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Bioorganic & Medicinal Chemistry Letters 18 (2008) 223–227
Bioorganic & Medicinal Chemistry Letters

Combinatorial synthesis of anilinoanthraquinone derivatives and evaluation as non-nucleotide-derived $P2Y_2$ receptor antagonists

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Received 29 September 2007; revised 23 October 2007; accepted 25 October 2007
Available online 30 October 2007

Abstract—A library of anilinoanthraquinone derivatives was synthesized by parallel Ullmann coupling reaction of bromaminic acid with aniline derivatives in solution using a compact parallel synthesizer. The products were purified by HPLC and evaluated as antagonists at mouse and human $P2Y_2$ receptors. 4-Phenylamino-substituted 1-amino-2-sulfoanthraquinones, for example, 1-amino-4-(2-methoxyphenyl)-2-sulfoanthraquinone (PSB-716), were potent $P2Y_2$ antagonists with IC_{50} values in the low micromolar range.

$P2Y_2$ receptors ($P2Y_2R$) belong to the family of G protein-coupled nucleotide (P2) receptors.[1–4] They are activated by the physiological nucleotides UTP and ATP, and by dinucleotides, such as diadenosine tetraphosphate (Ap_4A).[4] $P2Y_2R$ show a wide distribution in the body, including lung, heart, skeletal muscle, spleen, kidney, and brain.[1–4] There is a lack of potent and selective $P2Y_2R$ antagonists, which are required as pharmacological tools to elucidate the (patho)physiological roles of the receptors.[1–5] In addition, such compounds have potential as novel therapeutics, for example, as anti-inflammatory agents, for the treatment of coronary vasospastic disorders, as analgesics, or as neuroprotective drugs.[1–4,6]

The present study focuses on the development of non-nucleotide-derived $P2Y_2R$ antagonists using Reactive Blue 2 (RB-2, **1**, Fig. 1) as a lead structure. RB-2 is one of the most potent $P2Y_2$ antagonists known to date (IC_{50} 1–5 μM).[4] However, RB-2 also inhibits ectonucleotidases[7] and blocks other P2 receptor subtypes as well.[3,4,8,9] Furthermore, RB-2 has a relatively high molecular weight (MW = 840 g/mol) and bears three negatively charged sulfonate groups. Therefore, it does not exhibit properties that are desirable for a drug (MW < 500 g/mol, not permanently charged at pH 7.4).[10]

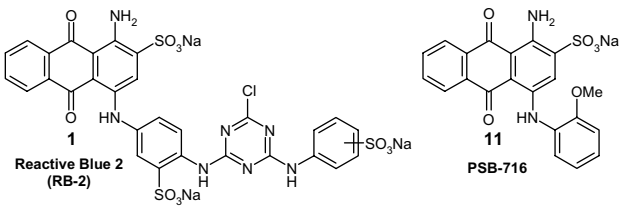

Figure 1. Structures of $P2Y_2$ receptor antagonists Reactive Blue 2 (RB-2) and compound **11** (PSB-716).

In previous studies anthraquinone derivatives related to RB-2, including Acid Blue 25 (AB-25, compound **3**, Table 2), have been evaluated as P2 receptor antagonists and ectonucleotidase inhibitors.[9,11] The substitution pattern on the aniline ring has been found to be important for activity and P2Y versus P2X receptor selectivity.[11b–d] However, no structure–activity relationships (SARs) have been reported for this class of compounds at $P2Y_2R$.

In the present study, we investigated whether a combinatorial synthetic approach using a compact parallel

Keywords: $P2Y_2$ receptors; Antagonists; Combinatorial synthesis; Anthraquinones; Ullmann coupling reaction.
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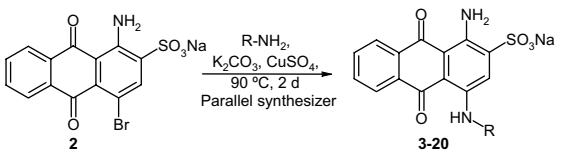
doi:10.1016/j.bmcl.2007.10.082

Table 1. Yields of anthraquinone derivatives synthesized in a compact parallel synthesizer in solution



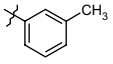
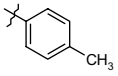
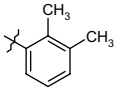
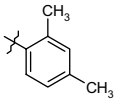
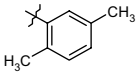
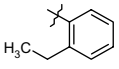
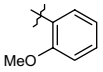
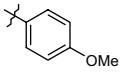
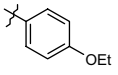
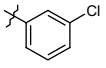
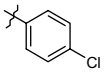
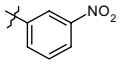
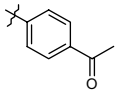
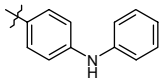
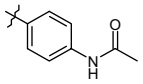
Compound	R	Yield[a]
3		60
4		74
5		83
6		26
7		72
8		42
9		26
10		76
11 (PSB-716)		67
12		82
13		52
15		61
15		16
16		56

Table 1 (*continued*)

Compound	R	Yield[a]
17		35
18		74
19		17
20		90

[a] Isolated yields (purity of products ⩾98%) calculated based on starting compound **2**; true yields were higher since commercial **2** was only 90% pure (main contaminant: desbromo derivative, 8%).[15]

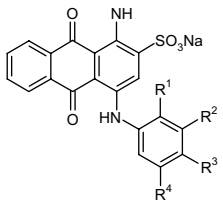
synthesizer for the preparation of a library of 4-phenylamino-substituted 1-amino-2-sulfoanthraquinones would be feasible. Furthermore, we developed an efficient HPLC method for the purification of the products. The compounds were evaluated as $P2Y_2R$ antagonists and SARs were analyzed.

The classical method to obtain the target compounds is the copper-catalyzed Ullmann coupling reaction.[12] Thus, bromaminic acid sodium salt (**2**) was reacted with aniline derivatives in the presence of copper(II) sulfate and sodium carbonate in water at 90 °C for 2 days. These conditions were found to be a good compromise for all the aniline derivatives employed, which possess very different reactivities. Parallel synthesis was performed in polypropylene vials using a MiniBlock™ synthesizer (Mettler Toledo, Switzerland).[13] After lyophilization the products were subjected to purification by HPLC on a Eurospher 100 C 18 column (10 μm, 250 × 20 mm).[14]

Examples for typical chromatograms are shown in Figure 2 for products **7**, **8**, and **13**. The starting compound bromaminic acid (**2**, red) and the side-product 1-amino-4-hydroxy-2-sulfoanthraquinone (**21**, dark red), which results from a substitution of the bromine atom by hydroxide, eluted within the first 5 min. In contrast, the somewhat less polar anilino derivatives **3–20** were eluted between 8 and 14 min thus allowing a baseline separation from the starting compound and the side-product (Fig. 2), which results in very pure products. Purities were determined by LCMS (for details, see Supplementary Information) and found to be >98% in all cases. The products were further characterized by ^{1}H- and ^{13}C NMR spectra, elemental analysis, or high resolution mass spectra, respectively; for a typical example, see footnote.[16]

As shown in Table 1 yields ranged from 16 to 90% depending on the substituents on the phenyl ring. In most cases, yields were greater than 50%; only for 6 products (**6**, **8**, **9**, **15**, **17**, and **19**) lower yields were ob-

Table 2. Pharmacological Evaluation of the synthesized anthraquinone derivatives as antagonists at mouse and human $P2Y_2$ receptors



Compound	R^1	R^2	R^3	R^4	IC_{50} ± SEM ($mP2Y_2$)[a] [μM]	IC_{50} ± SEM ($hP2Y_2$)[b] [μM]
1 (RB-2)	For structure, see Figure 1				**5.0** ± 0.5	**1.85** ± 0.39
2[c]	For structure, see Table 1				**>100**	nd[d]
3 (AB-25)	H	H	H	H	**11.1** ± 3.0	**5.61** ± 0.47
4	CH_3	H	H	H	**10** ± 2	**16.2** ± 2.2
5	H	CH_3	H	H	**22** ± 7	**3.04** ± 0.58
6	H	H	CH_3	H	**27** ± 5	**24.9** ± 8.19
7	CH_3	CH_3	H	H	**14** ± 9	**7.95** ± 1.05
8	CH_3	H	CH_3	H	**9** ± 1	**5.43** ± 1.78
9	CH_3	H	H	CH_3	**17** ± 5	**30.3** ± 9.79
10	C_2H_5	H	H	H	**24** ± 4	**9.26** ± 2.76
11 (PSB-716)	OCH_3	H	H	H	**9** ± 2	**9.82** ± 0.43
12	H	H	OCH_3	H	**17** ± 1	**11.5** ± 2.43
13	OC_2H_5	H	H	H	**17** ± 4	**15.0** ± 5.6
14	H	H	OC_2H_5	H	**9** ± 1	**11.5** ± 2.43
15	H	Cl	H	H	**>100**	**>30**
16	H	H	Cl	H	**20** ± 7	**5.31** ± 0.54
17	H	NO_2	H	H	**>100**	**7.52** ± 0.82
18	H	H	$COCH_3$	H	**>100**	nd[d]
19	H	H	NHC_6H_5	H	**30** ± 7	**3.93** ± 0.76
20	H	H	$NHCOCH_3$	H	**12** ± 4	**5.81** ± 1.57

[a] Inhibition of Ca^{2+} mobilization induced by 3 μM UTP in NG108-15 cells which natively express the mouse $P2Y_2$ receptor.
[b] Inhibition of Ca^{2+} mobilization induced by 1 μM UTP in 1321N1 astrocytoma cells recombinantly expressing the human $P2Y_2$ receptor. IC_{50} values for antagonists were calculated by nonlinear regression using Prism® 3.0.
[c] Bromaminic acid (see Table 1).
[d] nd, not determined.

tained. In case of compounds **15** and **17** electronic reasons (electron-withdrawing substituents) are likely, while steric hindrance might be an explanation for the low yields of **8** and **9**; however other ortho-substituted anilines provided higher yields. Nevertheless, all desired products were obtained in sufficient amount and purity for pharmacological testing.

$P2Y_2R$ are coupled to phospholipase $C\beta_1$ via $G\alpha_{q/11}$ protein mediating the production of inositol trisphosphate which in turn leads to intracellular calcium release.[3] All products were evaluated as antagonists at mouse $P2Y_2R$ natively expressed in neuroblastoma × glioma hybrid (NG108-15) cells[17,18] and at human $P2Y_2R$ heterologously expressed in 1321N1 astrocytoma cells.[19] Inhibition of UTP-induced calcium mobilization was determined using the calcium-complexing fluorescent dye Fura-2.[18,20] Assays were performed using a FLUOstar® plate reader (BMG LabTechnologies, Offenburg, Germany) in 96-well plates as previously described.[18a,21] UTP was used for activating the $P2Y_2R$ at a concentration where it exhibited ca. 50–80% of the maximal effect (1 μM for 1321N1 astrocytoma cells expressing the human $P2Y_2R$, 3 μM for NG108-15 cells) (for details, see Supporting Information).

The lead structure RB-2 (**1**) exhibited an IC_{50} value of 5.0 μM at mouse and 1.85 μM at human $P2Y_2R$ in agreement with literature data.[4] The much smaller, unsubstituted phenylamino-anthraquinone derivative **3** (AB-25) was nearly as potent as RB-2 (11.1 μM at mouse and 5.16 μM at human $P2Y_2R$). The phenyl residue was essential since the starting compound bromaminic acid (**2**) was inactive. Chloro-substitution in the *meta*-position abolished activity, while a number of other mono- and di-substitutions were tolerated. Potencies ranged from 9 to 30 μM at mouse and 3 to 30 μM at human $P2Y_2R$ for the majority of the compounds. Most anilinoanthraquinones were more potent at human as compared to mouse $P2Y_2R$. SARs appeared to be slightly different at mouse and at human receptors indicating subtle species-dependent differences of the binding site. The most potent compound of the present series at the human $P2Y_2R$ was the *m*-methyl derivative **5** (IC_{50} 3.04 μM); however, the compound was 7-fold weaker at mouse $P2Y_2R$ (IC_{50} 22.0 μM). The *o*-methoxy derivative **11**, which was nearly equipotent at mouse and human $P2Y_2R$, was selected for further preliminary characterization. It was much weaker in inhibiting rat NTPDase1 (K_i > 100 μM) and rat ecto-5′-nucleotidase (K_i > 100 μM), and in antagonizing the human $P2Y_4$ (IC_{50} ca. 50 μM) and the rat $P2Y_6$ receptor subtypes

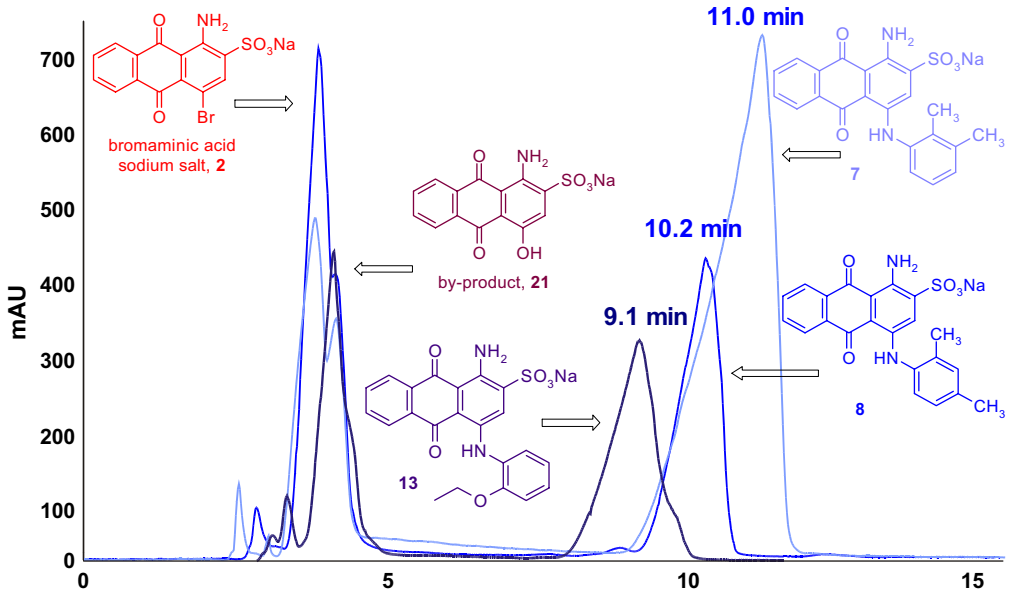


Figure 2. HPLC chromatograms of selected products (**7**, **8**, and **13**, blue). Raw products contain the starting compound (**2**, red-colored) and a by-product (**21**, dark red-colored), which can be removed by preparative HPLC. *X*-axis: time (min); *y*-axis: mAU, milli-absorbance units. For conditions, see Ref. 14.

($IC_{50} > 100\ \mu M$) (unpublished data) than as $P2Y_2$ antagonist. However, a comprehensive characterization has yet to be performed.

In conclusion, we established a combinatorial synthetic approach using a compact parallel synthesizer to obtain a small library of 1-amino-4-phenylamino-2-sulfoanthraquinone derivatives related to RB-2 by Ullmann coupling reaction. The method has been used to synthesize three new compounds (compounds **9**, **13**, and **19**) that have, to the best of our knowledge, not been described in the literature. In addition we have synthesized and characterized several compounds (**7**, **8**, **10**, **14**, **17**, and **18**) for which neither synthetic procedures nor spectral data have previously been reported. After having obtained the proof of principle, this reaction can now be transferred to an even larger, fully automated parallel synthesizer to produce extensive compound libraries. Numerous anilines and related amine derivatives are commercially available as building blocks. Furthermore, we have recently developed a synthetic approach to obtain a large variety of substituted *p*-(phenylamino)aniline derivatives[22] allowing us to synthesize derivatives of **19**, the compound with the second highest potency of the present series at human $P2Y_2R$. Very recently we have successfully improved the classical Ullmann coupling reaction by using phosphate buffer, pH 6–7, and performing the reaction under microwave irradiation, which led to dramatically accelerated reactions and improved yields.[15] The next step would be to apply these conditions to parallel combinatorial synthesis. For purification of the products, an efficient preparative HPLC method has been developed. Several small molecules with molecular weight of less than 500 g/mol proved to be $P2Y_2R$ antagonists with low micromolar IC_{50} values. Especially compound **11** (PSB-716) was found to be a promising candidate since—in contrast to the standard $P2Y_2$ antagonist Reactive Blue 2 (**1**, RB-2)—it appeared to be selective versus other P2Y subtypes as well as nucleotide-metabolizing enzymes including ecto-5′-NT and E-NTPDase1. Future efforts will be directed toward the replacement of the remaining sulfonate group by carboxylate in order to obtain drugs that will be able to penetrate membranes. Further modification may lead to more potent and selective $P2Y_2$ antagonists.

Acknowledgments

This work was funded by the DFG (GRK 804) and the DAAD (scholarship for Y.B.). We thank Karen Schmeling for skilfull technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.10.082.

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13. *Synthetic procedure.* Bromaminic acid sodium salt (**2**, 0.250 g, 0.62 mmol), anhydrous copper(II)sulfate (0.015 g, 0.09 mmol), and sodium carbonate (0.085 g, 0.80 mmol) were added to polypropylene reaction vials (MiniBlock^{TM} 48, Mettler Toledo). Then 2.48 mmol of the corresponding aniline derivative (see Table 2) and 2–3 mL of water were added. The reactor was locked and fixed on the shaking-washing unit (Mettler Toledo). The mixtures were shaken for 2 days at 90 °C. Then the vials were filtered and the filter residues were washed 3 times with 2 mL of dichloromethane each. Then the filter residues were suspended in a mixture of water and methanol (50:50), and the water–methanol solution was combined with the aqueous layer, put into round-bottom flasks and the solvent was evaporated under reduced pressure. The residue was taken up in water and washed several times with dichloromethane until the organic phase was clear and colorless. The volume of the aqueous phase was reduced by rotary evaporation and subsequently subjected to lyophilization.
14. *HPLC purification method.* The lyophilized solid products were dissolved in 50 mL of a mixture consisting of water:methanol = 65:35 and filtered through a microfilter (0.45 μm) to remove insoluble materials. The solutions were subsequently subjected to HPLC chromatography using a Knauer HPLC instrument equipped with a pump K1800, a UV detector K-2600, and a Eurospher 100 C 18 column, 10 μM, 250 × 20 mm (ID). An amount of 3 ml containing 30 mg of product was injected and the separation was performed for 15 min at a flow rate of 30 mL/min under the following conditions: isocratic elution (H_2O:methanol = 65:35) for 1 min, followed by a gradient from H_2O:methanol (65:35) to H_2O:methanol (26:74) for 14 min.
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16. Compound **13** was prepared according to the general procedure[13] using 2-ethoxyaniline (0.339 g, 0.324 mL). Yield: 52%; HPLC retention time: 9.1 min. ^{1}H NMR (DMSO-d_6, 500 MHz, 293 K): δ [ppm] = 1.33 (t, 3H, ^3J = 6.9 Hz, CH_3); 4.12 (q, 2H ^3J = 7.0 Hz, OCH_2); 6.96–7.02 (m, 1H, C4′H or C5′H); 7.13-7.16 (m, 2H C3′H or C6′H, C4′H or C5′H); 7.28 (d, 1H, ^3J = 7.6 Hz, C3′H or C6′H); 7.48 (br, 1H, NH_2); 7.83 (td, 1H, ^3J = 7.2 Hz, ^4J = 2.1 Hz, C6H or C7H); 7.85 (td, 1H, ^3J = 7.2 Hz, ^4J = 2.2 Hz, C6H or C7H); 7.95 (s, 1H, C3H); 8.25–8.29 (m, 2H, C5H,C8H); 10.11 (br, 1H, NH_2); 11.96 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 125 MHz, 293 K): δ [ppm] = 14.78 (CH_3); 64.14 (OCH_2); 109.30, 111.57 (C4a, C9a); 113.49 (C3′); 120.95 122.73 (C3); 123.35, 125.19 (C4′, C5′, C6′); 126.11, 126.15 (C5, C8); 128.42 (C1′); 132.86, 133.18, 133.81, 134.28 (C6, C7, C8a, C10a); 140.74, 142.58, 144.39 (C1, C2, C4); 151.16 (C2′); 181.93, 182.37 (C9, C10). LC-MS m/z (%) = 437.0 (100; $[C_{22}H_{17}N_2O_6S]^-$); 407.9 (47; $[C_{20}H_{12}N_2O_6S]^-$); 255.1 (29; $[C_{14}H_{11}N_2O_3]^-$). Purity by HPLC-UV (254 nm)-ESI-MS: 100%. Anal Calcd for $C_{22}H_{17}N_2O_6S{\cdot}Na{\cdot}3H_2O$: C, 51.36; H, 4.51; N, 5.44. Found: C, 51.39; H, 4.21; N, 5.28. UV absorption (CH_3OH): λ_{max} = 464 nm. Fluorescence (CH_3OH): λ_{max} = 740 nm.
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