New Iantherans from the Marine Sponge *Ianthella quadrangulata*: Novel Agonists of the $P2Y_{11}$ Receptor

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Three new iantherans, iso-iantheran A (**1**), 8-carboxy-iso-iantheran A (**2**), and iso-iantheran B (**4**) were isolated together with two further new brominated tyrosine-derived metabolites **5** and **6** from the polar extract of the Australian marine sponge *Ianthella quadrangulata*. Structures were elucidated on the basis of extensive spectral analysis. The dimeric benzofuran skeleton including a 2,3-dihydroxy-1,3-butadiene disulfate moiety found in **1**, **2**, and **4** is a unique feature of iantherans and to date only described for iantherans A and B. Iso-iantheran A (**1**) and 8-carboxy-iso-iantheran A (**2**) exhibited agonist activity at $P2Y_{11}$ receptors with EC_{50} values of 1.29 μM and 0.48 μM, respectively. Compound **2** showed some selectivity for $P2Y_{11}$ over $P2Y_1$ and $P2Y_2$ receptors. Compounds **1** and **2** represent a new structural type for the development of $P2Y_{11}$ receptor agonists.

Introduction

P2Y receptors belong to the purine receptors, which are divided into the P1 or adenosine receptors and the P2 receptors.[1] The P2 receptors can be further classified into the ionotropic P2X and the metabotropic (G protein-coupled) P2Y receptors.[2] To date, eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) subtypes have been cloned and functionally defined, and their physiological occurring agonists were characterized (e.g., ATP[a], ADP, UTP, UDP, and UDP-glucose).[3,4] $P2Y_{11}$ receptors are naturally activated by ATP and couple to both the phosphoinositide and the cAMP pathways (G_q and G_s coupled).[5] The physiological role of $P2Y_{11}$ receptors is still a matter of debate, which makes the need for selectively $P2Y_{11}$ receptor-modulatory compounds obvious. $P2Y_{11}$ receptors seem to play a role in the immune system, in that they are involved in the maturation and differentiation of dendritic cells.[6−8] There are many synthetic nucleotide analogues (e.g., ATPγS) described as $P2Y_{11}$ ligands but only very few non-nucleotide ligands.[9,10] We recently reported two non-nucleotide $P2Y_{11}$ antagonists; the suramin derivative 8,8′-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-fluoro-phenylene)carbonylimino))bis(naphthalene-1,3,5-trisulfonic acid) hexasodium salt (NF157) and the tetrasulfonic acid derivative 4,4′-(carbonylbis(imino-3,1-(4-methyl-phenylene)carbonylimino))-bis(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340) with K_i values of 44.3 nM and 19.9 nM, respectively.[11,12] To date, natural products were not evaluated as $P2Y_{11}$ receptor ligands.

Marine sponges are well-known to contain bioactive secondary metabolites, such as antitumor- and antiinflammatory compounds, e.g., discodermolide from *Discodermia dissoluta* and manoalide from *Luffariella variabilis*, respectively.[13] Moreover, metabolites occurring in sponges serve as lead structures for approved drugs, e.g., cytarabin, derived from spongothymidine and spongouridine, and for putative potent pharmaceuticals which are currently under clinical investigations, e.g., derivatives of hemiasterlin and contignasterol.[13,14]

Sponges of the species *Ianthella basta* have been shown to contain brominated macrocyclic alkaloids, named bastadins that exhibit remarkable tumor angiogenesis inhibitory properties.[15,16] Searching for new marine bioactive secondary metabolites, we performed chemical investigations of a less well investigated *Ianthella* species, *Ianthella quadrangulata*. This project resulted in the isolation of three new iantherans (**1**, **2**, and **4**) composed of a rare dimeric benzofuran skeleton including a 2,3-dihydroxy-1,3-butadiene disulfate moiety that is only found in iantherans A and B.[17,18] Biological assays have demonstrated an agonist effect of **1** and **2** at $P2Y_{11}$ receptors with EC_{50} values of 1.29 μM and 0.48 μM, respectively. Compounds **1** and **2**, isolated from the marine sponge *Ianthella quadrangulata*, are thus the first natural non-nucleotides acting as agonists at $P2Y_{11}$ receptors and provide a new structural skeleton for further ligand development.

Chemistry

The sample of *Ianthella quadrangulata* investigated in this study was collected from Wistari Reef near Heron Island, Great Barrier Reef, Australia. Exhaustive extraction of the sample with MeOH and a mixture of EtOH and H_2O, that was stored frozen at −20° prior to use, yielded three new iantherans (**1**, **2**, and **4**), as well as a dibromo-cinnamic acid derivative, **5**, and a dibromo-phenylacetic acid derivative, **6**, after repeated fractionation employing reversed phase chromatography.

The negative ESI-MS spectrum of iso-iantheran A (**1**) showed a characteristic isotope pattern at *m*/*z* 486, 487, 488, 489, and 490 $(M - 2Na)^{2-}$, 893, 895, 897, 899, and 901 $(M - SO_3Na - Na + H)^-$ and 813, 815, 817, 819, and 821 $(M - 2SO_3Na + H)^-$ corresponding to the presence of four bromine atoms and two sulfate groups in the molecule. The molecular formula

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[a] Abbreviations: ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); cAMP, cyclic 3′,5′-adenosine monophosphate; 2-MeSADP, 2-methylthio-adenosine-5′-diphosphate; SEM, standard error of the mean; UTP, uridine-5′- triphosphate; UDP, uridine-5′-diphosphate.

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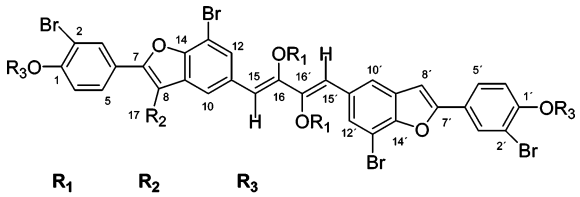
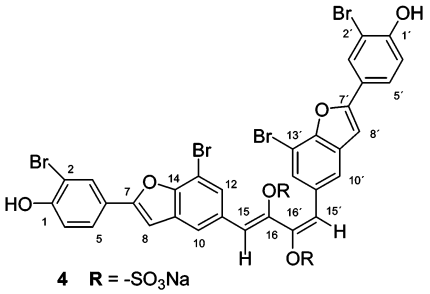
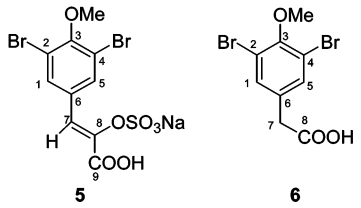


	R_1	R_2	R_3
1	-SO_3Na	H	H
2	-SO_3Na	COOH	H
3	-SO_3Na	COOMe	Me



4 **R** = -SO_3Na



5 **6**

of **1** was determined to be $C_{32}H_{16}Br_4Na_2O_{12}S_2$ by high-resolution mass measurements [HR-FT/ICR, *m/z* 485.8413 $(M - 2Na)^{2-}$, Δ +0.4 mmu]. The presence of the sulfate groups was supported by intense IR bands at 1241 and 1053 cm^{-1}.[19] All ^1H and ^{13}C NMR signals of **1** appeared in the olefinic and aromatic region of the spectra. Since only 7 proton and 16 carbon signals (7 × CH; 9 × C) were observed for **1**, it must be a symmetrical molecule. The proton spin−spin-coupling data evident from a ^1H-^1H-COSY experiment revealed the presence of a 1,2,4-trisubstituted benzene moiety by showing ortho-coupling ($^3J_{H5/5'-H6/6'} = 8.5$ Hz) between H-5/5′ and H-6/6′, and meta-coupling ($^4J_{H3/3'-H5/5'} = 2.1$ Hz) between H-3/3′ and H-5/5′ (see Table 1). Additionally, coupling between H-10/10′ and H-12/12′ ($^4J_{H10/10'-H12/12'} = 1.2$ Hz) was observed and pointed toward meta positioned protons attached to a second aromatic ring. The remaining proton signals were two singlets (H-15/15′, δ 7.04 and H-8/8′, δ 7.15) located according to their chemical shifts and coupling on a trisubstituted carbon−carbon double bond. All the protons were associated with their directly bonded carbon atoms from the results of a one bond $^1H-^{13}C$ 2D NMR shift correlated measurement (HSQC). The use of $^1H-^{13}C$ HMBC experiments (see Table 1) allowed us to deduce the remaining carbon−carbon connectivities of **1**. Thus, the 1,2,4-trisubstituted benzene moiety was confirmed from long-range CH couplings between H-3/3′ and C-1/1′, H-5/5′ and C-1/1′ as well as H-6/6′ and both C-2/2′ and C-4/4′. The deshielded ^{13}C NMR resonance for C-1/1′ (δ 156.4) indicated a substitution with oxygen whereas the ^{13}C NMR chemical shift of C-2/2′ (δ 111.4) was characteristic for a bromine-bearing aromatic carbon in ortho position to an oxygenated carbon.

Elucidation of the second aromatic substructure (C-7/7′ to C-14/14′) within **1** also resulted from interpretation of HMBC and ^{13}C NMR data (see Table 1). The meta positioned protons H-10/10′ and H-12/12′ all coupled with C-9/9′, C-13/13′, and C-14/14′, whereas H-10/10′ coupled additionally with C-8/8′. H-8/8′ (δ 7.15) correlated with C-7/7′, C-9/9′, C-10/10′, C-13/13′, and C-14/14′. These findings together with the ^{13}C NMR chemical shifts of C-7/7′ to C-14/14′ indicated the presence of a benzofuran moiety attached to the already deduced phenyl group.[17,18,20] The ^{13}C NMR chemical shift of C-13/13′ (δ 103.7) pointed toward a bromine substitution of this carbon. Long-range CH couplings observed between H-8/8′ and C-4/4′ and between both H-3/3′, H-5/5′, and C-7/7′ established the connection between the two aromatic units within each half of iso-iantheran A (**1**).

At this stage of the structural analysis, the methine group CH-15/15′ (δ_C 119.3, δ_H 7.04, s) was found to be connected to C-16/16′ (δ 145.4) based on the CH long-range coupling observed between H-15/15′ and C-16/16′. Furthermore, C-15/15′ was found to be linked to C-11/11′ due to weak $^1H-^1H$-COSY $^4J_{H-H}$ couplings between H-15/15′ and both H-10/10′ and H-12/12′, as well as the HMBC correlations between H-15/15′ and C-10/10′, C-11/11′, and C-12/12′. The ^{13}C NMR chemical shift of C-16/16′ (δ 145.4) showed this carbon to be attached to an oxygen substituent. Since C-16/16′ is a quaternary carbon and **1** a symmetrical molecule, the connection between the two symmetrical parts of **1** had to take place between C-16 and C-16′. Thus, **1** is composed of two benzofuran units, connected by a 2,3-dihydroxy-1,3-butadiene moiety.

The positions of the two *O*-sulfate ester functions still required assignment. On the basis of the symmetry within **1**, the two groups must be placed either both at the aromatic oxygen-bearing carbons C-1 and C-1′ (δ 156.4) or both at the olefinic oxygen-bearing carbons C-16 and C-16′ (δ 145.4). Since the ^{13}C NMR chemical shifts of C-1/1′, C-2/2′, C-6/6′, and C-16/16′ found in iso-iantheran A (**1**) are in good accordance with the corresponding values reported for the structurally related iantheran A and aplysillin A, the two sulfate groups were designated to C-16/16′.[17,21] This conclusion is supported by the work of Ragan and others who have documented that the ^{13}C NMR signals of the *ipso* carbon of a phenol undergoes an up field shift ($\Delta\delta$ 2.8−5.0 ppm) while the *ortho* carbon signals move downfield ($\Delta\delta$ 6.3−7.8 ppm) upon conversion of OH to O−SO_3H or the corresponding alkali metal salt O−SO_3M.[22−24]

Since the structure of iso-iantheran A (**1**) is that of a symmetrical dimer, the stereochemistry of its 1,3-butadiene substructure must be either *Z,Z* or *E,E*. In a gated decoupling experiment, the ^{13}C NMR signal for C-16 and C-16′ appeared as a triplet with the $^{13}C-^1H$ coupling constants $^3J_{C16-H15'} = {}^3J_{C16'-H15} = {}^2J_{C16-H15} = {}^2J_{C16'-H15'} = 3.6$ Hz (Figure 1).[25] As it was documented that *cis*-vicinal $^3J_{C-H}$ coupling constants (3−9 Hz) of substituted alkenes are usually smaller than the comparable *trans*-vicinal $^3J_{C-H}$ values (8−16 Hz), the double bond geometry of **1** was assigned as 15*Z*,15′*Z*.[26]

ROESY correlations (see Figure 2) confirmed the structure of **1**. These results also gave evidence for **1** being a positional isomer of iantheran A.[17] In **1** a connection between C-7/7′ and C-4/4′ is found, in contrast to a linkage between C-8/8′ and C-4/4′ in iantheran A.[17] Iso-iantheran A is proposed as the trivial name for **1**.

The second iantheran-like compound, 8-carboxy-iso-iantheran A (**2**), displayed isotopic clusters in the negative ESI-MS spectrum at *m/z* 1039, 1041, 1043, 1045, and 1047 $(M - Na)^-$, 937, 939, 941, 943, and 945 $(M - SO_3Na - Na + H)^-$, and 813, 815, 817, 819, and 821 $(M - 2SO_3Na - CO_2 + H)^-$, indicating the presence of four bromine atoms, two sulfate groups, and one carboxyl group. High-resolution FT/ICR mass measurements [*m/z* 507.8358 $(M - 2Na)^{2-}$, Δ +0.1 mmu] determined the molecular formula of **2** as $C_{33}H_{16}Br_4Na_2O_{14}S_2$. From ^1H and ^{13}C NMR data it became apparent that **2** was similar to **1** (see Table 1), but in contrast the ^1H and ^{13}C NMR

Table 1. ^{1}H and ^{13}C NMR Spectral Data for **1** (300 MHz, MeOD), **2**, and **4** (500 MHz, MeOD)

no.[a]	δ ^{13}C[b,c] (**1**)	δ ^{1}H[b] (**1**)	HMBC[d] (**1**)	δ ^{13}C[b,c] (**2**)	δ ^{1}H[b] (**2**)	δ ^{13}C[b,c] (**4**)	δ ^{1}H[b] (**4**)
1	156.4, C			156.7, C		156.5, C	
2	111.4, C			111.0, C		111.4, C	
3	130.7, CH	8.02 (d, 2.1)	1, 2, 4, 5, 6, 7	133.7, CH	8.35 (d, 2.2)	130.7, CH	8.02 (d, 2.1)
4	124.3, C			124.5, C		124.2, C	
5	126.6, CH	7.73 (dd, 2.1, 8.5)	1, 2, 3, 7	129.7, CH	8.03 (dd, 2.2, 8.6)	126.6, CH	7.73 (dd, 2.1, 8.5)
6	117.6, CH	7.03 (d, 8.5)[e]	1, 2, 4	117.1, CH	7.01 (d, 8.6)	117.6, CH	7.02 (d, 8.5)
7	157.0, C			154.6, C		157.1, C	
8	102.3, CH	7.15 (s)	4, 7, 9, 10, 13, 14	117.3, C		102.2, CH	7.14 (s)
9	131.8, C			131.5, C		131.9, C	
10	122.8, CH	8.17 (d, 1.2)	8, 9, 12, 13, 14, 15	124.4, CH	8.16 (brs)	122.5, CH	8.03 (d, 1.2)
11	133.5, C			133.8, C[f]		133.6, C[g]	
12	130.5, CH	8.00 (d, 1.2)	9, 10, 13, 14, 15	130.4, CH	8.38 (d, 1.4)	130.0, CH	7.85 (d, 1.2)
13	103.7, C			104.0, C		103.9, C	
14	152.3, C			151.2, C		152.5, C	
15	119.3, CH	7.04 (s)[e]	10, 11, 12, 13, 16	119.6, CH	7.07 (s)	123.4, CH	6.50 (s)
16	145.4, C			145.6, C[h]		146.7, C[i]	
17				171.7, C			
1′	156.4, C			156.7, C		156.4, C	
2′	111.4, C			111.7, C		111.4, C	
3′	130.7, CH	8.02 (d, 2.1)	1′, 2′, 4′, 5′, 6′, 7′	130.9, CH	8.04 (d, 2,2)	130.6, CH	8.00 (d, 2.1)
4′	124.3, C			124.4, C		124.3, C	
5′	126.6, CH	7.73 (dd, 2.1, 8.5)	1′, 2′, 3′, 7′	126.8, CH	7.76 (dd, 2.2, 8.3)	126.6, CH	7.71 (dd, 2.1, 8.5)
6′	117.6, CH	7.03 (d, 8.5)[e]	1′, 2′, 4′	117.9, CH	7.04 (d, 8.3)	117.6, CH	7.00 (d, 8.5)
7′	157.0, C			157.2, C		156.9, C	
8′	102.3, CH	7.15 (s)	4′, 7′, 9′, 10′, 13′, 14′	102.5, CH	7.17 (s)	102.2, CH	7.10 (s)
9′	131.8, C			132.0, C		131.8, C	
10′	122.8, CH	8.17 (d, 1.2)	8′, 9′, 12′, 13′, 14′, 15′	122.9, CH	8.19 (d, 1.3)	122.0, CH	7.93 (dd, 0.6, 1.5)
11′	133.5, C			133.7, C[f]		132.8, C[g]	
12′	130.5, CH	8.00 (d, 1.2)	9′, 10′, 13′, 14′, 15′	130.7, CH	7.99 (d, 1.3)	129.7, CH	7.76 (dd, 0.6, 1.5)
13′	103.7, C			103.9, C		103.6, C	
14′	152.3, C			152.5, C		152.1, C	
15′	119.3, CH	7.04 (s)[e]	10′, 11′, 12′, 13′, 16′	119.3, CH	7.03 (s)	121.0, CH	6.87 (brs)
16′	145.4, C			145.5, C[h]		143.2, C[i]	

[a] Position of carbon atom. [b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [c] Implied multiplicities determined by DEPT. [d] Numbers refer to carbon resonances. [e] Overlapping signals. [f–i] Tentative assignments within the same superscripts.

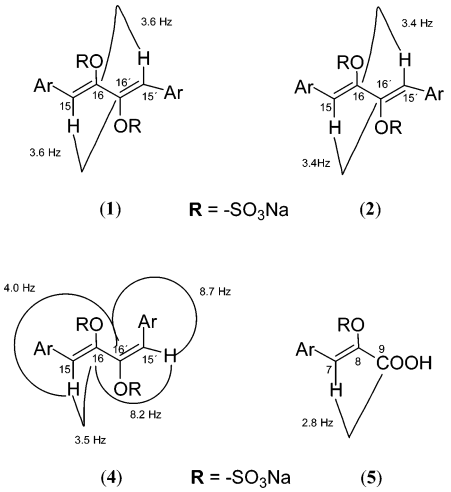


Figure 1. Multiple bond J_{C-H} values for **1**, **2**, **4**, and **5**.

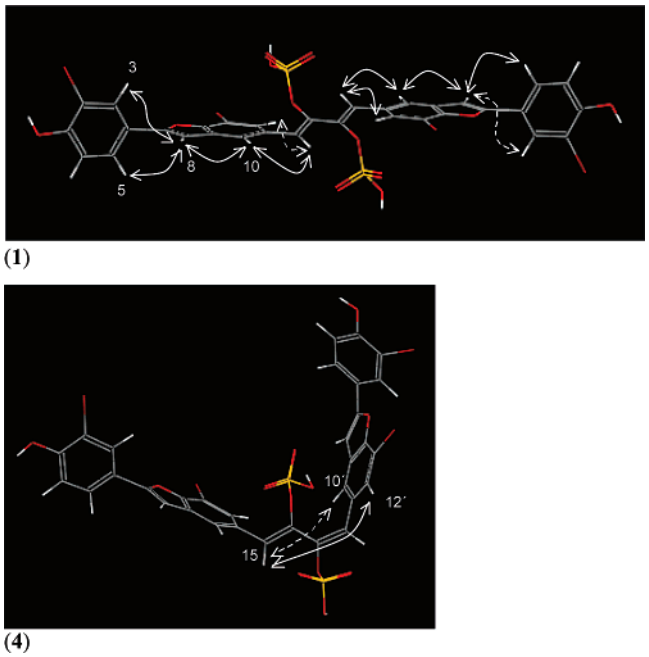


Figure 2. Selected ROESY correlations of **1** and **4**. Dashed lines are behind the image plane.

data of **2** contained individual resonances for each atom. These data suggested that **2** is an asymmetrical derivative of **1**. The only difference between the two molecules was the additional carboxyl group (C-17, δ 171.7) in compound **2**. On the basis of the absence of a H-8 resonance in the ^{1}H NMR spectrum, the carboxyl group was determined to be located at C-8 (δ 117.3). This deduction was supported by the significant downfield shift of C-8 (δ 117.3) compared to the unsubstituted C-8′ resonance (δ 102.5). The ^{13}C NMR chemical shift of the carboxylic acid substituted C-8 was also in accordance with that of a reported 3-carboxy-furano-sesquiterpene.[27] Methylation of **2** with CH_2N_2 provided further evidence for the presence of the carboxyl group (see Supporting Information). Analysis of the methylation product of **2** via HPLC-ESI-MS (negative mode) revealed the formation of the dimethoxy-methyl ester derivative **3**, as evident from isotope peak patterns at *m*/*z* 529, 530, 531, 532, and 533 $(M - 2Na)^{2-}$, 979, 981, 983, 985, and 987 $(M - SO_3Na - Na + H)^-$, and 899, 901, 903, 905, and 907 $(M - 2SO_3Na + H)^-$. These results were in agreement with those reported for the marine disulfate aplysillin A, in which methylation under the same conditions led to a trimethoxyl derivative

without any conversion of the sulfate groups.[21] Finally, the stereochemistry of **2** was found to be 15*Z*,15′*Z* inferred from the vicinal $^3J_{C16-H15'}$ (3.4 Hz) and $^3J_{C16'-H15}$ (3.4 Hz) coupling constants again obtained from a gated decoupling experiment (C-16, δ 145.6, t, 3.4 Hz; C-16′, δ 145.5, t, 3.4 Hz; see Figure 1). 8-Carboxy-iso-iantheran A (**2**) was hence defined as the 8-carboxy-derivative of iso-iantheran A (**1**).

The negative ESI-MS spectrum of the third compound isolated in the current study, iso-iantheran B (**4**), showed fragments and isotope peak patterns at *m*/*z* 486, 487, 488, 489, and 490 $(M - 2Na)^{2-}$, 973, 975, 977, 979, and 981 $(M - 2Na + H)^-$, 893, 895, 897, 899, and 901 $(M - SO_3Na - Na + H)^-$, and 813, 815, 817, 819, and 821 $(M - 2SO_3Na + H)^-$, suggesting the presence of two sulfate groups and four bromine atoms. Based on accurate mass analysis [HR-FT/ICR, *m*/*z* 994.6725 $(M - Na)^-$, Δ +1.1 mmu], the elemental composition of **4** was found to be identical to that of iso-iantheran A (**1**), $C_{32}H_{16}Br_4Na_2O_{12}S_2$. Furthermore, MS and NMR data revealed that both molecules, **1** and **4**, shared most of their structural features. The ^{1}H and ^{13}C NMR spectra of **4** are similar to those of **1**, but for **4** each ^{1}H and ^{13}C atom gave rise to an individual resonance, suggesting **4** to be an asymmetric isomer of **1** (see Table 1). After all proton and carbon resonaces had been assigned from the results of extensive 1D and 2D NMR experiments it became evident that **4** was a Δ^{15} or $\Delta^{15'}$ geometric isomer of **1**. ROESY correlations between H-15 and both H-10′ and H-12′ pointed toward the 15′*E* geometry of $\Delta^{15',16'}$ (see Figure 2). The 15*Z*,15′*E* geometry was confirmed based on the magnitude of the C−H vicinal coupling constants by combined use of gated decoupling and HMBC experiments. Unlike **1** and **2**, the two signals for C-16 (δ 146.7) and C-16′ (δ 143.2) in **4** both appeared as a doublet of doublets (dd) with J_{C-H} = 3.5, 8.2 Hz and J_{C-H} = 4.0, 8.7 Hz, respectively, in the gated decoupling experiment (see Figure 1). The small magnitude of $^3J_{C16'-H15}$ (4.0 Hz) and the large magnitude of $^3J_{C16-H15'}$ (8.2 Hz) implied the *cis*-vicinal and *trans*-vicinal C−H orientations, respectively, namely, the 15*Z*,15′*E* geometry.[26] The relatively large geminal $^2J_{C16'-H15'}$ value of 8.7 Hz in **4** can be explained by the *cis* relationship between H-15′ and the oxygen substituent at C-16′, further supporting the previous structural deductions.[28,29] Thus, iso-iantheran B (**4**) was identified as the 15*Z*,15′*E* geometric isomer of iso-iantheran A (**1**) and moreover as a positional isomer of iantheran B concerning the conjunction between the benzofuran substructure and the phenyl groups.[18]

Fragment ions in the negative ESI mass spectrum of component **5** indicated the presence of two bromine atoms and a single *O*-sulfate ester [*m*/*z* at 429, 431, 433 $(M - Na)^-$ and 349, 351, 353 $(M - SO_3Na)^-$]. The molecular formula $C_{10}H_7Br_2NaO_7S$ was established for **5** by HR-FT/ICR [*m*/*z* 428.8295 $(M - Na)^-$, Δ +1.6 mmu], implying six ring double bond equivalents aside from those present in the sulfate group. Its ^{1}H NMR spectrum showed the presence of one methoxyl-group (3-O-Me, δ 3.89, s, 3H) and a symmetrically 1,2,3,5-tetrasubstituted aromatic ring (H-1 and H-5, δ 8.16, s, 2H). The remaining olefinic singlet (H-7, δ 6.85, s, 1H) exhibited ^4J-^{1}H-^{1}H-COSY coupling to the aromatic two-proton singlet H-1/H-5. The ^{13}C NMR data contained in total only eight resonances, instead of the anticipated ten, as a result of the aromatic ring being symmetrically substituted (C-1/C-5, δ 135.2; C-2/C-4, δ 118.6). Compound **5** consequently contains one methoxyl (3-O−Me, δ 61.0), three methine groups, and six quaternary carbon atoms. Investigation of the direct proton−carbon and carbon−carbon connections was performed via one-bond (HSQC) and long-range (HMBC) ^{1}H−^{13}C 2D NMR shift correlated measurements, respectively (see Supporting Information). The aromatic two-proton singlet (H-1/H-5) showed HMBC correlations to the resonances for C-3 and C-2/C-4, whereas the methoxyl protons (3-O−Me) exhibited long-range CH coupling only to C-3 giving rise to the symmetrically 1,2,3,5-tetrasubstituted benzene moiety of **5**. Further long-range CH couplings were observed between H-7 and both C-8 and C-9. The olefinic methine group CH-7 (δ_C 119.7, δ_H 6.85) thus had to be connected to the sp^2-hybridized, oxygen-bearing quaternary carbon C-8 (δ 147.1), which in turn had also to bond to the carboxyl carbon C-9 (δ 171.2) resulting in a substituted acrylic acid moiety. Additional HMBC correlations, this time between H-7 and both aromatic meta positioned carbons C-1 (δ 135.2) and C-5 (δ 135.2) established the conjunction between the phenyl ring and the acrylic acid side chain. Finally, the position of the quaternary carbon C-6 (δ 134.6), that had no HMBC correlations, and the location of the sulfate group followed by deduction and were consistent with the molecular formula requirements and the NMR data of **1**, **2**, and **4**, at comparable centers. In a gated decoupling experiment the ^{13}C NMR signal for C-9 appeared as a doublet with $^3J_{C9-H7}$ = 2.8 Hz (see Figure 1). According to the stereochemistry of structures **1** and **2** the Δ^7 double bond geometry of **5** was concluded to be 7*Z*.

Compound **6** showed pseudomolecular ions at *m*/*z* 321, 323, and 325 $(M - H)^-$ and 277, 279, and 281 $(M - CO_2H)^-$ in the negative ESI-MS spectrum pointing at two bromine atoms and a carboxyl group in the molecule. The elemental composition was secured as $C_9H_8Br_2O_3$ by accurate mass measurements [HR-FT/ICR, *m*/*z* 642.7616 $(2M - H)^-$, Δ +1.4 mmu]. From its ^{1}H and ^{13}C NMR data it was evident that **6** had many structural features in common with **5**. The only difference between both compounds was the nature of the substituent at C-6: in **5** this was an acrylic acid moiety while in **6** it was an acetic acid function as demonstrated by the presence of a methylene group (CH_2-7, δ_H 3.43, δ_C 44.8) and a carboxyl carbon C-8 (δ 178.7). HMBC correlations confirmed the symmetrically 1,2,3,5-tetrasubstituted benzene residue as being identical to the one in **5**. Long-range CH couplings between the benzylic protons H_2-7 and the aromatic carbons C-1 (δ 134.6), C-5 (δ 134.6), C-6 (δ 138.7), and the carboxyl carbon C-8 (δ 178.7) clearly defined the side chain of **6** as being part of a substituted phenylacetic acid. Compound **6** has been previously documented as a synthetic intermediate but has never been reported as a natural product.[30,31] Compounds similar to **5** and **6** have been reported as products of acid hydrolysis and methylation of the extract of the red alga *Halopytis incurvus*; the original natural products remained unknown.[32]

Biological Results

Compounds **1**, **2**, **5**, and **6** were analyzed for their interactions with P2Y$_1$, P2Y$_2$, and P2Y$_{11}$ receptors, recombinantly expressed in 1321N1 astrocytoma cells by using a fluorescence calcium assay described by Kassack et al.[33] Functional activation of P2Y$_1$, P2Y$_2$, and P2Y$_{11}$ receptors by agonist stimulation resulted in increasing cytosolic Ca^{2+} levels and thus an increase in the recorded fluorescence intensity (see Supporting Information). Stimulating 1321N1 wild-type cells with 2-MeSADP, UTP, or ATPγS, respectively, did not yield Ca^{2+} signals (data not shown), demonstrating a valid test system. Compounds **1**, **2**, **5**, and **6** were screened for agonist activity at the three examined P2Y receptors in concentrations of 1 μM and 10 μM. Higher concentrations of the test compounds showed toxic effects on 1321N1 astrocytoma cells (Ca^{2+} mobilization through cell lysis) while toxic effects were not observed for any test compound at 1 μM and 10 μM (data not shown).

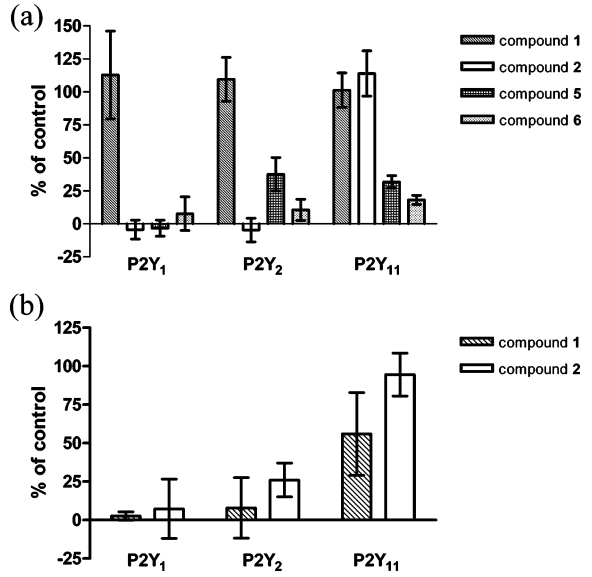


Figure 3. Activating potency by 10 μM (a) or 1 μM (b) of test compounds shown as % of respective standard agonist control at $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors recombinantly expressed in 1321N1 astrocytoma cells. Shown are mean ± SEM, $n = 3$.

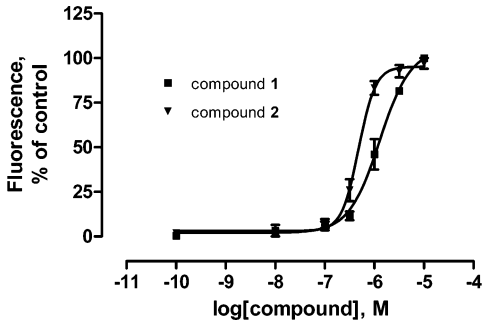


Figure 4. Concentration−response curves of test compounds **1** and **2** at $P2Y_{11}$ receptors recombinantly expressed in 1321N1 cells. Data shown are mean ± SEM of the pooled data of n independent experiments each with three replicates. pEC_{50} [compound **1**] = 5.89 ± 0.06, $n = 3$. pEC_{50} [compound **2**] = 6.32 ± 0.04, $n = 5$.

At all three examined P2Y receptors, the small molecules (**5**, **6**) displayed agonist effects of less than 37.5% of the standard agonist response at 10 μM (Figure 3a). A 10 μM concentration of compound **1** showed an agonistic effect at all three receptors comparable to the respective standard agonists (Figure 3a) but showed agonist activity exclusively at $P2Y_{11}$ receptors if the concentration was reduced to 1 μM (Figure 3b). Compound **2** exhibited an activating effect exclusively at $P2Y_{11}$ receptors at both concentrations (1 μM: 94.6%, 10 μM 113.9% of ATPγS control) while only negligible effects (<5.0%) were observed at $P2Y_1$ and $P2Y_2$ receptors (Figure 3a,b). These findings indicated that compound **2** has some selectivity for $P2Y_{11}$ over $P2Y_1$ and $P2Y_2$ receptors. In a control experiment, no effects of compounds **1** and **2** on 1321N1 wild-type cells were observed up to 10 μM (data not shown), confirming specific agonist activities of **1** and **2** at $P2Y_{11}$ receptors rather than unspecific cellular effects. Next, concentration−response curves were monitored for iso-iantheran A (**1**) and 8-carboxy-iso-iantheran A (**2**) at $P2Y_{11}$ receptors. Figure 4 contains the pooled normalized data from three (compound **1**) or five (compound **2**) independent experiments each with three replicates. The pEC_{50} values were as follows: compound **1**: $pEC_{50} = 5.89 \pm 0.06$; compound **2**: $pEC_{50} = 6.32 \pm 0.04$, revealing that **2** was about 2.7-fold more potent at $P2Y_{11}$ receptors than **1**. Additional

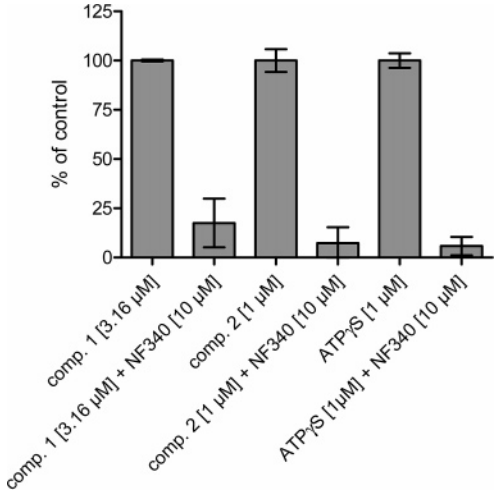


Figure 5. Effect on calcium mobilization after injection of compound **1**, compound **2**, and ATPγS at $P2Y_{11}$ receptors, expressed as % of control. The effect was tested after injection of compounds with or without 30 min preincubation of 10 μM NF340.

investigations demonstrated that the agonist effect of compounds **1** and **2** could be blocked with NF340, a $P2Y_{11}$ antagonist.[12] The effect at $P2Y_{11}$ receptors of 3.16 μM compound **1** and 1 μM **2** in comparison to the standard agonist ATPγS could be completely blocked by 10 μM NF340 (Figure 5), confirming effect mediation through $P2Y_{11}$ receptors rather than unspecific responses.

Discussion

The superfamily of G protein-coupled receptors (GPCRs) is a rich source for well-known and novel targets for pharmacotherapy. Natural products and natural product-derived compounds (e.g., morphine and ipratropium bromide, respectively) are prominent ligands of these receptors, and some of them are approved drugs. Secondary metabolites have also been described as ligands at G protein-coupled P2Y receptors and have been used as a scaffold for the rational design of P2Y ligands.[34−36] Some of the G protein-coupled P2Y receptors have shown to be promising drug targets: the $P2Y_{12}$ receptor is blocked by the active metabolite of the synthetic drug clopidogrel, resulting in inhibition of blood platelet aggregation; the nucleotide derived $P2Y_2$ receptor agonists diquafosol and denufosol are currently undergoing clinical trials for dry eye disease and cystic fibrosis, respectively.[37−40] Further P2Y receptors are discussed as therapeutic targets, but in many cases the pharmacological characterization of the involved P2Y-subtypes and therefore the identification of putative drug targets remains difficult due to a restricted availability of subtype selective ligands (e.g., $P2Y_{11}$ receptor).[41] The $P2Y_{11}$ receptor is highly expressed in immunocytes and is assumed to play a role in their maturation, suggesting it to be developed as a possible therapeutic target in the future.[6−8,41] Currently, there are only three non-nucleotide ligands at $P2Y_{11}$ receptors given in the literature.[11,12] The newly identified iso-iantheran A (**1**) and 8-carboxy-iso-iantheran A (**2**) are to our knowledge the first reported secondary metabolites acting as P2Y receptor agonists. Compound **2** but not compound **1** showed some selectivity for $P2Y_{11}$ over $P2Y_1$ and $P2Y_2$ receptors. Our compounds represent new structural types in the area of $P2Y_{11}$ ligands.

Concerning the analyzed compounds **1**, **2**, **5**, and **6** (compound **4** was not available for biological tests) exclusively the dimeric molecules **1** and **2** displayed agonist effects at $P2Y_{11}$ receptors. The additional carboxyl group of **2** ($EC_{50} = 0.48$ μM) compared

to **1** (EC_{50} = 1.29 μM) increases the selectivity for $P2Y_{11}$ over $P2Y_1$ and $P2Y_2$ receptors and moreover enhances the activating potency about 2.7-fold. The selectivity for $P2Y_{11}$ over other P2Y receptors was so far only estimated at $P2Y_1$ and $P2Y_2$, representing two major P2Y subtypes that are activated either by adenine nucleotides exclusively ($P2Y_1$) or by adenine and uracil nucleotides ($P2Y_2$).[3,4] Compound **5**, a small sulfated phenylpropionate structure, was not active at $P2Y_{11}$ receptors supporting the necessity of a more complex, dimeric skeleton. Similar results were obtained for the first $P2Y_{11}$ receptor antagonist NF157. NF157 is a dimeric compound with submicromolar inhibitory activity (K_i = 44 nM) whereas its monomers are inactive.[11]

The dimeric benzofuran skeleton of iantherans is unique in nature: it is only described for the two known iantherans A and B and finally in this study for **1**, **2**, and **4**.[17,18] The 2,3-dihydroxy-1,3-butadiene disulfate moiety within this carbon skeleton is also rare among natural products and apart from iantherans only found in the marine sponge metabolite aplysillin A.[17,18,21] Biosynthetically **1**, **2**, **4**, **5**, and **6** seem to derive from tyrosine. The dimeric compounds **1**, **2**, and **4** are probably formed via an enzymatically controlled biosynthesis involving radical pairing comparable to that found for lignans.[42]

Conclusion

This study presents the isolation, structure elucidation, and biological evaluation of five new brominated tyrosine-derived secondary metabolites (**1**, **2**, **4**, **5**, and **6**) from the polar extract of the marine sponge *Ianthella quadrangulata*. The new iantherans (**1**, **2** and **4**) are characterized by a rare dimeric benzofuran skeleton. More importantly, **1** and **2** are the first secondary metabolites that act as $P2Y_{11}$ receptor agonists with low micromolar potency and in the case of **2** with some selectivity for $P2Y_{11}$ over $P2Y_1$ and $P2Y_2$ receptors. Thus, we have identified a new structural type for the development of potent and selective ligands at $P2Y_{11}$ receptors.

Experimental Section

General Experimental Procedures. HPLC was carried out using a Waters M-6000A pump and a Knauer 298.00 differential refractometer as detector. ^{1}H, ^{13}C, COSY, ROESY, HSQC, and HMBC NMR spectra were recorded in CD_3OD either using a Bruker Avance 300 DPX or 500 DRX spectrometer operating at 300 or 500 MHz for proton and at 75 or 125 MHz for ^{13}C, respectively. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (CD_3OD). UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. Optical rotation was measured on a Jasco DIP 140 polarimeter. HPLC-ESI MS measurements were performed employing an Agilent 1100 Series HPLC including DAD, with reversed phase C18 column (Macherey-Nagel Nucleodur 100, 125 mm × 2 mm, 5 μm) and gradient elution (0.25 mL/min, NH_4Ac buffer 2 mmol, from MeOH 10:H_2O 90 in 20 min to 100% MeOH, then isocratic for 10 min), coupled with an API 2000, Triple quadrupole LC/MS/MS, Applied Biosystems/MDS Sciex and ESI source. HR ESI FT/ICR MS were recorded on a Bruker Daltonics APEX−III FT-ICR-MS spectrometer.

Animal Material. *Ianthella quadrangulata* was collected from Heron Island's, Wistari Reef, Australia at a depth of 12 m. and stored frozen at −20 °C until extraction. A voucher specimen is deposited at the Institute for Pharmaceutical Biology, University of Bonn, voucher number Her 20.

Extraction and Isolation. Thawed sponge tissue (82.5 g dry wt) was extracted with MeOH and a mixture of EtOH and H_2O to yield 31.2 g of brown extract, which was fractionated by vacuum liquid chromatography (VLC) over RP material (Macherey-Nagel, Polygoprep 60−50 C18) using gradient elution from H_2O containing increasing proportions of MeOH, followed by CH_2Cl_2, to yield 10 fractions. ^{1}H NMR investigations of these fractions indicated fractions 5, 6, and 7 to be of further interest, based on the presence of resonances attributable to aromatic protons. Fractions 5, 6, and 7 were further fractionated by reversed-phase (RP) HPLC (column: Knauer C18 Eurospher-100, 250 × 8 mm, 5 μm) using MeOH 20:H_2O 80 (1.7 mL/min) to yield pure compounds **5** (15.3 mg) and **6** (9.7 mg), applying MeOH 60:H_2O 40 (2.0 mL/min) to obtain a mixture of **2** and **4** (32.2 mg) and utilizing MeOH 65:H_2O 35 (2.0 mL/min) to afford component **1** as an amorphous solid (40.4 mg), respectively. Required purification of the mixture was achieved by RP HPLC (column: Macherey-Nagel Nucleodur 100−5 C18 EC, 250 × 4.6 mm, 5 μm; MeOH 50:H_2O 50, 0.8 mL/min) to give 6.8 mg of **2** and 7.3 mg of **4**.

Iso-iantheran A (1): brownish amorphous solid (40.4 mg, 0.049% dry wt); optically inactive, $[\alpha]_D^{23}$ −0.7 (*c* 0.39, MeOH); UV (MeOH) λ_{max} 206 (ϵ 36553), λ 280−360 nm (br), λ_{max} 325 nm (ϵ 41643); IR (ATR) ν_{max} 3363 (br), 1660, 1611, 1572, 1493, 1449, 1406, 1241, 1125, 1053, 1012, 975, 878, 827, and 735 cm^{-1}; ^{1}H and ^{13}C NMR data (see Table 1); ESIMS *m*/*z* 486, 487, 488, 489, and 490 $(M - 2Na)^{2-}$, 893, 895, 897, 899 and 901 $(M - SO_3Na - Na + H)^-$ and 813, 815, 817, 819, and 821 $(M - 2SO_3Na + H)^-$; HRMS (FT/ICR) calcd for $C_{32}H_{16}{}^{79}Br_4O_{12}S_2$ $(M - 2Na)^{2-}$ *m*/*z* 485.8409, found 485.8413.

8-Carboxy-iso-iantheran A (2): brownish amorphous solid (6.8 mg, 0.008% dry wt); UV (MeOH) λ_{max} 207 (ϵ 37285), λ 280−360 nm (br), λ_{max} 327 nm (ϵ 36719); IR (ATR) ν_{max} 3375 (br), 1605, 1403, 1242, 1052, 1021, 888, 826, and 736 cm^{-1}, ^{1}H and ^{13}C NMR data (see Table 1), ESIMS *m*/*z* 1039, 1041, 1043, 1045, and 1047 $(M - Na)^-$, 937, 939, 941, 943 and 945 $(M - SO_3Na - Na + H)^-$ and 813, 815, 817, 819, and 821 $(M - 2SO_3Na - CO_2 + H)^-$, HRMS (FT/ICR) calcd for $C_{33}H_{16}{}^{79}Br_4O_{14}S_2$ $(M - 2Na)^{2-}$ *m*/*z* 507.8357, found *m*/*z* 507.8358.

Iso-iantheran B (4): brownish amorphous solid (7.3 mg, 0.009% dry wt); UV (MeOH) λ_{max} 207 (ϵ 31336), λ 280−360 nm (br), λ_{max} 310 nm (ϵ 24305); IR (ATR) ν_{max} 3375 (br), 1609, 1572, 1493, 1450, 1404, 1239, 1126, 1052, 1012, 903, 825, and 737; ^{1}H and ^{13}C NMR data (see Table 1); ESIMS *m*/*z* 486, 487, 488, 489, and 490 $(M - 2Na)^{2-}$, 973, 975, 977, 979 and 981 $(M - 2Na + H)^-$, 893, 895, 897, 899, and 901 $(M - SO_3Na - Na + H)^-$, and 813, 815, 817, 819, and 821 $(M - 2SO_3Na + H)^-$; HRMS (FT/ICR) calcd for $C_{32}H_{16}{}^{79}Br_4NaO_{12}S_2$ $(M - Na)^-$ *m*/*z* 994.6714, found 994.6725.

(*Z*)-3-(3,5-Dibromo-4-methoxyphenyl)-2-(sulfooxy)-2-propenoic acid (5): brownish amorphous solid (15.3 mg, 0.019% dry wt); UV (MeOH) λ_{max} 226 (ϵ 18906), λ_{max} 277 nm (ϵ 16801); IR (ATR) ν_{max} 3382 (br), 1590, 1472, 1386, 1263, 1041, 997, 900, 804, and 741; ^{1}H and ^{13}C NMR data (see Supporting Information Table S3); ESIMS *m*/*z* 429, 431, 433 $(M - Na)^-$ and 349, 351, 353 $(M - SO_3Na)^-$, HRMS (FT/ICR) calcd for $C_{10}H_7{}^{79}Br_2O_7S$ $(M - Na)^-$ *m*/*z* 428.8279, found 428.8295.

2-(3,5-Dibromo-4-methoxyphenyl)acetic acid (6): brownish amorphous solid (9.7 mg, 0.012% dry wt); UV (MeOH) λ_{max} 208 (ϵ 40417), λ_{max} 276 nm (ϵ 1320); IR (ATR) ν_{max} 3316 (br), 1577, 1542, 1473, 1421, 1379, 1284, 1257, 1026, 992, and 738, ^{1}H and ^{13}C NMR data (see Supporting Information Table S3); ESIMS *m*/*z* 321, 323 and 325 $(M - H)^-$ and 277, 279 and 281 $(M - CO_2H)^-$; HRMS (FT/ICR) calcd for $C_{18}H_{15}{}^{79}Br_4O_6$ $(2M - H)^-$, *m*/*z* 642.7602, found 642.7616.

Methylation of 2: See Supporting Information.

Cell Culture and Measurements of Intracellular Calcium. All methods have been previously described in detail.[33] 1321N1-$P2Y_1$ astrocytoma cells stably transfected with a plasmid containing the human $P2Y_1$ coding sequence (GenBank ACC# AY136752), 1321N1-$P2Y_2$ astrocytoma cells stably transfected with a plasmid containing the human $P2Y_2$ coding sequence (GenBank ACC# AY136753) and 1321N1-$P2Y_{11}$ astrocytoma cells stably transfected with a plasmid containing the human $P2Y_{11}$ coding sequence (GenBank ACC# AY449733) were cultured in Dulbecco's modified Eagle Medium (DMEM) with sodium pyruvate, glucose (4500 mg/L), and pyridoxine (Sigma-Aldrich) supplemented with 5 mM

L-glutamine, 100 U/mL penicillin G, 100 μg/mL streptomycin, 10% fetal bovine serum (Sigma-Aldrich), and 400 μg/mL G418 (Carl-Roth, Karlsruhe, Germany). Cells were incubated at 37 °C in 5% CO_2.

Ca^{2+} fluorescence was measured as previously described using a fluorescence microplate reader with a pipettor system (NOVOstar; BMG LabTech, Offenburg, Germany).[33] Harvested cells (0.05% trypsin/0.02% EDTA, Sigma Aldrich) were rinsed with the appropriate culture medium. After centrifugation, the pelleted cells were resuspended in fresh medium and kept at 37 °C under 5% CO_2 for 20 min. After being washed two times with Krebs-HEPES buffer, cells were loaded with Oregon Green 488 BAPTA-1/AM (3 μM; Molecular Probes, Eugene, OR) for 60 min at 25 °C in the same buffer containing 1% Pluronic F-127 (Sigma-Aldrich). After being rinsed three times with Krebs-HEPES buffer, the cell suspension was diluted and evenly plated into 96-well plates (Greiner, Frickenhausen, Germany) at a density of 50−100,000 cells/well. Concentration−response curves of agonists were obtained by injection of increasing concentrations of the test compounds or 2-MeSADP (1321N1-$P2Y_1$ cells), UTP (1321N1-$P2Y_2$ cells), or ATPγS (1321N1-$P2Y_{11}$ cells). Excitation wavelength was 485 nm (bandwidth 12 nm) and fluorescence intensity was monitored at 520 nm (bandwidth 35 nm) for 30 s at 0.4 s intervals.

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Supporting Information Available: NMR spectra of **1**, **2**, **4**, **5**, and **6**; NMR data of **5** and **6**; purity data of **1**, **2**, **5**, and **6**; methylation of **2** and concentration−response curves of the respective agonist at $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors. This information is available free of charge via the Internet at http://pubs.acs.org.

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