

# Structure-activity relationships of anthraquinone derivatives derived from bromaminic acid as inhibitors of ectonucleoside triphosphate diphosphohydrolases (E-NTPDases)

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**Abstract** Reactive blue 2 (RB-2) had been characterized as a relatively potent ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) inhibitor with some selectivity for NTPDase3. In search for the pharmacophore and to analyze structure-activity relationships we synthesized a series of truncated derivatives and analogs of RB-2, including 1-amino-2-sulfo-4-*ar*(alk)ylaminoanthraquinones, 1-amino-2-methyl-4-arylaminanthraquinones, 1-amino-4-bromoanthraquinone 2-sulfonic acid esters and sulfonamides, and bis-(1-amino-4-bromoanthraquinone) sulfonamides, and investigated them in preparations of rat NTPDase1, 2, and 3 using a capillary electrophoresis assay. Several 1-amino-2-sulfo-4-*ar*(alk)ylaminanthraquinone derivatives inhibited E-NTPDases in a concentration-dependent manner. The 2-sulfonate group was found to be required for inhibitory activity, since 2-methyl-substituted derivatives were inactive. 1-Amino-2-sulfo-4-*p*-chloroanilinoanthraquinone (**18**) was identified as a nonselective competitive blocker of NTPDases1, 2, and 3 ( $K_i$  16–18  $\mu$ M), while 1-amino-2-sulfo-4-(2-naphthylamino)anthraquinone (**21**) was a potent inhibitor with preference for NTPDase1 ( $K_i$  0.328  $\mu$ M) and NTPDase3 ( $K_i$  2.22  $\mu$ M). Its isomer, 1-amino-2-sulfo-4-(1-naphthylamino)anthraquinone (**20**), was a potent and selective inhibitor of rat NTPDase3 ( $K_i$  1.5  $\mu$ M).

**Keywords** Anthraquinone · E-NTPDase · Inhibitor · Reactive blue 2 · Synthesis

## Abbreviations

|             |  |
|-------------|--|
| ARL 67156   | $N^6$ -diethyl- $\beta,\gamma$ -dibromomethylene-ATP         |
| CE          | capillary electrophoresis                                    |
| DMSO        | dimethyl sulfoxide   |
| EI          | electron impact  |
| ESI         | electrospray ionization                                      |
| HEPES       | $N$ -[2-hydroxyethyl]piperazine- $N'$ -2-ethanesulfonic acid |
| HPLC        | high-performance liquid chromatography                       |
| HRMS        | high-resolution mass spectrometry                            |
| NMR         | nuclear magnetic resonance                                   |
| (E)-NTPDase | (ecto)-nucleoside 5'-triphosphate diphosphohydrolase         |
| PPADS       | pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid        |
| RB-2        | reactive blue 2  |
| SAR(s)      | structure-activity relationship(s)                           |

## Introduction

Extracellular nucleotides such as ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP), and uridine diphosphate (UDP) act as extracellular signaling molecules in virtually all tissues and organs [1]. Especially ATP is involved in a variety of physiological and pathological functions in the body, e.g., as a neurotransmitter and neuro-modulator in the central and peripheral nervous system, as a key messenger in nociception, as a tumor-inhibiting agent, in the control of secretion from a variety of endocrine glands, in the modulation of platelet aggregation by ADP, in chloride

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transport in the airway epithelia, in renal function, in bone and cartilage disease, and as a proinflammatory agent in the immune system [2, 3]. Extracellular nucleotides are efficiently inactivated by surface-located enzymes which sequentially hydrolyze the phosphate groups eventually resulting in the formation of the respective nucleoside and inorganic phosphate [4]. Nucleoside 5'-triphosphates and 5'-diphosphates may be hydrolyzed by members of the E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family, the E-NPP (ectonucleotide pyrophosphatase/phosphodiesterase) family, and by alkaline phosphatases. Nucleoside 5'-monophosphates are subject to hydrolysis by ecto-5'-nucleotidase and also by alkaline phosphatases [5]. Members of the various ectonucleotidase families reveal overlapping substrate specificity and tissue distribution whose functional significance needs to be further elucidated [4].

E-NTPDases comprise eight members, four of which have two plasma membrane-spanning domains with an active site facing the extracellular milieu [4–6], namely, NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), and NTPDase8 [7, 8]. In contrast, NTPDase4, 5, 6, and 7 are intracellular membrane proteins. Even though NTPDases 5 and 6 may be present at the surface of the plasma membrane and secreted as soluble enzymes following a proteolytic cleavage, their high  $K_m$  values and low specific activities [9] make it unlikely that these enzymes regulate P2 receptor signaling. NTPDases share five highly conserved sequence domains (apyrase conserved regions) that are presumably of major relevance for their catalytic activity [10]. They contain the actin-HSP 70-hexokinase  $\beta$ - and  $\gamma$ -phosphate binding motif [11, 12].

Members of the E-NTPDase family can hydrolyze nucleoside 5'-triphosphates and nucleoside 5'-diphosphates albeit with varying preference for the individual type of nucleotide [10, 11]. NTPDase1 (CD39, ectoapyrase, ecto-ATP diphosphohydrolase) hydrolyzes ATP and ADP at a molecular ratio of about 1:0.5 to 1:0.9 [13]. In contrast, NTPDase2 has a strong preference for ATP with molecular ratios of ATP:ADP of 1:0.03 or less. NTPDase3 is a functional intermediate and reveals a molecular ratio of ATP:ADP of approximately 1:0.3. The activity of all three types of ectonucleotidases depends on millimolar concentrations of divalent cations such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  [11, 13, 14]. The enzymes hydrolyze not only ATP or ADP but have broad substrate specificity towards purine and pyrimidine nucleotides.

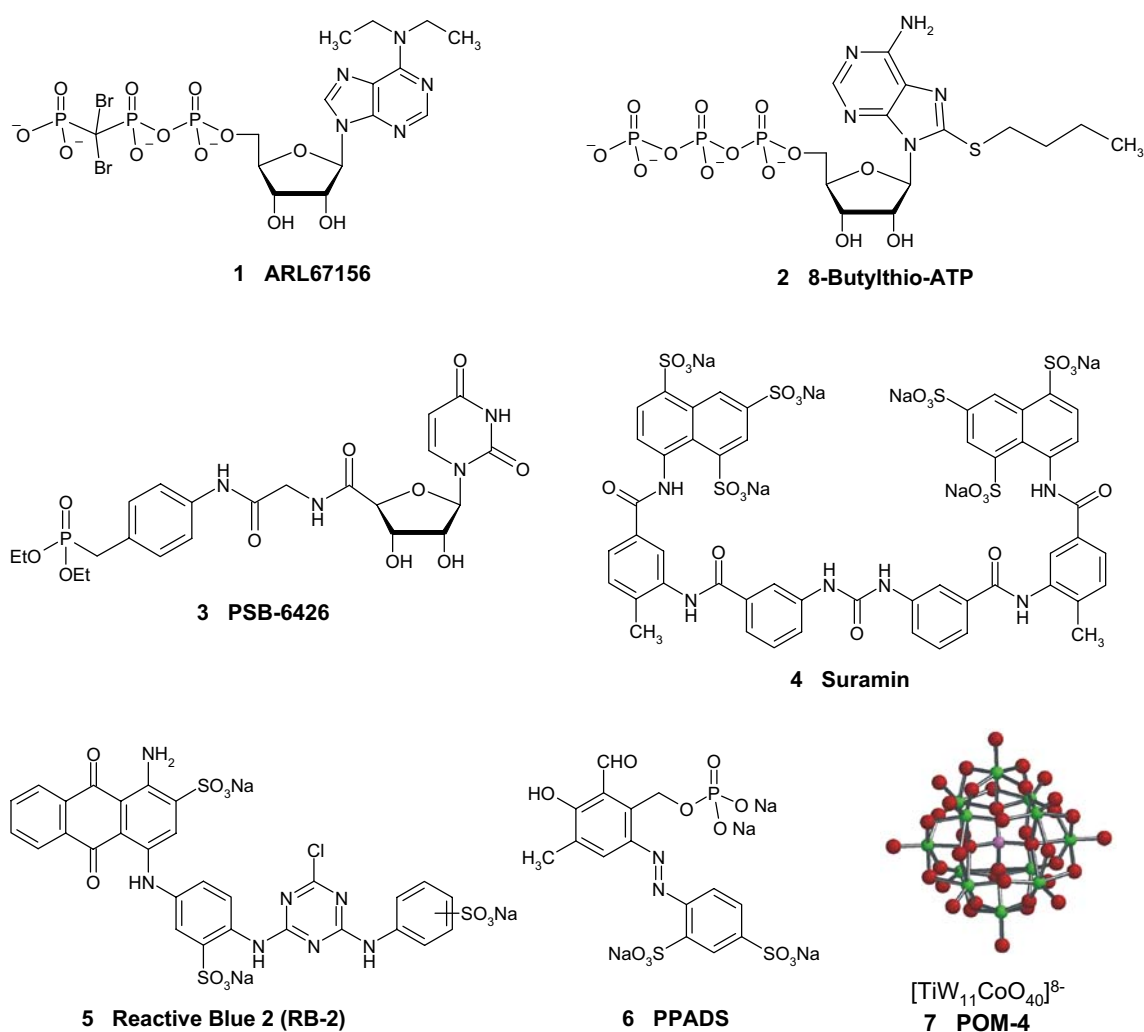
The analysis of nucleotide release requires the availability of inhibitors of ectonucleotidases that should ideally have no effect on P2 receptor activation [6]. It has been demonstrated that stable analogs of ATP can elicit tissue contractions up to 100 times more effectively than ATP. This suggests that the effects of exogenously applied ATP on P2 receptors are limited by its enzymatic degradation. Inhibitors of ectonucleotidases could thus serve as drugs that increase the life-

time of extracellular ATP (or ADP, UTP, or UDP) in situ. They could act in a site- and event-specific manner since they would only have an effect when nucleotides are present.

NTPDase1 is the major physiological E-NTPDase member showing a nearly ubiquitous tissue distribution. It is constitutively expressed in microvascular endothelium and certain immune cells and plays an important role in thromboregulation and immune function [15]. The distribution of NTPDase2 appears to be more restricted. NTPDase2 has been detected on blood vessels [16], cultured brain astrocytes [17], and on certain neuronal progenitor [18] and cancer cells [19, 20]. In contrast, NTPDase3 is expressed in the brain exclusively in neurons, with a predominantly axonal localization. Its colocalization with hypocretin-1/orexin-A indicates that it may be involved in the modulation of feeding, the sleep-wake cycle, or other behavioral components [21]. NTPDase3 has also been reported to be involved in auditory neurotransmission since it is highly expressed in the cochlea [22].

Potent and selective inhibitors for the individual NTPDase isoenzymes are required as pharmacological tools to investigate the (patho)physiological roles of NTPDases. Furthermore, such compounds are required to study their potential as novel drugs, e.g., for the treatment of cancer, as immunomodulatory agents, and for the treatment of cardiovascular or central nervous system disorders. So far only few classes of NTPDase inhibitors have been described [23] (Fig. 1).

These include nucleotide derivatives and analogs, mainly derived from ATP. Until recently, the only compound that has been shown to effectively inhibit the hydrolysis of ATP in a variety of tissues (albeit with moderate potency) without significantly acting on P2 receptors was the structural analog of ATP, ARL 67156 (**1**, FPL 67156) ( $N^6$ -diethyl- $\beta$ ,  $\gamma$ -dibromomethylene-ATP) [24–26]. However, ARL 67156 was recently found to be nearly inactive at rat, mouse, and human NTPDase2 [27, 28]. Although **1** is stable versus ecto-alkaline phosphatases and E-NTPDases, it might be hydrolyzed by E-NPPs, which directly attack the oxygen bridge between the  $\alpha$ - and  $\beta$ -phosphorus atom of ATP and related compounds. However, Sévigny and colleagues showed that human NPP1 and NPP3 were unable to hydrolyze ARL 67156 [28]. 8-Thioether-ATP derivatives, found inactive at P2Y and P2X receptors, and stable to NTPDase hydrolysis, have also been described as NTPDase inhibitors. 8-Butylthioadenosine-5'-triphosphate (compound **2**) acts as a competitive inhibitor of ATP with an estimated  $K_i$  value of 10  $\mu\text{M}$  at NTPDase from bovine spleen [29]. Although the compound appears to be stable against NTPDases, it is likely to be hydrolyzed by the ubiquitously present ectoalkaline phosphatases which accept a very broad variety of substrates. Very recently, we have developed metabolically stable, uncharged nucleotide mimetics derived



**Fig. 1** Structures of NTPDase inhibitors

from uridine 5'-carboxylate and identified PSB-6426 (**3**) as a selective inhibitor of human NTPDase2 [30].

Besides inhibitors derived from nucleotides, non-nucleotide NTPDase inhibitors have been described. These include suramin (**4**) and related compounds [13, 31–33], sulfonate dyes such as reactive blue 2 (RB-2, **5**) [34–37], and PPADS (**6**, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid) [13, 38, 39], which can all attenuate the hydrolysis of ATP. However, all of these compounds are nonselective, as they are also potent antagonists at P2 receptors. We recently identified a novel class of NTPDase inhibitors, the polyoxometalates (POMs), which are inorganic anionic metal complexes, e.g., POM-4 (**7**) [40]. These compounds have turned out to be useful pharmacological tools [41–43]. However, they have high molecular weights, bear several negative charges, and are not drug-like molecules.

The present study was aimed at identifying the pharmacophore of RB-2 for inhibiting NTPDases and at investigating the structure-activity relationships of truncated RB-2

derivatives. Since RB-2 shows some selectivity for rat NTPDase3 ( $K_i$  1.10  $\mu\text{M}$ , approximately 20-fold selectivity vs NTPDase1 and 2) [27, 44], we expected that this strategy might lead to the identification of an NTPDase3-selective inhibitor.

## Results and discussion

### Syntheses

The 1-amino-4-anilino-2-sulfoanthraquinone derivatives **11–19** were synthesized as recently described by combinatorial parallel synthesis using the Ullmann coupling reaction [45]. The new compounds **20–22** were obtained by two different methods. The 1-naphthyl (**20**) and the 3,4-dimethoxyphenyl derivative (**22**) were synthesized by the classic Ullmann reaction from bromaminic acid (**8**) and 1-naphthylamine, or 3,4-dimethoxyphenethylamine, respec-

tively, in water in the presence of potassium carbonate and copper sulfate at 100–105°C (see Fig. 2). The 2-naphthyl derivative (**21**) was obtained by a new microwave-catalyzed Ullmann reaction in phosphate buffer using elemental copper as a catalyst [46] within only 5 min reaction time (Fig. 2). The 1-amino-2-methyl-4-arylaminoanthraquinone derivatives **23–26** were obtained by solvent-free reaction of 1-amino-2-methyl-4-bromoanthraquinone (**9**) with the appropriate arylamine derivative in the presence of copper acetate and potassium acetate at 110°C for 5–24 h according to a procedure described by Harris et al. [47] (see Fig. 2).

Anthraquinone sulfonic acid esters **27–30** and sulfonamides (**31–33**) were obtained by chlorination of bromaminic acid (**8**) with phosphorus pentachloride in phosphorus oxychloride to yield the sulfonic acid chloride **10** as a reactive intermediate, which was subsequently treated with the appropriate alcohol or phenol, or ar(alkyl) amine under basic conditions (Fig. 3). When *p*-ethoxy- or *o*-methoxy-substituted aniline was used for the condensation with **10**, the desired simple sulfonamide derivatives were not obtained. Mass spectral and nuclear magnetic resonance (NMR) analysis revealed that bissulfonamides **34** and **35** had formed instead, although the anilines had been present in the reaction mixtures in excess. The explanation may be an increased nucleophilicity of the aniline nitrogen atom due to the positive mesomeric effects of the substituents (*o*-methoxy, *p*-ethoxy).

#### Solubility and stability of selected anthraquinone derivatives

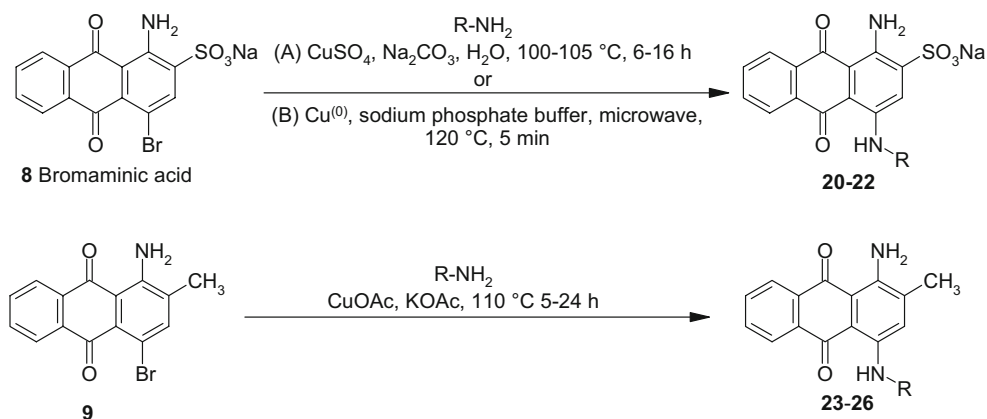
It was observed that some anthraquinone sulfonic acid sodium salts, which were initially well soluble in water, formed precipitates during the testing. One explanation may be that the high salt concentrations in the buffer solutions, e.g., the presence of calcium ions, which may form hardly soluble calcium sulfonates, reduced their solubility. Howev-

er, precipitation might also be due to another chemical reaction. Therefore, selected 1-amino-4-phenylamino-2-sulfoanthraquinone derivatives (**12**, **15**, **17**, **18**) bearing different substituents on the phenyl ring (*o*-methyl, *o*, *p*-dimethyl, *o*-ethoxy, and *p*-chloro residues) were investigated for their stability under the test conditions. Aqueous solutions (10 mM) were prepared and diluted with the *N*-[2-hydroxyethyl]piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer that was used in the assays, to obtain a compound concentration of 3 mM. After a short period of time after dilution, compound **18** precipitated, while the other compounds stayed in solution. After 15 h at room temperature, compound **17** also started to precipitate, while **12** precipitated after 48 h and **15** after about 1 week. The precipitated compounds were collected by filtration, dried, and NMR spectra were recorded showing that the precipitated compounds were structurally identical to the original anthraquinone derivatives. This result indicates that the compounds were not degraded, but precipitated upon dilution with buffer solutions due to the lower solubility in the presence of high salt concentrations probably due to the formation of poorly soluble salts of the anthraquinone sulfonic acids with cations in the buffer (HEPES and/or calcium). Since precipitation in most cases only occurred after several hours, testing of high concentrations was always performed with freshly prepared solutions.

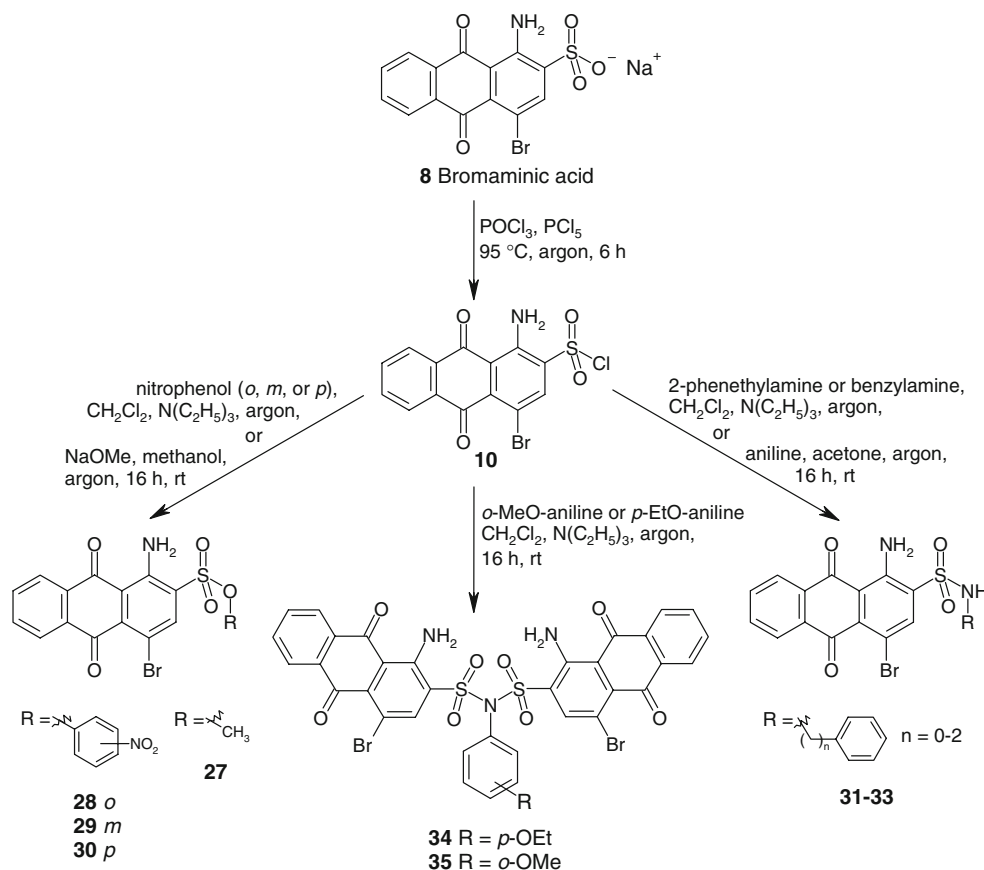
Investigation of anthraquinone derivatives as potential inhibitors of nucleoside triphosphate diphosphohydrolases by capillary electrophoresis

Inhibition of rat NTPDases1, 2, and 3 was performed essentially as previously described using a capillary electrophoresis (CE) method [27]. Membrane preparations derived from transfected cells containing rat NTPDase1, NTPDase2, or NTPDase3 were employed. A fixed substrate concentration of 400 μM was used. Test compounds were initially screened at a concentration of 1 mM. For compounds which showed about 50% inhibition or more, IC<sub>50</sub> values were

**Fig. 2** Synthesis of aryl- and arylalkylaminoanthraquinone derivatives **20–22** and **23–26** (for R see Table 1)



**Fig. 3** Synthesis of bromaminic acid esters **27–30**, bromaminic acid amides **31–33**, and bis(bromaminic acid) amides **34** and **35**



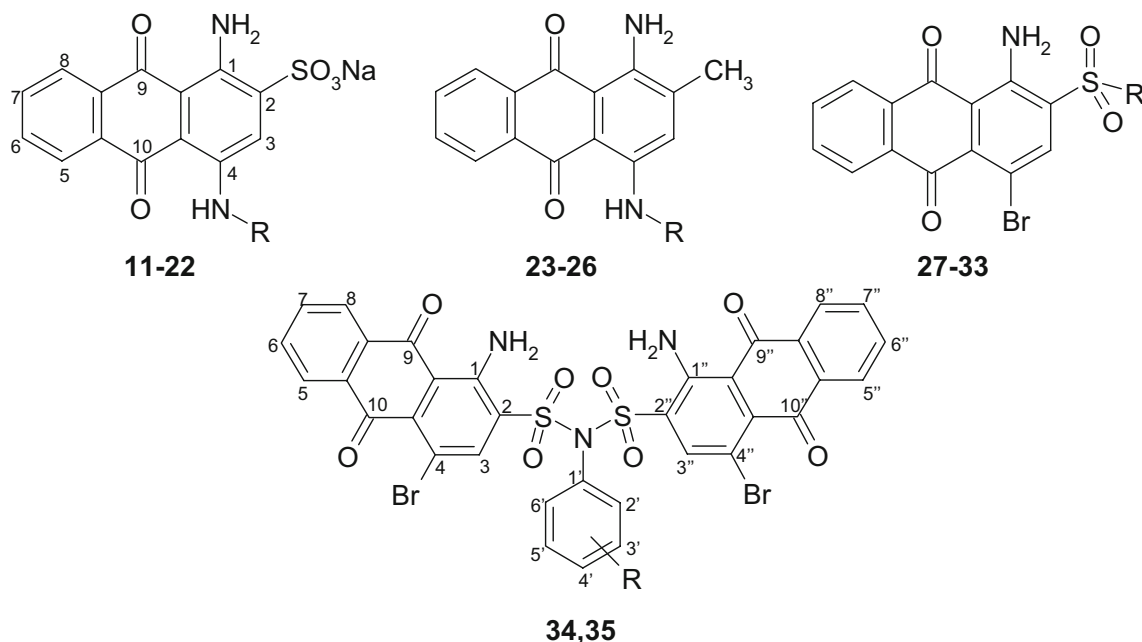
determined with six to eight different concentrations of inhibitor spanning about three orders of magnitude. After stopping the reaction by heating, an aliquot was subjected to CE analysis with UV detection at 210 nm. Under the applied conditions less than 10% of substrate was converted by the enzymes.  $K_i$  values were calculated from  $IC_{50}$  values as previously described assuming a competitive inhibition mechanism [27].

#### Structure-activity relationships

Even though the investigated 1-amino-2-sulfo-4-*ar*(alk)-ylaminoanthraquinone derivatives exhibited only a partial structure of RB-2 and were much smaller than the parent compound, many of them were similarly or even more active as NTPDase inhibitors (Table 1). For example, compound **18** bearing a *p*-chlorophenylamino residue was a potent, nonselective NTPDase inhibitor ( $K_i$  16–18  $\mu$ M). While NTPDase2 and 3 were quite tolerant with regard to the substituent in the 4-position accepting a large variety of differentially substituted arylamino residues and even a 3,4-dimethoxyphenethylamino residue, structure-activity relationships (SARs) of NTPDase1 were more restricted: phenylamino (**11**), *m*-methylphenylamino (**13**), and particularly *o,p*-dimethylphenylamino (**15**) and *p*-chlorophenylamino (**18**) were best tolerated, while monosubstitution in

the ortho-position of the phenylamino residue (**12**, **16**, **17**) or replacement of the phenyl ring by 1-naphthyl (**20**) or a 3,4-dimethoxyphenethyl residue (**22**) were not well tolerated by rat NTPDase1. On the other hand, a 2-naphthyl ring was very well tolerated by NTPDase1: compound **21** was the most potent NTPDase1 inhibitor of the present series with a  $K_i$  value of 0.328  $\mu$ M; it was less potent at NTPDase3 ( $K_i$  2.22  $\mu$ M) and much less potent at NTPDase2 (19.1  $\mu$ M). Small *p*-substituents on the phenyl ring (*p*-chloro, *p*-methyl) were well accepted by NTPDase1, while an acetylamino residue in the *p*-position was detrimental. Compound **20** was the most potent and selective inhibitor of rat NTPDase3 and not inhibitory to rat NTPDase1 and 2 even at high, millimolar concentrations. Rat NTPDase3 appears to have a lipophilic pocket which can accommodate a 1-naphthylamino residue in the 4-position. Concentration-inhibition curves of the most potent nonselective NTPDase inhibitor **18** (PSB-069) at the three investigated NTPDases and of the NTPDase3-selective inhibitor **20** (PSB-06126) are shown in Fig. 4.

An unsubstituted sulfonate group in the 2-position of the anthraquinone scaffold was essential for inhibitory activity. Replacement of the sulfonate by a methyl group (**23–26**) abolished activity. Bromaminic acid derivatives, in which the sulfonic acid group was converted to sulfonic acid esters (**27–30**) or sulfonamides (**31–33**), were also totally

**Table 1** Inhibitory potencies of anthraquinone derivatives at rat NTPDase1, 2, and 3 determined by capillary electrophoresis<sup>a</sup>

| Inhibitor  | R                           | $K_i$ ( $\mu\text{M}$ ) $\pm$ SEM (or percent inhibition at 1 mM) |                  |                 |
|--|-----------------------------|---|------------------|-----------------|
|  |                             | NTPDase1  | NTPDase2         | NTPDase3        |
| <b>1</b>   | ARL 67156 [27]              | 27.0 $\pm$ 0.0  | (50%)            | 112 $\pm$ 0     |
| <b>5</b>   | Reactive blue 2 (RB-2) [27] | 20.0 $\pm$ 0.0  | 24.20 $\pm$ 0.06 | 1.10 $\pm$ 0.03 |
| 1-Amino-4-aryl(alkyl)amino-2-sulfo-anthraquinones            |                             |   |                  |                 |
| <b>11</b>  | Phenyl                      | 49.1 $\pm$ 5.1  | 35.8 $\pm$ 6.1   | 14.3 $\pm$ 1.5  |
| <b>12</b>  | 2-Methylphenyl              | (25%)   | 25.7 $\pm$ 5.1   | 23.0 $\pm$ 2.5  |
| <b>13</b>  | 3-Methylphenyl              | 51.5 $\pm$ 0.4  | 12.8 $\pm$ 0.9   | 19.1 $\pm$ 6.0  |
| <b>14</b>  | 2,3-Dimethylphenyl          | (67%) <sup>b</sup>  | 22.7 $\pm$ 3.4   | 38.5 $\pm$ 5.0  |
| <b>15</b>  | 2,4-Dimethylphenyl          | 18.0 $\pm$ 3.5  | 15.6 $\pm$ 2.5   | 41.8 $\pm$ 5.5  |
| <b>16</b>  | 2-Methoxyphenyl             | (57%)   | 53.8 $\pm$ 5.7   | 17.6 $\pm$ 6.6  |
| <b>17</b>  | 2-Ethoxyphenyl              | (17%)   | 40.8 $\pm$ 11.1  | 58.0 $\pm$ 12.7 |
| <b>18</b>  | 4-Chlorophenyl              | 15.7 $\pm$ 3.4  | 18.0 $\pm$ 2.0   | 16.4 $\pm$ 1.6  |
| <b>19</b>  | 4-Acetylamino-phenyl        | (15%)   | 486 $\pm$ 18     | 343 $\pm$ 63    |
| <b>20</b>  | 1-Naphthyl                  | (0%)  | (0%)             | 1.5 $\pm$ 0.1   |
| <b>21</b>  | 2-Naphthyl                  | 0.328 $\pm$ 0.110   | 19.1 $\pm$ 1.6   | 2.22 $\pm$ 1.03 |
| <b>22</b>  | 3,4-Dimethoxy-phenethyl     | 173 $\pm$ 6   | 54.1 $\pm$ 6.8   | 23.4 $\pm$ 0.4  |
| 1-Amino-4-aryl(alkyl)amino-2-methyl-anthraquinones           |                             |   |                  |                 |
| <b>23</b>  | Phenyl                      | (0%)  | (0%)             | (0%)            |
| <b>24</b>  | 3-Methylphenyl              | (0%)  | (0%)             | (0%)            |
| <b>25</b>  | 4-Methylphenyl              | (0%)  | (0%)             | (0%)            |
| <b>26</b>  | 1-Naphthyl                  | (0%)  | (0%)             | (0%)            |
| 1-Amino-4-bromo-anthraquinone 2-sulfonates or 2-sulfonamides |                             |   |                  |                 |
| <b>27</b>  | Methoxy                     | (0%)  | (0%)             | (0%)            |
| <b>28</b>  | 2-Nitrophenoxy              | (0%)  | (0%)             | (0%)            |
| <b>29</b>  | 3-Nitrophenoxy              | (0%)  | (0%)             | (0%)            |
| <b>30</b>  | 4-Nitrophenoxy              | (0%)  | (0%)             | (0%)            |
| <b>31</b>  | Phenylamino                 | (0%)  | (0%)             | (0%)            |
| <b>32</b>  | Benzylamino                 | (0%)  | (0%)             | (0%)            |
| <b>33</b>  | Phenethylamino              | (0%)  | (0%)             | (0%)            |

**Table 1** (continued)

| Inhibitor  | R          | K <sub>i</sub> (μM)±SEM (or percent inhibition at 1 mM) |          |          |
|--|------------|---|----------|----------|
|  |            | NTPDase1  | NTPDase2 | NTPDase3 |
| Bis-(1-amino-4-bromo-anthraquinone) sulfonamides |            |   |          |          |
| <b>34</b>  | 4'-Ethoxy  | (0%)  | (0%)     | (0%)     |
| <b>35</b>  | 2'-Methoxy | (0%)  | (0%)     | (0%)     |

<sup>a</sup> The results are means±SEM of three separate experiments each run in duplicate

<sup>b</sup> Curve could not be determined: inhibition was not concentration dependent; there was no inhibition at 100 μM

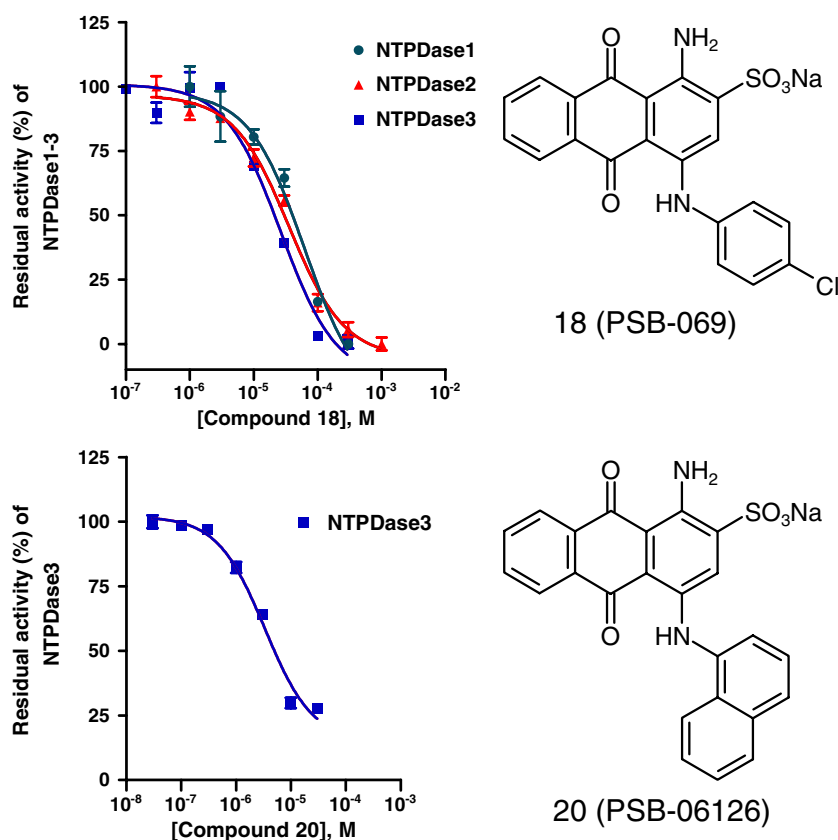
inactive. Thus, a negative charge (a free sulfonate group) appeared to be required for NTPDase inhibition. This is in agreement with previous studies on sulfonate dyes and related compounds, which showed that sulfonate groups in certain positions of the molecules were important for ectonucleotidase inhibitory activity [23, 34].

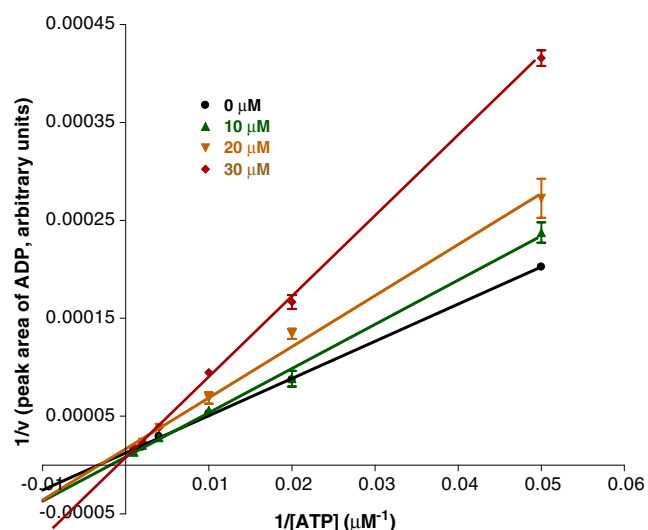
In order to investigate the inhibition mechanism of this class of NTPDase inhibitors, kinetics of NTPDase3 were exemplarily determined in the absence and in the presence of compound **18** (10, 20, and 30 μM). While the V<sub>max</sub> value was not affected by the inhibitor, the K<sub>m</sub> value increased with increasing concentration of inhibitor indicating a

competitive mechanism of inhibition (for Lineweaver-Burk plot see Fig. 5).

The present study is the first one to systematically investigate the SARs of anthraquinone derivatives derived from RB-2 at defined NTPDase isoenzymes. We could confirm previous findings by Tuluc et al. [37] that anthraquinone derivatives with only one substituted phenyl ring at the 4-amino group, which are much smaller than RB-2, can exhibit ectonucleotidase inhibitory activity. However, compound **11** (also known as acid blue 25) was inactive (at 100 μM) in their study investigating ATP degradation in rat vas deferens tissue [37]. Like RB-2, its truncated derivatives

**Fig. 4** Concentration-inhibition curves of selected anthraquinone derivatives. *Top*: curves of the nonselective NTPDase inhibitor **18** at NTPDase1, 2, and 3. *Bottom*: curve of the NTPDase3 inhibitor **20**





**Fig. 5** Lineweaver-Burk plot for rat NTPDase3 kinetics in the absence and presence of different concentrations of inhibitor **18**

also interact with P2 receptors [37, 45, 48]. However, the SARs are clearly different. For example, **16** (PSB-716) was shown to be a potent P2Y<sub>2</sub> antagonist (IC<sub>50</sub> ca. 9 μM) [45], but it is only a weak NTPDase1 inhibitor. Compound **19** was also a potent inhibitor at P2Y<sub>2</sub> (IC<sub>50</sub> 6–12 μM) [45], but nearly inactive at all three NTPDases tested.

## Conclusions

We have investigated 25 anthraquinone derivatives related to RB-2 for their potency at inhibiting rat NTPDases1, 2, and 3 in order to obtain initial information about their structure-activity relationships. While 11 of the compounds had previously been reported, 14 compounds are new and their syntheses and physicochemical characterization are described for the first time. Certain 1-amino-2-sulfo-4-arylaminoanthraquinones were identified as potent NTPDase inhibitors. While the 4-chlorophenylamino derivative **18** was a nonselective inhibitor (K<sub>i</sub> 16–18 μM), the 4-(1-naphthylamino) derivative **20** was very potent and selective for rat NTPDase3 versus rat NTPDase1 and 2. In contrast, its 2-naphthylamino isomer **21** was a very potent NTPDase1 inhibitor (K<sub>i</sub> 0.328 μM). For future studies it would be of interest to investigate potential species differences of these and other NTPDase inhibitors. Furthermore, it will be important to investigate the potency of some of these compounds at other ectonucleotidases, including ecto-5'-nucleotidase, nucleotide pyrophosphatases/phosphodiesterases (NPPs), and alkaline phosphatases, and to determine their profile at the various P2Y and P2X receptor subtypes, in order to be able to fully assess their usefulness as pharmacological tools. The compounds described will be good starting points for further optimization.

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## Appendix

### Experimental section

#### Reagents and chemicals

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), ATP, MgCl<sub>2</sub>·6H<sub>2</sub>O, and tris(hydroxymethyl)aminomethane (Trizma Base), penicillin and streptomycin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Culture medium was obtained from Invitrogen (Karlsruhe, Germany). Leupeptin, pepstatin A, chymostatin, and antipain were received from Calbiochem (Schwalbach, Germany).

Bromaminic acid (**8**) and 1-amino-4-bromo-2-methylanthraquinone (**9**) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Aniline derivatives were a gift from Bayer AG (through Fonds der Chemischen Industrie). Liquid aniline derivatives were purified by distillation before use in case they showed a dark brown color. Solvents were dried according to standard methods when required. All other chemicals were obtained from standard commercial sources or prepared as described below. Anthraquinone derivatives **11–19** were prepared as recently published [45, 46].

#### Instruments

NMR spectra were recorded on a Bruker Avance 500 spectrometer. Electron impact mass spectra (EI-MS) were obtained on an MS 50 instrument of the company A.E.I., or a MAT 95 XL instrument of Thermoquest. Polar compounds were analyzed by electrospray ionization using the API 2000 LC-MS instrument from Applied Biosystems. Elemental analyses were performed with a VarioEL instrument of Elementar Analysensysteme GmbH. Melting points were determined on a Gallenkamp apparatus, or a B530 or a B-545 apparatus from Büchi. Melting points of sodium sulfonates were usually not determined because they are >300°C. Reactions were monitored by thin-layer chromatography (TLC) using silica gel plates F<sub>254</sub> and/or RP silica gel plates F<sub>254</sub>. For microwave reactions a CEM Focused™ Microwave Synthesis type Discover apparatus was used. A freeze dryer (CHRIST ALPHA 1–4 LSC) was used for lyophilization.



Synthetic procedures and analytical data of anthraquinone derivatives

*Sodium 1-amino-4-(1-naphthylamino)-9,10-dioxo-9,10-dihydroanthracene 2-sulfonate (20)*

Bromaminic acid sodium salt (0.500 g, 1.25 mmol), Na<sub>2</sub>CO<sub>3</sub> (0.175 g), and CuSO<sub>4</sub> (0.030 g) were dissolved in 10 ml of water. Then 2 g (14 mmol) of 1-aminonaphthalene was added and the reaction mixture was stirred for 16 h at 105°C. After completion of the reaction the mixture was cooled for 4 h at 4°C, and the formed precipitate was filtered off. The precipitate was washed with 10 ml of water and subsequently with 200 ml of dichloromethane until the filtrate became colorless. The residue was dried under vacuum. Yield: 0.281 g (49%); mp>300°C. <sup>1</sup>H-NMR [dimethyl sulfoxide (DMSO)-d<sub>6</sub>, 500 MHz, 303 K]: δ (ppm)=7.49 (br, 1H, NH<sub>2</sub>); 7.50 (d, 1H, <sup>3</sup>J=7.3 Hz, naphthalene); 7.58–7.63 (m, 3H, naphthalene); 7.80 (s, 1H, C3H); 7.84–7.90 (m, 3H, C6H, C7H, naphthalene); 8.02, 8.08 (m, 2H, naphthalene); 8.32 (m, 2H, C5H, C8H); 10.12 (br, 1H, NH<sub>2</sub>); 12.51 (s, 1H, NH). <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 125 MHz, 303 K): δ (ppm)=109.23, 111.46 (C4a, C9a); 121.55, 121.97 (C2', C4'); 122.92 (C3); 125.76, 126.20 (2C), 126.24, 126.91, 127.12, 128.67, 129.00 (C5, C8, C5', C6', C7', C8', C4a', C8a'); 132.94, 133.38, 133.78, 134.40, 134.48, 135.21 (C6, C7, C8a, C10a, C1', C3'); 142.58, 143.15, 144.49 (C1, C2, C4); 181.92, 182.95 (C9, C10). FAB m/z (%)=466 (100, C<sub>25</sub>H<sub>15</sub>N<sub>2</sub>O<sub>5</sub>SNa); 444 (46, C<sub>25</sub>H<sub>15</sub>N<sub>2</sub>O<sub>5</sub>S).

*Sodium 1-amino-4-(2-naphthylamino)-9,10-dioxo-9,10-dihydroanthracene 2-sulfonate (21)*

The synthesis was performed in analogy to a recently described procedure [46]. To a 5-ml microwave reaction vial equipped with a magnetic stirring bar were added bromaminic acid sodium salt (0.081 g, 0.20 mmol) and 1-aminonaphthalene (80 mg, 0.40 mmol), followed by a buffer solution consisting of 4 ml (0.2 M) aq. Na<sub>2</sub>HPO<sub>4</sub> solution (pH 9.6) and 1 ml (0.12 M) of NaH<sub>2</sub>PO<sub>4</sub> (pH 4.2). A catalytic amount (ca. 0.002–0.003 g) of finely powdered elemental copper was added. The mixture was capped and irradiated in the microwave oven (100 W) for 5 min at 120°C. Then the reaction mixture was cooled to room temperature (RT), and the product was purified using the following procedure. The contents of the vial were filtered to remove the elemental copper. Then water (approximately 200 ml) was added to the filtrate and the aqueous solution was extracted with chloroform (200 ml). The extraction procedure was repeated until the organic layer became colorless (2–3 times). Then the aqueous layer was reduced by rotary evaporation to a volume of ca. 10 ml, which was subsequently submitted to flash column chromatography using RP-18 silica gel and water as

an eluent. The polarity of the eluent was then gradually decreased by the addition of methanol in the following steps: 20, 40, 60, 80, and 100%. Fractions containing blue product were collected. The pooled product-containing fractions were evaporated under reduced pressure to remove the methanol, and the remaining water was subsequently removed by lyophilization to yield 21 as blue powder. Yield: 0.046 g (50%); mp>300°C. <sup>1</sup>H-NMR: δ 7.49 (m, 3H, naphthalene); 7.76 (d, 1H, naphthalene); 7.86 (m, 2H, 6-H, 7-H); 7.92 (d, 1H, naphthalene); 8.00 (d, 1H, naphthalene); 8.09 (s, 1H, 3-H); 8.29 (m, 2H, 5-H, 8-H); 10.12 (br, 2H, 1-NH<sub>2</sub>); 12.16 (s, 1H, NH). <sup>13</sup>C-NMR: δ 109.4, 112.0 (C4a, C9a); 119.3, 123.1 (C2', C4'); 123.3 (C3); 125.3, 126.1, 126.2, 126.9, 127.3, 127.8, 129.6, 130.5 (C5, C8, C5', C6', C7', C8', C4a', C8a'); 133.0, 133.4, 133.7, 134.0, 134.3, 137.1 (C6, C7, C8a, C10a, C1', C3'); 140.7, 142.90, 144.6 (C1, C2, C4); 182.0 (C9), 182.8 (C10). LC-MS (m/z): 462 [M-Na+NH<sub>4</sub><sup>+</sup>]<sup>+</sup>, 445 [M-Na]<sup>+</sup>, 443 [M-Na]. Purity by high-performance liquid chromatography (HPLC)-UV (254 nm)-electrospray ionization (ESI)-MS: 98%.

*Sodium 1-amino-4-(3,4-dimethoxyphenethylamino)-9,10-dioxo-9,10-dihydroanthracene 2-sulfonate (22)*

Bromaminic acid sodium salt (1.0 g, 2.5 mmol), Na<sub>2</sub>CO<sub>3</sub> (0.210 g), and CuSO<sub>4</sub> (0.070 g) were suspended in 20 ml of water. Then 3,4-dimethoxyphenethylamine (670 μl, 0.725 g, 4.0 mmol) was added and the reaction mixture was heated at 60°C. Then another portion of 3,4-dimethoxyphenethylamine (1 ml, 1.09 g, 6 mmol) was added and the reaction mixture was stirred for 6 h at 100°C. After the reaction was completed the mixture was cooled and neutralized by the addition of diluted aq. HCl solution. Then 20 ml of water containing 1 ml of saturated aq. NaCl solution were added. The blue-colored solid material was filtered off and washed twice with 20 ml of a saturated NaCl solution, and subsequently three times with 20 ml of dichloromethane each. The residue was dissolved in 200 ml of a mixture of hot water:methanol (1:3) and filtered. The filtrate was evaporated to dryness yielding a blue-violet product, which was further purified by preparative HPLC in analogy to a described procedure [45]. Yield: 54%; mp>300°C. <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 303 K, 500 MHz): δ (ppm)=2.90 (t, 2H, <sup>3</sup>J=6.9 Hz, ArCH<sub>2</sub>); 3.63 (dt, <sup>3</sup>J<sub>NHCH</sub>=5.3 Hz, <sup>3</sup>J<sub>CH<sub>2</sub>CH<sub>2</sub></sub>=6.9 Hz NHCH<sub>2</sub>); 3.71 (s, 3H, C3'-OCH<sub>3</sub> or C4'-OCH<sub>3</sub>); 3.77 (s, 3H, C3'-OCH<sub>3</sub> or C4'-OCH<sub>3</sub>); 6.84 (dd, 1H, <sup>3</sup>J=8.0 Hz, <sup>4</sup>J=1.7 Hz, C6'H); 6.89 (d, 1H, <sup>3</sup>J=8.2 Hz, C5'H); 6.97 (d, 1H, <sup>4</sup>J=1.6 Hz, C2'H); 7.40 (br, 1H, NH<sub>2</sub>); 7.77–7.80 (m, 2H, C6H, C7H); 7.81 (s, 1H, C3H); 8.19–8.26 (m, 2H, C5H, C8H); 10.08 (br, 1H, NH<sub>2</sub>); 10.76 (t, 1H, <sup>3</sup>J=5.2 Hz, NH). <sup>13</sup>C-NMR (DMSO-d<sub>6</sub>, 303 K, 125 MHz): δ (ppm)=34.88 (ArCH<sub>2</sub>); 44.35 (NHCH<sub>2</sub>); 55.49, 55.68 (C3'-OCH<sub>3</sub>, C4'-OCH<sub>3</sub>); 109.07, 109.29 (C4a, C9a); 112.23,

112.94 (C2', C5'); 120.70, 121.02 (C3, C6'); 125.79, 126.01 (C5, C8); 131.52 (C1'); 132.56, 132.64, 134.05, 134.10 (C6, C7, C8a, C10a); 143.20, 143.72, 145.06 (C1, C2, C4); 147.57, 148.87 (C3', C4'); 180.97, 181.74 (C9, C10).

General procedure for the synthesis of 4-substituted 1-amino-2-methylantraquinone derivatives (23–26)

1-Amino-4-bromo-2-methylantraquinone (**9**), potassium acetate, and copper(I) acetate were put together into a mortar and pestled until a finely ground powder was obtained. The mixture was then transferred to a round-bottom flask and the appropriate aniline, or aminonaphthalene derivative, respectively, was added. The mixture was then heated at 100°C under an argon atmosphere upon stirring for 5–24 h. After cooling, ethanol (5–10 ml) was added and the solid residue was collected by filtration, washed with ethanol, 2 N aqueous HCl solution, and water (15 ml each) and finally recrystallized from pyridine (if necessary).

#### 1-Amino-2-methyl-4-phenylaminoanthraquinone (23)

The compound was synthesized from 500 mg (1.6 mmol) of **9** and 2 ml (2.04 g, 22 mmol) of aniline in the presence of 350 mg (3.6 mmol) of potassium acetate and 30 mg (0.16 mmol) of copper(I) acetate. Reaction time: 5 h.

Yield: 331 mg (64%); mp: 243°C. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ (ppm)=2.25 (d, 3H, <sup>4</sup>J=0.6 Hz, CH<sub>3</sub>); 7.18 (tt, 1H, <sup>3</sup>J=7.4 Hz, <sup>4</sup>J=1.2 Hz C4'H); 7.31 (dm, 2H, <sup>3</sup>J=7.6 Hz, C2'H, C6'H); 7.43 (tm, 2H, <sup>3</sup>J=7.9 Hz, C3'H, C5'H); 7.44 (s, 1H, <sup>4</sup>J=0.6 Hz, C3H); 7.82 (dd, 1H, <sup>3</sup>J=6.3 Hz, <sup>4</sup>J=2.1 Hz, C6H or C7H); 7.83 (dd, 1H, <sup>3</sup>J=6.3 Hz, <sup>4</sup>J=2.1 Hz, C6H or C7H); 8.24–8.29 (m, 2H, C5H, C8H), 8.49 (br, 2H, NH<sub>2</sub>); 12.25 (s, 1H, NH). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz, 303 K): δ (ppm)=18.68 (CH<sub>3</sub>); 108.08, 109.62 (C4a, C9a); 123.19 (2C, C2', C6'); 124.43 (C4'); 124.52 (C3); 126.00, 126.13 (C5, C8); 129.82 (2C, C3', C5'); 132.83, 133.03 (C6, C7); 133.96, 134.12 (C8a, C10a); 137.55 (C2); 139.40 (C1'); 142.02 (C4); 147.23 (C1); 181.97, 182.04 (C9, C10). The assignment of the signals was based on one- and two-dimensional NMR spectra; however, some signals could not be unambiguously assigned.

EI-MS: m/z (%)=328 (100; [C<sub>21</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>]<sup>+</sup>); 311 (8; [C<sub>21</sub>H<sub>13</sub>NO<sub>2</sub>]<sup>+</sup>). High-resolution mass spectrometry (HRMS) (70 eV, EI): Calcd.: 328.121178. Found 328.1209 ⇒ C<sub>21</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>. Anal. Calcd. for C<sub>21</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>·0.25H<sub>2</sub>O: C, 75.77; H, 5.00; N, 8.42. Found: C, 75.96; H, 4.86; N, 8.45.

#### 1-Amino-2-methyl-4-(3-methylphenylamino)anthraquinone (24)

The compound was synthesized from 500 mg (1.6 mmol) of **9** and 2 ml (1.96 g, 18.29 mmol) of 3-methylaniline in

the presence of 350 mg (3.6 mmol) of potassium acetate and 30 mg (0.16 mmol) of copper(I) acetate. Reaction time: 24 h.

Yield: 384 mg (71%); mp: 255°C. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ (ppm)=2.25 (d, 3H, <sup>4</sup>J=0.6 Hz, 2-CH<sub>3</sub>); 2.33 (s, 3H, 3'-CH<sub>3</sub>); 7.00 (d, 1H, <sup>3</sup>J=7.3 Hz, C4'H or C6'H); 7.11 (d, 1H, <sup>3</sup>J=7.3 Hz, C4'H or C6'H); 7.11 (s, 1H, C2'H); 7.31 (t, 1H, <sup>3</sup>J=7.9 Hz, C5'H); 7.43 (d, 1H, <sup>4</sup>J=0.6 Hz, C3H); 7.80–7.84 (m, 2H, C6H, C7H); 8.25–8.27 (m, 2H, C5H, C8H); 12.24 (s, 1H, NH). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz, 303 K): δ (ppm)=18.69 (2-CH<sub>3</sub>); 21.13 (3'-CH<sub>3</sub>); 108.05, 109.48 (C4a, C9a); 120.32 (C6'); 123.85 (C2' or C4'); 124.63 (C3); 125.27 (C2' or C4'); 126.00, 126.13 (C5, C8); 129.59 (C5'); 132.82, 133.00 (C6, C7); 133.99, 134.12 (C8a, C10a); 137.56 (C2); 139.27, 139.37 (C1', C3'); 142.22 (C4); 147.21 (C1); 181.86, 182.01 (C9, C10). EI-MS: m/z (%)=342.2 (100; C<sub>22</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub><sup>+</sup>); 325.2 [3; (C<sub>22</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>-NH<sub>3</sub>)<sup>+</sup>]. HRMS (70 eV, EI): Calcd.: 342.1368. Found 342.1367 ⇒ C<sub>22</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>. Anal. Calcd. for C<sub>22</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>·0.75H<sub>2</sub>O: C, 74.24; H, 5.52; N, 7.87. Found: C, 73.98; H, 5.28; N, 8.16.

#### 1-Amino-2-methyl-4-(4-methylphenylamino)anthraquinone (25) [49]

The compound was synthesized from 350 mg (1.1 mmol) of **9** and 2.6 g (24.3 mmol) of 4-methylaniline in the presence of 245 mg (3.6 mmol) of potassium acetate and 21 mg (0.16 mmol) of copper(I) acetate. Reaction time: 5 h.

Yield: 260 mg (68%). <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ (ppm)=2.24 (s, 3H, 2-CH<sub>3</sub>); 2.32 (s, 3H, 4'-CH<sub>3</sub>); 7.20 (dm, 2H, <sup>3</sup>J=8.5 Hz, C2'H, C6'H or C3'H, C5'H); 7.24 (dm, 1H, <sup>3</sup>J=8.2 Hz, C2'H, C6'H or C3'H, C5'H); 7.37 (s, 1H, C3H); 7.80–7.84 (m, 2H, C6H, C7H); 8.25–8.28 (m, 2H, C5H, C8H); 12.26 (s, 1H, NH). <sup>13</sup>C-NMR (DMSO-*d*<sub>6</sub>, 125 MHz, 303 K): δ (ppm)=18.69 (2-CH<sub>3</sub>); 20.65 (3'-CH<sub>3</sub>); 107.98, 109.11 (C4a, C9a); 123.70 (2C, C2', C6'); 124.38 (C3); 125.97, 126.12 (C5, C8); 130.30 (2C, C3', C5'); 132.78, 133.91 (C6, C7); 133.99, 134.03, 134.11 (C8a, C10a, C4'); 136.58, 137.64, (C2, C1'); 142.75 (C4); 147.12 (C1); 181.61, 182.94 (C9, C10).

#### 1-Amino-2-methyl-4-(1-naphthylamino)anthraquinone (26)

The compound was synthesized from 400 mg (1.3 mmol) of **9** and 2.5 g (17 mmol) of 1-aminonaphthalene in the presence of 280 mg (2.85 mmol) of potassium acetate and 25 mg (0.14 mmol) of copper(I) acetate. Reaction time: 12 h. The product is recrystallized from pyridine.

Yield: 123 mg (20%). <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>, 500 MHz) δ (ppm)=2.19 (d, 3H, <sup>4</sup>J=0.6 Hz, 2-CH<sub>3</sub>); 7.27 (d, 1H, <sup>4</sup>J=0.6 Hz C3H); 7.55–7.62 (m, 4H, naphthalene); 7.82–7.85 (m, 3H, C6H, C7H, naphthalene); 8.02 (m, 1H, naphtha-

lene); 8.07 (m, 1H, naphthalene); 8.31 (m, 2H, C5H, C8H); 12.74 (s, 1H, NH).  $^{13}\text{C}$ -NMR (DMSO- $d_6$ , 125 MHz):  $\delta$  (ppm)=18.63 (2- $\text{CH}_3$ ); 108.00, 109.61 (C4a, C9a); 121.35, 121.95 (C2', C4'); 124.67 (C3); 125.45, 126.10, 126.19, 126.27, 126.82, 127.00, 128.63, 128.81 (C5, C8, C5', C6', C7', C8', C4a', C8a'); 132.84, 133.09, 134.01, 134.22, 134.45, 135.16 (C6, C7, C8a, C10a, C1', C3'); 137.76 (C2); 143.48 (C4); 147.24 (C1); 181.99, 182.27 (C9, C10). EI-MS:  $m/z$  (%)=378.2 (100;  $\text{C}_{25}\text{H}_{18}\text{N}_2\text{O}_2^+$ ). HRMS (70 eV, EI): Calcd.: 378.1368. Found 378.1365  $\Rightarrow$   $\text{C}_{25}\text{H}_{18}\text{N}_2\text{O}_2$ .

#### Synthesis of the sulfonic acid chloride of bromaminic acid (10) [50]

Bromaminic acid sodium salt (**8**, 10 g, ca. 25 mmol) was suspended in 100 ml of phosphorus oxychloride ( $\text{POCl}_3$ ) and phosphorus pentachloride ( $\text{PCl}_5$ , 9 g, 43 mmol) was added. The reaction mixture was stirred at 95°C under argon for 6 h. The remaining  $\text{POCl}_3$  was distilled off under reduced pressure. Two different methods have been used to isolate the product: (1) the residue was cooled in an ice bath and the cold mixture was poured into 200 ml of ice water. The precipitated solid was filtered off and dried under vacuum and stored until use; (2) the residue was taken up in dichloromethane and the resulting solution was washed three times with 40 ml of ice water each. The organic phase was dried over anhydrous magnesium sulfate and after filtration, the solvent was distilled off under vacuum. The residue obtained was dried and used in the next step without further purification. Yield: 90–96% (raw product); mp approximately 190°C (decomposition).

#### *1-Amino-4-bromoanthraquinone-2-sulfonic acid methyl ester (27)*

The sulfonic acid chloride **10** (250 mg, 0.6 mmol) was suspended in 50 ml of methanol, and sodium methoxide (approximately 500 mg) was added. The reaction mixture was stirred under an argon atmosphere for 16 h at RT and subsequently evaporated to dryness. The residue was suspended in a mixture of cyclohexane and ethyl acetate (37:3), silica gel (5 g) was added, and the solvent was removed by rotary evaporation. The material absorbed on silica gel was put onto a silica gel column and the product was purified by column chromatography using cyclohexane:ethyl acetate (37:3) as eluent.

Yield: 143 mg (58%);  $^1\text{H}$ -NMR (DMSO- $d_6$ , 500 MHz, 303 K):  $\delta$  (ppm)=3.97 (s, 3H,  $\text{OCH}_3$ ); 7.52 (br, 1H,  $\text{NH}_2$ ); 7.83 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.6$  Hz, C6H or C7H); 7.87 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.7$  Hz, C6H or C7H); 8.04 (s, 1H, C3H); 8.09 (dd, 1H,  $^3J=7.6$  Hz,  $^4J=1.3$  Hz, C5H or C8H); 8.16 (dd, 1H,  $^3J=7.6$  Hz,  $^4J=1.3$  Hz, C5H or C8H); 9.56 (br, 1H,  $\text{NH}_2$ ).  $^{13}\text{C}$ -NMR (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$

(ppm)=62.24 ( $\text{OCH}_3$ ); 106.21, 114.79 (C4a, C9a); 126.17, 126.46 (C5, C8); 131.40 (C4); 133.10, 133.68, 133.76, 134.31 (C6, C7, C8a, C10a); 138.63, 138.93 (C2, C3); 148.4 (C1); 182.16, 183.70 (C9, C10).

#### General procedure for the synthesis of 1-amino-4-bromoanthraquinone-2-sulfonic acid nitrophenyl esters

The sulfonic acid chloride **10** (285 mg, 0.7 mmol) was suspended in 33 ml of freshly distilled dichloromethane and completely dissolved by stirring it under an argon atmosphere for 30 min. Then a solution of 430 mg (3.1 mmol) of the appropriate nitrophenol and 4 ml of triethylamine in 8 ml of dichloromethane were added (producing white fumes). The reaction mixture was stirred until the starting compound **10** had completely disappeared (TLC control). The solvent was evaporated and the residue was dissolved in 10 ml of a mixture of dichloromethane and cyclohexane (7:2). Subsequent column chromatography on silica gel using the same solvent mixture gave the pure products.

#### *1-Amino-4-bromoanthraquinone-2-sulfonic acid 2-nitrophenyl ester (28)*

Yield: 96 mg (27%); mp 213 – 214°C.  $^1\text{H}$ -NMR (DMSO- $d_6$ , 303 K, 500 MHz):  $\delta$  (ppm)=7.32 (dd, 1H,  $^3J=8.3$  Hz,  $^4J=1.2$  Hz, C6'H); 7.63 (ddd, 1H,  $^3J=8.2$  Hz,  $^3J=7.6$  Hz,  $^4J=1.3$  Hz, C5'H); 7.74 (ddd,  $^3J=8.2$  Hz,  $^3J=7.6$  Hz,  $^4J=1.6$  Hz, C4'H); 7.89 (s, 1H, C3H); 7.91 (td, 1H,  $^3J=7.3$  Hz,  $^4J=1.7$  Hz, C6H or C7H); 7.94 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.8$  Hz, C6H or C7H); 8.10 (ddm, 1H,  $^3J=7.1$  Hz,  $^4J=1.8$  Hz, C5H or C8H); 8.14 (dd, 1H,  $^3J=8.0$  Hz,  $^4J=1.7$  Hz, C3'H); 8.20 (ddm, 1H,  $^3J=7.1$  Hz,  $^4J=1.8$  Hz, C5H or C8H).  $^{13}\text{C}$ -NMR (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm)=104.08 (C9a); 118.13 (C4a); 120.62 (C4); 124.87 (C3' or C6'); 126.48, 126.58 (C5, C8); 126.60 (C3' or C6'); 129.44 (C4'); 132.85, 133.62, 134.54, 134.84 (C6, C7, C8a, C10a); 135.65 (C5'); 137.62 (C2); 139.95 (C2'); 142.23 (C3); 142.90 (C1'); 148.21 (C1); 182.15, 184.42 (C9 und C10). EI-MS:  $m/z$  (%)=502.0 + 504.0 (ratio 1:1; 100; [ $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ ] $^{+}$ ); 380.9+382.9 (ratio 1:1; 31; [ $\text{C}_{14}\text{H}_8\text{BrNO}_5\text{S}$ ] $^{+}$ ); 300.0+301.9 (ratio 1:1; 84; [ $\text{C}_{14}\text{H}_7\text{BrNO}_2$ ] $^{+}$ ); 221.1 (41; [ $\text{C}_{14}\text{H}_7\text{NO}_2$ ] $^{+}$ ). HRMS (70 eV, EI): Calcd.: 501.9470. Found: 501.9471  $\Rightarrow$   $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ . Anal. Calcd. for  $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ : C, 47.73; H, 2.20; N, 5.57. Found: C, 47.81; H, 2.26; N, 5.52

#### *1-Amino-4-bromoanthraquinone-2-sulfonic acid 3-nitrophenyl ester (29)*

Yield: 215 mg (61%); mp 189°C.  $^1\text{H}$ -NMR (DMSO- $d_6$ , 303 K, 500 MHz):  $\delta$  (ppm)=7.65 (ddd, 1H,  $^3J=8.2$  Hz,  $^4J=$

2.4 Hz,  $^5J=1.1$  Hz, C6'H); 7.71 (t, 1H,  $^3J=8.3$  Hz, C5'H); 7.89 (td, 1H,  $^3J=7.3$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 7.92 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 8.01 (s, 1H, C3'H); 8.07 (ddm, 1H,  $^3J=7.0$  Hz,  $^4J=2.0$  Hz, C5'H or C8'H); 8.10 (t,  $^4J=2.4$  Hz, C2'H); 8.16 (ddm, 1H,  $^3J=7.6$  Hz,  $^4J=1.6$  Hz, C5'H or C8'H); 8.22 (ddd, 1H,  $^3J=8.2$  Hz,  $^4J=2.1$  Hz,  $^5J=1.1$  Hz, C4'H).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm)=104.28 (C9a); 117.78 (C2'); 117.88 (C4a); 120.72 (C4); 123.15 (C4'); 126.44, 126.57 (C5, C8); 128.83 (C6'); 131.94 (C5'); 132.83, 133.21, 134.50, 134.78 (C6, C7, C8a, C10a); 137.44 (C2); 142.54 (C3); 148.11 (C1); 148.32, 148.67 (C1', C3'); 182.03, 184.40 (C9, C10). EI-MS:  $m/z$  (%)=502.0+504.0 (ratio 1:1; 46;  $[\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}]^+$ ); 424 (84;  $[\text{C}_{20}\text{H}_{11}\text{N}_2\text{O}_7\text{S}]^+$ ); 300.0+301.9 (ratio 1:1; 45;  $[\text{C}_{14}\text{H}_7\text{BrNO}_2]^+$ ); 286 (49;  $[\text{C}_{14}\text{H}_8\text{NO}_4\text{S}]^+$ ); 238.0 (61;  $[\text{C}_{14}\text{H}_8\text{NO}_3]^+$ ); 222.0 (100;  $[\text{C}_{14}\text{H}_7\text{NO}_2]^+$ ). HRMS (70 eV, EI): Calcd.: 501.9470. Found: 501.9469  $\Rightarrow$   $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ . Anal. Calcd. for  $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ : C, 47.73; H, 2.20; N, 5.57. Found: C, 50.98; H, 2.29; N, 5.88.

*1-Amino-4-bromoanthraquinone-2-sulfonic acid 4-nitrophenyl ester (30)*

Yield: 266 mg (76%); mp 196°C.  $^1\text{H-NMR}$  (DMSO- $d_6$ , 303 K, 500 MHz):  $\delta$  (ppm)=7.49 (dm, 2H,  $^3J=9.2$  Hz, C2'H, C6'H); 7.89 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.6$  Hz, C6'H or C7'H); 7.92 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.8$  Hz, C6'H or C7'H); 7.98 (s, 1H, C3'H); 8.08 (ddm, 1H,  $^3J=7.4$  Hz,  $^4J=1.6$  Hz, C5'H or C8'H); 8.17 (ddm, 1H,  $^3J=7.4$  Hz,  $^4J=1.3$  Hz, C5'H or C8'H); 8.26 (dm, 2H,  $^3J=9.2$  Hz, C3'H, C5'H).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm)=104.27 (C9a); 117.96 (C4a); 120.81 (C4); 123.54 (2C, C2', C6'); 126.19 (2C, C3', C5'); 126.45, 126.58 (C5, C8); 132.85, 133.23, 134.51, 134.79 (C6, C7, C8a, C10a); 137.47 (C2); 142.42 (C3); 146.61 (C4'); 148.03 (C1); 152.65 (C1'); 182.06, 184.44 (C9 und C10). EI-MS:  $m/z$  (%)=502.0+504.0 (ratio 1:1; 30;  $[\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}]^+$ ); 424 (84;  $[\text{C}_{20}\text{H}_{11}\text{N}_2\text{O}_7\text{S}]^+$ ); 300.0+301.9 (ratio 1:1; 18;  $[\text{C}_{14}\text{H}_7\text{BrNO}_2]^+$ ); 286 (52;  $[\text{C}_{14}\text{H}_8\text{NO}_4\text{S}]^+$ ); 238.0 (61;  $[\text{C}_{14}\text{H}_8\text{NO}_3]^+$ ); 222.0 (100;  $[\text{C}_{14}\text{H}_7\text{NO}_2]^+$ ). HRMS (70 eV, EI): Calcd.: 501.9470. Found: 501.9472  $\Rightarrow$   $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ . Anal. Calcd. for  $\text{C}_{20}\text{H}_{11}\text{BrN}_2\text{O}_7\text{S}$ : C, 47.73; H, 2.20; N, 5.57. Found: C, 47.55; H, 2.20; N, 5.52.

General procedure for the synthesis of bromaminic acid amides (sulfonamides 31–33)

The sulfonic acid chloride **10** was put into a round-bottom flask flushed with argon and freshly dried and distilled dichloromethane or acetone, respectively, was added. The appropriate amine dissolved in a mixture of triethylamine and dichloromethane was added to the suspension resulting in the evolution of white fumes. The reaction mixture was

stirred at RT under an argon atmosphere until starting compound **10** had completely disappeared [TLC monitoring on silica gel using cyclohexane:ethyl acetate (17:3) as an eluent]. Then the solvent was distilled off under reduced pressure and the residue was dissolved in a mixture of dichloromethane:cyclohexane:ethyl acetate (40:51:9 or 10:17:3, respectively) or in dichloromethane:cyclohexane (7:3), respectively, and purified by column chromatography on silica gel using the same solvent mixture.

*1-Amino-4-bromoanthraquinone-2-sulfonic acid phenyl amide (31) [49]*

The compound was prepared from 0.5 g (1.25 mmol) of **10** in 30 ml of dichloromethane and 1.5 ml (1.53 g, 16.5 mmol) of freshly distilled aniline in 10 ml of dichloromethane. Reaction time: 16 h at RT; purification by column chromatography with dichloromethane:cyclohexane:ethyl acetate (10:17:3) as eluent. Upon evaporation of the product-containing fractions an orange-red colored product precipitated, which was collected by filtration and dried.

Yield: 200 mg (18%); mp 214°C (lit. mp 220°C [48]).  $^1\text{H-NMR}$  (DMSO- $d_6$ , 500 MHz, 303 K):  $\delta$  (ppm)=7.08 (t,  $^3J=7.4$  Hz, C4'H); 7.11 (m, 2H, C2'H, C6'H); 7.28 (m, 2H, C3'H, C5'H); 7.81 (td, 1H,  $^3J=7.3$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 7.84 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 8.00 (dd, 1H,  $^3J=7.1$  Hz,  $^4J=1.8$  Hz, C5'H or C8'H); 8.08 (s, 1H, C3'H); 8.09 (dd, 1H,  $^3J=7.6$  Hz,  $^4J=1.6$  Hz, C5'H or C8'H); 10.81 (s, 1H, NH).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm)=104.67 (C9a); 116.82 (C4a); 120.68 (2C, C2', C6'); 125.13 (C4'); 126.26, 126.44 (C5, C8); 126.98 (C4); 129.65 (2C, C3', C5'); 132.86, 133.30 (C8a, C10a); 134.18, 134.50, 135.33 (C3, C6, C7); 136.51 (C1'); 141.88 (C2); 147.50 (C1); 181.95, 184.28 (C9, C10).

*1-Amino-4-bromoanthraquinone-2-sulfonic acid benzylamide (32)*

The compound was prepared from 0.5 g (1.25 mmol) of **10** in 20 ml of dichloromethane, and 0.3 ml (0.29 g, 2.75 mmol) of benzylamine and 0.3 ml of triethylamine in 30 ml of dichloromethane. Reaction time: 16 h at RT; purification by column chromatography with dichloromethane:cyclohexane:ethyl acetate (40:51:9) as eluent.

Yield: 215 mg (37%); mp 189°C.  $^1\text{H-NMR}$  (DMSO- $d_6$ , 303 K, 500 MHz):  $\delta$  (ppm)=4.12 (s, 2H,  $\text{CH}_2$ ); 7.02 (t,  $^3J=7.2$  Hz, C4'H); 7.14 (t, 2H,  $^3J=7.7$  Hz C3'H, C5'H); 7.18 (d, 2H,  $^3J=6.8$  Hz C2'H, C6'H); 7.87 (td, 1H,  $^3J=7.2$  Hz,  $^4J=1.4$  Hz, C6'H or C7'H); 7.90 (td, 1H,  $^3J=7.4$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 8.02 (s, 1H, C3'H); 8.07 (dd, 1H,  $^3J=7.4$  Hz,  $^4J=1.8$  Hz, C5'H or C8'H); 8.14 (dd, 1H,  $^3J=7.5$  Hz,  $^4J=1.4$  Hz, C5'H or C8'H); 8.81 (s, 1H, NH).  $^{13}\text{C-NMR}$  (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm)=46.06 ( $\text{CH}_2$ ); 105.19

(C9a); 116.41 (C4a); 126.30, 126.51 (C5, C8); 127.23 (C4'); 127.72, 128.18 (C2', C6', C3', C5'); 128.96 (C4); 132.83, 133.37 (C8a, C10a); 134.22, 134.62, 134.63 (C3, C6, C7); 136.69 (C1'); 141.51 (C2); 147.39 (C1); 182.07, 184.08 (C9, C10). EI-MS:  $m/z$  (%) = 471.9 + 469.9 (ratio 1:1; 8;  $[C_{21}H_{15}BrN_2O_4S]^+$ ); 392.0 (5;  $[C_{21}H_{15}N_2O_4S]^+$ ); 300.9 + 303 (ratio 1:1; 27;  $[C_{14}H_7BrNO_2]^+$ ); 223.1 (29;  $[C_{14}H_7NO_2]^+$ ); 106.1 (100;  $[C_7H_8N]^+$ ). HRMS (70 eV, EI): Calcd.: 469.993. Found: 469.992  $\Rightarrow$   $C_{21}H_{15}BrN_2O_4S$ . Anal. Calcd. for  $C_{21}H_{15}BrN_2O_4S \cdot 1.5 C_6H_{12}$ : C, 60.30; H, 5.57; N, 4.69. Found: C, 60.17; H, 5.21; N, 4.88.

*1-Amino-4-bromoanthraquinone-2-sulfonic acid phenethylamide (33)*

The compound was prepared from 0.5 g (1.25 mmol) of **10** in 10 ml of dichloromethane, and 0.3 ml (0.29 g, 2.4 mmol) of 2-phenethylamine and 0.3 ml of triethylamine in 30 ml of dichloromethane. Reaction time: 16 h at RT; purification by column chromatography with dichloromethane:cyclohexane:ethyl acetate (40:51:9) as eluent.

Yield: 296 mg (48%); mp 176°C.  $^1H$ -NMR (DMSO- $d_6$ , 303 K, 500 MHz):  $\delta$  (ppm) = 2.68 (t, 2H,  $^3J=7.1$  Hz,  $ArCH_2$ ); 3.15 (t, 2H,  $^3J=7.1$  Hz,  $NHCH_2$ ); 7.00 (tt,  $^3J=6.8$  Hz,  $^4J=1.9$  Hz, C4'H); 7.10–7.16 (m, 4H, C2'H, C3'H, C5'H, C6'H); 7.87 (td, 1H,  $^3J=7.2$  Hz,  $^4J=1.4$  Hz, C6'H or C7'H); 7.90 (td, 1H,  $^3J=7.3$  Hz,  $^4J=1.7$  Hz, C6'H or C7'H); 8.08 (ddd, 1H,  $^3J=7.3$  Hz,  $^4J=1.7$  Hz,  $^5J=0.4$  Hz, C5'H or C8'H); 8.09 (s, 1H, C3'H); 8.16 (ddd, 1H,  $^3J=7.3$  Hz,  $^4J=1.6$  Hz,  $^5J=0.3$  Hz, C5'H or C8'H); 8.33 (s, 1H, NH).  $^{13}C$ -NMR (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm) = 35.29 ( $ArCH_2$ ); 43.86 ( $NHCH_2$ ); 105.09 (C9a); 116.67 (C4a); 126.23, 126.31, 126.51 (C5, C8, C4'); 128.23 (2C, C2', C6' or C3', C5'); 128.69 (C4); 128.76 (2C, C2', C6' or C3', C5'); 132.90, 133.45 (C8a, C10a); 134.21, 134.60, 134.78 (C3, C6, C7); 138.49 (C1'); 141.24 (C2); 147.45 (C1); 182.15, 184.22 (C9, C10). EI-MS:  $m/z$  (%) = 484.0 + 486.0 (ratio 1:1; 21;  $[C_{22}H_{17}BrN_2O_4S]^+$ ); 392.9 + 394.9 (ratio 1:1; 35;  $[C_{15}H_{10}BrN_2O_4S]^+$ ); 380 + 381.9 (ratio 1:1; 68;  $[C_{14}H_8BrN_2O_4S]^+$ ); 300.0 + 302.0 (ratio 1:1; 100;  $[C_{14}H_7BrNO_2]^+$ ); 221.1 (40;  $[C_{14}H_7NO_2]^+$ ); 120.2 (27;  $[C_7H_8N]^+$ ). HRMS (70 eV, EI): Calcd.: 484.00924. Found: 484.0095  $\Rightarrow$   $C_{22}H_{17}BrN_2O_4S$ . Anal. Calcd. for  $C_{22}H_{17}BrN_2O_4S \cdot 0.5H_2O$ : C, 53.45; H, 3.67; N, 5.67. Found: C, 53.50; H, 3.74; N, 5.56.

*Bis-(1-amino-4-bromoanthraquinone-2-sulfonic acid 4-ethoxyphenylamide (34)*

The compound was prepared from 1.0 g (2.5 mmol) of **10** in 80 ml of dichloromethane, and 1.0 ml (1.05 g, 7.7 mmol) of 4-ethoxyaniline and 3 ml of triethylamine in 10 ml of dichloromethane. Reaction time: 16 h at RT; purification by

column chromatography with dichloromethane:cyclohexane (7:3) as eluent. Upon concentrating the product-containing fractions under reduced pressure the product precipitated and was filtered off.

Yield: 180 mg (17%); mp 262°C.  $^1H$ -NMR (DMSO- $d_6$ :  $CDCl_3$  (approx. 3:1)), 500 MHz, 303 K):  $\delta$  (ppm) = 1.34 (t, 3H,  $^3J=7.0$  Hz,  $CH_3$ ); 4.07 (d, 2H,  $^3J=7.0$  Hz,  $OCH_2$ ); 6.93 (dm, 2H,  $^3J=8.9$  Hz, C2'H, C6'H or C3'H, C5'H); 7.19 (dm, 2H,  $^3J=8.9$  Hz, C2'H, C6'H or C3'H, C5'H); 7.84 (td, 2H,  $^3J=6.4$  Hz,  $^4J=2.1$  Hz, C6'H, C6''H or C7'H, C7''H); 7.86 (td, 2H,  $^3J=6.4$  Hz,  $^4J=2.1$  Hz, C6'H, C6''H or C7'H, C7''H); 8.01 (s, 2H, C3'H, C3''H); 8.04–8.08 (m, 2H, C5'H, C5''H or C8'H, C8''H); 8.09–8.13 (m, 2H, C5'H, C5''H or C8'H, C8''H); 8.25 (br, 4H, 1-NH<sub>2</sub>, 1''-NH<sub>2</sub>).  $^{13}C$ -NMR (DMSO- $d_6$ :  $CDCl_3$  (ca. 3:1)), 125 MHz, 303 K):  $\delta$  (ppm) = 14.04 ( $CH_3$ ); 63.54 ( $OCH_2$ ); 104.23 (2C, C9a, C9a''); 115.42 (2C, C3', C5'); 117.63 (2C, C4a, C4a''); 123.15 (C1'); 124.00 (2C, C4, C4''); 126.03, 126.20 (2C each, C5, C5'', C8, C8''); 132.48 (C8a, C8a'' or C10a, C10a''); 132.76 (2C, C2', C6'); 132.85 (C8a, C8a'' or C10a, C10a''); 133.87, 134.10 (2C each, C6, C6'', C7, C7''); 136.74 (2C, C2, C2''); 143.03 (2C, C3, C3''); 147.82 (2C, C1, C1''); 160.46 (C4'); 181.26, 183.71 (2C each, C9, C9'', C10, C10''). EI-MS:  $m/z$  (%) = 867.0 + 865 + 863 (ratio 1:2:1; 11;  $[C_{36}H_{23}Br_2N_3O_9S]^+$ ); 500 + 502.0 (ratio 1:1; 34;  $[C_{22}H_{17}BrN_2O_5S]^+$ ); 301.0 + 303 (ratio 1:1; 25;  $[C_{14}H_8BrNO_2]^+$ ); 136.1 (100;  $[C_8H_{10}NO]^+$ ); 108.1 (69;  $[C_6H_6NO]^+$ ). IR (KBr):  $\nu$  [ $cm^{-1}$ ] = 3459, 3420 (NH<sub>2</sub>); 1669 (C=O); 1587 (Ar); 1499 (Ar); 1390, 1366 (SO<sub>2</sub>NR<sub>2</sub>); 1254 (C-O); 1160 (SO<sub>2</sub>NR<sub>2</sub>); 889 (Ar); 745, 724 (o-subst. Ar).

*Bis-(1-amino-4-bromoanthraquinone-2-sulfonic acid 2-methoxyphenylamide (35)*

The compound was prepared from 1.0 g (2.5 mmol) of **10** in 80 ml of dichloromethane, and 1.0 ml (1.092 g, 8.867 mmol) of 2-methoxyaniline and 3 ml of triethylamine in 10 ml of dichloromethane. Reaction time: 16 h at RT; purification by column chromatography with dichloromethane:cyclohexane (7:3) as eluent.

Yield: 180 mg (14%); mp 271°C.  $^1H$ -NMR (DMSO- $d_6$ , 500 MHz, 303 K):  $\delta$  (ppm) = 3.60 (s, 3H,  $CH_3$ ); 7.13 (dd, 1H,  $^3J=8.4$  Hz,  $^4J=1.3$  Hz, C3'H or C6'H); 7.13 (ddd, 1H,  $^3J=8.0$  Hz,  $^3J=7.6$  Hz,  $^4J=0.7$  Hz, C3'H or C4'H); 7.47 (dd, 1H,  $^3J=8.2$  Hz,  $^4J=1.6$  Hz, C3'H or C6'H); 7.57 (ddd, 1H,  $^3J=8.3$  Hz,  $^3J=7.5$  Hz,  $^4J=1.7$  Hz, C3'H or C4'H); 7.85 (td, 2H,  $^3J=7.2$  Hz,  $^4J=1.7$  Hz, C6'H, C6''H or C7'H, C7''H); 7.88 (td, 2H,  $^3J=7.2$  Hz,  $^4J=1.8$  Hz, C6'H, C6''H or C7'H, C7''H); 8.01 (ddm, 2H,  $^3J=6.8$  Hz,  $^4J=2.1$  Hz, C5'H, C5''H or C8'H, C8''H); 8.08 (ddm, 2H,  $^3J=6.4$  Hz,  $^4J=2.5$  Hz, C5'H, C5''H or C8'H, C8''H); 8.08 (s, 2H, C3'H, C3''H); 8.35 (br, 4H, 1-NH<sub>2</sub>, 1''-NH<sub>2</sub>).  $^{13}C$ -NMR (DMSO- $d_6$ , 125 MHz, 303 K):  $\delta$  (ppm) = 55.53 ( $OCH_3$ ); 103.84 (2C,

C9a, C9a"); 112.80 (C3'); 117.18 (2C, C4a, C4a"); 119.42, 121.15 (C1', C5'); 124.15 (2C, C4, C4"); 126.05, 126.20 (2C each, C5, C5", C8, C8"); 132.45, 132.86, 132.99, 133.19 (C8a, C8a", C10a, C10a", C4', C6'); 134.08, 134.35 (2C each, C6, C6", C7, C7"); 136.75 (2C, C2, C2"); 143.18 (2C, C3, C3"); 148.13 (2C, C1, C1"); 157.11 (C4'); 181.34, 183.66 (2C each, C9, C9", C10, C10"). EI-MS:  $m/z$  (%) = 848.9+850.9+853 (ratio 1:2: 1; 27;  $[C_{35}H_{21}Br_2N_3O_9S]^+$ ); 486 + 488.0 (ratio 1:1; 80;  $[C_{21}H_{15}BrN_2O_5S]^+$ ); 301.0+303 (ratio 1:1; 34;  $[C_{14}H_8BrNO_2]^+$ ); 122.1 (100;  $[C_7H_8NO]^+$ ). IR (KBr):  $\nu$  [ $cm^{-1}$ ]=3424 (NH<sub>2</sub>); 2845 (OCH<sub>3</sub>); 1675 (C=O); 1594 (Ar); 1366 (SO<sub>2</sub>NR<sub>2</sub>); 1257 (CO); 11.78 (SO<sub>2</sub>NR<sub>2</sub>); 891 (Ar); 724 (o-subst. Ar).

## NTPDase inhibition assays

### Enzyme preparations

The preparation of recombinant rat NTPDase1–3 was performed essentially as described by Iqbal et al. [27]. In brief, Chinese hamster ovary (CHO) cells were transfected by electroporation with plasmid DNA-containing rat NTPDase1 (GenBank Accession number U81295) [51] and NTPDase2 (Y11835) [51] and NTPDase3 (AJ437217) [52], all cloned into the pcDNA3 plasmid. Transfection with the empty plasmid pcDNA3 served as a control. Membrane fractions were prepared in the presence of protease inhibitors from transiently transfected CHO cells 48 h after electroporation. The final pellet fraction was resuspended in 50% (v/v) glycerol containing 2 mM iodoacetamide, 20 mM HEPES (pH 7.4) and stored at -20°C. The membrane preparations contained 4–6  $\mu$ g of protein/ $\mu$ l.

### Enzyme assay

Stock solutions (10 mM or 1 mM) of test compounds were prepared in water or water containing 10% DMSO for the more lipophilic compounds, respectively, and dilutions were made in assay buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.4). The reaction mixture for NTPDase inhibition assays contained ATP (final concentration 400  $\mu$ M) dissolved in assay buffer and test compound (10  $\mu$ l, different concentrations). The reaction was started by the addition of 10  $\mu$ l of enzyme-containing membrane preparation to obtain a final volume of 100  $\mu$ l. Control experiments were performed using membrane preparations of cells transfected with the empty plasmid (pcDNA3). The reaction was carried out at 37°C for 10 min and stopped by heating at 99°C for 3 min. Then 50  $\mu$ l were transferred to a CE vial and mixed with an UMP solution (final concentration 10  $\mu$ M, internal standard) in 450  $\mu$ l of water. The resulting solution was subjected to CE

analysis using a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA) equipped with a UV detection system coupled with a diode array detector. The capillary temperature was kept constant at 25°C. The temperature of the sample storing unit was adjusted to 25°C. The electrophoretic separations were carried out using an eCAP polyacrylamide-coated fused-silica capillary [30 cm (20 cm effective length)  $\times$  50  $\mu$ m internal diameter  $\times$  360  $\mu$ m outer diameter, obtained from CS-Chromatographie (Langerwehe, Germany)]. The separations were performed with phosphate buffer 50 mM, pH 6.5 using an applied current of -60  $\mu$ A and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 210 nm. The capillary was conditioned by rinsing with water for 2 min and subsequently with phosphate buffer for 1 min. Sample injections were made at the cathodic side of the capillary. Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT. Inhibition curves were analyzed by Prism 3.0 (GraphPad, San Diego, CA, USA).

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