found: | 45.69 | 3.07 | 15.65 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
3.23 (s, 3H, N1-$CH_3$); 7.38(d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.04 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.26 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 8.34 (d, J = 8.82 Hz, 2H, C2'H, C5'H); 12.0 (s, 1H, N1-H); 14.13 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
27.28 (N1-$CH_3$); 108.96 (C5); 123.61 (C3", C5"); 126.04 (C2", C6"); 127.46 (C3', C5'); 129.26 (C2', C6'); 134.26 (C1'); 135.04 (C4'); 146.34 (C4"); 147.39 (C4); 147.66 (C8); 151.30 (C2); 153.20 (C1"); 155.24 (C6).

### 6.3.2. 27 1-Propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (9d)



Compound **9d** was prepared from 660 mg (1.19 mmol) of 6-amino-3-propyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil **8d** and ca. 5 g of PPSE as described above for **9a**.

Yield: 416 mg (65 %)
m.p.: >300 °C

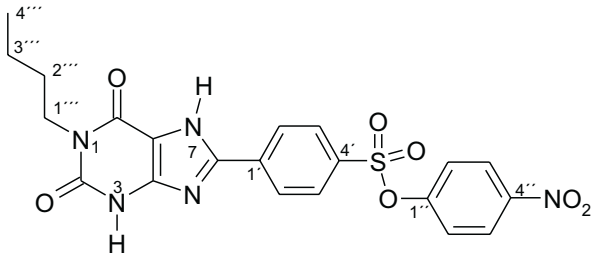
C,H,N analysis: $C_{20}H_{17}N_5O_7S$ [471.45 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 50.95 | 3.63 | 14.85 |
| Found: | 50.31 | 4.39 | 15.29 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.87 (t, J = 7.40 Hz, 3H, C3"'H); 1.58 (m, 2H, C2"'H); 3.84(t, J = 7.41 Hz, 2H, C1"'H); 7.39 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.03 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.26 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 8.33 (d, J = 8.82 Hz, 2H, C2'H, C5'H); 11.8 (s, 1H, N1-H); 14.1 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.3 (C3"'); 20.99 (C2"'); 41.66 (C1"'); 108.96 (C5); 123.60 (C3", C5"); 126.04 (C2", C6"); 127.49 (C3', C5'); 129.25 (C2', C6'); 134.30 (C1'); 135.02 (C4'); 146.33 (C4"); 147.48 (C4); 147.73 (C8); 151.07 (C2); 153.20 (C1"); 155.07 (C6).

### 6.3.2. 28 1-Butyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (9e)




Compound **9e** was prepared from 616 mg (1.19 mmol) of 6-amino-3-butyl-5-[4'-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil **8e** and ca. 5 g of PPSE as described above for **9a**.

Yield: 450 mg (76 %)
m.p.: >300 °C

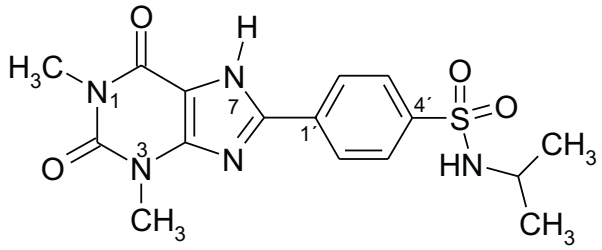
C,H,N analysis: $C_{21}H_{19}N_5O_7S$ [485.48 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 51.96 | 3.94 | 14.43 |
| Found: | 51.71 | 4.11 | 14.55 |

$^1$H-NMR ( DMSO-$d_6$) $\delta$ [ppm]:
0.9 (t, J = 7.41 Hz, 3H, C4"'H); 1.3 (m, 2H, C3"'H); 1.54 (m, 2H, C2"'H); 3.87 (t, J = 7.41 Hz, 2H, C1"'H); 7.39 (d, J = 9.14 Hz, 2H, C2"H, C5"H); 8.02 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.28 (d, J = 9.14 Hz, 2H, C3"H, C5"H); 8.35 (d, J = 8.2 Hz, 2H, C2'H, C5'H); 11.96 ( s, 1H, N1-H); 14.12 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
14.17 (C4""); 20.99 (C3"'); 30.18 (C2"'); 39.64 (C1"'); 123.91 (C3", C5"); 126.34 (C2", C6"); 127.76 (C3', C5'); 129.55 (C2', C6'); 134.50 (C1'); 135.50 (C4'); 146.65 (C4"); 151.37 (C2); 153.51 (C1"); 155.43 (C6).

### 6.3.2. 29 4'-(1,3-Dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-isopropyl-benzenesulfonamide (10a)




To a solution of 50 mg (0.11 mmol) 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]-xanthine **9a** in 5 mL of DMSO, 1 mL (12 mmol) of isopropylamine was added and the mixture was first stirred at r.t. for 40 min, then it was heated at 150 °C for 3 h. The remaining isopropylamine and DMSO was removed *in vacuo*. The residue was isolated by flash chromatography on silica gel with dichloromethane : methanol (50 : 1).

| | | |
|---|---|---|
| Yield: | 25 mg | (61 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.22 | (S 5) |

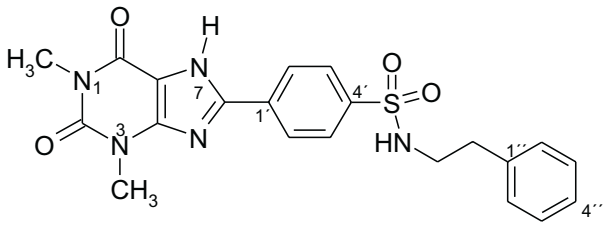
C,H,N analysis: $C_{16}H_{19}N_5O_4S$ [377.42 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 50.92 | 5.07 | 18.56 |
| Found: | 50.52 | 5.06 | 18.04 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.95 (d, J = 6.62 Hz, 6H, $CH_3$); 3.26 (s, 3H, N1-$CH_3$); 3.53 (s, 3H; N3-$CH_3$); 7.23 (d, J = 7.57 Hz, 1H, $SO_2NH$); 7.92 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8,82 Hz, 2H, C2'H, C6'H ); 14.1 (br, 1H, N7H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
23.35 ($CH_3$); 27.97 (N1-$CH_3$); 29.95 (N3-$CH_3$); 45.5 (CH); 108.9 (C5); 127 (C3', C5'); 127.15 (C2', C6'); 132.3 (C1'); 142.9 (C4'); 148.27, 148.62 (C8, C4); 151.33 (C2); 154.57 (C6).

### 6.3.2. 30 4'-(1,3-Dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-phenylethyl-benzenesulfonamide (10b)




Compound **10b** was obtained as described above for **10a**, from 50 mg (0.11 mmol) of 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9a** and 1 mL (8 mmol) of phenylethylamine in 5 mL of DMSO. The product was purified by flash chromatography on silica gel with dichloromethane : methanol (50 : 1).

| | | |
|---|---|---|
| Yield: | 21 mg | (44 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.19 | (S 5) |

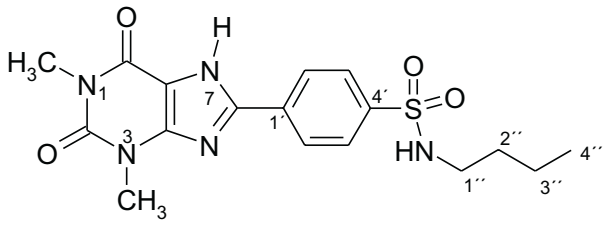
C,H,N analysis: $C_{21}H_{21}N_5O_4S$ [439.50 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 57.39 | 4.82 | 15.93 |
| Found: | 57.10 | 4.80 | 15.66 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
2.68 (t, J = 7.41 Hz, 2H, $CH_2$-phenyl); 3.0 (m, 2H, $CH_2$-NH); 3.25 (s, 3H, N1-$CH_3$); 3.62 (s, 3H; N3-$CH_3$); 7.15 (m, 3H, C2"H, C6"H, C4"H); 7.22 (m, 2H, 3"H, 5"H); 7.8 (t, J = 5.68 Hz, 1H, $SO_2$NH); 7.89 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.82 Hz, 2H, C2'H, C6'H); 14.1 (s, 1H, N7H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
28.71 (N1-$CH_3$); 30.71 (N3-$CH_3$); 36.17 ($CH_2$-phenyl); 44.93 ($CH_2$-NH); 127.12, 127.72, 128.01 (aromat-C); 129.19 (C3', C5'); 129.53 (C2', C6'); 139.50 (C1'); 141.98 (C4'); 152.09 (C2); 155.43 (C6).

### 6.3.2. 31 4'-(1,3-Dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-butyl-benzenesulfonamide (10c)



Compound **10c** was obtained as described above for **10a**, from 50 mg (0.11 mmol) of 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9a** and 1 mL (8 mmol) of butylamine in 5 mL of DMSO. The product was purified by flash chromatography on silica gel with dichloromethane : methanol (50 : 1).

| | | |
|---|---|---|
| Yield: | 21 mg | (44 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.28 | (S 5) |

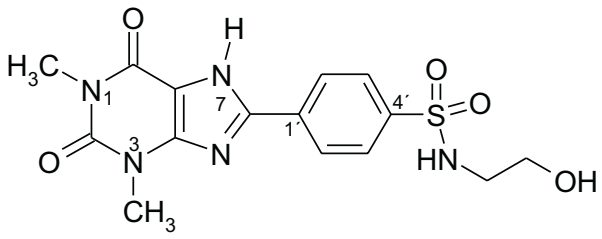
C,H,N analysis: $C_{17}H_{21}N_5O_4S$ [391.45 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 52.16 | 5.41 | 17.89 |
| Found: | 53.34 | 5.73 | 17.46 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.84 (t, J = 7.4 Hz, 3H, C4"H); 1.29 (m, 2H, C3"H); 1.32 (m, 2H, C2"H); 2.76 (m, 2H, C1"H); 3.34 (s, 3H, N1-$CH_3$); 3.58 (s, 3H; N3-$CH_3$); 7.73 (t, J = 5.83 Hz, 1H, $SO_2NH$); 7.89 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H, C6'H); 14.21 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
13.79 (C4"); 19.54 (C3"); 28.21 (N1-$CH_3$); 30.20 (N3-$CH_3$); 31.33 (C2"); 42.46 (C1"); 127.12(C3', C5'); 127.37 (C2', C6'). 141.75 (C4'); 148.36 (C8); 148.76 (C4); 151.44 (C2); 154.69 (C6).

### 6.3.2. 32 4'-(1,3-Dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-(2-hydroxy-ethyl)-benzenesulfonamide (10d)




Compound **10d** was obtained as described above for **10a**, from 66.8 mg (0.15 mmol) of 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9a** and 1 mL (8 mmol) of ethanolmaine in 5 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

| | | |
|---|---|---|
| Yield: | 28 mg | (51 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.09 | (S 3) |

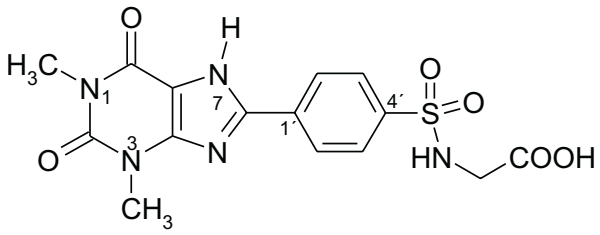
C,H,N analysis: $C_{15}H_{17}N_5O_5S \cdot 0.8\ H_2O$ [393.82 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 45.71 | 4.72 | 17.78 |
| Found: | 46.13 | 4.78 | 17.33 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
2,90 (m, 2H, $NHCH_2$); 3.33 (s, 3H, N1-$CH_3$); 3.52 (s, 3H; N3-$CH_3$); 7.76 (t, J = 5.83 Hz, 1H, $SO_2NH$); 7.89 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 14.00 (br, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
28.18 (N1-$CH_3$); 30.20 (N3-$CH_3$); 45.46 ($CH_2NH$); 60.26 ($CH_2OH$); 126.88 (C3', C5'); 127.25 (C2', C5'); 141.35 (C4'); 148.60 (C8); 148.78 (C4); 151.37 (C2); 154.78(C6).

### 6.3.2. 33 4'-(1,3-Dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-benzenesulfonylamino acetic acid (10e)




Compound **10e** was obtained as described above for **10a**, from 57.5 mg (0.13 mmol) of 1,3-dimethyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9a** and 88 mg (1.17 mmol) of glycine in 5 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

| | | |
|---|---|---|
| Yield: | 14 mg | (28 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.23 | (S 3) |

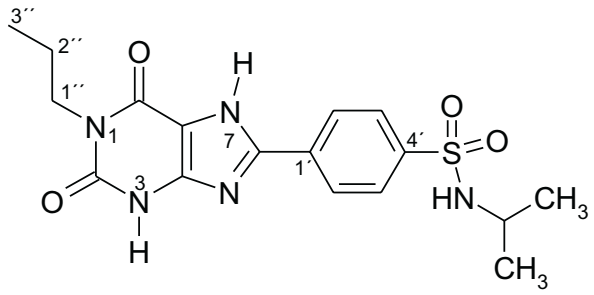
C,H,N analysis: $C_{15}H_{15}N_5O_6S$ [393.38 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 45.80 | 3.84 | 17.80 |
| Found: | 45.69 | 4.67 | 18.68 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
2.46 (d, J = 5.04 Hz, 2H, $CH_2$); 3.29 (s, 3H, N1-$CH_3$); 3.53 (s, 3H, N3-$CH_3$); 7.57 (t, J = 4.83 Hz, 1H, $SO_2NH$); 7.91 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 14.1 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm] :
27.99 ($CH_2$); 28.80 (N1-$CH_3$); 29.97 (N3-$CH_3$); 108.76 (C5); 127.08 (C3', C5'); 127.48 (C2', C6'); 132.40 (C1'); 140.35 (C4'); 148.14 (C8); 148.14 (C4); 151.32 (C2); 154.53 (C6).

### 6.3.2. 34 4'-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-isopropyl-benzenesulfonamide (11a)

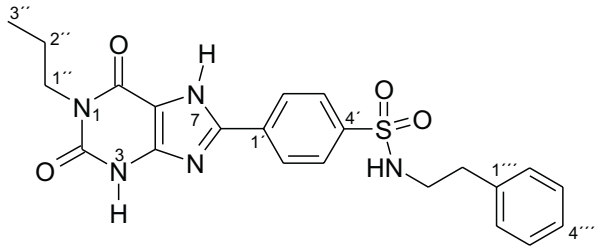



To a solution of 60 mg (0.12 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** in 5 mL of DMSO, 1 mL (12 mmol) of isopropylamine was added into. The mixture was first stirred at r.t. for 40 min, then it was heated at 150 °C for 5 h. The remaining isopropylamine and DMSO were distilled off *in vacuo*, the residue was suspended in methanol and isolated by flash chromatography on silica gel with dichloromethane: methanol (40 : 1).

| | | |
|---|---|---|
| Yield: | 32 mg | (65 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.12 | (S 4) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.70 (t, J = 7.41 Hz, 3H, C3"H); 0.77 (d, J = 6.62 Hz, 6H, $CH_3$); 1.38 (m, 2H, C2"H); 3.62 (t, J = 7.25 Hz, 2H, C1"H) ; 7.45 (d, J = 7.25 Hz, 1H, $SO_2NH$); 7.88 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 13.98 (s, 1H, N7-H).

### 6.3.2. 35 4'-(1-Propyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-phenethyl-benzenesulfonamide (11b)



Compound **11b** was obtained as described above for **11a**, from 79 mg (0.17 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** and 1 mL (8 mmol) of phenylethylamine in 5 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (40 : 1).

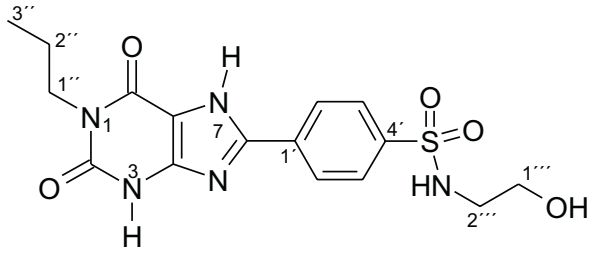
| | | |
|---|---|---|
| Yield: | 66 mg | (88 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.07 | (S 4) |

| | |
|---|---|
| Cal Mass: | 453.1470 g/mol ($C_{22}H_{23}N_5O_4S$) |
| High Resolution MS: | 453.1469 |
| EI-MS m/z (%): | 354 ($M^+$; 20.76); 362 ([M - 91]$^+$, 32); |
| | 333 ([M-$NHCH_2CH_2$-phenyl]$^+$, 48); |
| | 269 ([M-$SO_2NHCH_2CH_2$-phenyl]$^+$, 100) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.88 (t, J = 7.41 Hz, 3H, C3"H); 1.59 (m, 2H, C2"H); 2.71 (t, J = 7.57 Hz, 2H, $CH_2$-phenyl); 3.02 (t, J = 6.2 Hz, 2H, $CH_2$-N); 3.83 (t, J = 7.4 Hz, 2H, C1"H); 7.2 (m, 3H, C3"'H, C4"'H, C5"'H); 7.26 (m, 2H, C2"'H, C6"'H); 7.80 (t, J = 5.83 Hz, 1H, $SO_2NH$); 7.88 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 13.98 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.32 (C3"); 21.03 (C2"); 35.44 ($CH_2$-phenyl); 41.56 (C1"); 44.19 ($CH_2$-NH); 126.37, 126.89, 127.22 (aromat-C); 128.44 (C3', C5'); 128.79 (C2', C6'); 138.77 (C1'); 151.16 (C2).

### 6.3.2. 36 4'-(1-Propyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-(2-hydroxyethyl)-benzenesulfonamide (11c)




Method 1: To a solution of 75.2 mg (0.16 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9c** in 5 mL of DMSO, 1 mL (11 mmol) of ethanolamine was added. The mixture was stirred at r.t. under argon for ca. 72 h. Remaining ethanolamine and DMSO was distilled off *in vacuo*, the residue was suspended in methanol and purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

Method 2: To a solution of 43.7 mg (0.09 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** in 5 mL of DMSO, 1 mL (11 mmol) of ethanolamine was added. The mixture was stirred at r.t. for 30 min, then it was heated at 150 °C for 5 h, until the color turned red. Remaining ethanolamine and DMSO were distilled off *in vacuo*, the residue was suspended in methanol and isolated by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

| | | | |
|---|---|---|---|
| Yield: | method 1 : | 33 mg | (53 %) |
| | method 2 : | 10 mg | (28 %) |
| m.p.: | >300 °C | | |
| $R_f$: | 0.08 | | (S 3) |

C,H,N analysis: $C_{16}H_{19}N_5O_5S \cdot 2.5\ H_2O$ [438.47 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 43.78 | 5.47 | 15.96 |
| Found: | 44.22 | 4.77 | 15.78 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.88 (t, J = 7.41 Hz, 3H, C3"H); 1.57 (m, 2H, C2"H); 2.81 (t, J = 6.1Hz, 2H, C2"'H); 3.81 (t, J = 7.4 Hz, 2H, C1"H); 4.69 (t, J = 5.35 Hz, 2H, C1"'H); 7.69 (t, J = 5.83 Hz, 1H, $SO_2NH$); 7.88 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.51 Hz, 2H, C2'H,

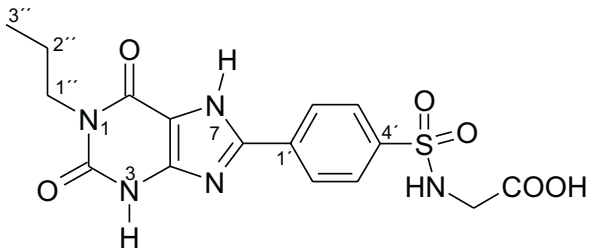
C6'H ); 11.94 (s, 1H, N1-H); 13.94 (s, 1H, N7-H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
11.32 (C3"); 21.01 (C2"); 41.61 (C1"); 45.25 (C2"'); 60.05 (C1"'); 126.95 (C3', C5'); 127.27 (C2', C6'); 132.55 (C1'); 141.55 (C4'); 147.76 (C4); 148.50 (C8); 151.12 (C2); 155.13 (C6).

Determination of purity by CE:

| | |
|---|---|
| Buffer: | Phosphate 20 mM, pH 7.4 + 100 mM SDS |
| Voltage: | 10 kV |
| Retention time: | 12.13 min |
| Purity: | 100 % |

### 6.3.2. 37 4'-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonylamino acetic acid (11d)




Compound **11d** was obtained as described above for **11a**, from 70 mg (0.146 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** and 76.7 mg (1.022 mmol) of glysine in 7 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

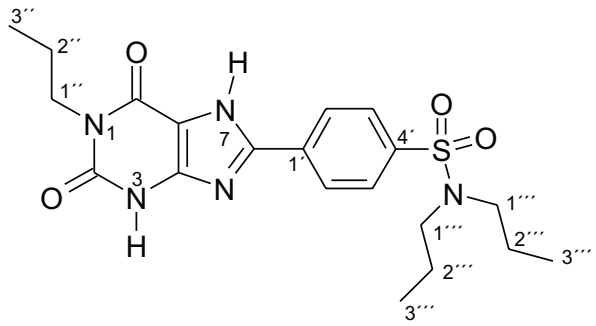
| | | |
|---|---|---|
| Yield: | 20 mg | (34 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.19 | (S 3) |

C,H,N analysis: $C_{16}H_{17}N_5O_6S$ [407.41 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 47.17 | 4.21 | 17.19 |
| Found: | 47.58 | 4.63 | 18.02 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.88 (t, J = 7.41 Hz, 3H, C3"H); 1.58 (m, 2H, C2"H); 2.44 (t, J = 5.04 Hz, 2H, $CH_2$); 3.84 (t, J = 7.40 Hz, 2H, C1"H); 7.51 (t, 1H, $SO_2NH$); 7.89 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.3 (d, J = 8.20 Hz, 2H, C2'H, C6'H ); 11.91 (s, 1H, N1-H).

### 6.3.2. 38 4'-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-N,N-dipropylbenzenesulfoamide (11e)



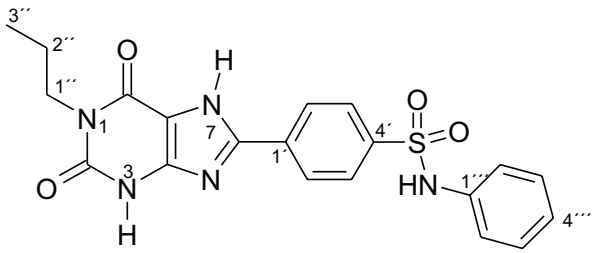

Compound **11e** was obtained as described above for **11a**, from 50 mg (0.106 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** and 1 mL (7.32 mmol) of dipropylamino in 7 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (40 : 1).

| | | |
|---|---|---|
| Yield: | 15 mg | (33 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.26 | (S 4) |

| | |
|---|---|
| Cal Mass: | 433.1784 g/mol ($C_{20}H_{27}N_5O_4S$) |
| High Resolution MS: | 433.1787 |
| EI-MS m/z (%): | 433.2 ($M^+$; 30); 404.2 ([M-$CH_3CH_2$]$^+$, 100); 269.1 ([M-$SO_2N(CH_3CH_2CH_3)_2$]$^+$, 60) |

$^1$H-NMR (DMSO-d$_6$) $\delta$ [ppm]:
0.81 (t, J = 7.25 Hz, 6H, C3"'H); 0.88 (t, J = 7.41 Hz, 3H, C3"H); 1.47 (m, 4H, C2"'H); 1.58 (m, 2H, C2"H); 3.12 (m, 4H, C1"'H); 3.82 (t, J = 7.41 Hz, 2H, C1"H); 7.89 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.27 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 11.91 (bs, 1H, N1-H); 13.96 (bs, 1H, N7-H).

### 6.3.2. 39 4'-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-N-phenyl-benzenesulfonamide (11f)




Compound **11f** was obtained as described above for **11a**, from 48 mg (0.1 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** and 1 mL (11 mmol) of aniline in 7 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

| | | |
|---|---|---|
| Yield: | 17 mg | (40 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.24 | (S 5) |

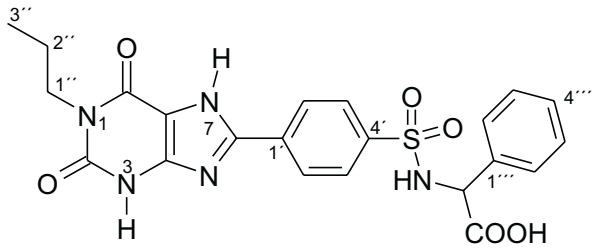
| | |
|---|---|
| Cal Mass: | 425.1157 g/mol ($C_{20}H_{19}N_5O_4S$) |
| High Resolution MS: | 425.1145 |
| EI-MS m/z (%): | 425.2($M^+$; 100); 426.2 ($[M+1]^+$, 20); 383.2 ($[M- CH_3CH_2CH_2+3]^+$, 45) |

$^1$H-NMR (DMSO-d$_6$) $\delta$ [ppm]:
0.85 (t, J = 7.4 Hz, 3H, C3"H); 1.58 (m, 2H, C2"'H); 3.82 (t, J = 7.41 Hz, 2H, C1"H); 7.02 (m, 1H, C4"'H); 7.08 (m, 2H, C2"'H, C6"'H); 7.21 (m, 2H, C3"'H, C5"'H); 7.79 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.15 (d, J = 8.83 Hz, 2H, C2'H, C6'H ); 11.91 (bs, 1H, N1-H); 13.96 (bs, 1H, N7-H).

$^{13}$C-NMR (DMSO-d$_6$) $\delta$ [ppm]:
11.32 (C3"); 21.07 (C2"); 41.45 (C1"); 120.40, 124.28 ($C_{aromat}$); 126.53 (C3', C5'); 127.32

(C2', C6'); 129.29, 137.77 ($C_{aromat}$); 148.10 (C4); 151.24 (C2); 155.17 (C6).

### 6.3.2. 40 4'-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-benzenesulfonylaminophenyl acetic acid (11g)




Compound **11g** was obtained as described above for **11a**, from 52 mg (0.1 mmol) of 1-propyl-8-[4'-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine **9d** and 120 mg (1.07 mmol) of $\alpha$-aminophenylacetic acid in 7 mL of DMSO. Product was purified by flash chromatography on silica gel with dichloromethane : methanol (20 : 1).

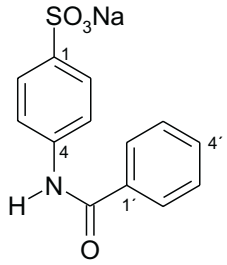
| | | |
|---|---|---|
| Yield: | 90 mg | (44 %) |
| m.p.: | >300 °C | |
| $R_f$: | 0.13 | (S 5) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
0.85 (t, J = 7.4 Hz, 3H, C3"H); 1.58 (m, 2H, C2"'H); 3.82 (t, J = 7.41 Hz, 2H, C1"H); 4.02 (d, J = 5.99 Hz, 1H, CH); 7.24 (m, 5H, CH-phenyl); 7.87 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 8.22 (d, J = 8.51 Hz, 2H, C2'H, C6'H ); 11.84 (bs, 1H, N1-H); 13.96 (bs, 1H, N7-H).

#### 6.3.2. 41 Sodium *m*-nitrophenoxide (12)

To a solution of 5 g (36 mmol) *m*-nitrophenol in 20 mL of methanol, 1.5 g (37.5 mmol) of sodium hydroxide in 15 mL 85 % aq. methanol was added. The flask was stirred under the protection of nitrogen for 5 hours. Red sodium *m*-nitrophenoxide was obtained after the solvent was removed *in vacuo*.

#### 6.3.2. 42 4-Benzoylamino-1-benzenesulfonic acid sodium salt (13a)




To a solution of 2 g (12 mmol) sulfanilic acid in 30 mL of water, 2.1 mL (18 mmol) of benzoyl chloride in 10 mL of toluene was slowly added under vigorous stirring. During the whole reaction time, a pH of 3 was maintained by manual addition of aqueous 1 M aqueous $Na_2CO_3$ solution, the reaction was continued until no more sulfanilic acid could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more benzoic acid could be detected by TLC. The aqueous phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 2.5 g | (72 %) |
| $R_f$: | 0.74 | (S 6) |

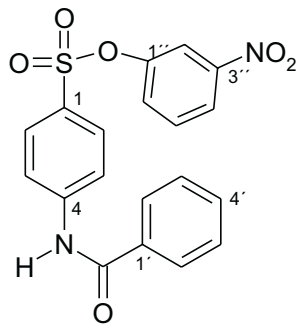
C,H,N analysis: $C_{13}H_{10}NNaO_4S \cdot 1.5\ H_2O$ [326.31 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 47.81 | 3.98 | 4.29 |
| Found: | 48.02 | 4.41 | 4.31 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.52 (m, 2H, C3'H, C5'H); 7.6 (m, 3H, C2'H, C6'H, C4'H); 7.73 (d, J = 8.51 Hz, 2H, C3H, C5H); 7.95 (d, J = 7.88 Hz, 2H, C2H, C6H); 10.25 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
119.47 (C3, C5); 126.17 (C2, C6); 127.84 (C3', C5'); 128.54 (C2', C6'); 131.76 (C4'); 135.03 (C1'); 139.39 (C1); 143.78 (C4); 165.7 (CONH).

### 6.3.2. 43 4-Benzoylamino-1-benzenesulfonic acid *m*-nitrophenyl ester (14a)




Compound **13a** (430 mg, 1.33 mmol) was dissolved in 10 mL of pyridine and 20 mL of acetic anhydride. The mixture was stirred at r.t. overnight. A white precipitate was obtained after 40 mL of diethyl ether was added into the above solution. The precipitate was mixed with 4 g of $PCl_5$, and heated at 60 °C for 2 h. A white powder was obtained after the mixture was carefully poured into ice-cold water. The residue was recrystallized from toluene, yielding 4-benzamido-1-benzenesulfonylchloride.

To a solution of 92 mg (0.57 mmol) sodium *m*-nitrophenoxide in 10 mL of dichloromethane, 2 mL of TEA and 170 mg (0.57 mmol) of 4-benzamido-1-benzenesulfonylchloride in 10 mL dichlormethane were added. The color of the reaction turned to white after one hour of stirring. The reaction was completed when there was no more starting material detectable by TLC. A white solid was obtained after the solvent was removed *in vacuo*, acetone : water (1 : 1) was used for the recrystallization.

| | | |
|---|---|---|
| Yield: | 273 mg | (48 %) |
| m.p.: | 177 °C | |
| $R_f$: | 0.36 | (S 10) |

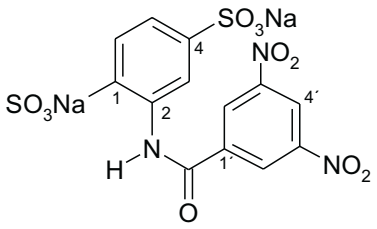
C,H,N analysis: $C_{19}H_{14}N_2O_6S$ [398.40 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 57.28 | 3.54 | 7.03 |
| Found: | 57.35 | 3.57 | 11.30 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.55 (m, 3H, C3'H, C5'H, C6"H); 7.62 (t, J = 7.25 Hz, 1H, C4'H); 7.7 (t, J = 8.35 Hz, 1H, C5"H); 7.9 (m, 3H, C3H, C5H, C2"H); 7.96 (d, J = 7.25 Hz, 2H, C2'H, C6'H); 8.09 (d, J = 9.14 Hz, 2H, C2H, C6H); 8.2 (m, 1H, C4"H); 10.65 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.55 (C2"), 120.24 (C3, C5); 122.57 (C4"); 127.24 (C1); 128.03 (C3', C5'); 128.65 (C2', C6'); 129.15 (C5"); 129.89 (C4'); 131.66 (C2, C6); 132.30 (C6"); 134.36 (C3"); 145.54 (C4); 148.48 (C1"); 149.12 (C1'); 166.50(CONH).

### 6.3.2. 44 2-(3',5'-Dinitrobenzoylamino)benzene-1,4-disulfonic acid monosodium salt (13b)




To a solution of 1.5 g (5.5 mmol) 2-amino-1,4-benzenedisulfonic acid monosodium salt in 50 mL of water, 2.5 g (10.8 mmol) of 3,5-dinitrobenzoyl chloride in 30 mL of toluene was added under vigorous stirring. During the whole reaction time, a pH of 4.5 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. the reaction was continued until no more 2-amino-1,4-benzenedisulfonic acid monosodium salt could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more 3,5-dinitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the yellow residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 1.66 g | (62 %) |
| $R_f$: | 0.58 | (S 8) |

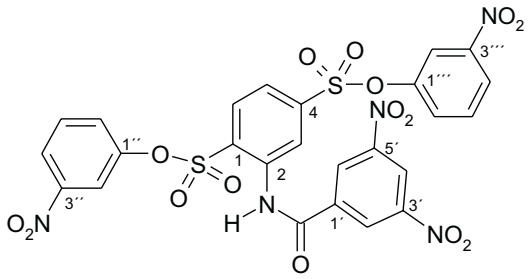
C,H,N analysis: $C_{13}H_7N_3Na_2O_{11}S \cdot 3\ H_2O$ [545.38 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 28.60 | 2.38 | 7.70 |
| Found: | 28.54 | 2.39 | 7.73 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.4 (m, 1H, C5H); 7.7 (d, 1H, C6H); 8.75 (s, 1H, C3H); 9.01 (d, 1H, C4'H); 9.05 (d, 2H, C2'H, C6'H); 12.03 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.71 (C3); 121.12 (C5); 121.55 (C4'); 126.77 (C6); 127.27 (C2', C6'); 133.87 (C1); 135.95 (C2); 137.5 (C1'); 148.74 (C3', C5'); 149.94 (C4 ); 160.03 (NHCO).

### 6.3.2. 45 2-(3',5'-Dinitrobenzoylamino)-benzene-1,4-disulfonic acid - bis-(*m*-nitrophenyl)ester (14b)




Compound **13b** (300 mg, 0.61 mmol) and ca. 5 g of $PCl_5$ were heated at 150 °C for two hours. A yellow brown solid was obtained after the mixture was poured into ice-cold water. This brown 2-(3',5'-dinitrobenzyolamino)benzene-1,4-disulfonyl dichloride was directly used for the subsequent reaction without purification.

To a solution of 222 mg (2.48 mmol) sodium *m*-nitrophenoxide in 20 mL of dichloromethane, 2 mL of TEA and sulfonylchloride in 20 mL of dichloromethane were added drop by drop under vigorous stirring. The reaction was monitored by TLC and continued until no more starting compound were detectable. The solvent was distilled off *in vacuo* and the residue was purified by flash chromatography on silica gel using acetone : petroleum ether = 1 : 3.5 as eluent.

| | | |
|---|---|---|
| Yield: | 125 mg | (18 %) |
| m.p. | 159 °C | |
| $R_f$: | 0.33 | (S 9) |

C,H,N analysis: $C_{25}H_{15}N_5O_{15}S_2$ [689.55 g/mol]

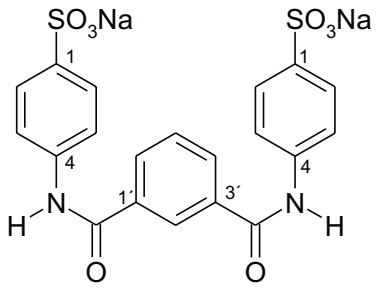
| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 43.55 | 2.19 | 10.16 |
| Found: | 44.93 | 3.20 | 8.09 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.32 (m, 1H, C6"H); 7.58 (t, J = 8.4 Hz, 1H, C5"H); 7.65 (m, 1H, C5H); 7.69 (m, 1H,

C6"'H); 7.75 (t, J = 8.2 Hz, 1H, C5"'H); 7.80 (t, J = 2.2 Hz, 1H, C2"H ); 7.89 (d, J = 1.9 Hz, 1H, C3H); 8.05 (t, J = 2.2 Hz, 1H, C2"'H); 8.05 (d, J = 7.5 Hz; 1H, C6H); 8,18 (m, 1H, C4"H ); 8.23 (m, 1H, C4"'H); 8.57 (d, J = 2.2 Hz, 2H, C2'H, C6'H); 8.8 (t, J = 2.2 Hz, 1H, C4'H).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.25, 117.22 (C2"', C2"); 120.71 (C4'); 122.83 (C4"); 123.04 (C5); 123.08 (C4"'); 123.28 (C3); 128.43 (C6"); 128.73 (C6"'); 128.93 (C2', C6'); 129. 87 (C1); 131.61 (C5"); 132.04 (C5"'); 132.64 (C6); 133.29 (C2); 140.70 (C1'); 147.79 (C4); 147.98 (C3', C5'); 148.4 (C3"); 148.50 (C3"'); 148.60 (C1"); 148.76 (C1"'); 157.02 (CONH).

### 6.3.2. 46 Isophthaloyl-N,N-bis-*p*-benenesulfonic acid sodium salt (15a)




To a solution of 1.0 g (5.78 mmol) sulfanilic acid in 20 mL of water, 0.58 g (2.8 mmol) of isophthaloyl dichloride in 10 mL of toluene was added under vigorous stirring. During the whole reaction, a pH of 3 was controlled by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more sulfanilic acid could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more isophthalic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the yellow residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 997 mg | (66 %) |
| $R_f$: | 0.5 | (S 7) |

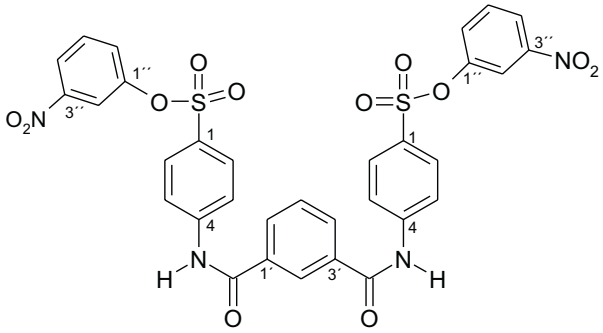
C,H,N analysis: $C_{20}H_{14}N_2Na_2O_8S_2$ [520.45 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 46.16 | 2.71 | 5.38 |
| Found: | 45.62 | 2.96 | 5.21 |

$^{1}$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.6 (d, J = 8.51 Hz, 4H, C3H, C5H); 7.67 (t, J = 7.72 Hz, 1H, C5'H ); 7.75 (d, J = 8.82 Hz, 4H, C2H, C6H); 8.14 (m, 2H; C4'H, C6'H ); 8.53 (t, J = 1.73 Hz, 1H, C2'H ); 10.5 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
119.49 (C3, C5); 126.22 (C2, C6); 127.12 (C5'); 128.77 (C2'); 130.89 (C4', C6'); 135.22 (C1', C3'); 139.24 (C1); 143.91 (C4); 165.21 (CONH).

### 6.3.2. 47 Isophthaloyl-N,N-bis-(*p*-benenesulfonic acid)-*m*-nitrophenyl ester (16a)




Compound **15a** (600 mg, 1.26 mmol) and ca. 6 g of $PCl_5$ was heated at 150 °C for two hours. A white solid was precipitated after the mixture was poured into ice-cold water. This white isophthaloyl-N,N-bis(*p*-benenesulfonyl)dichloride was directly used for the subsequent reaction without purification.

To a solution of 493 mg (3.06 mmol) sodium *m*-nitrophenoxide in 20 mL of dichloromethane, 2 mL of TEA and sulfonyldichloride in 20 mL of dichloromethane were added drop by drop into the above solution. The reaction was monitored by TLC until no starting compound was detectable anymore. The solvent was distilled off *in vacuo*, and the residue was purified by flash chromatography on silica gel using acetone : petroleum ether = 1 : 2 as eluent.

| | | |
|---|---|---|
| Yield: | 150 mg | (17 %) |
| m.p. | 240 °C | |
| $R_f$: | 0.28 | (S 10) |

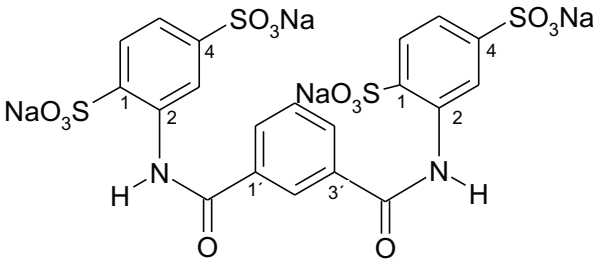
C,H,N analysis: $C_{32}H_{22}N_4O_{12}S_2 \cdot H_2O$ [736.72 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 52.12 | 3.26 | 7.60 |
| Found: | 53.84 | 3.62 | 7.18 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.55 (m, 2H, C6"H); 7.7 (m, 3H, C5"H, C5'H); 7.89 (t, J = 2.36 Hz, 2H, C2"H); 7.92 (d, J = 9.14 Hz, 4H, C3H, C5H); 8.1 (d, J = 9.14 Hz, 4H, C2H, C6H,); 8.2 (m, 4H, C4'H, C6'H, C4"H); 8.55 (t, J = 1.58 Hz, 1H, C2'H); 10.9 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.55 (C2"); 120.34 (C3, C5); 122.6 (C4"); 127.51 (C5'); 127.58 (C1); 129.05 (C2'); 129.19 (C5"); 129.97 (C2, C6); 131.58 (C4'); 131.68 (C6"); 134.67 (C3"); 145.34 (C4); 148.48 (C1"); 149.11 (C1'); 165.85 (CONH).

### 6.3.2. 48 Isophthaloylamino-N,N'-bis-benzene-1,4-disulfonic acid sodium salt (15b)




To a solution of 1.0 g (3.6 mmol) 2-amino-1,4-benzenedisulfonic acid monosodium salt in 20 mL of water, 0.74 g (3.6 mmol) of isophthaloyl dichloride in 20 mL of toluene was added into the aqueous solution under vigorous stirring. During the whole reaction time, a pH of 3 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued for more than 12 hours, until no more 2-amino-1,4-benzene-disulfonic acid monosodium salt could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more isophthalic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the yellow residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 350 mg | (23 %) |
| $R_f$: | 0.78 | (S 7) |

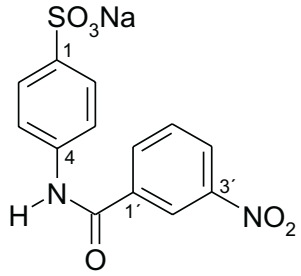
C,H,N analysis: $C_{20}H_{12}N_2Na_4O_{14}S_4 \cdot 3\ H_2O$ [778.6 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 30.82 | 3.59 | 2.31 |
| Found: | 30.74 | 3.59 | 2.47 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm] :
7.25 (m, 2H, C5H); 7.6 (m, 2H, C6H); 7.78 (t, J = 7.72 Hz, 1H, C5'H); 8.14 (m, 2H, C4'H, C6'H); 8.58 (s, 1H, C2'H); 8.82 (d, 2H, C3H); 11.54 (s, 2H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm] :
117.54 (C3); 120.25 (C5); 126.54 (C6); 127.22 (C5'); 129.57 (C4', C6');129.61 (C2'); 134.59 (C1); 135.6 (C2); 135.66 (C1', C3'); 149,69 (C4); 163.26 (CONH).

### 6.3.2. 49 4-(3'-Nitro-benzoylamino)-1-benzenesulfonic acid sodium salt (17a)




To a solution of 2 g (1.2 mmol) sulfanilic acid in 20 mL of water, 2.5 g (1.35 mmol) of 3-nitrobenzoyl chloride in 10 mL of toluene was added under vigorous stirring. During the whole reaction time, a pH of 3 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more sulfanilic acid could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more 3-nitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 4.4 g | (72 %) |
| $R_f$: | 0.61 | (S 7) |

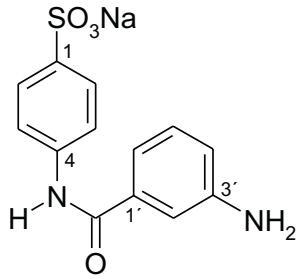
C,H,N analysis: $C_{13}H_9N_2NaO_6S \cdot 2\ H_2O$ [380.3 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 41.02 | 3.42 | 7.36 |
| Found: | 40.76 | 3.44 | 7.25 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.6 (d, J = 8.82 Hz, 2H, C3H, C5H); 7.8 (m, 3H, C5'H, C2H, C6H); 8.4 (m, 1H, C6'H); 8.47 (m, 1H, C4'H); 8.8 (s, 1H, C2'H); 10.75 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
119.84 (C3, C5);122.73 (C2'); 126.28 (C2, C6); 126.33 (C4'); 130.35 (C5'); 134.42 (C6'); 136.3 (C1'); 139.16 (C1); 143.85 (C4); 147.93 (C3'); 163.59 (CONH).

### 6.3.2. 50 4-(3'-Aminobenzoylamino)benzenesulfonic acid sodium salt (18a)



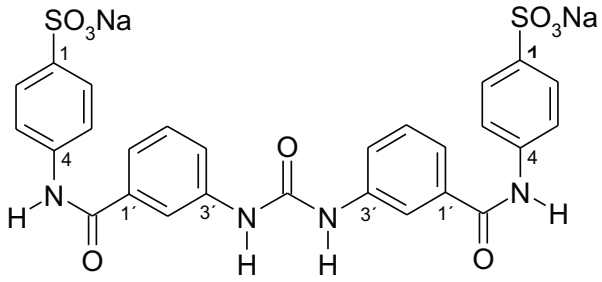

A suspension of 1.8 g (5.3 mmol) **17a** in 100 mL of $H_2O$ was adjusted to pH 8 by 2 M aqueous $Na_2CO_3$ solution, the solution was hydrogenated at a hydrogen pressure of 1 bar with Pd/C (10 % Pd) as a catalyst. The reaction mixture was filtered. The filtrate was brought to pH 6 by 1 M HCl solution, then the aqueous solution was dried to yield **18a** as white powder .

| | | |
|---|---|---|
| Yield: | 1.6 g | (95 %) |
| $R_f$: | 0.68 | (S 7) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
5.27 (s, 2H, $NH_2$); 6.7 (m, 1H, C4'H); 7.04 (m, 1H, C6'H); 7.08 (t, J = 2.04 Hz, 1H, C2'H); 7.12 (t, J = 7.88 Hz, 1H, C5'H); 7.65 (d, J = 8.82 Hz, 2H, C3H, C5H); 7.7 (d, J = 8.82 Hz, 2H, C2H, C6H); 10.1 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
113.16 (C2); 114.92 (C6'); 116.95 (C4'); 119.22 (C3, C5); 126.01 (C2, C6); 128.88 (C5'); 136.0 (C1'); 139.54 (C1); 143.61 (C4); 148.89 (C3'); 166.49 (CONH).

### 6.3.2. 51 4,4-(Carbonylbis(imino-1',3'-phenylene)carbonylimino)bis-1-sulfonic acid sodium salt (19a)




A 20 % solution of phosgene in toluene (20.5 mL, 41 mmol $COCl_2$) was added dropwise over 1 h at r.t. to a vigorously stirred solution of 1.3 g (4.1 mmol) **18a** in 200 mL of $H_2O$. During the whole reaction, the mixture was strictly maintained at pH 3 by automatic addition of 2 M aqueous $Na_2CO_3$ solution. Soon large amounts of precipitate came out of the solution. After ca. 3 h of stirring, the precipitate was filtered off and washed with methanol.

| | | |
|---|---|---|
| Yield: | 606 mg | (22 %) |
| $R_f$: | 0.48 | (S 7) |

C,H,N analysis: $C_{27}H_{20}N_4Na_2O_9S_2 \cdot 1.5\ H_2O$ [681.62 g/mol]

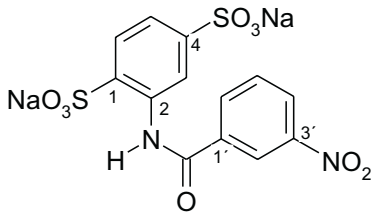
| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 47.53 | 3.37 | 8.22 |
| Found: | 47.68 | 3.50 | 8.28 |

$^{1}$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.42 (t, J = 8.04 Hz, 1H, C5'H); 7.55 (m, 1H, C4'H); 7.59 (d, J = 8.51 Hz, 2H, C3H, C5H); 7.71 (m, 1H, C6'H); 7.73 (d, J = 8.82 Hz, 2H, C2H, C6H); 7.98 (t, J = 1.89 Hz, 1H, C2'H); 9.1 (s, 1H; NHCONH); 10.3 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm] :
117.99 (C2'); 119.49 (C3, C5); 121.23 (C6'); 121.58 (C4'); 126.22 (C2, C6); 129.02 (C5');

135.89 (C1'); 139.49 (C3'); 139.61 (C1); 143.61 (C4); 152.79 (NHCONH); 165.86 (CONH).

### 6.3.2. 52 2-(3'-Nitrobenzoylamino)-benzene-1,4-disulfonic acid sodium salt (17b)




To a solution of 1.5 g (5.5 mmol) 2-amino-1,4-benzenedisulfonic acid monosodium salt in 40 mL of water, 1.5 g (8.1 mmol) of 3-nitrobenzoyl chloride in 30 mL of toluene was added under vigorous stirring. During the whole reaction time, a pH of 3 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more 2-amino-1,4-benzene-disulfonic acid monosodium salt could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more 3-nitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 1.72 g | (71 %) |
| $R_f$: | 0.62 | (S 7) |

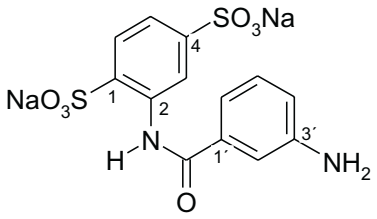
C,H,N analysis: $C_{13}H_8N_2Na_2O_9S_2 \cdot 2\ H_2O$ [482.36 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 32.34 | 2.49 | 5.81 |
| Found: | 32.79 | 2.46 | 5.92 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.3 (m, 1H, C5H); 7.68 (d, J = 7.88 Hz, 1H, C6H); 7.9 (t, J = 8.03 Hz, 1H, C5'H); 8.34 (m, 1H, C6'H); 8.45 (m, 1H, C4'H); 8.71 (t, J = 1.89 Hz, 1H, C3H); 8.8 (t, 1H, C2'H); 11.7 (s, 1H, CONH).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.55 (C3); 120.59 (C5); 122.09 (C2'); 126.58 (C4'); 126.61 (C6); 130.96 (C5'); 132.99 (C6'); 134.2 (C1'); 135.73 (C2); 136.31 (C1); 148.37 (C3'); 149.84 (C4); 161.85 (CONH).

### 6.3.2. 53 2-(3'-Aminobenzoylamino)benzene-1,4-disulfonic acid sodium salt (18b)



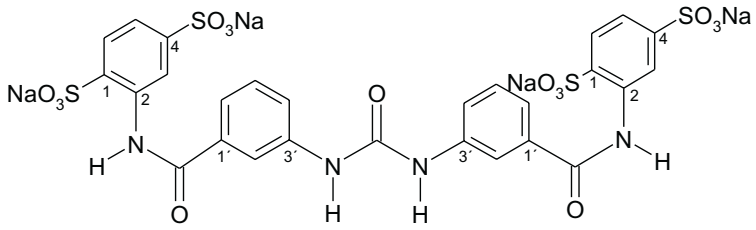

A suspension of **17b** (1.22 g, 2.7mmol) in 50 mL of $H_2O$ was brought to pH 8 by 2 M aqueous $Na_2CO_3$ solution. The mixture was hydrogenated at a hydrogen pressure of 1 bar with Pd/C (10 % Pd) as a catalyst. The reaction mixture was filtered, the filtrate was adjusted to pH 6 by 1 M HCl and the aqueous solution was dried to get light-yellow powder of **18b**.

| | | |
|---|---|---|
| Yield: | 1.6 g | (95 %) |
| $R_f$: | 0.74 | (S 7) |

$^1$H-NMR ( DMSO-$d_6$) $\delta$ [ppm]:
6.75 (m, 1H, C4'H); 7.04 (m, 1H, C6'H); 7.14 (m, 2H, C2'H, C5'H); 7.3 (m, 1H, C5H); 7.64 (d, J = 8.2 Hz, 1H, C6H); 8.77 (t, J = 1.58 Hz, 1H, C3H); 11.1 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
112.84 (C2'). 113.88 (C6'); 117.23 (C4'); 117.36 (C3); 119.66 (C5); 126.4 (C6); 129.2 (C5'); 134.99 (C1'); 135.45 (C2); 135.75 (C1); 149.26 (C3'); 149.55 (C4); 164.83 (CONH).

### 6.3.2. 54 2,2-(Carbonylbis(imino-1',3'-phenylene)carbonylimino)-bis-1,4- disulfonic acid sodium salt (19b)




A 20 % solution of phosgene in toluene (8 mL, 16 mmol $COCl_2$) was added dropwise over 1 h at r.t. to a vigorously stirred solution of 1.14 g (2.7 mmol) of **18b** in 100 mL of

$H_2O$. During the whole reaction time, the mixture was maintained at pH 3 by automatic addition of 2 M aqueous $Na_2CO_3$ solution. After all phosgene had been added and no more amine could be detected by TLC, stirring was continued for 30 min and the pH was adjusted to 6 by 1 M HCl. A light-yellow solids was obtained after the mixture was evaporated to dryness.

| | | |
|---|---|---|
| Yield: | 1.59 g | (68 %) |
| $R_f$: | 0.58 | (S 7) |

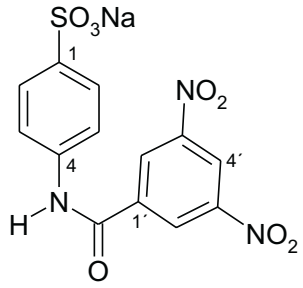
$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.32 (m, 1H, C5H); 7.45 (t, J = 7.88 Hz, 1H, C5'H); 7.52 (m, 1H, C6'H); 7.64 (d, J = 8.20 Hz, 1H, C6H); 7.84 (m, 2H, C4'H); 7.98 (t, J = 1.89 Hz, 1H, C2'H); 8.8 (t, J = 1.58 Hz, 1H, C3H); 9.7 (s, 1H, NHCONH); 11.3 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.36 (C2'); 117.46 (C3); 119.72 (C5); 119.98 (C6'); 121.43 (C4'); 126.5 (C6); 129.38 (C5'); 134.81 (C1'); 135.55 (C2); 123.59 (C1); 140.57 (C3'); 149.57 (C4); 152.84 (NHCONH); 164.02 (NHCO).

Determination of purity by CE:

| | |
|---|---|
| Buffer: | Phosphate 50 mM, pH 7.4 |
| Voltage: | 10 kV |
| Retention time: | 20.58 min |
| Purity: | 95.5 % |

### 6.3.2. 55 4-(3',5'-Dinitro-benzoylamino)-1-benzenesulfonic acid sodium salt (20)




To a solution of 1 g (5.78 mmol) sulfanilic acid in 20 mL of water, 2.66 g (11.5 mmol) of 3,5-dinitrobenzoyl chloride in 10 mL of toluene was added under vigorous stirring. During the whole reaction time, a pH of 4.5 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more sulfanilic acid could be detected by TLC. The aqueous phase was separated and washed with toluene until no more 3,5-dinitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 2.1 g | (92 %) |
| $R_f$: | 0.6 | (S 8) |

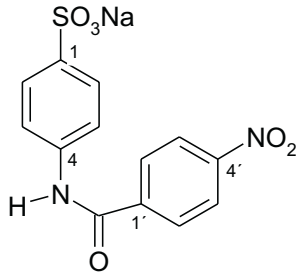
C,H,N analysis: $C_{13}H_8N_3Na_2O_8S \cdot 4\ H_2O$ [461.36 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 34.49 | 3.49 | 9.10 |
| Found: | 32.79 | 3.21 | 9.13 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.6 (d, J = 8.83 Hz, 2H, C3H, C5H); 7.75 (d, J = 8.83 Hz, 2H, C2H, C6H); 8.98 (t, J = 2.21 Hz, 1H, C4'H); 9.2 (d, J = 1,89 Hz, 2H, C2'H, C6'H); 10.9 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
119.94 (C3, C5); 121.27 (C4'); 126.33 (C2, C6); 128.26 (C2', C6'); 137.59 (C1'); 138.54 (C1); 144..55 (C4); 148.29 (C3', C5'); 161.54 (CONH).

### 6.3.2.56 4-(4'-Nitro-benzoylamino)-1-benzenesulfonic acid sodium salt (21a)




To a solution of 1 g (5.8 mmol) sulfanilic acid in 30 mL of water, 1.5 g (8.1 mmol) of 4-nitrobenzoyl chloride in 20 mL of toluene was added under vigorous stirring. During the whole reaction, a pH of 3 was controlled by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more sulfanilic acid was detectable by TLC. The aqueous phase was separated, and washed with toluene until no more 4-nitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 1.72 g | (87 %) |
| $R_f$: | 0.6 | (S 8) |

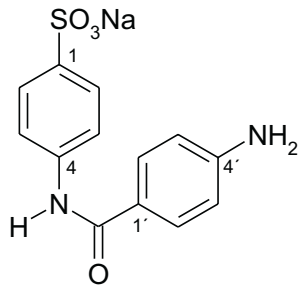
C,H,N analysis: $C_{13}H_9N_2NaO_6S \cdot 2\ H_2O$ [380.32 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 41.02 | 3.42 | 7.36 |
| Found: | 41.54 | 3.31 | 7.33 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.60 (d, J = 8.51 Hz, 2H, C3H, C5H); 7.72 (d, J = 8.82 Hz, C2H, C6H); 8.19 (d, J = 8.82 Hz; C2'H, C6'H); 8.34 (d, J = 8.82 Hz, C3'H, C5'H); 10.61 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
119.64 (C3, C5); 123.67 (C3', C5'); 126.27 (C2, C6); 129.41 (C2', C6'); 138.92 (C1'); 140.68 (C1); 144.23 (C4); 149.33 (C4'); 164.06 (CONH).

### 6.3.2. 57 4-(4'-Amino-benzoylamino)-1-benzenesulfonic acid sodium salt (22a)




A suspension of 4-(4'-nitro-benzoylamino)-1-benzenesulfonic acid sodium salt **21a** (0.8 g, 2.3 mmol) in 80 mL of $H_2O$ was adjusted to pH 8 by 2 M aqueous $Na_2CO_3$ solution. It was hydrogenated at a hydrogen pressure of 1 bar with Pd/C (10 % Pd) as a catalyst. The reaction mixture was filtered, and the filtrate was adjusted to pH 6 by 1 M HCl, then the aqueous solution was dried to yield **22a** as white powder .

| | | |
|---|---|---|
| Yield: | 0.5 g | (69 %) |
| $R_f$: | 0.62 | (S 7) |

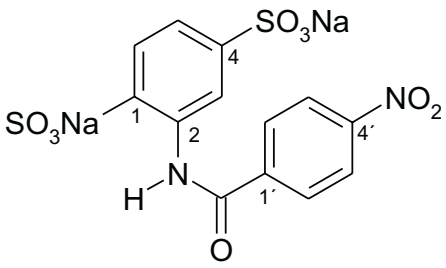
C,H,N analysis: $C_{13}H_{12}N_2NaO_4S \cdot 0.5\ H_2O$ [323.33 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 48.25 | 3.71 | 8.66 |
| Found: | 48.61 | 3.83 | 8.61 |

$^1$H-NMR ( DMSO-$d_6$) $\delta$ [ppm]:
5.72 (s, 2H, $NH_2$); 6.59 (d, J = 8.51 Hz, 2H, C3'H, C5'H ); 7.52 (d, J = 8.51 Hz, 2H, C2'H, C6'H); 7.68 (d, J = 8.51 Hz, 2H, C3H, C5H); 7.72 (d, J = 8.82 Hz, C2H, C6H); 9.78 (s, 1H, NHCO).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
112.71 (C3', C5'); 119.1 (C3, C5); 121.14 (C1'); 126.0 (C2, C6); 129.51 (C2', C6'); 139.97 (C1); 143.08 (C4); 152.31 (C4'); 165.41 (CONH).

### 6.3.2. 58 2-(4'-Nitro-benzoylamino)benzene-1,4-disulfonic acid sodium salt (21b)




To a solution of 1 g (3.6 mmol) of 2-amino-1,4-benzene disulfonic acid monosodium salt in 20 mL of water, 0.5 g (4.3 mmol) of 4-nitrobenzoylchloride in 10 mL of toluene was added under vigorous stirring. During the whole reaction time, a pH of 3 was maintained by the manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more 2-amino-1,4-benzenedisulfonic acid monosodium salt was detectable by TLC. The aqueous phase was separated, and washed with toluene until no more 4-nitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 0.99 g | (61 %) |
| $R_f$: | 0.56 | (S 8) |

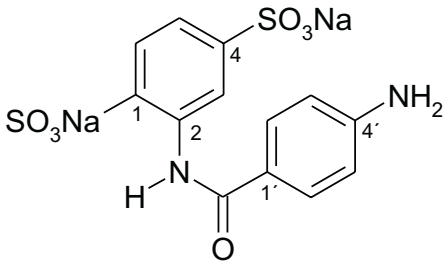
C,H,N analysis: $C_{13}H_8N_2Na_2O_9S_2 \cdot 3\ H_2O$ [500.39 g/mol]

| | C % | H % | N % |
|---|---|---|---|
| Calcd.: | 31.17 | 2.80 | 5.60 |
| Found: | 31.03 | 2.77 | 5.60 |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.38 (m, 1H, C5H); 7.69 (d, J = 8.20 Hz, 1H, C6H); 8.26 (d, J = 9.14 Hz, 2H, C2'H, C6'H); 8.42 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.78 (t, J = 1.58 Hz, 1H, C3H); 11.63 (s, 1H, CONH).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.59 (C3); 120.68 (C5); 124.33 (C3', C5'); 126.64 (C6); 128.65 (C2', C6'); 134.25 (C2); 135.82 (C1); 140.39 (C1'); 149.62 (C4); 149.77 (C4'); 162.36 (CONH).

### 6.3.2. 59 2-(4'-Aminobenzoylamino)benzene-1,4-disulfonic acid sodium salt (22b)



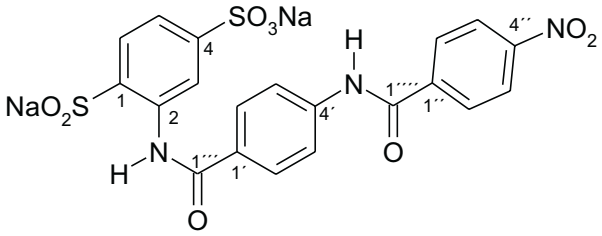

A suspension of 2.82 g (6.3 mmol) 2-(4'-nitro-benzoylamino)benzene-1,4-disulfonic acid sodium salt **21b** in 50 mL of $H_2O$ was adjusted to pH 8 by 2 M aqueous $Na_2CO_3$ solution. It was hydrogenated at a hydrogen pressure of 1 bar with Pd/C (10 % Pd) as a catalyst. The reaction mixture was filtered, and the filtrate was adjusted to pH 6 by 1 M HCl, then the aqueous solution was dried to get a light-yellow powder.

| | | |
|---|---|---|
| Yield: | 2.6 g | (98 %) |
| $R_f$: | 0.54 | (S 7) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
5,81 (s, 2H, $NH_2$); 6.61 (d, J = 8.82 Hz, 2H, C3'H, C5'H); 7.25 (m, 1H, C5H); 7.63 (m, 3H, C6H, C2'H, C6'H); 8.79 (t, J = 1.58 Hz, 1H, C3H); 11.01 (s, 1H, CONH).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
113.04 (C3', C5'); 117.16 (C3); 119.13 (C5); 121.18 (C1'); 126.45 (C6); 128.96 (C2', C6'); 134.96 (C2); 135.57 (C1); 149.52 (C4); 152.57 (C4'); 164.22 (CONH).

### 6.3.2. 60 2-(4'-(4"-Nitrobenzoylamino)benzoylamino)benzene-1,4-disulfonic acid sodium salt (23b)



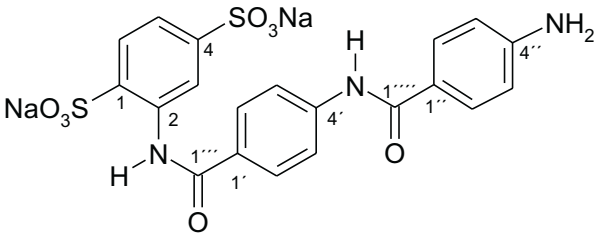

To a solution of 1.05 g (2.52 mmol) 2-(4'-amino-benzoylamino)-benzene-1,4-disulfonic acid sodium salt **22b** in 20 mL of $H_2O$, 0.9 g (4.8 mmol) of 4-nitrobenzoyl chloride in 10 mL of toluene was added under vigorous stirring. During the whole reaction, a pH of 3 was maintained by manual addition of 2 M aqueous $Na_2CO_3$ solution. The reaction was continued until no more **22b** could be detected by TLC. The aqueous phase was separated, and washed with toluene until no more 4-nitrobenzoic acid could be detected by TLC. The water phase was evaporated *in vacuo* to dryness and the residue was recrystallized from water : ethanol (1 : 1).

| | | |
|---|---|---|
| Yield: | 1.14 g | (80 %) |
| $R_f$: | 0.72 | (S 7) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
7.38 (m, 1H, C5H); 7.69 ( d, J = 8.20 Hz, 1H, C6H); 7.95 ( d, J = 8.82 Hz, 2H, C3'H, C5'H); 8.00 (d, J = 8.82 Hz, 2H, C2'H, C6'H); 8.26 (d, J = 8.82 Hz, 2H, C2"H, C6"H); 8.36 (d, J = 8.82 Hz, 2H, C3"H, C5"H); 8.78 (t, J = 1.58 Hz, 1H, C3H); 10.96 (s, 1H, NHCO); 11.37 (s, 1H, NHCO-arom-$NO_2$).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
117.32 (C3); 119.86 (C5); 120.21 (C3'); 123.67 (C3"); 126.49 (C6); 127.98 (C2'); 129.62 (C2"); 130.1(C1'); 134.87 (C2); 135.37 (C1); 140.44 (C1"); 142.18 (C4'); 149.44 (C4); 149.71 (C4"); 163.46 (C1""); 164.45 (C1"').

### 6.3.2. 61 2-(4'-(4"-Aminobenzoylamino)benzoylamino)benzene-1,4-disulfonic acid sodium salt (24b)




A suspension of 1 g (1.77 mmol) of 2-(4-(4-nitrobenzoylamino)-benzoylamino)benzene-1,4-disulfonic acid sodium salt **23b** in 50 mL of $H_2O$ was adjusted to pH 8 by 2 M aqueous $Na_2CO_3$ solution. It was hydrogenated at a hydrogen pressure of 1 bar with Pd/C (10 % Pd) as a catalyst. The reaction mixture was filtered, the filtrate was adjusted to pH 6 by 1 M HCl, then the aqueous solution was dried to yield **24b**.

| | | |
|---|---|---|
| Yield: | 0.9 g | (95 %) |
| $R_f$: | 0.59 | (S 7) |

$^1$H-NMR (DMSO-$d_6$) $\delta$ [ppm]:
5.81 (s, 2H, $NH_2$); 6.61 (d, J = 8.82 Hz, 2H, C3"H, C5"H); 7.66 (d, J = 7.88 Hz, 2H, C6H); 7.76 (d, J = 8.82 Hz, 2H, C2"H, C6"H); 7.89 (d, J = 8.83 Hz, 2H, C3'H, C5'H); 7.96 (d, J = 8.82 Hz, 2H, C2'H, C6'H); 8.81 (t, J = 1.58 Hz, 1H, C3H);10.96 (s, 1H, NHCO); 11.37 (s, 1H, NHCO-arom-$NO_2$).

$^{13}$C-NMR (DMSO-$d_6$) $\delta$ [ppm]:
112.73 (C3", C5"); 117.3 (C3); 119.61 (C3', C5'); 119.73 (C5); 120.79 (C1"); 126.48 (C6); 127.82 (C2', C6'); 128.73 (C1'); 129.75 (C2", C6"); 135.0 (C2); 135.33 (C1); 143.40 (C4'); 149.65 (C4); 152.61 (C4"); 163.46 (C1""); 164.45 (C1"').

# Chapter 7

# Summary

Synthesis, stability tests and pharmacological tests of the sulfonate derivatives of P1 (adenosine) receptor antagonists and P2 receptor antagonists were successfully performed.

**P1 (adenosine) receptor antagonists**

- *m*-Nitrophenyl sulfonate esters of *p*-sulfophenylxanthine derivatives were designed and synthesized. It was proven that the modified synthetic route was a very convenient way for the synthesis of this kind of xanthine sulfonate esters, since the yield of the ring closure, which normally is the most difficult step during the synthesis of xanthine derivatives, reached more than 50 % on average. The purification of the synthesized sulfonate esters could be easily performed by recrystallization with DMF/water.

- A new synthetic strategy which is suitable for the preparation of gram amounts of 1-substituted-8-*p*-sulfophenylxanthine derivatives was developed. That is, hydrolysis of the 1-substituted-8-[4'-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine to produce the corresponding 1-substituted-8-*p*-sulfophenylxanthine derivatives, yields of the isolated hydrolysis products reached ca. 80 %. Altogether more than 8 g of 1-propyl-8-sulfophenylxanthine were synthesized by using the new strategy.

- 8-*p*-Sulfonamidophenylxanthine derivatives were also synthesized by using *p*-nitrophenyl as a new leaving group for the aminolysis, which has never been reported before. Altogether 12 differently N-substituted 8-*p*-sulfonamidophenylxanthine derivatives were synthesized, these amines ranging from relatively reactive primary amines to unreactive secondary amines. It was proven that the newly developed method is a very efficient and practical synthetic method, since the synthetic route is easy and the yields are satisfying.

- *In vitro* stability tests of these *m*-nitrophenyl sulfonate esters of *p*-sulfophenylxanthine derivatives were performed in chemical and biological media. It was concluded

that these synthesized xanthine *m*-nitrophenyl sulfonate esters are relatively stable, since the half-lives of these esters in 0.001 M NaOH at 37 °C reach 20 h on average. In the biological *in vitro* test, the 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** did not hydrolyze in simulated gastric acid within 4 hours of incubation at 37 °C. That means it can resist to acidic media before it reaches the blood stream across the gastrointestinal wall. It remains as an intact ester when it reaches in the blood stream since the half-life of it in fetal calf serum reaches 16.4 h. At last it would be hydrolyzed in the liver through enzymatic biotransformation to the parent *m*-nitrophenol and 8-*p*-sulfophenylxanthine drug, the half-life in rat liver homogenate being about 41 min.

- All of the synthesized sulfonate esters and sulfonamidoxanthine derivatives were tested in radioligand binding assays for their affinity to $A_1$, $A_{2A}$ and $A_3$ adenosine receptor. It was proven that compared to the 8-*p*-sulfophenylxanthine, all the sulfonate esters and sulfonamide derivatives are more potent at $A_1$ and $A_{2A}$ adenosine receptors, but the selectivity $A_1/A_{2A}$ of these sulfonate xanthine derivatives is not high, ranging from 3-fold (**5e**) to 30-fold (**9e**). Most of the compounds are not active at $A_3$ ARs. Two compounds (**5d**, **9d**) have been tested at $A_{2B}$ ARs, and it was proven that they possess high $A_{2B}$ affinity.

**P2 receptor antagonists**

- New XAMR and NF023 analogues using sulfanilic acid and 2-amino-1,4-benzenedisulfonic acid monosodium salt as the starting materials were synthesized. The *m*-nitrophenyl sulfonate esters of the XAMR analogues and some NF023 analogues were subsequently synthesized by chlorination and esterification of the sulfonic acid derivatives.

- The inhibitory potency of some synthesized potential P2 receptor antagonists was tested at $P2Y_2$ receptor of NG108-15 cells. Compound 4-benzoylamino-benzenesulfonic acid *m*-nitrophenyl ester **14a** has an $IC_{50}$ of 8.6 $\mu M$. The other sulfonic acids and sulfonate esters were virtually inactive at $P2Y_2$ receptors.

- Like the pharmacological properties of the P1 antagonists, the sulfonate esters of XAMR and NF023 analogues show a higher antagonistic affinity as the corresponding sulfonic acids. That means that with the increase in lipophilicity of the sulfonate derivatives, the antagonistic activity of these derivatives increased as well.

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# Abbreviations

| | |
|---|---|
| AC | Adenylate cyclase |
| ADA | Adenosine deaminase |
| ADP | Adenosine diphosphate |
| ADP$\beta^{35}$S | [$^{35}S$]Adenosine 5'-O-2-thiodiphosphate |
| AMP | Adenosine monophosphate |
| ARs | Adenosine receptors |
| ATP | Adenosine triphosphate |
| CADO | 2-Chloroadenosine |
| cAMP | Cyclic adenosine monophosphate |
| [$^3H$]CCPA | [$^3H$]2-Chlor-N$^6$-cyclopentyladenosine |
| CE | Capillary electrophoresis |
| [$^3H$]CHA | [$^3H$]N$^6$-Cyclohexyladenosine |
| CHO | Chinese hamster ovary |
| CNS | Central nervous system |
| COSY | Correlation spectroscopy |
| DEPT | Distortionless enhancement of polarization transfer |
| DIDS | 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid |
| DMF | N,N-Dimethylformamide |
| DMSO | Dimethylsulfoxide |
| DPBS | Dulbeccco's phosphate buffered saline |
| DPCPX | 1,3-Dipropyl-8-Cyclopentylxanthine |
| EDC | N-(Dimethylaminopropyl)-N'-ethylcarbodiimide-HCl |
| EDTA | Ethylenediamine tetraacetic acid |
| HMDS | 1,1,1,3,3,3-Hexamethyldisilazane |
| HMQC | Heteronuclear multiple quantum correlation |
| HPLC | High pressure liquid chromatography |
| IU | International unit |
| KN-62 | 1-N,O-bis-1,5-isoquinolinesulfonyl-N-methyl-L-tyrosly-4-phenylpiperazine |
| MRS 2179 | 2'-Deoxy-N$^6$-methyladenosine-3',5'-bisphosphate |
| MRS 2220 | Cyclic pyridoxine-4,5-monophosphate-6-azophenyl-2',5'-disulfonic acid |

| | |
|---|---|
| [$^3H$]MSX-2 | [$^3H$]3-((3-Hydroxypropyl)-8-*m*-methoxystyryl)-7-methyl-1-propargyl xanthine |
| NECA | N-Ethylcarboxamidoadenosine |
| NF023 | 8,8'-(Carbonylbis(imino-3,1-phenylene))bis-1,3,5-naphthalene trisulfonic acid |
| NMR | Nuclear magnetic resonance |
| PBS-buffer | Phosphate buffered saline buffer |
| PEI | Polyethylenimine |
| [$^3H$]PIA | [$^3H$]-N$^6$-Phenylisopropyladenosine |
| PPADS | pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid |
| PPSE | Polyphosphoric acid trimetylsilyl ester |
| PSB-1115 | 1-Propyl-8-p-sulfophenylxanthine |
| [$^3H$]PSB-11 | [$^3H$]2-(2',3',5'-trichlorophenyl)8-ethyl-4-methyl-(8R)-4,5,7,8-tetrahydro -*1H*-imidazo-[2,1.i]purin-5-one |
| r.t. | Room temperature |
| SDS | Sodium dodecylsulfate |
| SEM | Standard error of the mean |
| Suramin | 8-(3-Benzamido-4-methylbenzamido)naphthalene-1,3,5-trisulfonic acid |
| TEA | Triethylamine |
| TFA | Trifluoroacetic acid |
| THF | Tetrahydrofuran |
| TLC | Thin layer chromatography |
| TMS | Tetramethylsilane |
| TNP-ATP | 2',3'-O-(2,4,6-Trinitrophenyl)adenosine triphosphate |
| TRIS | Tris(hydroxymethyl)aminomethane |
| UDP | Uridin-5'-diphosphate |
| UMP | Uridin-5'-monophosphate |
| UTP | Uridin-5'-triphosphate |
| Uracil | 1,2,3,4-Tetrahydro-2,4-pyrimidine |
| UV | Ultraviolet |
| XAMR0716 | 8-(Phenylenecarbonylimino)-1,3,5-naphthalene trisulfonic acid sodium salt |
| XAMR0721 | 8-(3,5-Dinitrophenylenecarbonylimino)-1,3,5-naphthalene trisulfonic acid sodium salt |
| Xanthine | 2,3,6,7-Tetrahydro-2,6(*1H*,*3H*)-purindione |
| [$^3H$]ZM214385 | [$^3H$]4-(2-((7-Amino-2-(furyl)1,2,4-triazolo[2,3-a]-1,3,5-triazin-5-yl)amino)- -ethyl)phenol |

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