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Toward the Discovery of New Agents Able to Inhibit the Expression of Microsomal Prostaglandin E Synthase-1 Enzyme as Promising Tools in Drug Development

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In our recent studies, we focused our attention on the synthesis of several γ-hydroxybutenolides designed on the basis of petrosaspongiolide M 1 (PM) structure that has been recognized to potently inhibit the inflammatory process through the selective PLA_2 enzyme inhibition. By means of a combination of computational methods and efficient synthetic strategies, we generated small collections of PM modified analogs to identify new potent PLA_2 inhibitors, suitable for clinical development. In the course of the biological screening of our compounds, we discovered a potent and selective inhibitor of *m*PGES-1 expression, the benzothiophene γ-hydroxybutenolide 2, which so far represents the only product, together with resveratrol, able to reduce PGE_2 production through the selective downregulation of mPGES-1 enzyme. In consideration that microsomal prostaglandin E synthase 1 (*m*PGES-1) is one of the most strategic target involved both in inflammation and in carcinogenesis processes, we decided to explore the biological effects of some structural changes of the γ-hydroxybutenolide 2, hoping to improve its biological profile. This optimization process led to the identification of three strictly correlated compounds 14g, 16g, and 18 with higher inhibitory potency on PGE_2 production on mouse macrophage cell line RAW264.7 through the selective modulation of *m*PGES-1 enzyme expression.

Key words: COX-2, *m*PGES-1, PGE_2, γ-Hydroxybutenolide derivatives

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In the last few years, we have been involved in the discovery of new lead candidates suitable for the development of promising anti-inflammatory agents. Our research work was based on a sesterterpene marine metabolite, petrosaspongiolide M (PM) **1**, which was proved to potently inhibit the human synovial PLA_2 type IIA enzyme, responsible for the triggering of the inflammation pathway (1). Encouraged by its interesting biological profile, PM (Figure 1) was selected by our research group for extensive investigations in terms of chemical, synthetical, and biological aspects. In fact, computational methods allowed us to propose molecular details of its mode of interaction with the target enzyme (2). The high densely functionalized scaffold, represented by the γ-hydroxybutenolide moiety, forced us to find efficient synthetic strategies to generate focused collections of simplified analogs of the parent molecule suitable for a structure–activity relationship study. Despite we did not find for the synthetic molecules satisfying level of activity toward our selected target, PLA_2 enzyme, nevertheless we had the chance to discover a very interesting product, the benzothiophene γ-hydroxybutenolide **2**, which has been proved to be a potent and selective modulator of *m*PGES-1 expression (3,4). This downstream PG synthesizing enzyme, glutathione dependent, is of inducible type and that is one of the reasons why it has established itself as a novel drug target in the areas of inflammation (5,6), atherosclerosis (7), stroke (8), and cancer (9–11). On the basis of these premises, we decided to rely on some well-reasoned structural changes of the basic molecule **2**, in the attempt to improve its pharmacological behavior. For this purpose, we synthesized a series of analogs closely related with the leader compound **2**, whose biological activity was also investigated (Figure 2).

Methods and Materials

General methods

All water and air sensitive reactions were carried out under an inert atmosphere (N_2) in oven- or flame-dried glassware. Ethyl acetate, dichloromethane (DCM) and tetrahydrofuran (THF) were distilled from CaH_2 immediately prior to use. Water was degassed under vacuum (10 mbar). All reagents were used from commercial sources without any further purification. Organic extracts were dried over anhydrous Na_2SO_4. Reactions were monitored on silica gel 60 F254 (Merck) plates and visualized with potassium permanganate, cerium sulfate or ninhydrin and under UV (λ = 254 nm,

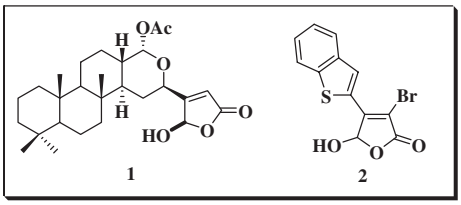


Figure 1: Petrosaspongiolide M (**1**) and 4-benzo[b]thiophen-2-yl-3-bromo-5-hydroxy-5H-furan-2-one (**2**).

365 nm). Flash column chromatography was performed using Merck 60/230–400 mesh silica gel. Analytical and semi-preparative reverse-phase HPLC purifications were performed on a Waters instrument using Jupiter C-18 column (250 × 4.60 mm, 5 μm, 300 Å; 250 × 10.00 mm, 10 μm, 300 Å, respectively). Purity grade of final products was determined on a Agilent 1100 HPLC using two different analytical reverse-phase columns (Method A: Jupiter C-18, 250 × 4.60 mm, 5 μm, 300 Å; Method B: Jupiter C-4, 250 × 4.60 mm, 5 μm, 300 Å). Reaction yields refer to chromatographically and spectroscopically pure products. Proton-detected (1H, HMBC, HSQC) and carbon-detected NMR spectra were recorded on Bruker instruments of Avance series operating at 300 and 600 MHz and 75 and 150 MHz, respectively. Chemical shifts are expressed in parts per million (ppm) on the delta (δ) scale. The solvent peak was used as internal reference: for 1H NMR $CDCl_3$ = 7.26 ppm; for ^{13}C NMR: $CDCl_3$ = 77.0 ppm. Multiplicities are reported as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. High-resolution mass spectra (HRMS) were recorded on a Q/TOF Premier WATERS (Milford, MA, USA) mass spectrometer using an electrospray ion source (ESI-MS).

[5,6,8,11,12,14,15(*n*)-^{3}H] PGE_2 and [9,10-^{3}H]oleic acid were purchased from Amersham Biosciences (Barcelona, Spain). The rest of reagents were from Sigma (St. Louis, MO, USA). *Escherichia coli* strain CECT 101 was a gift from Professor Uruburu, Department of Microbiology, University of Valencia, Spain.

Synthesis of 3,4-dibromo-5-(2-methoxy-ethoxymethoxy)-5H-furan-2-one (4)

Mucobromic acid **3** (100 mg, 0.387 mmol) was dissolved in 10 mL of dry dichloromethane, and MEM-Cl (66 μL, 0.581 mmol) was added to the solution. Diisopropylethylamine (101 μL, 0.581 mmol) was added dropwise over a period of 15 min. After 4 h, the reaction mixture was quenched with 20 mL of HCl 1 M. The aqueous layer was extracted with dichloromethane (3 × 30 mL), and the organics were dried with Na_2SO_4, filtered and concentrated in vacuo affording dark oil. The crude oil was purified by flash chromatography (10% diethyl ether/hexane) to give **4** (115 mg, 86% yield): ^{1}H NMR δ (300 MHz; $CDCl_3$): δ 6.10 (1H, s, OC*H*O), 5.20 (1H, d, *J* = 7 Hz, OC*H*HO), 4.87 (1H, d, *J* = 7 Hz, OCH*H*O), 3.79 (1H, m, OC*H*HCH$_2$O), 3.40 (3H, s, OC*H*$_3$), 3.60 (1H, m, OCH*H*CH$_2$O), 3.54 (2H, dd, OCH$_2$C*H*$_2$O); ^{13}C NMR δ (75 MHz; $CDCl_3$): δ 167.4, 144.2, 119.1, 100.4, 95.2, 72.1, 69.0, 59.8; HRMS calcd. for $C_8H_{11}Br_2O_5$: $[M+H]^+$ 344.8973, 346.8953, 348.8932 (ratio 1:2:1); found $[M+H]^+$ 344.8942, 346.8925, 348.8918 (ratio 1:2:1).

Esterification of boronic acid 7 and 8

The boronic acids **7** and **8** (0.667 mmol) were dissolved in 6 mL of ethyl acetate, and under stirring, pinacol (0.667 mmol) was added. After 4 h, the reaction was stopped adding anhydrous Na_2SO_4 (1 g) and $CaCl_2$ (1 g). The mixture was filtered and concentrated in *vacuo* (yield: 90% of **9** and 92% of **10**).

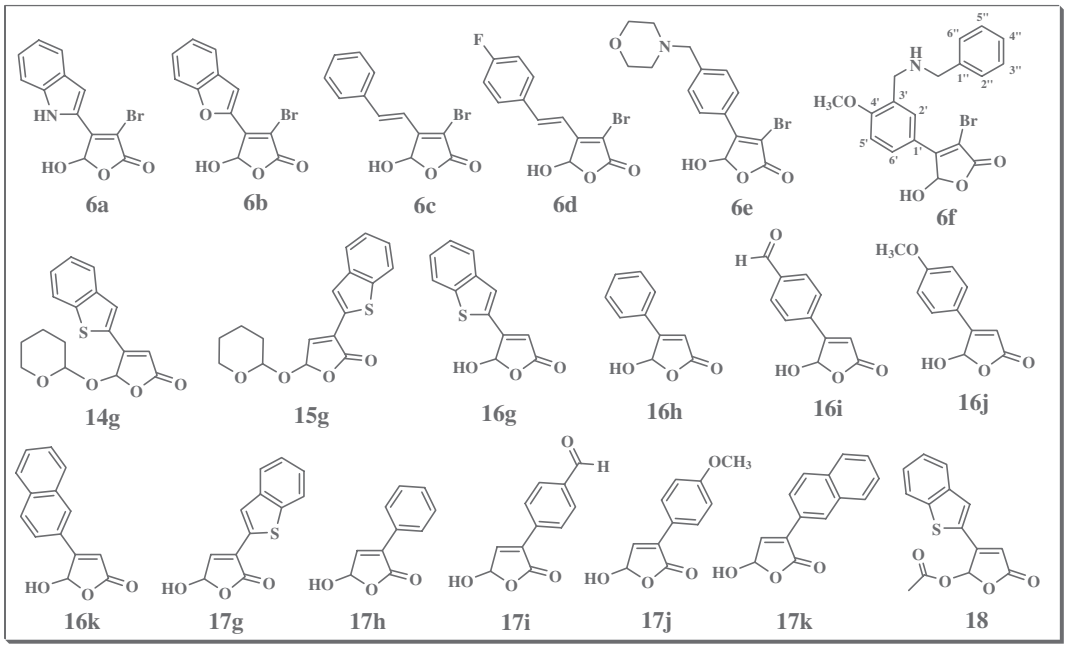


Figure 2: Collection of synthesized compounds.

Reductive amination: general procedure

Under inert atmosphere (N_2), 1 equiv. of boronic ester **9** or **10** was dissolved in anhydrous methanol (1 mL/0.18 mmol of ester). The mixture was kept under stirring at room temperature; anhydrous amine **11** or **12** (4 equiv.), $ZnCl_2$ (0.5 equiv.), and $NaCNBH_3$ (1 equiv.) were added. After 4 h, when the reagents disappeared, the reaction was stopped and 10 mL of an aqueous solution of NaOH 0.1 M was added. After concentration of methanol in *vacuo*, the aqueous phase was extracted with ethyl acetate (3 × 10 mL) and the organics were dried over Na_2SO_4, filtered and concentrated in *vacuo*.

The obtained oil was purified on silica gel by flash chromatography (10% ethyl acetate/n-hexane; yield: 79% of **e**).

BOC_2O protection of amine 13

At 0 °C, 1 equiv. of compound **13** was dissolved in a mixture of triethylamine/methanol 1:7 (v/v). The reaction was stirred for 10 min, and then a solution of BOC_2O (1.5 equiv.) was added dropwise. After 1 h, the ice bath was removed and the reaction was kept on stirring at room temperature overnight. Finally, the reaction material was first concentrated in *vacuo* and then dissolved in 20 mL of dichloromethane and 20 mL of distilled water. The aqueous phase was extracted with dichloromethane (3 × 20 mL); the organics were dried over Na_2SO_4, filtered and concentrated in *vacuo* (yield: 57% of **N-BOC-f**).

Microwave-assisted Suzuki coupling: general procedure

Compound **4** (1 equiv.), the boronic ester (**e** or **N-BOC-f**) or boronic acid **a-d** (1.5 equiv.), $Pd(dppf)_2Cl_2$ (0.03 equiv.), TBAB (0.5 equiv.), and CsF (4 equiv.) were placed in a CEM Discover vial. Water (500 μL) and THF (500 μL) were added under argon. The mixture was irradiated for 3–6 min, setting the power at 200 W, the temperature at 120 °C, the pressure at 250 psi, and the Power Max ON. After diluting (10 mL) with dichloromethane (DCM), 10 mL of an aqueous solution of HCl 1 N was added. The aqueous layer was extracted with DCM (3 × 10 mL). The organics were then dried over Na_2SO_4, filtered and concentrated in *vacuo*. The crude was purified by flash chromatography (from 10% diethyl ether/n-hexane to 40% diethyl ether/n-hexane), yield: 40–60%.

General procedure for MEM cleavage

The protected compounds **5a-e and N-BOC-f** were dissolved in a solution of trifluoroacetic acid (95%), triisopropylsilan (2.5%), and water (2.5%). The mixture was stirred at room temperature for 1.5 h and concentrated in *vacuo* at the end of the reaction.

General procedure for acetylation of 16g

Compound **16g** was dissolved in dichloromethane, the mixture was stirred at room temperature and then acetic anhydride (5 equiv.) and diisopropylethylamine (5 equiv.) were added. After 2 h, the mixture was diluted with 10 mL of HCl 1 N and the aqueous phase was extracted with dichloromethane (3 × 10 mL). The organics were dried over Na_2SO_4, filtered and concentrated *in vacuo*.

3-Bromo-5-hydroxy-4-(1H-indol-2-yl)-5H-furan-2-one (6a)

Yield: 90%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 9.25 (1H, s, N*H*), 7.72 (1H, d, *H*-8′), 7.46 (2H, m, *H*-3′, *H*-7′), 7.38 (1H, t, *H*-6′), 7.20 (1H, d, *H*-5′), 6.48 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.7, 149.1, 139.6, 135.5, 134.8, 129.1, 124.0, 124.4, 124.3, 122.9, 112.0, 98.1; HRMS calcd. for $C_{12}H_7BrNO_3$: $[M - H]^-$ 293.1 and 291.1 (1:1); found 293.1 and 291.1 (1:1).

4-Benzofuran-2-yl-3-bromo-5-hydroxy-5H-furan-2-one (6b)

Yield: 88%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.74 (1H, s, *H*-3′), 7.73 (1H, d, *H*-8′), 7.59 (1H, d, *H*-5′), 7.47 (1H, t, *H*-7′), 7.35 (1H, t, *H*-6′), 6.55 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.8, 151.1, 141.2, 137.0, 135.8, 130.8, 126.2, 125.8, 124.6, 124.1, 113.2, 99.1; HRMS calcd. for $C_{12}H_6BrO_4$: $[M - H]^-$ 294.1 and 292.1 (1:1); found 294.1 and 292.1 (1:1).

3-Bromo-5-hydroxy-4-styryl-5H-furan-2-one (6c)

Yield: 89%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.59 (3H, m, *H*-3′, *H*-4′, *H*-5′), 7.42 (2H, d, *H*-2′, *H*-6′), 7.38 (1H, d, *H*-1), 6.98 (1H, d, *H*-2), 6.34 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.3, 156.3, 136.2, 130.5, 130.4, 130.2, 129.7, 128.2, 128.1, 127.1, 107.1, 103.8; HRMS calcd. for $C_{12}H_8BrO_3$: $[M - H]^-$ 280.1 and 278.1 (1:1); found 280.1 and 278.1 (1:1).

3-Bromo-4-[2-(4-fluoro-phenyl)-vinyl]-5-hydroxy-5H-furan-2-one (6d)

Yield: 88%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.58 (2H, d, *H*-3′, *H*-5′), 7.36 (1H, d, *H*-1), 7.11 (2H, d, *H*-2′, *H*-6′), 6.90 (1H, s, *H*-2), 6.34 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.5, 103.8, 156.7, 107.6, 127.9, 130.8, 132.5, 130.1, 117.1, 163.5; HRMS calcd. for $C_{12}H_7BrFO_3$: $[M - H]^-$ 298.1 and 296.1 (1:1); found 298.1 and 296.1 (1:1).

3-Bromo-5-hydroxy-4-(3-morpholin-4-ylmethyl-phenyl)-5H-furan-2-one (6e)

Yield: 93%; ^{1}H-NMR δ (600 MHz; MeOD): 8.12 (1H, d, *H*-6′), 8.10 (1H, d, *H*-2′), 7.73–7.68 (2H, d, *H*-3′, *H*-5′), 6.62 (1H, s, OC*H*OH), 4.46 (2H, s, -CH_2N), 3.99–3.81 (4H, m, O-(CH_2)$_2$), 2.99 (2H, m, N-CH_2), 2.85 (2H, m, N-CH_2); ^{13}C-NMR (150 MHz, MeOD): 166.6, 155.7, 138.1, 133.2, 130.8, 130.4, 129.7, 129.3, 110.4, 98.8, 69.2, 69.1, 56.6, 56.4, 51.3; ESI-MS calcd. for $C_{15}H_{17}BrNO_4$: $[M+H]^+$ 354.0 and 356.0 (1:1); found 354.1 and 356.1 (1:1).

4-[3-(Benzylamino-methyl)-4-methoxy-phenyl]-3-bromo-5-hydroxy-5H-furan-2-one (6f)

Yield: 97%; ^{1}H-NMR δ (300 MHz; MeOD): 8.20 (1H, d, *H*-6′), 8.05 (1H, s, *H*-2′), 7.49 (5H, m, *H*-2″, *H*-3″, *H*-4″, *H*-5″, *H*-6″), 7.27 (1H,

d, *H*-5′), 6.55 (1H, s, OC*H*OH), 4.27–4.25 (4H, s, -CH_2NCH_2), 3.97 (3H, s, OCH_3); ^{13}C-NMR (150 MHz, MeOD): 167.5, 160.3, 155.6, 134.3, 132.7, 132.3, 130.9, 130.6, 129.2, 128.6, 125.5, 124.9, 112.3, 119.5, 111.1, 107.4, 98.4, 55.5, 53.7; ESI-MS calcd. for $C_{19}H_{19}BrNO_4$: $[M + H]^+$ 374.0 and 376.0 (1:1); found 374.0 and 376.0 (1:1).

Acetic acid 3-benzo[b]thiophen-2-yl-5-oxo-2,5-dihydro-furan-2-yl ester (18)

Yield: 81%; ^{1}H-NMR δ (300 MHz, $CDCl_3$): 7.86 (1H, d, *H*-8′), 7.50 (1H, s, *H*-3′), 7.44 (3H, m, *H*-5′, *H*-6′, *H*-7′), 6.35 (1H, s, OC*H*OC-OCH$_3$), 2.23 (3H, s, CH_3); ^{13}C-NMR δ (75 MHz, $CDCl_3$): 170.5, 170.1, 157.1, 141.1 108.5, 139.3, 132.8, 128.6, 127.7, 125.4, 125.2, 122.3, 115.2, 22.1; HRMS calcd. for $C_{14}H_9O_4S$: $[M - H]^-$ 273.3, found 273.3.

Assay of sPLA$_2$

*s*PLA$_2$ activity was assayed using [^{3}H]oleate-labeled membranes of *E. coli*, following a modification of the method of Franson *et al.* (29,30). *E. coli* strain CECT 101 was grown for 6–8 h at 37 /C in the presence of 5 μCi/mL [^{3}H]oleic acid (specific activity 10 Ci/mmol) until the end of the logarithmic phase. After centrifugation at 1800 *g* for 10 min at 4 /C, the membranes were washed, resuspended in phosphate-buffered saline (PBS), and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into the phospholipid fraction. Human recombinant synovial (group IIA *s*PLA$_2$) enzyme was used as source of *s*PLA$_2$. Enzyme was diluted in 10 μL of 100 mM Tris–HCl, 1 mM $CaCl_2$ buffer, pH 7.5, and preincubated at 37 /C for 5 min with 2.5 μL of test compound dissolved in ethanol or 2.5 μL of ethanol (control group) to get a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 20 μL of [^{3}H]oleic-*E. coli* membranes and was terminated by addition of 100 μL ice-cold solution of 0.25% bovine serum albumin (BSA) solution in saline to a final concentration of 0.07% (w/v). After centrifugation at 2500 *g* for 10 min at 4 /C, the radioactivity (cpm) in the supernatants was determined by liquid scintillation counting.

Western blot assay of COX-2 and mPGES-1

Cellular lysates from RAW 264.7 (murine macrophages, 1.5×10^6 cells/mL) incubated for 18 h with LPS (1 μg/mL) were obtained with lysis buffer A [10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N*′- tetraacetic acid (EGTA), 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 1 μg/mL leupeptin, 0.1 μg/mL aprotinin and 0.5 mM phenylmethanesulfonyl fluoride]. Following centrifugation (10 000 *g*, 15 min, 4 °C), supernatant protein was determined by the Bradford method with BSA as standard. COX-2 or *m*PGES-1 protein expression was studied in the total fraction or microsomal fractions, respectively. Equal amounts of protein (50 μg for both COX-2 and *m*PGES-1) were loaded on SDS–15% PAGE and transferred onto poly(vinylidene difluoride) membranes for 90 min at 125 mA. Membranes were blocked in PBS (0.02M pH 7.0)-Tween 20 (0.1%), containing 3% (w/v) nonfat milk and incubated with specific polyclonal antibody against COX-2 (1/1000) or *m*PGES-1 (1/200). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/10,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences).

Culture of murine macrophage RAW 264.7 cell line

The mouse macrophage cell line RAW 264.7 (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) was cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Cultures were maintained at 37 °C in 5% CO_2 (air:CO_2 95:5) humidified incubator. Cells were resuspended at a concentration of 1.5×10^6 cells/mL.

PGE$_2$ production in RAW 264.7 macrophages

RAW 264.7 macrophages (1.5×10^6 cells/mL) were co-incubated in 96-well culture plate (200 μL) with 1 μg/mL of *E. coli* [serotype 0111:B4] lipopolysaccharide (LPS) at 37 °C for 20 h in the presence of 2.0 μL of test compound dissolved in ethanol or 2.0 μL of ethanol (control group). PGE$_2$ levels were determined in culture supernatants by radioimmunoassay (31). The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (32) was used to assess the possible cytotoxic effects of compounds.

Statistical analysis

The results are presented as means ± SEM; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Significance was assumed at a p value of 0.05 or less.

Results and Discussion

Chemistry

Considering that compound **2**, our target molecule, consists of a 3-bromo 4-substituted hydroxybutenolide, we decided to start with the substitution of the benzothiophene appendage with bioisosteric moieties, such as indole and benzofurane units **6a** and **6b** (see chemistry section); as a result, we observed a clear loss of activity, especially for benzofurane derivative (Table 1). Then, we took into consideration another small array of 4-differently substituted 3-bromo-hydroxybutenolides, **6c-f**. Among these, compounds **6e** and **6f** belong to a small collection of products generated by *Ludi* (12–14) in the course of our previous medicinal chemistry project focused on the discovery of potential PLA$_2$ inhibitors. On the contrary, **6c** and **6d** were conceived to mimic the same structural moiety present in resveratrol, which has recently emerged as a potent modulator of *m*PGES-1 expression (15). Unfortunately, we did not observe an activity increase with respect to the lead compound **2** for any of the previous molecules. Afterward, we decided to investigate the role played by the bromine atom, and for this purpose, we

Table 1: Inhibitory activity and cytotoxic effect of the γ-hydroxybutenolide derivatives at 10 μM on the production of PGE_2 in LPS-stimulated RAW 264.7 cells.

Compound 10 μM	% Inhibition	IC_{50} (μM)	% Toxicity
6a	81.6 ± 4.7**	4.20	<5.0
6b	46.7 ± 6.1**	n.d.	<5.0
6c	45.1 ± 9.7**	n.d.	<5.0
6d	46.5 ± 11.1**	n.d.	<5.0
6e	61.7 ± 8.1**	7.30	<5.0
6f	72.2 ± 9.6**	4.80	<5.0
14g	100.0 ± 0.0**	0.85	28.9 ± 3.0**
15g	85.1 ± 3.5**	3.40	<5.0
16g	87.1 ± 3.9**	1.25	<5.0
16h	<20.0	n.d.	<5.0
16i	76.0 ± 5.7**	3.17	<5.0
16j	<20.0	n.d.	<5.0
16k	<20.0	n.d.	<5.0
17g	65.9 ± 8.1**	4.46	<5.0
17h	<20.0	n.d.	<5.0
17i	n.d.	n.d.	89.9 ± 0.2**
17j	<20.0	n.d.	<5.0
17k	<20.0	n.d.	<5.0
18	100.0 ± 0.0**	0.79	<5.0
2	72.2 ± 5.7**	1.80	<5.0

Results show means ± SEM (n = 6). Statistical significances: **p < 0.01, with respect to the LPS-stimulated control group. PGE_2 (non-stimulated cells = 0.6 ± 0.2 ng/mL; LPS-stimulated cells = 16.0 ± 1.6 ng/mL). n.d. = not determined.

examined the effects of two arrays of regioisomeric debrominated γ-hydroxybutenolides **16g-k** and **17g-k,** respectively, previously synthesized by us, in the frame of a project focused on a synthetic implementation task and re-synthesized, for the present purpose, using the same strategy reported by us, the photooxidation of 3-bromofuran with singlet oxygen in the presence of a suitable base (16). In the case of **16g**, the debrominated **2**, we observed a moderate activity increase. On the contrary, the other 4-substituted butenolides **16h-k** (except **16i**, which showed a discrete potency) were found completely inactive. The only consideration emerged actually from these preliminary results is that the only structural change proved to be effective for the activity was the elimination of bromine atom from the C-3 position of the lead compound **2**. In line with these findings, the other array of regioisomeric compounds **17g-k,** all presenting substitutions on C-3 position of the scaffold, did not give satisfactory results. Finally, we decided to perform on compound **16g,** the most active one so far obtained, the protection of OH either with acetyl **18** or with THP group **14g** to verify whether the masked aldehyde was crucial for the activity, as it was proved to be for the inhibition mechanism of the other known natural butenolides (2,17,18). Compounds **18** and **14g** at last displayed a higher potency in inhibiting the expression of *m*PGES-1, in comparison with the reference structure **2**, which is of relevance in consideration that only very few compounds are known to affect the level of *m*PGES-1 enzyme, so that the availability of pharmacologically active molecules capable of selective modulate *m*PGES-1 expression may provide new tools for better therapeutic approach.

For the synthesis of compounds **6a-f**, according to Scheme 1, we first protected the mucobromic acid **3** with methoxy-ethoxy-methyl-chloride (MEM-Cl) (19) and then we connected the MEM-protected butenolide **4** with the boronic acids $R\text{-}B(OH)_2$ or their respective pinacolic esters (20), using the Suzuki coupling reaction (21), previously optimized by us (22). To speed up the reaction, we took advantages of microwaves heating strategy (23), which gave compounds **5a-f** in good yields, together with some by-products, mainly consisting of bis-coupling and homo-coupling adducts. Finally, the removal of MEM protecting group afforded the desired compounds **6a-f**.

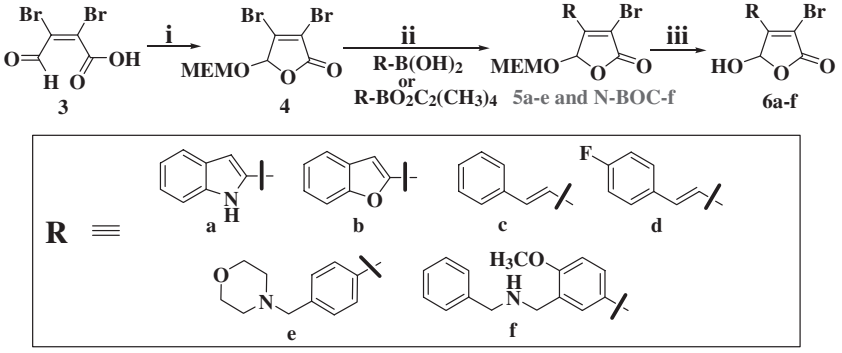
All the boronic partners utilized for the synthesis of **6a-f** are commercially available except for **e** and **N-BOC-f**. These last were obtained, according to Scheme 2, through a convergent synthetic approach. Based on this, we first assembled the molecular appendages by a reductive amination between the pinacolic esters **9** and **10** with the amines **11** and **12**, respectively, and then we performed the Suzuki coupling to connect these fragments to the butenolide scaffold **4**.

The two regioisomeric arrays of compounds **16g-k** and **17g-k** (Figure 2), respectively, were obtained utilizing the optimized protocol of photooxidation reaction performed on 3-bromofuran in the presence of a suitable base, as previously reported by us (16).

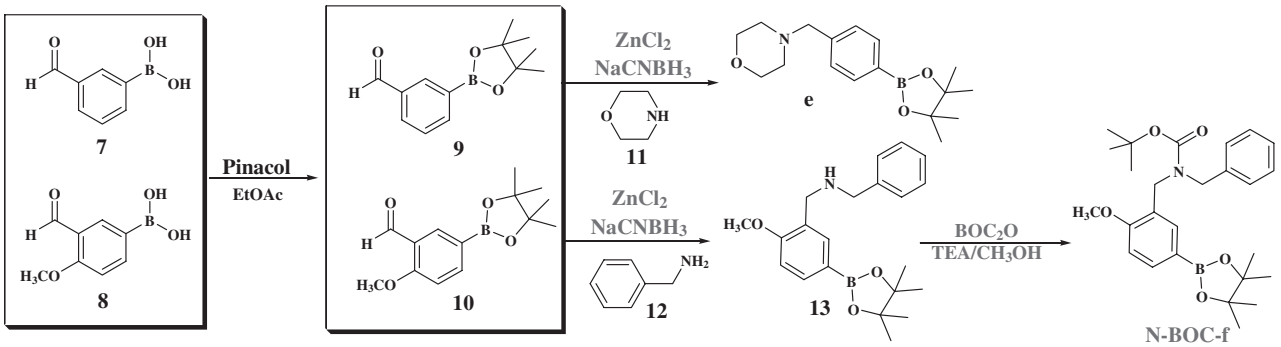
Finally, the acetylation of compound **16g** furnished the desired compound **18** (Scheme 3).

Biology

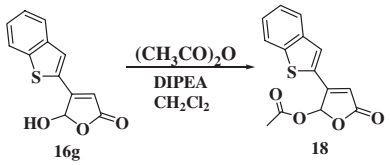
Enzymatic inhibition of $sPLA_2$, mainly type IIA $sPLA_2$, is a pharmacological approach that can modulate the availability of arachidonic



Scheme 1: Synthesis of compounds **6a-f**. [a]Reagents and conditions: (i) MEM-Cl, DIPEA, CH_2Cl_2, rt, 4h; (ii) $Pd(dppf)_2Cl_2$, TBAB, CsF, THF/H_2O 1:1, MW, 120°C, 3-6 min; (iii) TFA (95%), TIS (2.5%), H_2O (2.5%).



Scheme 2: Synthesis of appendages **e** and **N-BOC-f**.



Scheme 3: Acetylation of compound **16g**.

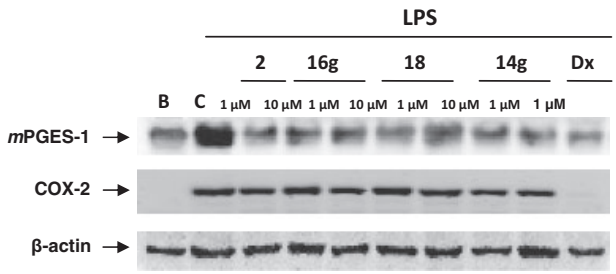


Figure 3: Effect of γ-hydroxybutenolide derivatives on *m*PGES-1 and COX-2 expression in LPS-stimulated RAW 264.7 cells. The figure is representative of two similar experiments. B: normal cells. C: LPS-stimulated cells. Dx. dexamethasone.

acid and consequently the production of PGE_2 and the inflammatory process (24). Among the nineteen new γ-hydroxybutenolide derivatives tested at 10 μM, under the same experimental conditions, on *s*PLA_2 belonging to the group IIA (human synovial recombinant), only compound **6e** exerted a weak inhibitory profile (30.4%). All the other γ-hydroxybutenolide derivatives tested were devoid of significant inhibition (data not shown) against this pro-inflammatory enzyme, unlike did the reference inhibitor LY311727 (96.3%) used as a reference tool (25).

We determined the effect of the nineteen γ-hydroxybutenolide derivatives on PGE_2 production on mouse macrophage cell line RAW264.7 stimulated with LPS (Table 1).

After 18-h stimulation, compounds **2**, **6a**, **6e**, **6f**, **14g**, **15g**, **16g**, **16i**, **17g**, and **18** were able to inhibit PGE_2 production with a percentage of inhibition higher than 50% at 10 μM, showing IC_{50} values in the micromolar range. Only compounds **18, 14g**, and **16g** exerted an inhibitory potency higher than the leader compound **2**. This profile is especially relevant for compounds **18** and **14g**, the two protected derivatives of the debrominated **2.** On the other hand, all the derivatives except **17i,** which was discarded, were devoid of significant cytotoxic effects on RAW264.7 at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Table 1). Compound **14g** showed a slight cytotoxic effect that disappeared at lower concentrations.

Western blot analysis for *m*PGES-1 and COX-2 proteins using 18-h LPS-stimulated RAW 264.7 cells (Figure 3) shows clearly that compounds **18, 14g**, and **16g** and the leader compound **2** inhibit *m*PGES-1 expression, without any effect on COX-2 expression, whereas dexamethasone, as expected, reduced the expression of both inducible proteins.

COX-2 and *m*PGES-1 are the main inducible enzymes responsible for PGE_2 synthesis (26), and both of them are downregulated by glucocorticoids in various cells (27,28). The use of Non Steroidal Anti-Inflammatory Drugs (NSADs) and glucocorticoids is a mainstay of anti-inflammatory therapy. Inhibition of PGE_2 formation by COX-2 inhibitors is effective in ameliorating symptoms of inflammation. However, the cardiovascular side effects associated with COX-2 inhibitors have limited their use. The concomitant increased cardiovascular safety observed in *m*PGES-1-deficient animals compared with COX-2 inhibition under similar conditions makes *m*PGES-1 an attractive target for development of a new class of therapeutic agents. The selective pharmacological profile exerted by these compounds could be of great interest to discover new promising drugs, as well as to provide pharmacological tools to discern the role of *m*PGES-1 and COX-2 in a great variety of inflammatory disorders.

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