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European Journal of Medicinal Chemistry 39 (2004) 345–357
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

Structure–activity relationships of analogues of NF449 confirm NF449 as the most potent and selective known $P2X_1$ receptor antagonist

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Received 17 July 2003; received in revised form 19 January 2004; accepted 19 January 2004

Abstract

NF449 [4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakisbenzene-1,3-disulfonic acid-octasodiumsalt)] was recently described to inhibit recombinant $rP2X_1$ receptors (Naunyn Schmiedeberg's Arch. Pharmacol. 364 (2001) 285). The purpose of this study was to examine structure–activity-relationships at P2 receptors of a series of NF449 analogues. Thus, compounds containing various arylaminemono-, di-, or trisulfonic acids and a replacement of the central urea bridge were synthesized. NF449 displayed a pIC_{50} at $P2X_1$ receptors (rat vas deferens) of 6.31 ± 0.04 being at least 19-fold more potent at $P2X_1$ than at $P2X_3$, $P2Y_1$, $P2Y_2$, or $P2Y_{11}$. Any deletion or change of position of sulfonic acid groups or replacing the central urea bond by the bisamide of terephthalic acid reduced the potency at $P2X_1$ by at least 90%. All compounds were very weak antagonists at $P2Y_2$ or $P2Y_{11}$ receptors ($pIC_{50} < 4.5$). In conclusion, NF449 remains the most potent and selective $P2X_1$ antagonist known. Potential lead compounds among the suramin class for $P2X_3$ (**16d**) and $P2Y_1$ (**16a**) receptors were identified.

Keywords: P2X; P2Y; NF449; NF279; NF023; Suramin

1.. Introduction

A series of adenine and uracil 5′-nucleotide (P2) receptors have been cloned [1–3]. P2 receptors can be divided into different classes according to their structure (ionotropic P2X and metabotropic G-protein coupled P2Y receptors) or to their responsiveness to different nucleotides, i.e., purine or pyrimidine nucleotides [4,5]. So far, seven P2X and eight P2Y receptors (including the UDP-glucose receptor) have been cloned [2,3,5,6]. P2 receptors display a wide expression profile (CNS and periphery) and are involved in a variety of physiological functions such as neurotransmission, secretion, vasodilation, urinary bladder control, nociception, thrombosis induction, proliferation and cell death. Thus, P2 receptors emerge as interesting potential therapeutic targets [5,7,8]. Three subunits may assemble to build one functional ATP-gated P2X ion channel [5]. P2X receptors consist of homo- or heterooligomers (except $P2X_6$ which builds heteromers only) [9] and can be divided into three groups [5]: $P2X_1$ and $P2X_3$ which show rapid activation upon agonist stimulation and equally rapid inactivation. The second group ($P2X_2$, $P2X_{2/3}$, $P2X_{2/6}$, and $P2X_5$) displays rapid activation but slow inactivation. The third group ($P2X_{1/5}$, $P2X_4$, $P2X_{4/6}$, and $P2X_7$) are rapidly activated and show both, fast and slow desensitization. P2Y receptors can be divided into subgroups by structural similarity ($P2Y_{1,\,2,\,4,\,6,\,11}$ and $P2Y_{12,\,13,\,14}$) [10], or by activation by uracil 5′-nucleotides ($P2Y_{2,\,4,\,6,\,14}$) or adenine 5′-nucleotides ($P2Y_{1,\,2,\,11,\,12,\,13}$) [5,11] or by coupling to different G-proteins ($G_{q/11}$: $P2Y_{1,\,2,\,4,\,6}$; G_q and G_s: $P2Y_{11}$; G_i: $P2Y_{12,\,13}$) [5].

Abbreviations: PPADS, pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulfonic acid; PPNDS, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate); IsoPPADS, pyridoxal-5′-phosphate-6-azophenyl-2′,5′-disulfonic acid; BzATP, 2′,3′-*O*-(4-benzoyl-benzoyl)-ATP; TNP-ATP, trinitrophenyl-ATP; Ip5I, diinosinepentaphosphate pentasodiumsalt; αβMeATP, α,β-methylene-ATP; ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); cAMP, cyclic 3′,5′-adenosinemonophosphate.

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doi:10.1016/j.ejmech.2004.01.007

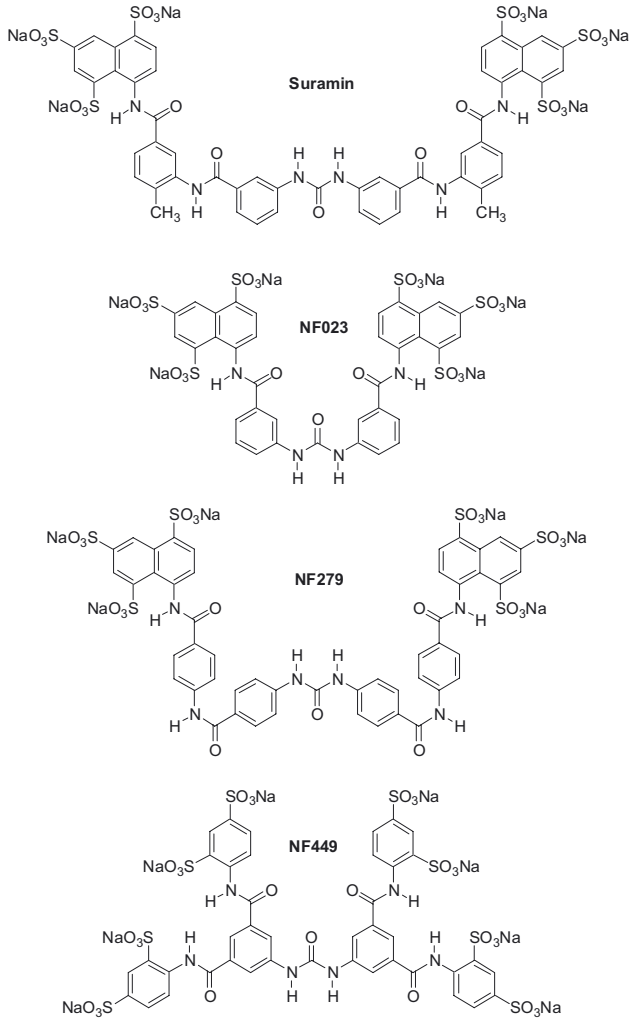

Fig. 1. Structural formula of suramin, NF023 (**10**), NF279 (**11**), and NF449 (**16b**).

The physiological function of P2 receptors has been explored with pharmacological and genetic approaches [1,5]. However, this evaluation has been hampered by a lack of subtype selective ligands, especially selective antagonists. Our group has provided an important advance by introducing the potent and selective $P2X_1$ antagonist NF449 and its "first-runners" NF023 and NF279 (Fig. 1), as well as PPADS and PPNDS [12–22]. Other potent $P2X_1$ antagonists are TNP-ATP and Ip_5I [5]. The suramin analogues NF023, NF279, and NF449 are at least 10-fold selective antagonists for $P2X_1$ receptors compared to $P2X_3$ whereas PPADS is about twofold selective for $P2X_1$, and TNP-ATP is a nanomolar, approximately sevenfold selective antagonist for $P2X_3$ vs. $P2X_1$ [5]. Very recently, a novel potent and selective nonnucleotide antagonist of $P2X_3$ receptors has been reported [23]. Except for $P2Y_1$ and $P2Y_{12}$ receptors, no selective and potent antagonists are available for P2Y receptors [5,11].

The purpose of this study was a systematic analysis of structural analogues of NF449 at various P2X and P2Y receptors. Since $P2X_1$ and $P2X_3$ receptors show functional

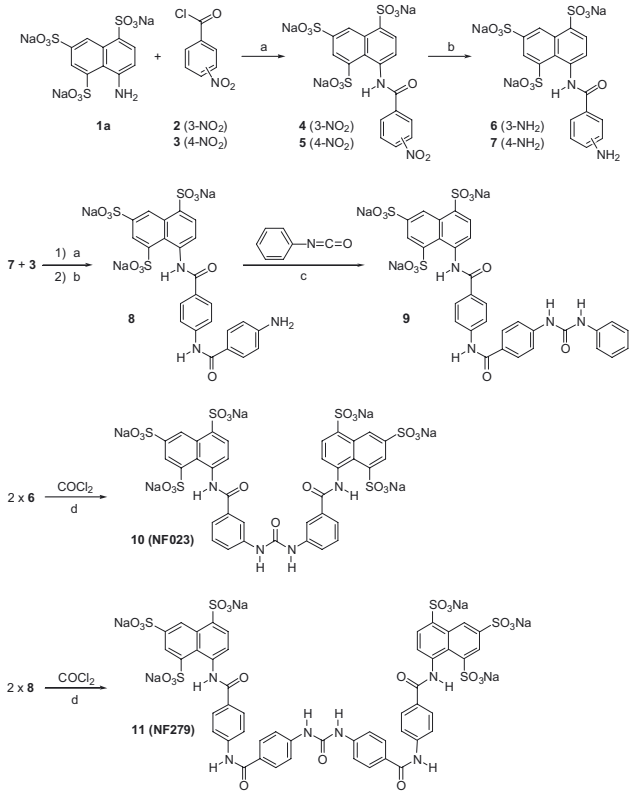

Fig. 2. Syntheses of compounds **9**, NF023 (**10**), and NF279 (**11**). Reagents/reaction conditions: (a) H_2O, pH 3.8 (**4**), pH 3.0 (**5** and **nitroprecursor of 8**); **2, 3** dissolved in toluene, 82.6% (**4**), 85% (**5**), 75% (**nitroprecursor of 8**). (b) Palladium (10%) on charcoal, H_2O, 98.2% (**6**), 85% (**7**), 74% (**8**). (c) H_2O, triethylamine, phenylisocyanate, diethylether, 70.8% (**9**). (d) H_2O, pH 3.5, phosgene (20%) in toluene, 84.6% (**10**), 84.6% (**11**).

similarity (rapid activation and inactivation) and may be of therapeutic importance as drug targets [5], the antagonistic properties of NF449 analogues were tested at these P2X receptor subtypes. Further, the selectivity of the test compounds was evaluated at adenine- and uracil-5′- nucleotide sensitive P2Y receptors, i.e., $P2Y_1$, $P2Y_2$, and $P2Y_{11}$. Besides the pharmacological results of the new NF449 analogues at P2X and P2Y receptors, the synthesis and analytical data of NF023, NF279, NF449 and analogues are presented which have not been published before.

2. Chemistry

Compounds derived from suramin and NF449 (**16b**) were synthesized. Variations in the arylsulfonic acid moieties (mono-, di-, or trisulfonic acids of benzene or naphthalene residues) and of the central urea bridge (replacement of the urea against the bisamide of terephthalic acid) have been introduced. Four types of compounds were synthesized: first, the suramin-like but asymmetric urea **9** (Fig. 2); second, the suramin-like symmetric ureas NF023 (**10**) and NF279 (**11**) (Fig. 2) which were described earlier but without providing analytical data [24]; third, NF449 (**16b**) and other symmetric ureas containing various arylmono-, di-, or trisulfonic acid

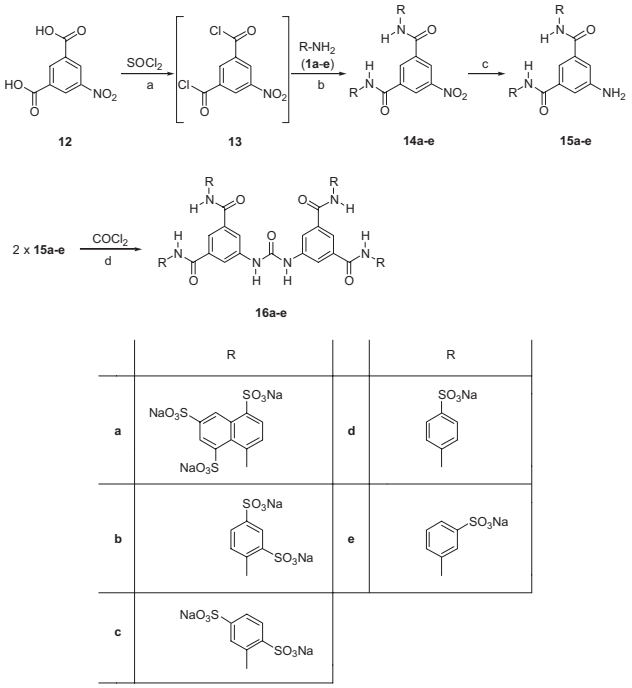

Fig. 3. Syntheses of NF449 (**16b**) and analogues with variations of the sulfonic acid residues. **13** was synthesized from **12** according to standard procedures and not isolated. Reagents/reaction conditions: (a) **12** (29.6 g, 140 mmol), toluene (60 ml), DMF (0.2 ml), thionylchloride (50 ml, 690 mmol), heating under reflux, excessive thionylchloride was removed under vacuum. (b) H_2O, pH 3.5 (**14a, 14b**, and **14c**), pH 5.0 (**14d** and **14e**), **13** dissolved in toluene, 76% (**14a**), 33.2% (**14b**), 58.7% (**14c**), 85% (**14d**), 79.6% (**14e**). (c) Palladium (10%) on charcoal, H_2O, 76% (**15a**), 82.5% (**15b**), 97.7% (**15c**), 89.4% (**15d**), 80.8% (**15e**). (d) H_2O, pH 4.0 (**16a, 16b, 16d**, and **16e**), pH 3.7 (**16c**), phosgene (20%) in toluene, 54.9% (**16a**), 54.6% (**16b**), 93.8% (**16c**), 87.5% (**16d**), 66.7% (**16e**).

groups (**16a–e**, Fig. 3); fourth, replacement of the central urea bridge in NF449 (**16b**) by terephthalic acid (**17**) resulting in the bisamide **19b** (Fig. 4). Syntheses of carboxylic acid chlorides (**13, 18**), carboxamides (**4, 5, 8, 14a–e, 19b**), reduction of nitro- to amine-groups (**6, 7, 8, 15a–e**), and syntheses of the ureas NF023 (**10**), NF279 (**11**), and **16a–e** by use of phosgene in toluene were all performed according to methods previously published [24,25]. The asymmetric urea **9** was synthesized according to Findeisen et al. [26]and Petersen et al. [27].

Identity of compounds was confirmed by nuclear magnetic resonance (NMR, ^1H, and ^{13}C), and IR spectroscopy. Protons and carbons were assigned to their respective ^1H and ^{13}C NMR signals as previously performed and published for other suramin analogues [25]. Further, electron spray mass spectrometry was performed. All synthesized compounds were obtained as sodium salts. Electron spray mass spectra were however monitored in a 5 mM ammonium acetate solution in methanol/H_2O (1:1). Thus, various anions (1–4 negative charges) with up to four sodium ions could be identified in the mass spectra of all compounds. Purity of compounds was demonstrated by elemental analysis (C, H, N) and by using thin layer chromatography (TLC) and a high-performance liquid chromatography method previously

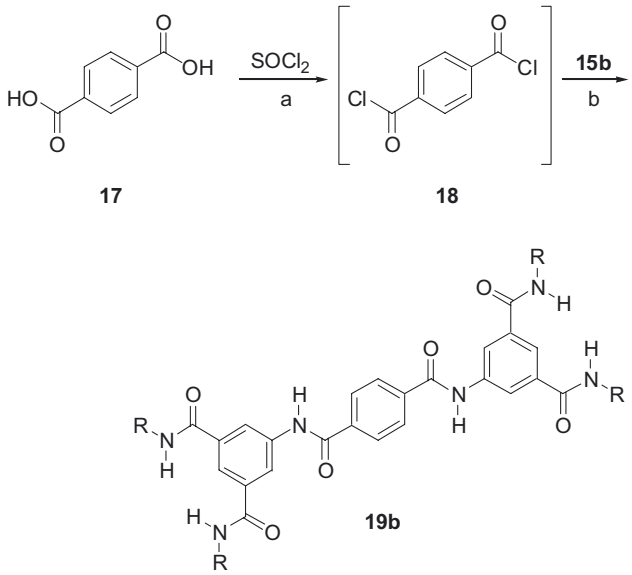

Fig. 4. Synthesis of the NF449 (**16b**) analogue **19b**. For residue "R", refer to table in Fig. 3. **18** was synthesized from **17** according to standard procedures and not isolated. Reagents/reaction conditions: (a) **17** (23.3 g, 140 mmol), toluene (50 ml), DMF (0.1 ml), thionylchloride (40 ml, 550 mmol), heating under reflux, excessive thionylchloride was removed under vacuum. (b) H_2O, pH 4.5, **18** dissolved in toluene, 22.8 % (**19b**).

published [28]. Due to the hygroscopic character of the synthesized compounds, the water content was estimated by Karl-Fischer titration analysis. Further, the sodium chloride content was estimated by titration analysis because sodium chloride may be present from the synthesis of the carboxamides and ureas. Sodium chloride content in all compounds was <5% except in the precursor compounds **14a** and **15a**. Water content estimated by Karl-Fischer titration was subtracted to evaluate elemental analysis data. Then, all elemental analysis data were within ± 0.4%, and HPLC purity was >95% for all compounds.

3. Pharmacology

All target compounds (ureas and bisamide of terephthalic acid) and the nitro- and amineprecursors of **16a–c** (**14a–c, 15a–c**, Fig. 3) were tested for blockade of various P2X and P2Y receptors. Test systems included the αβmeATP (10 μM) -stimulated rat vas deferens (native $P2X_1$ receptors), the αβmeATP (1 μM) -stimulated guinea pig ileum (native $P2X_3$), the ADPβS (10 μM) -stimulated guinea-pig ileum (native $P2Y_1$), UTP or ATP (3 μM) -stimulated HEK293 wildtype cells (native $P2Y_2$), and ATPγS (3 μM) -stimulated CHO cells recombinantly expressing human $P2Y_{11}$ receptors. The $P2X_1$, $P2X_3$, and $P2Y_1$ test systems have been widely used for the characterization of various test compounds including the suramin analogues NF023 (**10**), NF279 (**11**), and NF449 (**16b**) [13,14,29,30]. The used agonist concentrations of αβmeATP and ADPβS were their approximative corresponding EC_{50} values. HEK293 cells are known to

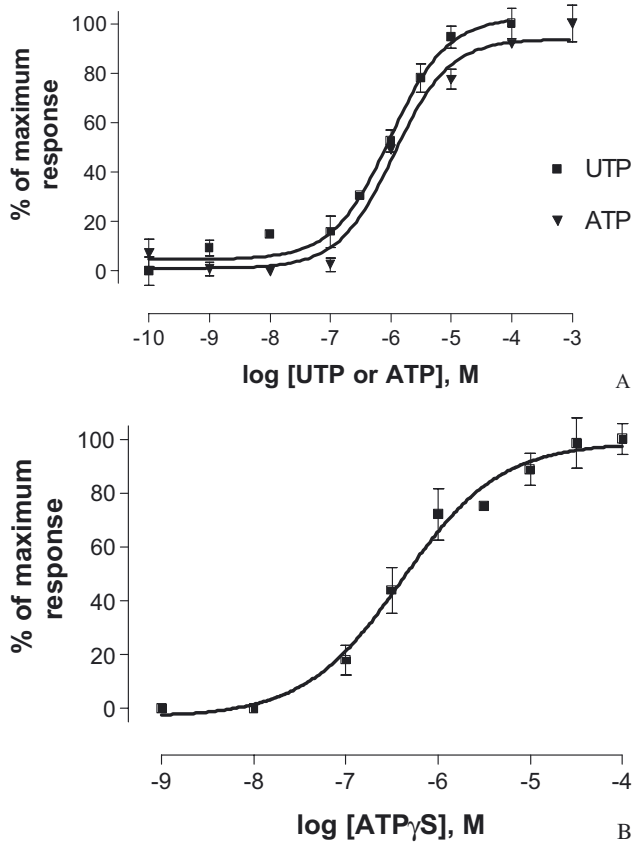

Fig. 5. Concentration–response curves of UTP and ATP in HEK293 wildtype cells (A) and of ATPγS in CHO cells recombinantly expressing $hP2Y_{11}$ receptors (B) using a recently described calcium assay [32]. Maximum increase in intracellular calcium was set as 100%. EC_{50} values were as follows (mean ± S.D.): UTP: 0.96 ± 0.20 μM; ATP: 1.13 ± 0.62 μM; ATPγS: 0.41 ± 0.07 μM. Slopes of all curves were not significantly different from unity.

express $P2Y_1$ and $P2Y_2$ receptors [31]. However, $P2Y_2$ receptors are about 10-fold more sensitive to ATP than $P2Y_1$ receptors, and UTP is selective for $P2Y_2$ receptors [5]. Fig. 5A shows that ATP and UTP were equipotent at native $P2Y_2$ receptors in HEK293 wildtype cells increasing intracellular calcium concentration. The same calcium assay [32] was used to monitore functional activation of $P2Y_{11}$ receptors recombinantly expressed in CHO cells after agonist stimulation with ATPγS. Fig. 5B shows a concentration–response curve of ATPγS at $P2Y_{11}$ receptors.

4. Results

Nitro- and amine-precursors **14a–c** and **15a–c** (10 μM) of the ureas **16a–c** did not display appreciable activities at any of the receptors under investigation (Table 1) except for the naphthalenetrisulfonic acid derivatives **14a** and **15a** which blocked the $P2Y_1$ receptor with pIC_{50} values of 5.05 and 5.03, respectively (Table 1). Even 100 μM **14a–c** and **15a–c** did not show any effect at $P2Y_2$ or $P2Y_{11}$ receptors (Table 1). Also, urea **16e** containing a benzene-3- sulfonic acid residue

Table 1
% Inhibition ± S.E.M. by precursors **14a–c** and **15a–c** and urea **16e** at various P2X and P2Y receptors

Compound	$P2X_1$ [a]	$P2X_3$ [b]	$P2Y_1$ [c]	$P2Y_2$ [d]	$P2Y_{11}$ [e]
14a	9.0 ± 4.9	15.4 ± 1.3	47.7 ± 11 [f]	NE	NE
14b	NE	5.8 ± 5.8	14.3 ± 4.9	NE	NE
14c	3.2 ± 1.7	5.1 ± 5.0	7.8 ± 3.5	NE	NE
15a	3.6 ± 2.0	32.9 ± 2.9	35.6 ± 8.5 [g]	NE	NE
15b	1.8 ± 0.4	NE	14.4 ± 1.5	NE	NE
15c	NE	NE	6.5 ± 3.3	NE	NE
16e	3.3 ± 2.9	ND	16.9 ± 5.9	NE	NE

NE means "no effect at 10 ($P2X_1$/$P2X_3$) or 100 ($P2Y_2$/$P2Y_{11}$) μM of test compound".
ND means "not determined".
[a] Rat vas deferens, 10 μM αβmeATP as agonist, 10 μM test compound.
[b] Guinea-pig ileum, 1 μM αβmeATP as agonist, 10 μM test compound.
[c] Guinea-pig ileum, 10 μM ADPβS as agonist, 10 μM test compound.
[d] HEK293 wildtype cells, 3 μM UTP or ATP as agonists, 100 μM test compound.
[e] Human recombinant receptors expressed in CHO cells, 3 μM ATPγS as agonist, 100 μM test compound.
[f] pIC_{50} = 5.05 ± 0.06.
[g] pIC_{50} = 5.03 ± 0.19.

showed weak antagonist potency only (Table 1) when tested at a concentration of 10 μM ($P2X_1$, $P2Y_1$) or 100 μM ($P2Y_2$, $P2Y_{11}$). pIC_{50} values of the corresponding ureas **16a–c**, the benzene-4-sulfonic acid derivative of NF449, **16d**, the terephthalic acid bisamide analogue of NF449, **19b**, along with pIC_{50} values of suramin and the suramin analogues NF023 (**10**), NF279 (**11**), and the asymmetric urea **9** are listed in Table 2. All synthesized compounds (precursors, ureas, or bisamide of terephthalic acid) were inactive or very weak antagonists at $P2Y_2$ or $P2Y_{11}$ receptors (Tables 1 and 2). The most potent compound at $P2Y_{11}$ was suramin (pIC_{50} = 4.73) which is however a non-selective and weak antagonist. Starting from NF023 (**10**), the selectivity and potency of the compounds for $P2X_1$ was increased with NF279 (**11**) and NF449 (**16b**) (Table 2) [13,14,16]. For the sake of a better comparison of potencies, Table 3 displays the relative potencies of the ureas and bisamide **19b** from Table 2 normalized to the activity of NF449 (**16b**) at $P2X_1$ receptors. Formal exchange of one half of the NF279 (**11**) molecule by a benzene residue gave the asymmetric urea **9**, and reduced the activity at $P2X_1$ by eightfold and further resulted in a loss of $P2X_1$ vs. $P2Y_1$ selectivity (Tables 2 and 3). Among the analogues of NF449 (**16b**) with different arylsulfonic acid moieties (**16a, 16c–e**), none achieved the potency and selectivity for $P2X_1$ of NF449 (**16b**) (Tables 1–3). NF449 displayed a pIC_{50} at $P2X_1$ receptors of 6.31 (using 10 μM αβmeATP, Table 2) and 7.15 (using 1 μM αβmeATP) [13], thus being at least 19-fold more potent at $P2X_1$ than at $P2X_3$, $P2Y_1$, $P2Y_2$, or $P2Y_{11}$ receptors (Table 3). Any deletion or change of position of sulfonic acid groups (**16c–e**) led to a decreased potency at $P2X_1$ receptors (Tables 1 and 2). However, introducing a naphthalenetrisulfonic acid residue (**16a**) instead of benzenemono- or disulfonic acids, led to a certain $P2Y_1$ selectivity [pIC_{50} ($P2Y_1$): 5.95; pIC_{50} ($P2X_1$): 5.32] whereas introducing a benzene-4-sulfonic acid moiety in-

Table 2
pIC_{50} ± S.E.M. values of suramin, the asymmetric urea **9**, NF023 (**10**), NF279 (**11**), NF449 (**16b**), and analogues of NF449 (**16a, 16c–d**, and **19b**) at various P2X and P2Y receptors

Compound	$P2X_1$ [a]	$P2X_3$ [b]	$P2Y_1$ [c]	$P2Y_2$ [d]	$P2Y_{11}$ [e]
Suramin	4.68 ± 0.02	4.36 ± 0.06	4.85 ± 0.08	4.32 ± 0.15 [h]	4.73 ± 0.27 [h]
9	4.80 ± 0.01	ND [f]	4.81 ± 0.01	<4	<4
10 (NF023)	4.93 ± 0.03	4.07 ± 0.20	4.52 ± 0.05	<4	3.98 ± 0.04 [h]
11 (NF279)	5.71 ± 0.09 [g]	4.71 ± 0.07	4.42 ± 0.01 [g]	~4 [g]	4.32 ± 0.01 [h]
16a	5.32 ± 0.02 [g]	4.87 ± 0.02	5.95 ± 0.13 [g]	<4	<4
16b (NF449)	6.31 ± 0.04 [g]	5.04 ± 0.08 [g]	4.85 ± 0.16 [g]	4.47 ± 0.22 [h]	<4
16c	4.40 ± 0.02	4.13 ± 0.01	5.08 ± 0.04	<4	<4
16d	4.80 ± 0.01	5.77 ± 0.12	3.55 ± 0.09	<4	<4
19b	5.47 ± 0.04	5.50 ± 0.02	4.39 ± 0.06	<4	<4

[a] Rat vas deferens, 10 μM αβmeATP as agonist.
[b] Guinea-pig ileum, 1 μM αβmeATP as agonist.
[c] Guinea-pig ileum, 10 μM ADPβS as agonist.
[d] HEK293 wildtype cells, 3 μM UTP or ATP as agonists.
[e] Human recombinant receptors expressed in CHO cells, 3 μM ATP γS as agonist.
[f] ND means "not determined".
[g] Data taken from Damer et al. [14], Braun et al. [13], and Lambrecht et al. [20].
[h] ±S.D. instead of S.E.M. values.

stead of aryldi-, or trisulfonic acids, yielded a $P2X_3$ preference of **16d** [pIC_{50} ($P2X_3$): 5.77; pIC_{50} ($P2X_1$): 4.80] (Table 2). Replacing the central urea bridge in NF449 (**16b**) by the bisamide of terephthalic acid resulting in compound **19b**, reduced the activity at $P2X_1$ by sevenfold and also led to a loss of selectivity for $P2X_1$ vs. $P2X_3$ receptors (Tables 2 and 3).

5. Discussion

Suramin has long been known to inhibit various P2 receptors [20,33]. However, suramin is neither potent nor selective for any of the P2X or P2Y receptor subtypes [5,20]. Our group has introduced NF023 (**10**), NF279 (**11**), and NF449 (**16b**) as selective and increasingly potent $P2X_1$ receptor antagonists (Table 2) but analytical data for these compounds have not yet been published [12–14,16]. In this study, structural variations of NF449 (**16b**), the so far most potent antagonist at $P2X_1$ receptors, have been synthesized and biologically evaluated at various P2 receptors to unravel structure–activity relationships. Furthermore, the synthesis of NF023 (**10**), NF279 (**11**), and an asymmetric urea derivative of NF279, compound **9**, and their analytical data are presented. NF449 (**16b**) derivatives include comprehensive variations of the benzene- or naphthalene-mono-, di-, or trisulfonic acid moieties (Fig. 3) and one variation of the urea bridge (replacement by the bisamide of terephthalic acid) but retaining the essential benzenedisulfonic acid residue of NF449, namely **1b**, resulting in compound **19b** (Fig. 4).

Table 3
Relative potencies of the ureas from Table 2 normalized to the potency of **16b** (NF449) at $P2X_1$ which was set as 100

Compound	$P2X_1$ [a]	$P2X_3$ [b]	$P2Y_1$ [c]	$P2Y_2$ [d]	$P2Y_{11}$ [e]
Suramin	2.3	1.1	3.5	1.0	2.6
9	3.1	ND [f]	3.2	<0.5	<0.5
10 (NF023)	4.2	0.6	1.6	<0.5	~0.5
11 (NF279)	25.1	2.5	1.3	~0.5	1.0
16a	10.2	3.6	43.7	<0.5	<0.5
16b (NF449)	100	5.4	3.5	1.4	<0.5
16c	1.2	0.7	5.9	<0.5	<0.5
16d	3.1	28.8	0.2	<0.5	<0.5
19b	14.5	15.5	1.2	<0.5	<0.5

[a] Rat vas deferens, 10 μM αβbmeATP as agonist.
[b] Guinea-pig ileum, 1 μM αβmeATP as agonist.
[c] Guinea-pig ileum, 10 μM ADPβS as agonist.
[d] HEK293 wildtype cells, 3 μM UTP or ATP as agonists.
[e] Human recombinant receptors expressed in CHO cells, 3 μM ATPγS as agonist.
[f] ND means "not determined".

Evaluation at P2 receptors of compounds **16a–e** with arylsulfonic acid variations and the NF449-like linker (5-nitroisophthalic acid, urea bridge) revealed that even slight variations of the aniline-2,4-disulfonic acid residue (deletion or change of position of sulfonic acid groups) resulted in a massive loss of potency at $P2X_1$ and/or selectivity for $P2X_1$ over $P2X_3$ or $P2Y_1$ receptors (Table 3). Furthermore, the linker region of the NF449 molecule is also sensitive to variations in terms of $P2X_1$ potency and selectivity: compound **19b** contains the aniline-2,4-disulfonic acid but the urea linker from NF449 (**16b**) has been exchanged against the bisamide of terephthalic acid (**17**). This exchange resulted in a sevenfold loss of potency at $P2X_1$ and a loss of selectivity for $P2X_1$ vs. $P2X_3$: **19b** was about equipotent at $P2X_1$ and $P2X_3$ receptors (pIC_{50} = 5.47 and 5.50, respectively, Table 2). Thus, two regions in the NF449 (**16b**) molecule have been identified in this study that are critical for a potent and selective antagonism at $P2X_1$ receptors: the benzene-2,4-disulfonic acid moieties and the urea linker.

A further question to be addressed was whether symmetric ureas or bisamides with two in the case of NF023 (**10**) and NF279 (**11**) or four in the case of NF449 (**16b**) arylsulfonic acid residues have to be present to antagonize potently P2X

receptors. There is a report from Bültmann et al. [29] that the compound BSt101, differing from NF023 (**10**) by removal of the three sulfonic acid groups from one of the naphthalene rings, is about equipotent to NF023 (**10**). The authors conclude that a second naphthalene-trisulfonic acid moiety as present in NF023 (**10**) may not be a precondition for "relatively high affinity" [29]. However, since both compounds, NF023 (**10**) and BSt101, are rather weak antagonists at $P2X_1$ receptors (pIC_{50}s < 5), the results of Bültmann et al. may be limited to NF023 (**10**) and BSt101. Furthermore, Lambrecht et al. [17,30] have introduced the P2 antagonists PPADS and SB9, hybrid compounds containing arylsulfonic acids and pyridoxal-5′-phosphate also bearing two anionic moieties. In the present study, we also found that among our compounds only those with at least two moieties containing anionic charges achieved a potent and selective blockade of $P2X_1$ receptors. The replacement of one half of NF279 (**11**) by a simple benzene moiety, resulting in the asymmetric urea **9**, led—compared to NF279 (**11**)—to a eightfold decrease in $P2X_1$ potency and loss of selectivity for $P2X_1$ vs. $P2Y_1$ receptors (Table 3). Furthermore, the precursor compounds **14a–c** and **15a–c** lacking a second anionic moiety, i.e., arylsulfonic acid residue, compared to their corresponding ureas **16a–c** or bisamide **19b** turned out to be inactive or very weak inhibitors at P2X or P2Y receptors (pIC_{50}s ≤ 5, Table 1). In contrast to Bültmann et al., in our study not only the sulfonic acid groups were removed from one naphthalene ring in NF279 but also the naphthalene and a further benzene residue resulting in compound **9**, and three aromatic residues and the urea bridge were missing in case of the precursor compounds **14a–c** and **15a–c**. The removed aromatic residues may however contribute to P2 receptor blockade. Thus, asymmetric NF449-derived ureas containing benzene instead of benzene-2,4-disulfonic acid residues or a NF279 analogue where only three sulfonic acid groups are removed from one of the naphthalene rings are necessary to clarify whether at least two anionic moieties in a distinct distance are compulsory for potent and selective $P2X_1$ receptor blockade.

NF023 (**10**) was the first $P2X_1$-selective suramin analogue displaying a sevenfold selectivity for $P2X_1$ over $P2X_3$ and a 2.6-fold selectivity for $P2X_1$ over $P2Y_1$ receptors (Table 3) [16]. An improvement in terms of $P2X_1$ selectivity and potency was achieved by introducing NF279 (**11**) (Table 3) [14]. NF449 (**16b**) showed a further improved potency at $P2X_1$ receptors and comparable selectivity to NF279 (**11**) (Table 3) [13]. All suramin analogues tested in this study are very weak or inactive at UTP and/or ATP preferring $P2Y_2$/$P2Y_{11}$ receptors (Tables 1 and 2). At $P2Y_1$ receptors which prefer nucleoside-diphosphates (ADP) over nucleosidetriphosphates as agonists, some of the tested compounds displayed an appreciable potency (Tables 2 and 3). The symmetrical urea **16a** with a urea spacer identical to that of NF449 (**16b**) but a naphthalene-trisulfonic acid residue like suramin, NF023 (**10**) and NF279 (**11**), showed a surprising fourfold selectivity for $P2Y_1$ over $P2X_1$ and 12-fold

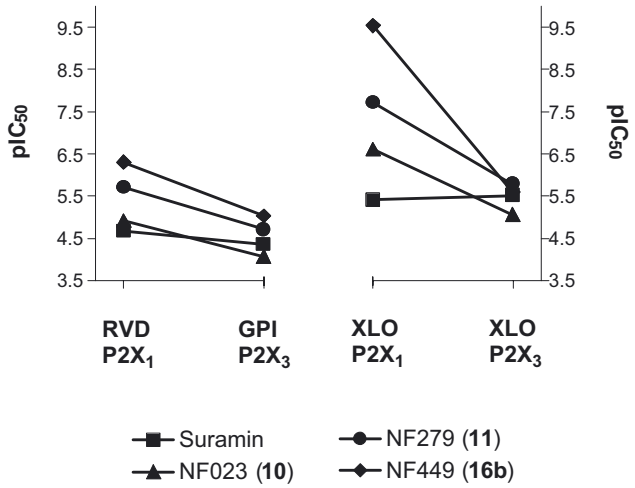

Fig. 6. Comparison of pIC_{50} values of suramin, NF023 (**10**), NF279 (**11**), and NF449 (**16b**) at native (RVD, GPI) vs. recombinant (XLO) rat $P2X_1$ and rat $P2X_3$ receptors. Data for RVD/$P2X_1$ and GPI/$P2X_3$ are from Table 2, data for XLO/$P2X_1$ and $P2X_3$ are taken from Refs. [13,21,22,34].

selectivity for $P2Y_1$ over $P2X_3$ receptors with a low μM-potency [pIC_{50} ($P2Y_1$): 5.95, Table 2]. $P2Y_1$ antagonists with nanomolar potency and highly selective for $P2Y_1$ receptors are already available: MRS2179 and MRS2279 [5]. However, so far selective and potent $P2Y_1$ antagonists among suramin derivatives have not yet been identified [5,20]. A $P2X_3$-selective compound was also found among the derivatives of NF449 (**16b**) namely **16d** (ninefold selective for $P2X_3$ over $P2X_1$, 144-fold selective for $P2X_3$ over $P2Y_1$ receptors, pIC_{50} ($P2X_3$): 5.77, Tables 2 and 3). Besides TNP-ATP which is about sevenfold selective for $P2X_3$ over $P2X_1$ receptors [5,20], compound A-317491 was recently described as a novel potent and selective non-nucleotide antagonist of $P2X_3$ and $P2X_{2/3}$ receptors (selectivity over other P2-receptors >100-fold) [23]. PPADS and isoPPADS are antagonists in the micromolar range, however they show no selectivity for $P2X_3$ but rather a small selectivity for $P2X_1$ receptors [19]. Thus, **16d** can serve as a lead compound for the development of $P2X_3$ selective antagonists derived from the suramin/NF449 (**16b**) family. All compounds tested for inhibition of ecto-nucleotidases (ecto-N), namely suramin, NF023 (**10**), NF279 (**11**), **16a**, NF449 (**16b**), **16c**, and **16d** were weak inhibitors of ecto-N (less than 45% inhibition of ecto-N at a concentration of 300 μM in folliculated *Xenopus laevis* oocytes) [20], thus confirming the $P2X_1$ (NF449-**16b**), $P2X_3$ (**16d**), or $P2Y_1$ (**16a**) selectivity of these three compounds.

$P2X_1$ and $P2X_3$ potencies and selectivities for suramin, NF023 (**10**), NF279 (**11**), and NF449 (**16b**) have previously also been evaluated at rat and human $P2X_1$ and rat $P2X_3$ receptors recombinantly expressed in *Xenopus laevis* oocytes (XLO) [12,13,21,22,34]. All compounds were 10–1000-fold more potent in the recombinant XLO assays than in the native test systems, rat vas deferens (RVD) for $P2X_1$, and guinea pig ileum (GPI) for $P2X_3$ (Fig. 6). With increasing potency at recombinant rat $P2X_1$ receptors, the selectivity

for $P2X_1$ over $P2X_3$ increased as well (Fig. 6). Whereas suramin remained approximately equipotent at rat $P2X_1$ and rat $P2X_3$ receptors in both the physiological RVD/GPI and the recombinant XLO assays, the selectivity for $P2X_1$ over $P2X_3$ increased in the recombinant system for NF023 (**10**) (RVD/GPI: sevenfold, XLO: 35-fold), NF279 (**11**) (RVD/GPI: 10-fold, XLO: 85-fold), and for NF449 (**16b**) (RVD/GPI: 19-fold, XLO: 8710-fold) (Fig. 6) [13,21,22,34]. The effect of increased potencies at recombinant P2 receptors may be due to depletion of suramin analogues in the RVD/GPI tissues used for estimation of pIC_{50} values at native P2 receptors [13]. However, further experiments are needed to clarify this issue. A similar apparent loss in potency has been observed for TNP-ATP and IP_5I which can be attributed to metabolic breakdown of these nucleotides in whole tissues [19].

6. Conclusion

In conclusion, in this study we present the synthesis and analytical data of the previously described $P2X_1$-selective antagonists NF023 (**10**), NF279 (**11**), and NF449 (**16b**) (Figs. 2 and 3). Furthermore, a series of NF449 (**16b**) analogues has been synthesized and evaluated at $P2X_1$, $P2X_3$, $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors (Figs. 3 and 4, Tables 1–3). New pharmacological data for NF023 (**10**), NF279 (**11**), and NF449 (**16b**) at $P2Y_2$ and $P2Y_{11}$ receptors are also presented (Table 2). NF449 (**16b**) remains the most potent so far known $P2X_1$ antagonist with submicromolar potency at rat vas deferens $P2X_1$ receptors (Table 2) and with subnanomolar potency at recombinant rat $P2X_1$ receptors (Fig. 6). Any deletions or shifts of the sulfonic acid residues or exchange of the central urea bridge by a bisamide of terephthalic acid in NF449 (**16b**) led to decreased activity at $P2X_1$ receptors and/or a loss of $P2X_1$ selectivity (Tables 2 and 3). Compounds **16a** and **16d** differing from NF449 (**16b**) only in the sulfonic acid residues showed a fourfold selectivity for $P2Y_1$ (**16a**) and a ninefold selectivity for $P2X_3$ (**16d**) compared to $P2X_1$ receptors, respectively (Tables 2 and 3). Thus, potential lead compounds for $P2Y_1$ and $P2X_3$ receptors related to suramin were identified.

7. Experimental protocols

7.1. Chemistry

Suramin, arylaminemono-, di-, and trisulfonic acids were gifts from Bayer AG (Leverkusen, Germany). All other reagents were purchased from Fluka, Aldrich, or Sigma (all: Taufkirchen, Germany).

^{1}H-NMR spectra were obtained with a Varian T 60 (60 MHz), a Varian XL 300 (300 MHz), and a Bruker DRX 500 (500 MHz) spectrometer (Karlsruhe, Germany) using DMSO-d_6 as a solvent and D_2O for H–D exchange. ^{13}C-NMR spectra were recorded by use of a Varian XL 300 (75 MHz) spectrometer using DMSO-d_6 as a solvent. NMR experiments were carried out at 15 °C. NMR chemical shifts are reported as δ values (ppm) downfield relative to Me_4Si which was used as internal standard (0 ppm). The following abbreviations are used: s (singlet), d (doublet), dd (double of doublet), pt (pseudotriplet), m (multiplet), ar (aromatic), br (broad), ex (exchangeable with D_2O), J (coupling constant in hertz). IR spectra were recorded with a FT-IR-spectrophotometer "Paragon 1000" from Perkin Elmer. Purity of compounds was checked by a previously published HPLC method [28]. Briefly, a Hewlett-Packard 1050 series HPLC apparatus equipped with a Hewlett-Packard MOS-Hypersil RP-C8 analytical column (5 μM, 100 × 2.1 mm) and a Hewlett-Packard MOS-Hypersil RP-C8 as precolumn (5 μM, 20 × 2.1 mm) was used. Temperature of the column was kept at 37 °C. The gradient solvent system consisted of a mixture of 6.25 mM tetrabutylammonium hydrogensulfate in 0.02 M phosphate buffer pH 6.5 and methanol starting at 80:20. A linear gradient was applied reaching a mixture of 46:54 within 8 min. The flow rate was maintained at 0.6 ml/min. Peaks were detected by UV absorption using a Hewlett-Packard 1040A diode array detector. All compounds tested for biological activity showed ≥95% purity in the HPLC analysis. Thin layer chromatography (TLC) was performed with all compounds on 20 × 20 cm aluminium sheets precoated with silica gel 60 F254 (Merck, Darmstadt, Germany). Elution solvent mixture was 2-propanol:ammonia (25%) = 5:2. TLC confirmed ≥95% purity for all compounds.

Low-resolution ES (electron spray) mass spectra were carried out with API2000 Applied Biosystems/MDS SCIEX LC/MS mass spectrometer from Applied Biosystems (Darmstadt, Germany). Solvent for the measurement was a solution of 5 mM ammonium acetate in a mixture of H_2O:methanol (1:1; pH 7). Elemental analyses (C, H, N) were performed on a Vario EL apparatus from Elementar (Hanau, Germany). Melting points of all compounds were greater than 300 °C (Mettler FP 61 apparatus, Giessen, Germany). NaCl content was estimated by potentiometric titration analysis on a titroprocessor 672 from Metrohm (Herisau, Switzerland) and found to be between 0.7% and 28.1%. Water content was measured by Karl-Fischer titration analysis using a Titrino 701 KT from Metrohm (Herisau, Switzerland). Between 2 and 16 mol H_2O per mol of compound were found. Yields are calculated for the pure compounds (without NaCl).

7.2. General acylation procedure. Synthesis of nitro derivatives *4, 5*, nitroprecursor of *8*, and *14a–e*

7.2.1. 8-(3-Nitrobenzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 2 H_2O (*4*)

1a (42.7 g, 100 mmol) was dissolved in water (400 ml) and the pH was adjusted to 3.8. To the stirred solution,

3-nitrobenzoylchloride **2** (26.2 g, 141 mmol) dissolved in 75 ml of toluene was slowly added. The reaction mixture was kept at a constant pH of 3.8 by automatic addition of a 2 M Na_2CO_3 solution. After separating the toluene from the water phase, pH was adjusted to 2.0, and the aqueous phase was extracted four times with 70 ml diethylether, respectively. After neutralisation (pH 7.0), the water was removed under vacuum. The crude product was purified by recrystallization from methanol. Yield: 49.4 g (82.6%). ^{1}H NMR (DMSO-d_6): δ 12.91 (br s,1H, NH, ex), 9.41 (d, 1H, ar, J = 1.9 Hz), 8.91 (pt, 1H, ar, J = 1.9 Hz), 8.62 (d, 1H, ar, J = 2.2 Hz), 8.60 (m, 1H, ar), 8.41 (dd, 1H, ar, J = 8.0, 2.2 Hz), 8.09 (d, 1H, ar, J = 8.2 Hz), 8.04 (d, 1H, ar, J = 7.9 Hz), 7.82 (pt, 1H, ar, J = 7.9 Hz). ^{13}C NMR (DMSO-d_6): δ 163.6 (C–O), 147.9 (ar, C–N), 143.0 (ar, C–S), 142.8 (ar, C–S), 141.3 (ar, C–S), 137.4 (ar, C–C), 134.5 (ar, C–H), 133.8 (ar, C–N), 131.5 (ar, C–C), 130.0 (ar, C–H), 127.0 (ar, C–H), 126.1 (ar, C–H), 125.7 (ar, C–H), 124.9 (ar, C–H), 123.4 (ar, C–C), 123.1 (ar, C–H), 123.0 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3468, 1632, 1534, 1353, 1245, 1193, 1044. NaCl: 1.4%. TLC: R_f 0.47. Anal. $C_{17}H_9N_2Na_3O_{12}S_3$ (C, H, N).

*7.2.2. 8-(4-Nitrobenzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 3 H_2O (**5**)*

Compound **5** was synthesized from **1a** and 4-nitrobenzoylchloride **3** according to Section 7.2.1. pH was kept constant at 3.0. Purification of the crude product was done by washing twice with ethanol (50 ml). Yield: 85%. ^{1}H NMR (DMSO-d_6): δ 12.86 (br s, 1H, NH, ex), 9.32 (d, 1H, ar, J = 1.9 Hz), 8.58 (d, 1H, ar, J = 2.0 Hz), 8.31 (dd, 2H, ar, J = 8.8, 2.2 Hz), 8.21 (dd, 2H, ar, J = 9.0, 2.2 Hz), 8.13 (d, 1H, ar, J = 8.3 Hz), 8.01 (d, 1H, ar, J = 8.1 Hz). ^{13}C NMR (DMSO-d_6): δ 163.7 (C–O), 148.6 (ar, C–N), 142.5 (ar, C–S), 142.2 (ar, C–C), 141.3 (ar, C–S), 141.0 (ar, C–S), 133.5 (ar, C–N), 131.1 (ar, C–C), 129.2 (ar, 2 C–H), 126.6 (ar, C–H), 125.6 (ar, C–H), 124.6 (ar, C–H), 123.0 (ar, C–C), 122.9 (ar, 2 C–H), 122.6 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3468, 1680, 1538, 1511, 1357, 1208, 1045. NaCl: 15.3%. TLC: R_f 0.43. Anal. $C_{17}H_9N_2Na_3O_{12}S_3$ (C, H, N).

*7.2.3. 8-(4-(4-Nitrobenzamido)-benzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 4 H_2O (nitroprecursor of **8**)*

The nitroprecursor of **8** was synthesized from **7** and 4-nitrobenzoylchloride **3** according to Section 7.2.1. pH was kept at 3.0. The crude product was purified by washing twice with ethanol (70 ml). Yield: 75%. ^{1}H NMR (DMSO-d_6): δ 12.59 (br s, 1H, NH, ex), 10.90 (br s, 1H, NH, ex), 9.41 (d, 1H, ar, J = 2.0 Hz), 8.64 (d, 1H, ar, J = 2.0 Hz), 8.37 (dd, 2H, ar, J = 9.0, 2.3 Hz), 8.26 (dd, 2H, ar, J = 8.8, 2.2 Hz), 8.17 (dd, 2H, ar, J = 9.0, 2.3 Hz), 8.10 (d, 1H, ar, J = 8.0 Hz), 8.01 (d, 1H, ar, J = 8.3 Hz), 7.95 (dd, 2H, ar, J = 8.8, 2.4 Hz). ^{13}C NMR (DMSO-d_6): δ 164.7 (C–O), 163.9 (C–O), 149.0 (ar, C–N), 142.3 (ar, C–S), 141.4 (ar, C–S), 141.2 (ar, C–N), 141.0 (ar, C–S), 140.2 (ar, C–C), 134.3 (ar, C–N), 131.1 (ar, C–C), 130.8 (ar, C–C), 129.2 (ar, 2 C–H), 128.6 (ar, 2 C–H), 126.5 (ar, C–H), 125.4 (ar, C–H), 124.7 (ar, C–H), 123.2 (ar, 2 C–H), 123.0 (ar, C–C), 122.2 (ar, C–H), 119.2 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3448, 1665, 1534, 1333, 1233, 1195, 1041. NaCl: 24.6%. TLC: R_f 0.33. Anal. $C_{24}H_{14}N_3Na_3O_{13}S_3$ (C, H, N).

*7.2.4. 8,8′-(5-Nitro-1,3-benzenediyl-bis(carbonylimino))-bis(naphthalene-1,3,5-trisulfonic acid) hexasodium salt × 4.5 H_2O (**14a**)*

14a was synthesized from **1a** and **13** according to Section 7.2.1. pH was kept at 3.5. Yield: 76%. ^{1}H NMR (DMSO-d_6): δ 12.98 (br s, 2H, NH, ex), 9.43 (d, 2H, ar, J = 2.2 Hz), 9.24 (d, 2H, ar, J = 1.3 Hz), 9.03 (pt, 1H, ar, J = 1.3 Hz), 8.64 (d, 2H, ar, J = 1.9 Hz), 8.12 (d, 2H, ar, J = 8.2 Hz), 8.06 (d, 2H, ar, J = 8.2 Hz). ^{13}C NMR (DMSO-d_6): δ 163.4 (2 C–O), 147.8 (ar, C–N), 143.0 (ar, 2 C–N), 142.7 (ar, 2 C–S), 141.4 (ar, 2 C–S), 137.4 (ar, 2 C–C), 134.3 (ar, 2 C–S), 133.8 (ar, C–H), 131.5 (ar, 2 C–C), 127.0 (ar, 2 C–H), 126.1 (ar, 2 C–H), 125.1 (ar, 2 C–H), 124.9 (ar, 2 C–H), 123.5 (ar, 2 C–C), 123.4 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3444, 3099, 1652, 1532, 1342, 1197, 1149, 1044. NaCl: 22.8%. TLC: R_f 0.14. Anal. $C_{28}H_{13}N_3Na_6O_{22}S_6$ (C, H, N).

*7.2.5. 4,4′-(5-Nitro-1,3-benzenediyl-bis(carbonylimino))-bis(benzene-1,3-disulfonic acid) tetrasodium salt × 3 H_2O (**14b**)*

14b was synthesized from **1b** and **13** according to Section 7.2.1. pH was kept at 3.5. Purification was done by recrystallization from water. Yield: 33.2%. ^{1}H NMR (DMSO-d_6): δ 12.00 (br s, 2H, NH, ex), 8.94 (pt, 1H, ar, J = 1.6 Hz), 8.91 (d, 2H, ar, J = 1.3 Hz), 8.47 (d, 2H, ar, J = 8.5 Hz), 8.06 (d, 2H, ar, J = 2.2 Hz), 7.65 (dd, 2H, ar, J = 8.5, 2.2 Hz). ^{13}C NMR (DMSO-d_6): δ 161.1 (2 C–O), 148.4 (ar, C–N), 143.0 (ar, 2 C–N), 136.6 (ar, 2 C–S), 134.7 (ar, 2 C–S), 134.4 (ar, 2 C–C), 132.1 (ar, C–H), 127.0 (ar, 2 C–H), 124.8 (ar, 2 C–H), 124.2 (ar, 2 C–H), 119.0 (ar, 2C–H). IR ν_{max} (KBr, cm^{-1}): 3468, 1696, 1625, 1591, 1540, 1229, 1187, 1040. NaCl: 1.7%. TLC: R_f 0.40. Anal. $C_{20}H_{11}N_3Na_4O_{16}S_4$ (C, H, N).

*7.2.6. 2,2′-(5-Nitro-1,3-benzenediyl-bis(carbonylimino))-bis(benzene-1,4-disulfonic acid) tetrasodium salt × 3 H_2O (**14c**)*

14c was synthesized from **1c** and **13** according to Section 7.2.1. pH was kept at 3.5. Purification was done by recrystallization in water. Yield: 58.7%. ^{1}H NMR (DMSO-d_6): δ 11.89 (br s, 2H, NH, ex), 8.94 (pt, 1H, J = 1.6 Hz), 8.92 (d, 2H, ar, J = 1.3 Hz), 8.81 (d, 2H, ar, J = 1.6 Hz), 7.71 (d, 2H, ar, J = 7.9 Hz), 7.41 (dd, 2H, ar, J = 7.9, 1.6 Hz). ^{13}C NMR (DMSO-d_6): δ 161.2 (2 C–O), 149.7 (ar, C–N), 148.6 (ar, 2 C–S), 137.0 (ar, 2 C–N), 135.9 (ar, 2 C–S), 134.1 (ar, 2 C–C), 132.4 (ar, C–H), 126.7 (ar, 2 C–H), 124.3 (ar, 2 C–H), 120.8 (ar, 2 C–H), 117.7 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3468, 1692, 1579, 1531, 1407, 1221, 1190. NaCl: 1.3%. TLC: R_f 0.43. Anal. $C_{20}H_{11}N_3Na_4O_{16}S_4$ (C, H, N).

*7.2.7. 4,4′-(5-Nitroisophthaloylbisimino)-bis(benzenesulfonic acid) disodium salt × 5 H_2O (**14d**)*

14d was synthesized from **1d** and **13** according to Section 7.2.1. pH was kept at 5. During the reaction, a precipitate of pure **14d** occurred. Yield: 85%. ^{1}H NMR (DMSO-d_6): δ 10.77 (br s, 2H, NH, ex), 9.00 (pt, 1H, ar, J = 1.6 Hz), 8.94 (d, 2H, ar, J = 1.6 Hz), 7.75 (dd, 4H, ar, J = 8.7, 1.8 Hz), 7.62 (dd, 4H, ar, J = 8.7, 1.8 Hz). ^{13}C NMR (DMSO-d_6): δ 163.1 (2 C–O), 147.9 (ar, C–N), 143.4 (ar, 2 C–S), 139.1 (ar, 2 C–N), 136.5 (ar, 2 C–C), 133.0 (ar, C–H), 126.2 (ar, 4 C–H), 125.2 (ar, 2 C–H), 119.8 (ar, 4 C–H). IR ν_{max} (KBr, cm^{-1}): 3440, 1670, 1590, 1530, 1400, 1230, 1190. NaCl: 1.0%. TLC: R_f 0.59. Anal. $C_{20}H_{13}N_3Na_2O_{10}S_2$ (C, H, N).

*7.2.8. 3,3′-(5-Nitroisophthaloylbisimino)-bis(benzenesulfonic acid) disodium salt × 3.5 H_2O (**14e**)*

14e was synthesized from **1e** and **13** according to Section 7.2.1. pH was kept constant at 5. Yield: 79.6%. ^{1}H NMR (DMSO-d_6): δ 10.84 (br s, 2H, NH, ex), 9.09 (pt, 1H, ar, J = 1.3 Hz), 8.96 (d, 2H, ar, J = 1.6 Hz), 8.09 (pt, 2H, ar, J = 1.3 Hz), 7.88 (dd, 2H, ar, J = 8.1, 1.3 Hz), 7.40 (dd, 2H, ar, J = 7.6, 1.3 Hz), 7.34 (pt, 2H, ar, J = 7.6 Hz). ^{13}C NMR (DMSO-d_6): δ 162.8 (2 C–O), 148.5 (ar, 2 C–S), 147.9 (ar, C–N), 138.1 (ar, 2 C–N), 136.4 (ar, 2 C–C), 132.9 (ar, C–H), 128.0 (ar, 2 C–H), 125.1 (ar, 2 C–H), 121.5 (ar, 2 C–H), 120.7 (ar, 2 C–H), 118.0 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3450, 1680, 1600, 1540, 1430, 1200, 1040. NaCl: 9.5%. TLC: R_f 0.66. Anal. $C_{20}H_{13}N_3Na_2O_{10}S_2$ (C, H, N).

*7.3. General hydrogenation procedure. Synthesis of amino derivatives **6, 7, 8, and 15a-e***

*7.3.1. 8-(3-Aminobenzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 3.5 H_2O (**6**)*

To a neutral solution of the nitro derivative **4** (31.3 g, 52 mmol) in 300 ml of water, palladium (10%) on charcoal was added as catalyst (350 mg; general: between 1% and 5% of the weight of the nitro compound). Under heavy stirring, the reaction mixture was hydrogenated under pressure (4.0 bar) in a Parr apparatus (Bonn, Germany). After the end of hydrogen absorption, the catalyst was removed by filtration. The aqueous phase was evaporated under vacuum yielding 29.2 g (98.2%) of **6**. ^{1}H NMR (DMSO-d_6): δ 12.44 (br s, 1H, NH, ex), 9.37 (d, 1H, ar, J = 1.9 Hz), 8.59 (d, 1H, ar, J = 1.9 Hz), 8.03 (d, 1H, ar, J = 7.9 Hz), 7.98 (d, 1H, ar, J = 8.2 Hz), 7.32 (dd, 1H, ar, J = 7.9, 1.6 Hz), 7.24 (pt, 1H, ar, J = 1.9 Hz), 7.09 (pt, 1H, ar, J = 7.9 Hz), 6.71 (dd, 1H, ar, J = 7.9, 2.2 Hz), 5.13 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 166.4 (C–O), 148.5 (ar, C–N), 142.8 (ar, C–S), 141.8 (ar, C–S), 141.6 (ar, C–S), 136.6 (ar, C–C), 134.9 (ar, C–N), 131.4 (ar, C–C), 128.4 (ar, C–H), 126.8 (ar, C–H), 125.7 (ar, C–H), 124.9 (ar, C–H), 123.3 (ar, C–H), 122.6 (ar, C–C), 116.6 (ar, C–H), 115.8 (ar, C–H), 114.0 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3436, 1652, 1528, 1489, 1338, 1199, 1043. NaCl: 1.3%. TLC: R_f 0.38. Anal. $C_{17}H_{11}N_2Na_3O_{10}S_3$ (C, H, N).

*7.3.2. 8-(4-Aminobenzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 1.5 H_2O (**7**)*

7 was synthesized from **5** according to Section 7.3.1. Yield: 85.1%. ^{1}H NMR (DMSO-d_6): δ 12.24 (br s, 1H, NH, ex), 9.28 (d, 1H, ar, J = 2.0 Hz), 8.57 (d, 1H, ar, J = 2.0 Hz), 8.09 (d, 1H, ar, J = 8.2 Hz), 7.92 (d, 1H, ar, J = 8.4 Hz), 7.75 (dd, 2H, ar, J = 8.7, 1.9 Hz), 6.65 (dd, 2H, ar, J = 8.7, 1.9 Hz), 5.60 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 165.2 (C–O), 151.3 (ar, C–N), 142.1 (ar, C–S), 141.3 (ar, C–S), 140.7 (ar, C–S), 135.0 (ar, C–N), 131.0 (ar, C–C), 129.6 (ar, 2 C–H), 126.4 (ar, C–H), 125.2 (ar, C–H), 124.7 (ar, C–H), 122.9 (ar, C–C), 122.2 (ar, C–C), 121.7 (ar, C–H), 112.2 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3527, 1624, 1508, 1287, 1204, 1188, 1043. NaCl: 14.9%. TLC: R_f 0.24. Anal. $C_{17}H_{11}N_2Na_3O_{10}S_3$ (C, H, N).

*7.3.3. 8-(4-(4-Aminobenzamido)-benzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 8 H_2O (**8**)*

8 was synthesized from the nitroprecursor **of 8** according to Section 7.3.1. Yield: 74%. ^{1}H NMR (DMSO-d_6): δ 12.53 (br s, 1H, NH, ex), 10.01 (br s, 1H, NH, ex), 9.32 (d, 1H, ar, J = 2.0 Hz), 8.60 (d, 1H, ar, J = 1.8 Hz), 8.12 (d, 1H, ar, J = 8.2 Hz), 8.01 (dd, 2H, ar, J = 8.8, 2.4 Hz), 7.99 (d, 1H, ar, J = 8.2 Hz), 7.79 (dd, 2H, ar, J = 8.8, 2.4 Hz), 7.70 (dd, 2H, ar, J = 8.6, 2.7 Hz), 6.63 (dd, 2H, ar, J = 8.8, 2.6 Hz), 5.78 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 165.3 (C–O), 164.8 (C–O), 152.1 (ar, C–N), 142.3 (ar, C–N), 142.2 (ar, C–S), 141.3 (ar, C–S), 141.2 (ar, C–S), 134.4 (ar, C–N), 131.1 (ar, C–C), 129.5 (ar, C–C), 129.3 (ar, 2 C–H), 128.5 (ar, 2 C–H), 126.5 (ar, C–H), 125.4 (ar, C–H), 124.7 (ar, C–H), 123.0 (ar, C–C), 122.2 (ar, C–H), 120.7 (ar, C–C), 118.7 (ar, 2 C–H), 112.4 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3436, 1636, 1513, 1232, 1188, 1044. NaCl: 25.2%. TLC: R_f 0.25. Anal. $C_{24}H_{16}N_3Na_3O_{11}S_3$ (C, H, N).

*7.3.4. 8,8′-(5-Amino-1,3-benzenediyl-bis(carbonylimino))-bis(naphthalene-1,3,5-trisulfonic acid) hexasodium salt × 4 H_2O (**15a**)*

15a was synthesized from **14a** according to Section 7.3.1. Yield: 76%. ^{1}H NMR (DMSO-d_6): δ 12.49 (br s, 2H, NH, ex), 9.39 (d, 2H, ar, J = 1.9 Hz), 8.61 (d, 2H, ar, J = 1.9 Hz), 8.06 (d, 2H, ar, J = 8.2 Hz), 7.96 (d, 2H, ar, J = 8.2 Hz), 7.75 (pt, 1H, ar, J = 1.6 Hz), 7.44 (d, 2H, ar, J = 1.3 Hz), 5.25 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 166.5 (2 C–O), 148.2 (ar, C–N), 142.7 (ar, 2 C–N), 141.9 (ar, 2 C–S), 141.5 (ar, 2 C–S), 136.5 (ar, 2 C–C), 134.9 (ar, 2 C–S), 131.4 (ar, 2 C–C), 126.8 (ar, 2 C–H), 125.7 (ar, 2 C–H), 125.1 (ar, 2 C–H), 123.5 (ar, 2 C–C), 123.1 (ar, 2 C–H), 116.3 (ar, C–H), 116.2 (ar, 2 C–H). IR ν_{max} (KBr, cm^{-1}): 3435, 1651, 1601, 1530, 1337, 1188, 1044. NaCl: 23.2%. TLC: R_f 0.11. Anal. $C_{28}H_{15}N_3Na_6O_{20}S_6$ (C, H, N).

*7.3.5. 4,4′-(5-Amino-1,3-benzenediyl-bis(carbonylimino))-bis(benzene-1,3-disulfonic acid) tetrasodium salt × 2 H_2O (**15b**)*

15b was synthesized from **14b** according to Section 7.3.1. Yield: 82.5%. ^{1}H NMR (DMSO-d_6): δ 11.36 (br s, 2H, NH, ex), 8.42 (d, 2H, ar, J = 8.5 Hz), 8.04 (d, 2H, ar, J = 2.2 Hz), 7.61 (pt, 1H, ar, J = 1.6 Hz), 7.59 (dd, 2H, ar, J = 8.5, 2.2 Hz), 7.27 (d, 2H, ar, J = 1.6 Hz), 5.67 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 164.6 (2 C–O), 149.6 (ar, C–N), 142.5 (ar, 2 C–N), 136.4 (ar, 2 C–S), 135.3 (ar, 2 C–S), 134.8 (ar, 2 C–C), 127.0 (ar, 2 C–H), 125.0 (ar, 2 C–H), 119.1 (ar, 2 C–H), 115.0 (ar, 2 C–H), 113.6 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3450, 1686, 1613, 1588, 1535, 1191, 1037. NaCl: 1.7%. TLC: R_f 0.37. Anal. $C_{20}H_{13}N_3Na_4O_{14}S_4$ (C, H, N).

*7.3.6. 2,2′-(5-Amino-1,3-benzenediyl-bis(carbonylimino))-bis(benzene-1,4-disulfonic acid) tetrasodium salt × 3.5 H_2O (**15c**)*

15c was synthesized from **14c** according to Section 7.3.1. Yield: 97.7%. ^{1}H NMR (DMSO-d_6): δ 11.25 (br s, 2H, NH, ex), 8.78 (d, 2H, ar, J = 1.6 Hz), 7.68 (d, 2H, ar, J = 7.9 Hz), 7.60 (pt, 1H, ar, J = 1.4 Hz), 7.34 (dd, 2H, ar, J = 7.9, 1.6 Hz), 7.27 (d, 2H, ar, J = 1.6 Hz), 5.67 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 164.5 (2 C–O), 149.6 (ar, 2 C–S), 149.4 (ar, C–N), 136.6 (ar, 2 C–N), 135.6 (ar, 2 C–S), 134.8 (ar, 2 C–C), 126.6 (ar, 2 C–H), 120.0 (ar, 2 C–H), 117.7 (ar, 2 C–H), 115.0 (ar, 2 C–H), 113.5 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3466, 1614, 1574, 1537, 1406, 1279, 1191, 1049, 1022. NaCl: 1.4%. TLC: R_f 0.40. Anal. $C_{20}H_{13}N_3Na_4O_{14}S_4$ (C, H, N).

*7.3.7. 4,4′-(5-Aminoisophthaloylbisimino)-bis(benzene-sulfonic acid) disodium salt × 6 H_2O (**15d**)*

15d was synthesized from **14d** according to Section 7.3.1. Yield: 89.4%. ^{1}H NMR (DMSO-d_6): δ 10.29 (br s, 2H, NH, ex), 7.73 (dd, 4H, ar, J = 8.9, 1.8 Hz), 7.67 (pt, 1H, ar, J = 1.3 Hz), 7.57 (dd, 4H, ar, J = 8.7, 1.8 Hz), 7.27 (d, 2H, ar, J = 1.6 Hz), 5.57 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 165.8 (2 C–O), 148.9 (ar, C–N), 143.2 (ar, 2 C–S), 139.4 (ar, 2 C–N), 135.9 (ar, 2 C–C), 126.0 (ar, 4 C–H), 119.2 (ar, 4 C–H), 115.8 (ar, 2 C–H), 113.9 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3400, 1660, 1640, 1590, 1530, 1400, 1200, 1030. NaCl: 2.8%. TLC: R_f 0.49. Anal. $C_{20}H_{15}N_3Na_2O_8S_2$ (C, H, N).

*7.3.8. 3,3′-(5-Aminoisophthaloylbisimino)-bis(benzene-sulfonic acid) disodium salt × 1.5 H_2O (**15e**)*

15e was synthesized from **14e** according to Section 7.3.1. Yield: 78%. ^{1}H NMR (DMSO-d_6): δ 10.27 (br s, 2H, NH, ex), 8.09 (s, 2H, ar), 7.79 (dd, 2H, ar, J = 8.1, 1.3 Hz), 7.73 (s, 1H, ar), 7.34 (dd, 2H, ar, J = 7.6, 1.3 Hz), 7.29 (d, 2H, ar, J = 1.6 Hz), 7.28 (pt, 2H, ar, J = 7.6 Hz), 5.55 (br s, 2H, NH, ex). ^{13}C NMR (DMSO-d_6): δ 165.7 (2 C–O), 149.0 (ar, C–H), 148.2 (ar, 2 C–S), 138.7 (ar, 2 C–N), 135.7 (ar, 2 C–C), 127.7 (ar, 2 C–H), 120.8 (ar, 2 C–H), 120.5 (ar, 2 C–H), 117.8 (ar, 2 C–H), 115.9 (ar, 2 C–H), 114.0 (ar, C–H). IR ν_{max} (KBr, cm^{-1}): 3350, 1650, 1590, 1540, 1420, 1200, 1040. NaCl: 21.8%. TLC: R_f 0.60. Anal. $C_{20}H_{15}N_3Na_2O_8S_2$ (C, H, N).

*7.4. General phosgenation procedure. Synthesis of urea derivatives **10, 11**, and **16a–e***

*7.4.1. 8,8′-(Carbonylbis(imino-3,1-phenylenecarbonylimino))bis(naphthalene-1,3,5-trisulfonic acid) hexasodium salt × 10 H_2O (**10**)*

To a solution of **6** (27.5 g, 48 mmol) in water (300 ml; pH was adjusted between 3.0 and 4.0 with 5 N NaOH) a solution of phosgene (20% in toluene, 50 ml, 100 mmol) was slowly added under heavy stirring at room temperature. The reaction mixture was maintained at pH 3.5 by automatic addition of 2 M Na_2CO_3 solution. The aqueous phase was then neutralised with 2 M Na_2CO_3. Water was evaporated under vacuum. NaCl was removed by stirring the crude product three times in methanol (100 ml). **10** was nearly insoluble in methanol. Yield: 23.6 g (84.6%). ^{1}H NMR (DMSO-d_6): δ 12.62 (br s, 2H, NH, ex), 9.40 (d, 2H, ar, J = 1.9 Hz), 8.96 (br s, 2H, NH, ex), 8.63 (d, 2H, ar, J = 1.9 Hz), 8.07 (d, 2H, ar, J = 8.2 Hz), 8.04 (d, 2H, ar, J = 8.2 Hz), 7.98 (pt, 2H, ar, J = 1.9 Hz), 7.85 (dd, 2H, ar, J = 8.2, 1.9 Hz), 7.82 (dd, 2H, ar, J = 8.2, 1.9 Hz), 7.40 (pt, 2H, ar, J = 8.2 Hz). ^{13}C NMR (DMSO-d_6): δ 165.6 (2 C–O), 152.8 (C–O), 142.8 (ar, 2 C–S), 142.1 (ar, 2 C–S), 141.5 (ar, 2 C–S), 139.7 (ar, 2 C–N), 136.5 (ar, 2 C–C), 134.6 (ar, 2 C–N), 131.5 (ar, 2 C–C), 128.6 (ar, 2 C–H), 126.9 (ar, 2 C–H), 125.9 (ar, 2 C–H), 125.0 (ar, 2 C–H), 123.4 (ar, 2 C–H), 122.9 (ar, 2 C–C), 121.7 (ar, 2 C–H), 121.0 (ar, 2 C–H), 118.3 (ar, 2 C–H). ES-MS: calcd./found (*m/z*): 1028.8/1028.6 [M–H]$^-$, 1050.9/1050.7 [M+Na–2H]$^-$, 514.0/514.2 [M–2H]$^{2-}$. IR ν_{max} (KBr, cm^{-1}): 3466, 1652, 1589, 1538, 1338, 1201, 1043. NaCl: 1.8%. TLC: R_f 0.11. Anal. $C_{35}H_{20}N_4Na_6O_{21}S_6$ (C, H, N).

*7.4.2. 8,8′-(Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonyl imino))bis (naphthalene-1,3,5-trisulfonic acid) hexasodium salt × 8.5 H_2O (**11**)*

11 was synthesized from **8** according to Section 7.4.1. Yield: 84.6%. ^{1}H NMR (DMSO-d_6): δ 12.57 (br s, 2H, NH, ex), 10.31 (br s, 2H, NH, ex), 9.53 (br s, 2H, NH, ex), 9.33 (d, 2H, ar, J = 1.8 Hz), 8.61 (d, 2H, ar, J = 2.0 Hz), 8.15 (d, 2H, ar, J = 8.3 Hz), 7.94 (d, 4H, ar, J = 8.7 Hz), 7.91 (d, 2H, ar, J = 8.4 Hz), 7.77 (d, 4H, ar, J = 8.7 Hz), 7.71 (d, 4H, ar, J = 8.7 Hz), 7.36 (d, 4H, ar, J = 8.7 Hz). ^{13}C NMR (DMSO-d_6): δ 165.0 (2 C–O), 164.8 (2 C–O), 152.0 (C–O), 142.7 (ar, 2 C–N), 142.3 (ar, 2 C–S), 141.8 (ar, 2 C–N), 141.3 (ar, 2 C–S), 141.2 (ar, 2 C–S), 134.5 (ar, 2 C–N), 131.1 (ar, 2 C–C), 130.0 (ar, 2 C–C), 128.7 (ar, 4 C–H), 128.6 (ar, 4 C–H), 127.6 (ar, 2 C–C), 126.6 (ar, 2 C–H), 125.5 (ar, 2 C–H), 124.8 (ar, 2 C–H), 123.1 (ar, 2 C–C), 122.4 (ar, 2 C–H), 119.0 (ar, 4 C–H), 117.2 (ar, 4 C–H). ES-MS: calcd./found (*m/z*): 1267.0/1266.7 [M–H]$^-$, 1289.0/1288.6 [M+Na–2H]$^-$, 633.0/633.0 [M–2H]$^{2-}$. IR ν_{max} (KBr, cm^{-1}):

3436, 1655, 1594, 1529, 1321, 1236, 1185, 1042. NaCl: 1.0%. TLC: R_f 0.06. Anal. $C_{49}H_{30}N_6Na_6O_{23}S_6$ (C, H, N).

*7.4.3. 8,8′,8″,8‴-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))tetrakis-(naphthalene-1,3,5-trisulfonic acid) dodecasodium salt × 9.5 H_2O (**16a**)*

16a was synthesized from **15a** according to Section 7.4.1. pH was kept at 4.0. Yield: 54.9%. ^{1}H NMR (DMSO-d_6): δ 12.66 (br s, 4H, NH, ex), 9.42 (d, 4H, ar, J = 1.6 Hz), 9.17 (br s, 2H, NH, ex), 8.63 (d, 4H, ar, J = 1.9 Hz), 8.38 (s, 4H, ar), 8.29 (s, 2H, ar), 8.09 (d, 4H, ar, J = 8.2 Hz), 8.00 (d, 4H, ar, J = 8.2 Hz). ^{13}C NMR (DMSO-d_6): δ 166.4 (4 C–O), 153.5 (ar, C–O), 143.5 (ar, 2 C–N), 142.8 (ar, 4 C–N), 142.1 (ar, 4 C–S), 140.1 (ar, 4 C–S), 137.2 (ar, 4 C–C), 135.3 (ar, 4 C–S), 132.2 (ar, 4 C–C), 127.6 (ar, 4 C–H), 126.6 (ar, 4 C–H), 125.8 (ar, 4 C–H), 124.3 (ar, 4 C–C), 124.0 (ar, 4 C–H), 122.6 (ar, 2 C–H), 121.4 (ar, 4 C–H). ES-MS: calcd./found (*m/z*): 614.9/614.9 $[M–3H]^{3–}$, 622.3/622.2 $[M+Na–4H]^{3–}$, 629.6/629.5 $[M+2Na–5H]^{3–}$. IR ν_{max} (KBr, cm^{-1}): 3446, 1653, 1533, 1339, 1199, 1047. NaCl: 0.7%. TLC: R_f 0.01. Anal. $C_{57}H_{28}N_6Na_{12}O_{41}S_{12}$ (C, H, N).

*7.4.4. 4,4′,4″,4‴-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakisbenzene-1,3-disulfonic acid octasodium salt × 7 H_2O (**16b**)*

16b was synthesized from **15b** according to Section 7.4.1. pH was kept at 4.0. Yield: 54.6%. ^{1}H NMR (DMSO-d_6): δ 11.59 (br s, 4H, NH, ex), 9.43 (br s, 2H, NH, ex), 8.47 (d, 4H, ar, J = 8.4 Hz), 8.24 (s, 4H, ar), 8.12 (s, 2H, ar), 8.05 (d, 4H, ar, J = 2.2 Hz), 7.61 (dd, 4H, ar, J = 8.4, 2.2 Hz). ^{13}C NMR (DMSO-d_6): δ 163.7 (4 C–O), 152.6 (C–O), 142.8 (ar, 4 C–N), 140.6 (ar, 2 C–N), 136.4 (ar, 4 C–S), 135.2 (ar, 4 C–S), 134.8 (ar, 4 C–C), 127.1 (ar, 4 C–H), 125.0 (ar, 4 C–H), 120.0 (ar, 4 C–H), 119.6 (ar, 4 C–H), 119.2 (ar, 2 C–H). ES-MS: calcd./found (*m/z*): 1326.9/1327.1 $[M–H]^–$, 1348.9/1348.8 $[M+Na–2H]^–$, 662.9/663.2 $[M–2H]^{2–}$. IR ν_{max} (KBr, cm^{-1}): 3435, 1684, 1589, 1533, 1184, 1134, 1040. NaCl: 0.8%. TLC: R_f 0.13. Anal. $C_{41}H_{24}N_6Na_8O_{29}S_8$ (C, H, N).

*7.4.5. 2,2′,2″,2‴-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakisbenzene-1,4-disulfonic acid octasodium salt × 9 H_2O (**16c**)*

16c was synthesized from **15c** according to Section 7.4.1. pH was kept at 3.7. Yield: 93.8%. ^{1}H NMR (DMSO-d_6): δ 11.48 (br s, 4H, NH, ex), 9.44 (br s, 2H, NH, ex), 8.82 (d, 4H, ar, J = 1.6 Hz), 8.24 (d, 4H, ar, J = 1.3 Hz), 8.14 (pt, 2H, ar, J = 1.2 Hz), 7.72 (d, 4H, ar, J = 8.2 Hz), 7.38 (dd, 4H, ar, J = 8.2 Hz). ^{13}C NMR (DMSO-d_6): δ 163.6 (4 C–O), 152.6 (C–O), 149.6 (ar, 4 C–S), 140.5 (ar, 4 C–N), 136.5 (ar, 4 C–S), 135.7 (ar, 2 C–N), 134.6 (ar, 4 C–C), 126.6 (ar, 4 C–H), 120.3 (ar, 4 C–H), 120.0 (ar, 4 C–H), 119.7 (ar, 2 C–H), 117.8 (ar, 4 C–H). ES-MS: calcd./found (*m/z*): 662.9/663.0 $[M–2H]^{2–}$, 673.9/674.0 $[M+Na–3H]^{2–}$, 441.6/441.8 $[M–3H]^{3–}$. IR ν_{max} (KBr, cm^{-1}): 3444, 1678, 1608, 1578, 1532, 1409, 1188, 1121, 1019. NaCl: 3.2%. TLC: R_f 0.18. Anal. $C_{41}H_{24}N_6Na_8O_{29}S_8$ (C, H, N).

*7.4.6. 4,4′,4″,4‴-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakisbenzene-sulfonic acid tetrasodium salt × 9.5 H_2O (**16d**)*

16d was synthesized from **15d** according to Section 7.4.1. pH was kept at 4.0. Yield: 87.5%. ^{1}H NMR (DMSO-d_6): δ 10.46 (br s, 4H, NH, ex), 9.30 (br s, 2H, NH, ex), 8.22 (d, 4H, ar, J = 1.3 Hz), 8.18 (pt, 2H, ar, J = 1.3 Hz), 7.75 (dd, 8H, ar, J = 8.7, 1.8 Hz), 7.59 (dd, 8H, ar, J = 8.7, 1.8 Hz). ^{13}C NMR (DMSO-d_6): δ 165.1 (4 C–O), 152.6 (C–O), 143.4 (ar, 4 C–S), 139.9 (ar, 2 C–N), 139.2 (ar, 4 C–N), 135.7 (ar, 4 C–C), 126.0 (ar, 8 C–H), 120.6 (ar, 4 C–H), 120.1 (ar, 2 C–H), 119.3 (ar, 8 C–H). ES-MS: calcd./found (*m/z*): 1007.1/1006.9 $[M–H]^–$, 1029.1/1029.0 $[M+Na–2H]^–$, 503.0/503.1 $[M–2H]^{2–}$. IR ν_{max} (KBr, cm^{-1}): 3400, 1670, 1600, 1580, 1540, 1400, 1330, 1195, 1130, 1040, 1010. NaCl: 1.8%. TLC: R_f 0.40. Anal. $C_{41}H_{28}N_6Na_4O_{17}S_4$ (C, H, N).

*7.4.7. 3,3′,3″,3‴-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino)))tetrakisbenzene-sulfonic acid tetrasodium salt × 10 H_2O (**16e**)*

16e was synthesized from **15e** according to Section 7.4.1. pH was kept at 4.0. The product precipitated during the reaction. Yield: 66.7%. ^{1}H NMR (DMSO-d_6): δ 10.56 (br s, 4H, NH, ex), 10.02 (br s, 2H, NH, ex), 8.29 (s, 2H, ar), 8.24 (d, 4H, ar, J = 1.3 Hz), 8.13 (s, 4H, ar), 7.86 (dd, 4H, ar, J = 8.2, 1.3 Hz), 7.37 (dd, 4H, ar, J = 7.8, 1.3 Hz), 7.31 (pt, 4H, ar, J = 7.9 Hz). ^{13}C NMR (DMSO-d_6): δ 165.0 (4 C–O), 152.8 (C–O), 148.4 (ar, 4 C–S), 140.1 (ar, 2 C–N), 138.5 (ar, 4 C–N), 135.6 (ar, 4 C–C), 127.8 (ar, 4 C–H), 121.0 (ar, 4 C–H), 120.5 (ar, 4 C–H), 120.4 (ar, 4 C–H), 120.1 (ar, 2 C–H), 117.9 (ar, 4 C–H). ES-MS: calcd./found (*m/z*): 1007.1/1006.8 $[M–H]^–$, 1029.1/1028.8 $[M+Na–2H]^–$, 503.0/503.2 $[M–2H]^{2–}$. IR ν_{max} (KBr, cm^{-1}): 3440, 1660, 1590, 1535, 1200, 1030. NaCl: 28.1%. TLC: R_f 0.53. Anal. $C_{41}H_{28}N_6Na_4O_{17}S_4$ (C, H, N).

*7.5. Synthesis of 8-(4-(4-((N-Phenylcarbamoyl)-amino))-benzamido)-benzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt × 4.5 H_2O (**9**)*

Compound **8** (0.5 g, 0.7 mmol) was dissolved in water (40 ml) and 0.5 ml triethylamine were added. Phenylisocyanate (0.9 ml, 8.4 mmol) was added in drops over a period of 36 h. Subsequently, the aqueous phase was exhaustively extracted with diethylether. Compound was dried under vacuum, and the crude product was stirred in methanol for purification. Yield: 0.4 g of **9** (70.8%). ^{1}H NMR (DMSO-d_6): δ 12.57 (br s, 1H, NH, ex), 10.27 (br s, 1H, NH, ex), 9.28 (br s, 1H, NH, ex), 9.04 (br s, 1H, NH, ex), 9.33 (d, 1H, ar, J = 2.0 Hz), 8.61 (d, 1H, ar, J = 1.8 Hz), 8.13 (d, 1H, ar, J = 8.3 Hz), 8.01 (dd, 2H, ar, J = 8.7, 2.0 Hz), 7.97 (d, 1H, ar, J = 8.2 Hz), 7.86 (dd, 2H, ar, J = 8.7, 1.9 Hz), 7.79 (dd, 2H, ar, J = 8.9, 2.0 Hz), 7.48 (dd, 2H, ar, J = 8.8, 1.8 Hz), 7.35 (dd, 2H, ar, J = 8.5, 1.2 Hz), 7.25 (pt, 2H, ar, J = 8.4 Hz), 6.99 (pt, 1H, ar, J = 7.6 Hz). ^{13}C NMR (DMSO-d_6): δ 164.9 (C–O),

164.7 (C–O), 152.1 (C–O), 142.9 (ar, C–N), 142.3 (ar, C–S), 141.7 (ar, C–N), 141.3 (ar, C–S), 141.2 (ar, C–S), 139.3 (ar, C–N), 134.4 (ar, C–N), 131.1 (ar, C–C), 130.0 (ar, C–C), 128.6 (ar, 2 C–H), 128.5 (ar, 4 C–H), 127.3 (ar, C–C), 126.5 (ar, C–H), 125.4 (ar, C–H), 124.7 (ar, C–H), 123.0 (ar, C–C), 122.2 (ar, C–H), 121.8 (ar, C–H), 118.9 (ar, 2 C–H), 118.2 (ar, 2 C–H), 116.9 (ar, 2 C–H). ES-MS: calcd./found (*m/z*): 739.1/739.2 $[M–H]^-$, 761.0/761.2 $[M+Na–2H]^-$, 369.0/369.2 $[M–2H]^{2-}$. IR ν_{max} (KBr, cm^{-1}): 3467, 1671, 1652, 1596, 1531, 1512, 1326, 1187, 1046. NaCl: 4.9%. TLC: R_f 0.27. Anal. $C_{31}H_{21}N_4Na_3O_{12}S_3$ (C, H, N).

*7.6. Synthesis of 4,4′,4″,4‴-(Terephthaloylbis(imino-5,1,3-benzenetriylbis-(carbonylimino-methylene)))tetrakis-benzene-1,3-disulfonic acid octasodium salt × 16 H_2O (**19b**)*

15b (5.0 g, 6.8 mmol) was dissolved in 60 ml water. Terephthaloyldichloride **18** (1.4 g, 7 mmol, dissolved in toluene, 20 ml) was slowly dropped into the aqueous solution of **15b**, and the pH was kept at 4.5 by automatic addition of a 2 M Na_2CO_3 solution. After finishing of the reaction, the water phase was separated from the toluene and extracted four times with diethylether (each 20 ml) at pH 2. After neutralisation, water was removed under vacuum. The crude product was purified by recrystallization in water. Yield: 1.1 g of **19b** (22.8%). ^{1}H NMR (DMSO-d_6): δ 11.61 (br s, 4H, NH, ex), 10.96 (br s, 2H, NH, ex), 8.56 (d, 4H, ar, J = 1.3 Hz), 8.44 (d, 4H, ar, J = 8.7 Hz), 8.23 (s, 2H, ar), 8.21 (s, 4H, ar), 8.05 (d, 4H, ar, J = 2.1 Hz), 7.62 (dd, 4H, ar, J = 8.4, 2.1 Hz). ^{13}C NMR (DMSO-d_6): δ 166.3 (2 C–O), 164.6 (4 C–O), 143.6 (ar, 4 C–S), 140.9 (ar, 2 C–N), 138.1 (ar, 4 C–C), 137.1 (ar, 4 C–N), 136.0 (ar, 4 C–S), 135.7 (ar, 2 C–C), 129.0 (ar, 4 C–H), 128.0 (ar, 4 C–H), 125.9 (ar, 4 C–H), 123.5 (ar, 4 C–H), 121.4 (ar, 2 C–H), 120.2 (ar, 4 C–H). ES-MS: calcd./found (*m/e*): 715.0/715.1 $[M–2H]^{2-}$, 476.3/476.6 $[M–3H]^{3-}$, 483.6/483.7 $[M+Na–4H]^{3-}$. IR ν_{max} (KBr, cm^{-1}): 3470, 1680, 1590, 1530, 1320, 1200, 1040. NaCl: 0.9%. TLC: R_f 0.27. Anal. $C_{48}H_{28}N_6Na_8O_{30}S_8$ (C, H, N).

7.7. Pharmacology

7.7.1. Contractions in the rat vas deferens and guinea-pig ileum

The methods used have been described in detail previously [13,18,30,35]. Briefly, prostatic segments of vasa deferentia from rats were set up for isometric tension measurement in modified Krebs solution (37 °C, pH 7.4, gassed with 95% O_2/5% CO_2) as were strips of guinea-pig ileum longitudinal smooth muscle. Contractions were elicited to single doses of αβmeATP in rat vas deferens (10 μM) or guinea-pig ileum (1 μM) and to ADPβS in ileum (10 μM) leaving 15–60 min before the next dose of agonist was added. Time-matched controls demonstrated that during the course of experiments there was no significant change in tissue sensitivity towards the nucleotide agonists. The antagonists were allowed to equilibrate for 60–120 min, and preliminary experiments indicated that these intervals were sufficient for equilibration of the antagonist concentrations used. Each concentration of antagonists to inhibit the contractile responses to αβmeATP or ADPβS was tested at least three times on rat vas deferens or guinea-pig ileum from at least two different animals. αβmeATP and ADPβS were obtained from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals were of the highest grade available, and used as purchased.

7.7.2. Cell culture and measurements of intracellular calcium

Wildtype HEK293 cells were cultured in Dulbecco's modified Eagle Medium Nutrient Mixture F-12 Ham (DMEM/F12 1:1 Mixture) (Sigma-Aldrich) containing 100 μg/ml streptomycin, 100 U/ml penicillin G, 10% fetal bovine serum (Sigma-Aldrich), and 5 mM L-glutamine (Sigma-Aldrich). CHO-$P2Y_{11}$ cells (chinese hamster ovary cells stably transfected with a plasmid containing the human $P2Y_{11}$ coding sequence) [36] were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin G, 5 mM L-glutamine, and 200 μg/ml G418 (Sigma-Aldrich). Cells were incubated at 37 °C in 5% CO_2. Ca^{2+} fluorescence measurements were performed as previously described using a NOVOstar with a pipettor system (BMG LabTechnologies, Offenburg, Germany) [32]. Briefly, cells were harvested with 0.05% trypsin/0.02% EDTA (Sigma-Aldrich) and rinsed with culture medium containing 10% fetal bovine serum. Pelleted cells were then resuspended in fresh medium, kept under 5% CO_2 at 37 °C for 30 min and vortexed every 15 min. After two washes with Krebs-HEPES buffer, cells were loaded with 3 μM Oregon Green 488 BAPTA-1/AM (Molecular Probes, Eugene, OR, USA) for 45 min at 25 °C in the same buffer containing 1% Pluronic F-127 (Sigma- Aldrich). Then, cells were rinsed three times with Krebs-HEPES buffer, diluted, and evenly plated into 96 well plates (Greiner, Frickenhausen, Germany) at a density of 35,000 cells/well. Agonist concentration–response curves were obtained by injection of increasing concentrations of UTP or ATP (HEK293 cells) or ATPγS (CHO-$P2Y_{11}$) and monitoring fluorescence intensity at 520 nm (bandwidth 35 nm) for 30 s at 0.4 s intervals. Excitation wavelength was 485 nm (bandwidth 12 nm). Concentration-inhibition curves of antagonists were obtained by preincubating the cells with the compounds for 30 min at 37 °C (HEK293 cells) and 25 °C (CHO-$P2Y_{11}$ cells), respectively, prior to injection of agonist (3 μM UTP or ATP in HEK293 cells or 3 μM ATPγS in CHO-$P2Y_{11}$ cells).

7.7.3. Data analysis

Effects of single doses of antagonists (10 μM) were expressed as a percentage of the agonist control responses.

Antagonist IC_{50} values (pIC_{50} = –log IC_{50}) represent the concentration needed to inhibit by 50% the effect elicited by single doses of agonists. EC_{50} values for agonists and IC_{50} values for antagonists were derived from –log concentration—effect (inhibition) curves fitted to the data by logistic, non-linear regression analysis (Prism 3.0, GraphPad Software, San Diego, CA, USA). All data are presented as arithmetic means ± S.E.M. from at least three independent experiments, unless otherwise stated. Differences between mean values were assessed using Student's *t*-test and considered significant when $P < 0.05$.

Acknowledgements

This work was supported by DFG (Deutsche Forschungsgemeinschaft) grants GRK677/1, GRK137/2, GRK137/3, and La350/7-3, as well as by the Fonds der Chemischen Industrie (Germany). The authors gratefully acknowledge the assistence of Mrs. M. Schneider in obtaining the mass spectra. CHO-cells recombinantly expressing the $P2Y_{11}$ receptor were kindly provided by Drs. D. Communi and J.M. Boeynaems, Brussels, Belgium.

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