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Semi-synthetic derivatives of natural isoflavones from *Maclura pomifera* as a novel class of PDE-5A inhibitors

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ABSTRACT

Natural (iso)flavonoids have been recently reported to inhibit cyclic nucleotide phosphodiesterases (PDEs) and induce vasorelaxation, albeit the results described in the literature are discordant. The cGMP-selective isoform PDE-5A, in particular, represents the target of sildenafil and its analogues in the treatment of erectile dysfunction (ED) and pulmonary hypertension by promoting relaxation in vascular smooth muscle through the activation of the NO/cGMP pathway. We undertook this study to verify if osajin and pomiferin, two natural prenylated isoflavones and major constituents of *Maclura pomifera* extracts previously investigated for their anticancer, antibacterial and antidiabetic properties, show inhibitory activity on PDE-5A. These two isoflavones were isolated from the plant extracts and then synthetically modified to obtain a set of semi-synthetic derivatives with slight and focused modifications on the natural scaffold. The compounds were at first screened against PDE-5A in vitro and, based on the encouraging results, further tested for their relaxant effect on isolated rat artery rings. Computational docking studies were also carried out to explore the mode of interaction with the target protein. The obtained data were compared to the behaviour of the well-known PDE-5A inhibitor sildenafil. Our results demonstrate that semi-synthetic derivatives of osajin and pomiferin show an inhibitory effect on the isolated enzyme that, for some of the compounds, is accompanied by a vasorelaxant activity. Based on our findings, we propose the here described isoflavones as potential lead compounds for the development, starting from natural scaffolds, of a new class of PDE-5A inhibitors with vasorelaxant properties.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) are a class of enzymes ubiquitously present in mammalian tissues which inactivate cyclic guanosine monophosphate (cGMP) and/or cyclic adenosine monophosphate (cAMP) by stereospecifically hydrolysing an intramolecular phosphodiester bond, thus influencing different cellular signalling pathways [1]. Purification and characterization of PDE activity was firstly reported by Butcher and Sutherland [2] whereas later studies (reviewed in [3]) underlined the relevant role of the individual phosphodiesterase isozymes in the regulation of physiological and pathological conditions such as inflammation, cardiovascular disease and immune disorders [4–6]. On the basis of these observations, PDEs have been proposed as possible pharmacological targets in asthma, chronic obstructive pulmonary disease, systemic hypertension and pulmonary arterial hypertension [7–9]. Currently, PDE-5 inhibitors constitute a widespread class of drugs that successfully find application in the pharmacological treatment of erectile dysfunction (ED). Concerning their mechanism of action, these drugs are non-hydrolysable cGMP analogues that act by slowing the degradation of cGMP by PDE-5 and, thus, promote relaxation of penile smooth muscle, sustaining the natural pathway that leads to the erectile effect [10].

Sildenafil, vardenafil, tadalafil and avanafil are worldwide-approved PDE-5 inhibitors used in the treatment of ED while udenafil and mirodenafil are approved only in Korea [11]. According to their efficacy, overall safety and different pharmacokinetic behaviours, these molecules are used both in chronic and "on demand" ED cases [12]. On the other hand, refractoriness or poor responses in some patients, such as those affected by diabetes and nerve-injury [13,14], represent unavoidable limitations stimulating the quest for novel candidates (or also novel targets) in the treatment of ED, especially for those of a natural origin [15–17].

Natural flavonoids represent a wide, well documented class of biologically active molecules deeply investigated for their potential application in a large number of fields (reviewed in [18]) and some isoflavones, in particular, have been recently reported to inhibit

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phosphodiesterases [1,19] and promote relaxant effect on isolated rat aortic and vein preparations [19–21]. It is also well known that prenylated flavonoids can show an interesting degree of activity towards PDEs: as an example, tests on rats *corpus cavernosum*, previously treated with sodium nitroprusside, showed that icariin seems to inhibit both PDE5 and PDE4, enhancing cGMP levels [22]. Icariin is one of the main constituents of *Epimedium* herbs (also known as "horny goat weed") that have been used, among other remedies reviewed in [23], for over 2000 years as a treatment against infertility and erectile dysfunction. We focused on the extraction and evaluation of osajin and pomiferin, two isoflavones from *Maclura pomifera* (Osage Orange) [24,25] endowed with anticancer, antibacterial and antidiabetic properties [26–28] but whose activity on phosphodiesterase, to our knowledge, is yet to be evaluated. Therefore, we optimized their extraction protocol and prepared a set of semi-synthetic derivatives of both osajin and pomiferin by modifying different portions of the isoflavonic scaffold. Natural and semi-synthetic compounds were properly characterized (NMR, high resolution mass spectrometry) and screened for their inhibitory activity against PDE-5 in comparison with sildenafil.

2. Materials and methods

2.1. Chemistry

Commercially available chemicals were purchased from Aldrich and used as received, unless otherwise stated. Semi-preparative and preparative purification of the derivatives were carried out using preparative TLC plates (Merck) or on Isolera One, an automated flash chromatography system provided by Biotage (Upsala, Sweden); chromatography was carried out using disposable cartridges made of silica gel as stationary phase and bench solvents as mobile phase. ^{1}H and $^{13}C\{1H\}$ NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer and on a Bruker AMX 300 MHz spectrometer. All spectra were recorded at room temperature, the solvent for each spectrum is given in parentheses. Chemical shifts are reported in ppm and are relative to TMS internally referenced to the residual solvent peak. Datasets were edited with Bruker TopSpin suite and iNMR. The multiplicity of signals is reported as singlet(s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br) or a combination of any of these. High resolution mass spectra were recorded on a ESI–TOF Mariner from Perseptive Biosystem (Stratford, Texas, USA), using electrospray (ES) ionization. Melting points were measured with a Buchi Melting point MP-560 apparatus. The purity profile of the compounds was assayed by HPLC using a Varian Pro-Star system equipped with a Biorad 1706 UV–VIS detector and an Agilent C-18 column (5 μm, 4.6 × 150 mm). An appropriate ratio of water (A) and acetonitrile (B) was used as mobile phase with an overall flow rate of 1 mL min^{-1}; the general method for the analyses is reported here: 0 min (90% A–10% B), 15 min (10% A–90% B), 20 min (10% A–90% B), 21 min (90% A–10% B), and 25 min (90% A–10% B). The purity of all compounds was ≥97%, unless otherwise stated.

2.1.1. Extraction of osajin and pomiferin from M. pomifera fruits

M. pomifera fruits were cut into slices (1 cm) and set in an oven for two days at 50 °C. The obtained material was then triturated and 200 g of the resulting powder was charged in a soxhlet extractor. A first extraction cycle was carried out with 1 L of petroleum ether allowing the solvent to reflux for 48 h. After discarding this first extract, the same solid was used for a further extraction using 1 L of diethyl ether over a period of 16 h. The liquid extract was collected and the solvent was removed in rotavapor. The resulting residue was dissolved in 500 mL of 95% ethanol and the addition of 50 mL of a 0.1 M solution of Pb(II) acetate in methanol through a dropping funnel led to the precipitation of the pomiferin-Pb(II) complex. This solid was collected by filtration; the filtrate was concentrated to dryness to give a yellow residue that was recrystallized from 95% ethanol providing 4.0 g of osajin as a light yellow solid. The previously collected precipitate (pomiferin-Pb(II) complex) was treated with acetic acid and the resulting solution was poured into 1 L of cold water to give a yellow solid that was collected by filtration. 0.7 g of pomiferin was obtained. The separation by precipitation was used since it is reliable and is easy to scale-up. An alternative procedure is the separation of the two compounds by flash chromatography on silica gel (hexane/ethyl acetate 4:1).

2.1.2. Characterization of 9-hydroxy-7-(p-hydroxyphenyl)-2,2-dimethyl-10-(3-methyl-2-butenyl)-1,5-dioxa-2H-phenanthren-8-one (1, osajin)

mp 191 °C. δ_H (400 MHz, $CDCl_3$) 1.49 (6H, s, Me), 1.70 (3H, s, Me), 1.83 (3H, s, Me), 3.37 (2H, d *J* 7.3 Hz, CH_2), 5.00 (1H, s, OH), 5.26 (1H, m *J* 7.3 Hz, C═CH), 5.61 (1H, d *J* 10 Hz, C═CH), 6.72 (1H, d *J* 10 Hz, C═CH), 6.90 (2H, m *J* 8.1 Hz, PhH), 7.41 (2H, m *J* 8.1 Hz, PhH), 7.88 (1H, s, C═CH), 13.14 (1H, s, OH). δ_C (100 MHz, $CDCl_3$) 18.0, 21.4, 26.0, 28.3, 77.9, 100.8, 105.6, 113.0, 115.1, 115.8, 122.0, 123.0, 123.7, 127.3, 130.5, 131.7, 150.6, 152.4, 155.0, 157.4, 159.3, 181.1. HMRS (ESI) found 405.1669 ($C_{25}H_{25}O_5$, $[M + H]^+$), calc. 406.1697. Anal. found C 74.1; H 6.0. Calc. for $C_{25}H_{24}O_5$: C 74.2; H 6.0%.

2.1.3. Characterization of 7-(3,4-dihydroxyphenyl)-9-hydroxy-2,2-dimethyl-10-(3-methyl-2-butenyl)-1,5-dioxa-2H-phenanthren-8-one (2, Pomiferin)

mp 200 °C. δ_H (400 MHz, $CDCl_3$) 1.41 (6H, s, Me). 1.63 (3H, s, Me), 1.76 (3H, s, Me), 3.32 (2H, d *J* 7.0 Hz, CH_2), 5.03 (1H, s, OH), 5.16 (1H, m, C═CH), 5.56 (1H, d *J* 10.0 Hz, C═CH), 6.15 (1H, s, OH), 6.65 (1H, d *J* 10.0 Hz, C═CH), 6.68 (1H, dd *J* 7.1 Hz *J* 1.1 Hz, PhH), 6.73 (1H, d *J* 7.1 Hz, PhH), 6.91 (1H, d *J* 1.1 Hz, PhH), 7.80 (1H, s, C═CH), 12.90 (1H, s, OH). δ_C (100 MHz, $CDCl_3$) 18.1, 21.6, 26.2, 28.3, 77.8, 100.9, 105.6, 113.2, 115.2, 115.6, 116.6, 121.8, 121.9, 122.8, 123.8, 127.4, 131.6, 144.1, 144.8, 150.4, 152.6, 157.4, 159.3, 181.2. HMRS (ESI) found 421.1653 ($C_{25}H_{25}O_6$, $[M + H]^+$), calc. 421.1646. Anal. found C 71.5; H 5.8. Calc. for $C_{25}H_{24}O_6$: C 71.4; H 5.8%.

2.1.4. Synthesis of 10-[(3,3-dimethyl-2,3-oxiranyl)methyl]-9-hydroxy-3,4-epoxy-3,4-dihydro-7-(p-hydroxyphenyl)-2,2-dimethyl-1,5-dioxa-2H-phenanthren-8-one (3)

A round-bottom flask was charged with osajin (50.0 mg, 0.12 mmol) and 10 mL of dichloromethane. The solution was allowed to stir at room temperature under a nitrogen atmosphere. A m-chloro perbenzoic acid solution (85.5 mg, 0.49 mmol in 10 mL of dichloromethane) was added drop by drop over 5 min. After 2 h an additional portion of m-chloro perbenzoic acid (85.5 mg, 0.49 mmol) was added to the reaction and the resulting mixture was stirred at room temperature for 48 h and monitored by TLC (cyclohexan/ethyl acetate 4:1). After the consumption of the starting material the mixture was washed with a 20% aqueous solution of $NaHSO_3$ and saturated Na_2CO_3. Organic layer was collected and evaporated to give 3.1 mg of the desired product as a brown solid. Yield: 6%, mp. 172 °C. δ_H (400 MHz, $CDCl_3$) 1.26 (6H, s, Me), 1.48 (3H, s, Me), 1.53 (3H, s, Me), 3.9–3.7 (5H, m, CH, CH_2), 7.39 (1H, s, C═CH), 7.7–7.5 (2H, dd, PhH), 8.0–7.9 (2H, dd, PhH), 8.08 (1H, s, PhOH). δ_C (100 MHz, $CDCl_3$) 20.0, 25.3, 28.6, 34.2, 61.3, 64.2, 67.2, 69.3, 115.0, 120.1, 127.3, 128.8, 129.0, 136.1, 144.3, 145.1, 153.9, 154.3, 157.9, 160.1, 175.9. HMRS (ESI) found 469.1864 ($C_{26}H_{29}O_8$, $[M + MeOH + H]^+$), calc. 469.1857. Anal. found C 68.9; H 5.5. Calc. for $C_{25}H_{24}O_7$: C 68.8; H 5.5%.

2.1.5. Synthesis of p-[9-acetoxy-2,2-dimethyl-10-(3-methyl-2-butenyl)-8-oxo-1,5-dioxa-2H-phenanthr-7-yl]phenyl acetate (4)

A round-bottom flask was charged with osajin (100.0 mg, 0.25 mmol) and 10 mL of pyridine. Acetyl chloride (5 mL, 70 mmol) was slowly added to the solution and the resulting mixture was allowed to stir at room temperature and monitored by TLC (cyclohexan, ethyl acetate 1:1). A water/dichloromethane extraction was then performed and the organic layer was collected and then washed with 0.6 M HCl before rotavapor evaporation. The obtained residue was re-crystallized