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Identification of new γ-hydroxybutenolides that preferentially inhibit the activity of mPGES-1

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ABSTRACT

Microsomal prostaglandin E_2 synthase-1 (mPGES-1) has been recognized as novel, promising drug target for anti-inflammatory and anticancer drugs. mPGES-1 catalyzes the synthesis of the inducible prostaglandin E_2 in response to pro-inflammatory stimuli, rendering this enzyme extremely interesting in drug discovery process owing to the drastic reduction of the severe side effects typical for traditional non-steroidal anti-inflammatory drugs. In the course of our investigations focused on this topic, we identified two interesting molecules bearing the γ-hydroxybutenolide scaffold which potently inhibit the activity of mPGES-1. Notably, the lead compound **2c** that inhibited mPGES-1 with IC_{50} = 0.9 μM, did not affect other related enzymes within the arachidonic acid cascade.

1. Introduction

Several recent epidemiological and clinical studies confirmed that the most widespread pathologies among population, in particular those afflicting aged people, have their base in inflammation.[1–7] Hence, there is a growing need for effective and safe anti-inflammatory drugs that in most cases, as in chronic affections, require a long-term use. Accordingly, the identification of novel strategic targets to address therapeutic intervention is considered extremely urgent. In this perspective in the last years microsomal prostaglandin E_2 synthase-1 (mPGES-1) has been considered of great interest for new efficient anti-inflammatory and anticancer drug discovery and development.[8] This enzyme is responsible, along the arachidonic acid cascade, for the conversion of the cyclooxygenase (COX)-derived unstable endoperoxide prostaglandin H_2 (PGH_2) into prostaglandin E_2 (PGE_2), and it is over-expressed in several inflammatory disorders as well as in many human tumors.[9] mPGES-1 is a glutathione dependent transmembrane enzyme belonging to membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) family and it represents the COX-2-coupled isoform that is more strictly involved in pathologies.[10] Pharmacological intervention with the enzyme levels or activity has been indicated as a promising approach for the development of safer drugs in inflammation disorders, because mPGES-1 seems not to affect the constitutive prostaglandins involved in gastro-protection and in several important physiological functions.[10] Moreover, inhibition of mPGES-1 activity could also represent a valid strategy in the chemotherapy field.[11] For example, it was shown that cell proliferation and invasive activity in vitro as well as xenograft formation in vivo were reduced by mPGES-1 knockdown and conversely enhanced by mPGES-1 overexpression in lung and prostate cancer cells.[12,13] Several mPGES-1 inhibitors of natural or synthetic origin have been recently identified, for review see,[8,14] for example also through structure-based virtual screening.[15]

Continuing our investigations in medicinal chemistry area, in the recent years our studies[16–19] have been directed towards the discovery of new molecules targeting this interesting enzyme as promising candidates for the development of innovative therapeutics.[8,14,20]

In our approaches two main strategies were followed to affect mPGES-1 levels: on one hand the direct inhibition of its catalytic activity and, on the other hand, the modulation of the enzyme expression. In the first case, recently, on the basis of an accurate drug-receptor analysis, we rationally designed and synthesized a small collection of fast synthetically accessible triazole-based compounds as potential mPGES-1 inhibitors, some of which showed an interesting pharmacological profile that encouraged us to continue our investigations on this topic.[19] As alternative approach, in the course of another previous project we were able to identify compound **1** (Chart 1) as a potent modulator of mPGES-1

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expression.[16,17] Note that compound **1** selectively reduced the LPS-induced expression of mPGES-1 protein in intact cells leading to reduced PGE_2 generation but not by affecting COX-2 or mPGES-1 enzymatic activity.[16,17]

This finding is of great relevance in consideration that this compound represents one of the few molecules able to produce a selective down-regulation of the expression of mPGES-1 enzyme so far identified.

On the basis of this premises, we decided to explore more chemical space generating a new collection of butenolides bearing, as side chain, structurally different appendages (Chart 2) with the aim of increasing the biological activity. The collection of desired compounds has been successfully synthesized and subjected to pharmacological screening. Even though none of the compounds reduced the expression of mPGES-1 protein, we identified two very interesting compounds able to efficiently inhibit the catalytic activity of mPGES-1. Our data revealed that molecules belonging to a chemical platform never experienced against this target can fit the structural demand of enzyme catalytic pocket, hence paving the way for the development of an innovative class of drug candidates.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **2a–f** and **3a–e**, characterized by complex amido–aromatic fragments linked to the γ-hydroxybutenolide scaffold, required the synthetic procedure outlined in Scheme 2.

Specifically, the retro-synthetic analysis (Scheme 1) suggested us to construct the amido-aromatic appendages through an amidation reaction, starting from the appropriate amines **a–f** and carboxylic acids **4–5**. As next step, the advanced intermediates obtained **9a–f** and **10a–e**, were linked to the mucobromic acid **13** through a Suzuki reaction.

In more detail, in order to reduce the polarity of the amido-aromatic intermediates and make easier the purification step on silica columns, we first converted the boronic acids **4** and **5** into the corresponding pinacol esters **6** and **7**. These last were subjected to amidation reaction with the appropriate amines **a–f**, using the same protocol applied in peptide synthesis. In our specific case, we used triethylamine (TEA) as base, *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-dicyclohexylcarbodiimide (DIC) as carboxylic acid activators and *N*,*N*-dimethylformamide (DMF) as solvent.

After purification on silica gel, the Suzuki coupling between these advanced intermediates and the methoxy-ethoxy-methyl-ether (MEM)-protected 3-bromo-γ-hydroxybutenolide scaffold **8** afforded the protected adducts **11a–f** and **12a–e** (Scheme 2). Indeed, because of the instability of the butenolide ring under basic conditions required from the Suzuki coupling reaction, it was necessary to protect the mucobromic acid **13** as MEM-derivative. For the Suzuki coupling, we followed the experimental conditions previously optimized in our laboratory[21] providing [1,10-bis-(diphenylphosphino)ferrocene]-dichloropalladium (II) (Pd(dppf)-Cl_2) for the use as a catalyst and CsF as a base in a mixture of tetrahydrofuran/water (THF/H_2O) 1:1, under microwave irradiation.

The last step, consisting in the removal of the protecting MEM group, using a solution of trifluoroacetic acid/triisopropylsilane and water (TFA/TIS/H_2O) 95:2.5:2.5, afforded our products **2a–f** and **3a–e** in good yields together with lower amounts of bis-substituted adducts as major by-products.

Before submitting the synthesized compounds **2a–f** and **3a–e** to the biological screening, their purities (>95%) were verified by Agilent Technologies 1200 series high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 280 nm (method: Jupiter C-18 column, 250–4.60 mm, 5 μm, 300 Å; 1.0 mL/min flow rate; 5–100% in 35 min of 0.1% TFA/CH_3CN-0.1% TFA/H_2O).

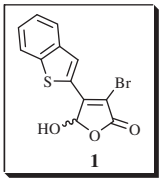


Chart 1. Chemical structure of compound **1**.

2.2. Biological evaluation

In order to investigate whether or not the test compounds **2a–f** and **3a–e** modulate the expression of mPGES-1, A549 cells were pretreated with the test compounds (10 μM, each) or with dexamethasone (1 μM, positive control) and then stimulated for 48 hours with IL-1β (or DMSO as vehicle) to induce the expression of mPGES-1. Compared to vehicle treatment, IL-1β up-regulated mPGES-1 protein as determined by Western blot and this was

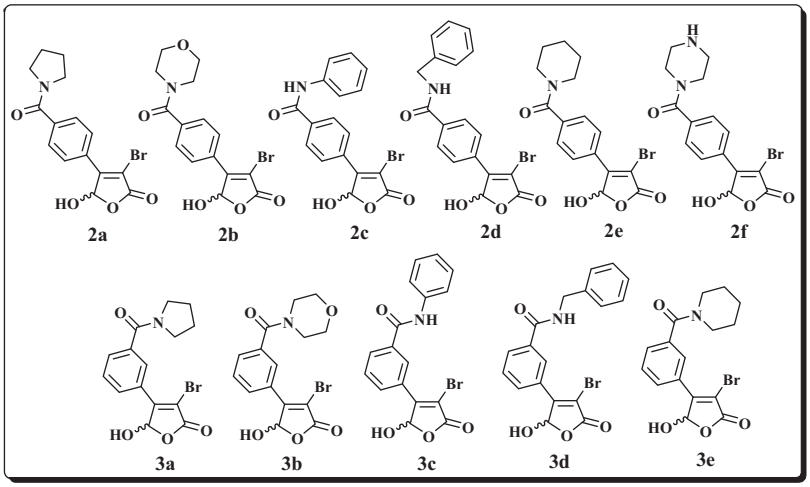
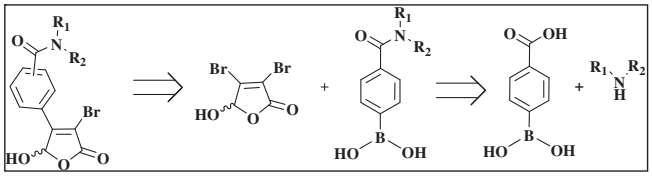


Chart 2. Collection of compound **1** derivatives.



Scheme 1. Retro-synthetic approach for the synthesis of **2a–f** and **3a–e**.

prevented by 1 μM dexamethasone. However, as shown in Figure 1, none of the test compounds **2a–f** and **3a–e** markedly reduced the expression of mPGES-1 protein.

Next, we evaluated the ability of the test compounds to interfere with the activity of mPGES-1. Microsomes of IL-1β-stimulated A549 cells expressing mPGES-1 were pre-incubated with the test compounds (10 μM) or with the reference inhibitor MK-886 (compound **14**, Chart 3, 10 μM) and PGE_2 formed by enzymatic conversion of PGH_2 (20 μM as exogenous substrate) was analyzed by RP-HPLC.

The reference mPGES-1 inhibitor **14** (10 μM) suppressed mPGES-1 activity by 82% (Fig. 2A). The structurally related compounds **2c** and **2d,** carrying a phenyl- or benzylcarboxamide in *p*-position of the linking benzene, significantly inhibited mPGES-1 activity, while the other compounds failed in this respect (Fig. 2). For compound **1**, only moderate mPGES-1 inhibition was evident (IC_{50} >10 μM, Fig. 2A). More detailed concentration-response analysis revealed IC_{50} values of 5.6 ± 0.4 and 0.9 ± 0.2 μM for **2d** and **2c**, respectively (Fig. 2). While **2c** as *N*-phenyl-substituted amide is quite potent, the related **2d** differs only by its *N*-benzyl moiety in structure which is seemingly detrimental. Also, relocation of the γ-hydroxybutenolide scaffold at the benzamide of **2c** and **2d** from 4- to 3-position (yielding **3c** and **3d,** respectively) led to inactive derivatives. Also, compounds where the benzamide nitrogen was incorporated into a pyrolidine, morpholine, piperidine or piperazine moiety failed to inhibit mPGES-1. Together, concrete structure activity relationships for these γ-hydroxybutenolides seem evident, and comparison of the overall structures of **2c** and **2d** reveal no obvious similarity to other known mPGES-1 inhibitors identified thus far.

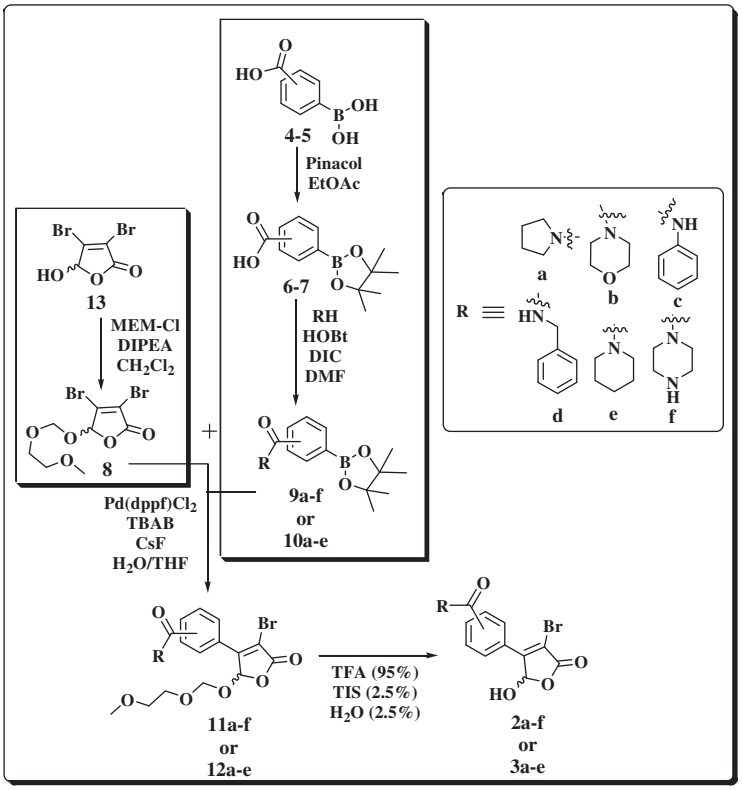
Because many structural diverse inhibitors of mPGES-1 also inhibited other enzymes within eicosanoid biosynthesis, in particular 5-LO and COX enzymes, we analyzed if the lead compound **2c** could inhibit the activities of 5-, 12-, and 15-LOs, COX-1/2, and $cPLA_2$. At a concentration of 10 μM, compound **2c** failed to affect the activity of COX-1 and COX-2 (isolated enzymes), of 12-LO and COX-1 in human platelets and of 15-LO in eosinophils (Supplementary data Fig. S1). Similarly, 5-LO activity in neutrophils was reduced by only 22%, and the activity of human recombinant $cPLA_2\alpha$ in cell-free assays was not suppressed by 10 μM of compound **2c** (Supplementary data Fig. S1). Nevertheless, **2c** might act as non-specific mPGES-1 binder, as reported for other mPGES-1 inhibitors,[22] and more detailed analysis of the molecular interactions (e.g. by surface plasmon resonance spectroscopy) are currently under investigation. Finally, analysis of **2c** (10 μM) in a cell viability assay using either RAW267.4 cells or primary human monocytes treated for 24 h revealed no significant cytotoxic effects (see Supplementary data). Taken together, compound **2c** is a potent and rather selective inhibitor of mPGES-1, and constitutes a promising candidate as anti-inflammatory drug candidate, suitable for further preclinical analysis. Ongoing distinct biological cell-based experiments cellular studies and animal experiments will further reveal the pharmacological potential of compound **2c**.

3. Experimental Section

3.1. Compounds and chemistry

3.1.1. Methods and materials

All water and air sensitive reactions were carried out under an inert atmosphere (N_2) in oven- or flame-dried glassware. CH_2Cl_2



Scheme 2. Synthetic protocol to generate derivatives **2a–f** and **3a–e**.

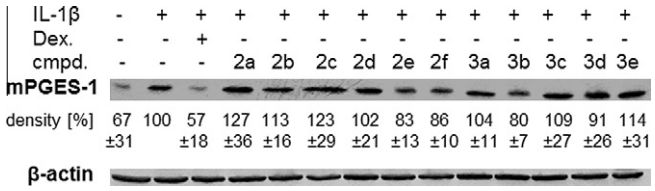


Figure 1. Effects of the test compounds on mPGES-1 expression. A549 cells were pre-incubated with the test compounds **2a–f** and **3a–e** (10 μM, each), dexamethasone (1 μM), or vehicle (DMSO, 0.3%) for 30 min and then IL-1β (2 ng/mL) was added. After 48 h, cells were harvested, lysed and mPGES-1 protein (and β-actin as control) was analyzed by Western blot. Data shown are representative for at least 3 experiments. Semi-quantification by densitometry of the mPGES-1 protein bands (in%) is given as mean ± SD, $n = 3$; the 100% value corresponds to the uninhibited vehicle (DMSO) control.

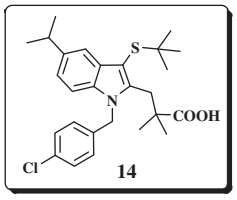


Chart 3. Chemical structures of MK886 (**14**).

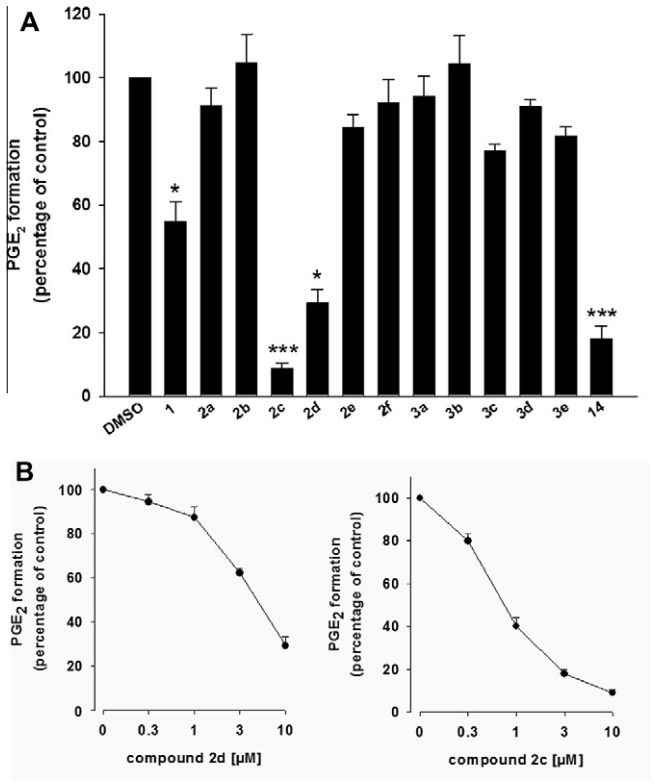


Figure 2. Inhibition of mPGES-1 activity by the test compounds. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with (A) the test compounds or the reference **14** at a concentration of 10 μM, each, or (B) with the indicated concentrations of compound **2d** (left panel) or **2c** (right panel). After 10 min on ice, 20 μM PGH_2 was added to start mPGES-1 mediated PGE_2 formation. Data are given as mean ± S.E., $n = 3$–4. The 100% value corresponds to the uninhibited vehicle (DMSO) control with an average of 0.8 nmol PGE_2.

and THF were distilled from CaH_2 immediately prior to use. Water was degassed under vacuum (10 mbar). All reagents were used from commercial source (Sigma–Aldrich) without any further purification.

Microwave reactions were performed on a CEM Discover® single mode platform using 10 mL pressurized vials.

Reactions were monitored on silica gel 60 F254 (Merck) plates and visualized with potassium permanganate or ninhydrine and under UV ($\lambda = 254$ nm, 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 an Agilent Technologies 1200 series using Jupiter C-18 column (250 4.60 mm, 5 μm, 300 Å; 250 10.00 mm, 10 μm, 300 Å, respectively).

3.1.2. Synthesis of 3,4-dibromo-5-(2-methoxy-ethoxymethoxy)-5H-furan-2-one 8

Mucobromic acid **13** (100 mg, 0.387 mmol) was dissolved in 10 mL of dry CH_2Cl_2 and MEM-Cl (66 μL, 0.581 mmol) was added to the solution. *N*,*N*-diisopropylethylamine (DIPEA, 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 CH_2Cl_2 (3 × 30 mL) and the organics were dried with Na_2SO_4, filtered and concentrated in vacuo. The crude dark oil obtained was purified by flash chromatography (5% diethyl ether/*n*-hexane to 20% diethyl ether/*n*-hexane) to give compound **8** (115 mg, 85% yield).

3.1.3. Esterification of boronic acids 4 and 5

The boronic acids **4** and **5** (0.667 mmol) were dissolved in 6 mL of ethyl acetate and, stirring the solution, pinacol (0.667 mmol) was added. After 4 h the reaction was stopped by adding anhydrous Na_2SO_4 (1 g) and $CaCl_2$ (1 g). The mixture was filtered and concentrated in vacuo (Yield: 91% of **6** and 90% of **7**).

3.1.4. Synthesis of amides 9a–f and 10a–e

The pinacol ester **6** or **7** (1 equiv) and the appropriate amine **a–f** (2 equiv) were dissolved in *N*,*N*-dimethylformamide (DMF). Triethylamine (TEA), *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-dicyclohexylcarbodiimide (DIC) (2 equiv of each) were added. The mixture was left at room temperature for 48 h under stirring. When TLC showed the consumption of the pinacol ester **6** or **7**, the reaction was stopped by adding HCl 1 N (10 mL). The aqueous phase was extracted with ethyl acetate (3 × 10 mL) and the organic phase was washed firstly with a saturated solution of $NaHCO_3$ and then with brine. Afterward, the organics were dried over Na_2SO_4, filtered and concentrated in vacuo. The crude was purified by flash chromatography (10% diethyl ether/*n*-hexane to 70% diethyl ether/*n*-hexane).

3.1.5. Microwave-assisted Suzuki coupling

In a CEM Discover vial, intermediate **8** (1 equiv), the appropriate boronic esters **9a–f** or **10a–e** (1.5 equiv), $Pd(dppf)Cl_2$ (0.03 equiv), TBAB (0.5 equiv) and CsF (4 equiv) were placed. Under argon, water (500 μL) and THF (500 μL) were added. 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. At the end of the reaction, the vial was cooled to 50 °C by gas jet cooling before it was opened. After diluting (10 mL) with CH_2Cl_2, 10 mL of an aqueous solution of HCl 1 N was added. The aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The organics were then dried over Na_2SO_4, filtered and concentrated in vacuo. The crude products were purified by flash chromatography (10% diethyl ether/*n*-hexane to 40% diethyl ether/*n*-hexane) to furnish compounds **11a–f** or **12a–e**.

3.1.6. MEM-cleavage to generate desired products 2a–f and 3a–e

The MEM-protected intermediates **11a–f** and **12a–e** were dissolved in a solution of TFA/TIS/H_2O 95:2.5:2.5. The mixture was stirred at room temperature for 1.5 h and concentrated in vacuo to leave a dark oil purified by flash chromatography (100% *n*-hexane to 30% diethyl ether/*n*-hexane). All the products **2a–f** and **3a–e** were obtained as white solids.

3.2. Biological assays

3.2.1. Materials and cells

The antibody against human mPGES-1 was from Cayman Chemical (Ann Arbor, MI), the antibody against COX-2 was obtained from Enzo Life Sciences (Loerrach, Germany).

Materials used: DMEM/High Glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution, PAA Laboratories (Linz, Austria); PGH_2, Larodan (Malmö, Sweden); 11β-PGE_2, PGB_1, MK-886 (compound **14**, 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), 6-keto $PGF_{1\alpha}$, Cayman Chemical (Ann Arbor, MI); Ultima Gold™ XR, Perkin Elmer (Boston, MA). All other chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) unless stated otherwise.

A549 cells were cultured in DMEM/High Glucose (4.5 g/L) medium supplemented with heat-inactivated fetal calf serum (FCS, 10%, v/v), penicillin (100 U/mL), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator. After 3 days, confluent cells were detached using 1 × trypsin/EDTA solution and reseeded at 2×10^6 cells in 20 mL medium in 175 cm^2 flasks.

3.2.2. Preparation of crude mPGES-1 in microsomes of A549 cells and determination of PGE_2 synthase activity

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously.[23] In brief, cells were treated with 1 ng/mL IL-1β for 48 h at 37 °C and 5% CO_2. After sonication, the homogenate was subjected to differential centrifugation at 10,000×*g* for 10 min and 174,000×*g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 mL homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethanesulphonyl fluoride, 60 μg/mL soybean trypsin inhibitor, 1 μg/mL leupeptin, 2.5 mM glutathione, and 250 mM sucrose), and the total protein concentration was determined. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4 °C, the reaction (100 μL total volume) was initiated by addition of PGH_2 (20 μM). After 1 min at 4 °C, the reaction was terminated using stop solution (100 μL; 40 mM $FeCl_2$, 80 mM citric acid, and 10 μM of 11β-PGE_2 as internal standard). PGE_2 was separated by solid phase extraction and analyzed by RP-HPLC as described.[23]

Acknowledgments

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Supplementary data

Supplementary data (HPLC conditions, mass spectrometry (MS) and nuclear magnetic resonance (NMR) data for the tested compounds **2a–f** and **3a–e** and their precursors; protocol of the additional bioactivity assays) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.032. These data include MOL files and InChiKeys of the most important compounds described in this article.

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