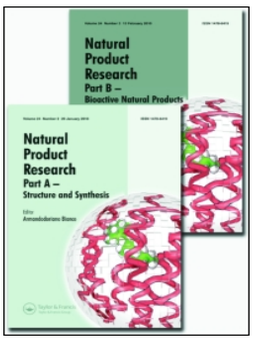

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Isoflavones from *Maclura pomifera*: structural elucidation and *in silico* evaluation of their interaction with PDE5

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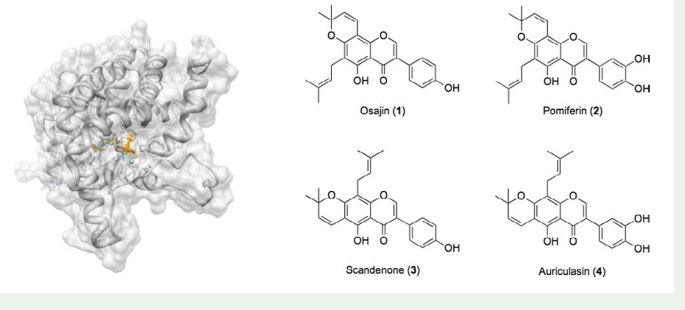
ABSTRACT

While osajin and pomiferin are known for their anticancer, antibacterial and antidiabetic properties, scandenone and auriculasin have been proposed as anti-inflammatory and antinociceptive agents. Curiously, these two couples of molecules are, from a chemical point of view, structural isomers which can all be extracted from *Maclura pomifera*. Although previous works described, separately, the isolation in reasonable amounts of the sole osajin/pomiferin couple or of scandenone/auriculasin, we report the extraction and characterization using direct spectral and chromatographical comparison of the four compounds. 2D NMR allowed to unambiguously assign the correct structures to the isomers. The compounds were screened *in silico* against PDE5 and their interaction pattern with the protein was compared with that of icarisid II, a natural PDE5 inhibitor.



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PDE5; isoflavonoids; icarisid II; sildenafil

1. Introduction

Natural flavonoids represent an attractive class of molecules, which have been widely investigated for their potential application in a large number of biological fields. In this context, it is known that *Maclura pomifera* ('Osage orange') is a convenient source of isoflavones such as osajin and pomiferin (Peterson et al. 2000; Tsao et al. 2003), which were reported to express anticancer, antibacterial and antidiabetic properties (Zhao et al. 2013; Mohamed et al. 2014; Moon 2014). Moreover, Kupeli et al. (2006) investigated the activity of scandenone and auriculasin, two major components of the chloroform extract of *M. pomifera*,

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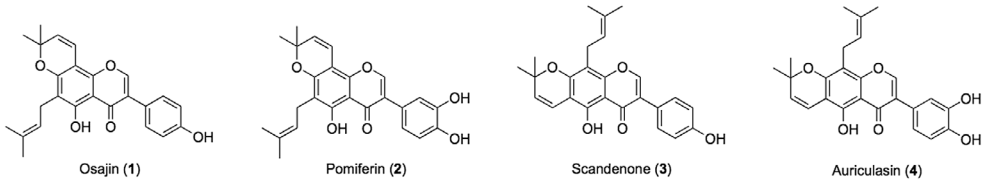

Figure 1. Chemical structures of the cited compounds.

as anti-inflammatory and antinociceptive agents. Scandenone and auriculasin are linear isomers of osajin and pomiferin, respectively (Figure 1).

We describe and compare the efficiency of the procedures for extracting osajin/pomiferin (Tsao et al. 2003; Ribaudo et al. 2015) and scandenone/auriculasin (Kupeli et al. 2006). All the compounds were isolated from *M. pomifera* and characterized by NMR and high-resolution mass spectrometry whilst assessing their purity by HPLC. In particular, taking advantage of 2D NMR experiments, we unambiguously identified and elucidated the structure of the isolated isomers.

One of the known and most attractive effects of flavonoids is the induction of vasorelaxation (possibly mediated by phosphodiesterase 5, PDE5), with eventual outcomes on pathologies such as erectile dysfunction (ED) and pulmonary hypertension (PH). With the aid of computational studies, we investigated the potential binding mode of the isolated compounds to PDE5.

2. Results and discussion

2.1. Extraction and structural elucidation

Kupeli et al. defined osajin and pomiferin (as) 'chemically constitutional isomers' of scandenone and auriculasin (Kupeli et al. 2006). According to literature, fruits of *M. pomifera* are sources of both sets of isomers. Extractions with ethyl acetate or diethyl ether are reported to give osajin and pomiferin (Tsao et al. 2003; Ribaudo et al. 2015), while chloroform extracts are said to be rich in scandenone and auriculasin (Kupeli et al. 2006), which were extracted in comparable yields (see Supplementary material for the detailed procedure and yields). Thus, previous works described the isolation of only small amounts of scandenone and auriculasin from *M. pomifera*, ranging from 'minor contaminants' to 15% of their respective angular isomers (Peterson et al. 2000; Delle Monache et al. 1994). For this research work, samples of *M. pomifera* fruits were collected by the authors in October 2015 in the Veneto region, Italy.

The isomer pair osajin and scandenone share the molecular formula ($C_{25}H_{24}O_5$), whilst both pomiferin and auriculasin have the molecular formula $C_{25}H_{24}O_6$. High-resolution mass spectrometry confirmed the calculated mass values without providing, as expected, any further clue for the structural elucidation. Melting points, ^{1}H and ^{13}C NMR analysis were in agreement with literature data for the four molecules. Delle Monache et al. described some 'diagnostic peaks' in the ^{1}H NMR spectrum (Delle Monache et al. 1994), which should help in distinguishing between linear and angular isomers. Thus, our direct comparison of ^{1}H NMR spectra within a couple of isomers showed only minor differences in the chemical shift values of some signals, mainly in the aromatic portion (see Supplementary material). We

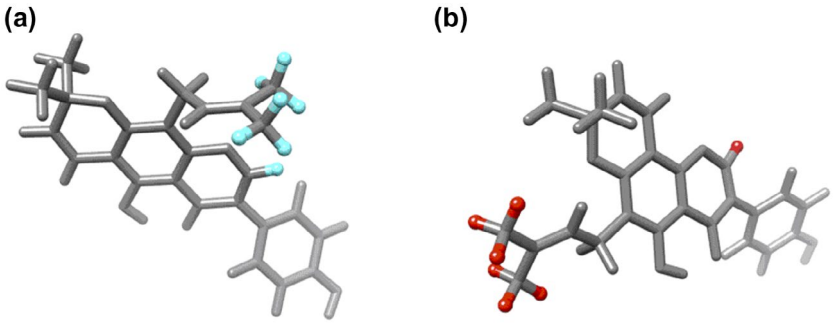

Figure 2. Comparison between minimized 3D arrangements (force field: MMFF94) of scandenone (a) and osajin (b). In scandenone, highlited functional groups are located in closer proximity. See Supplementary material for the 3D structures of the isomers.

identified in the NOESY NMR experiment a tool for unambiguously distinguishing the compounds and attributing the correct structures. Solution NOESY NMR highlights as cross-peaks in a 2D surface any non-scalar interactions between groups of protons which are in a close proximity within a molecule: we focused on two proton groups that relate very differently in the isomers and that could be used as probes. As shown in Figure 2, while the proton and the methyl groups highlighted in 'ball and sticks' are close enough in scandenone, they are well separated in its isomer osajin. This difference, which can also be encountered in pomiferin/auriculasin, causes that scandenone and auriculasin give a NOE signal that cannot be detected in the spectra of osajin and pomiferin (see Supplementary material for NOESY NMR spectra).

HPLC analysis was carried out on the isolated compounds. Different retention times were measured within the couples of isomers, with a marked separation between osajin and scandenone under the tested experimental conditions (see Supplementary material).

2.2. Docking studies and in silico analysis of the interaction with PDE5

Osajin, pomiferin and other flavonoids are being investigated as novel vasorelaxant agents and the involvement of the PDE5 inhibition in this effect is discussed thoroughly (Rodríguez-Ramos et al. 2013; Ribaudo et al. 2015). While osajin weakly inhibits PDE5 with an IC_{50} slightly under 50 µM (Ribaudo et al. 2015), icarisid II, a flavonoid from *Epimedium wanshanense*, has an IC_{50} of about 2 µM with selectivity against other PDEs (Wang et al. 2006). Moreover, a crystal structure of PDE5 in complex with icarisid II is also available (PDB ID: 2H44). The four natural compounds extracted from *M. pomifera* were docked to a crystal structure of PDE5 and their binding mode was explored. The interaction pattern with the protein was then compared to that observed in the icarisid II-PDE5 complex to highlight possible, shared exchanges.

The crystal structure of PDE5 in complex with its inhibitor sildenafil (PDB ID: 2H42) was used as target for the docking experiments. Protein and ligands were prepared with Avogadro 1.0.3 (http://avogadro.openmolecules.net) and dockings were performed using SwissDock (Grosdidier 2011a, 2011b) using default parameters for the algorithm. Docking results and interaction poses were analyzed with Chimera 1.6.2 (Huang et al. 1996; http://www.cgl.ucsf.edu/chimera).

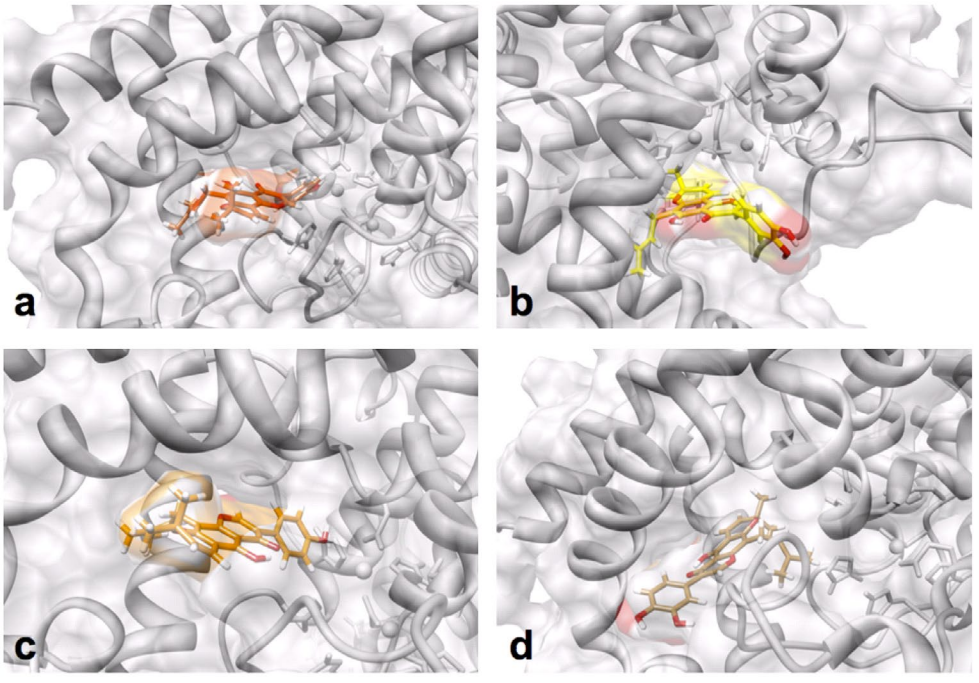

Figure 3. 3D interaction poses of osajin (a), pomiferin (b), scandenone (c) and auriculasin (d) docked to PDE5 (PDB ID: 2H42). See Supplementary material for full resolution color images of the docked compounds.

Table 1. Estimated ΔG (kcal/mol) values for the computed interactions and interacting residues with PDE5. Icarisid II is shown as reference.

Compound	Estimated ΔG (kcal/mol)	Interacting residues of PDE5
Osajin (**1**)	−8.37	Tyr612, His613, His617, Ile665, Asp764, Leu804, Ile813, Met816, Gln817, Phe820
Pomiferin (**2**)	−8.04	Tyr664, Gln666, Leu725, Leu765, Ala779, Ala783, Leu804, Ile813, Met816, Phe820
Scandenone (**3**)	−8.94	Tyr612, His613, His617, Ile665, Asp764, Ala783, Leu804, Ile813, Met816, Phe820
Auriculasin (**4**)	−8.15	Tyr664, Leu725, Ala767, Val782, Leu804, Met816, Gln817, Phe820
Icarisid II (**5**)	/	His613, His617, Asn661, Ser668, Ala767, Gln775, Leu804, Phe820

The compounds bearing a cathecol group, i.e. pomiferin and auriculasin, do not completely enter the binding pocket (Figure 3). This moiety is directed towards the solvent in the most stable conformational pose. On the other hand, osajin and scandenone penetrate more efficiently and the phenol ring is firmly pointed towards the metal atoms in the internal part of the enzyme under the same experimental conditions. Interestingly, the sole presence of a hydroxyl group radically changes the binding mode.

Docking results showed that scandenone gives the most efficient interaction *in silico*, followed by its isomer osajin (Table 1). This suggests that the pose assumed by the two compounds, with the phenol ring inside the enzyme and the hydrophobic chain towards the solvent, is favourable.

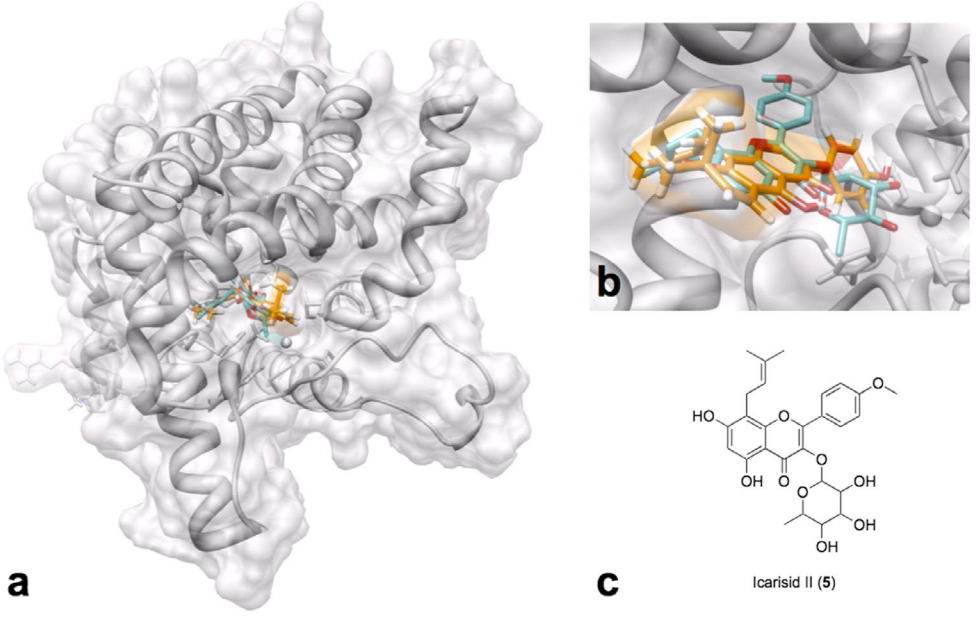

Figure 4. Comparison of the binding pose of scandenone, docked to PDE5 (PDB ID: 2H42) with that of icarisid II co-crystallized with the same enzyme (PDB ID: 2H44). The figure shows how scandenone binds to the same pocket of icarisid II (a). The residues involved in the ligand-protein interactions are highlighted (b). Chemical structure of icarisid II (c). See Supplementary material for full resolution color images of the docked compounds.

Confirming this preliminary observation, the binding pose predicted *in silico* for scandenone is extremely similar to that of icarisid II in its complex with PDE5 (PDB ID: 2H44). The compounds show a nearly complete superimposition of the scaffold and of the isoprenyl chain, which is due to a pose that only this isomer could assume (Figure 4).

The residues involved in the protein-lingand interaction (<5 Å) were also examined for all the docked compounds and icarisid II; the results are shown in Table 1. This analysis confirmed that osajin and scandenone have an interaction pattern that is very similar to that of icarisid II, involving many shared residues. Pomiferin and auriculasin, as pointed out above, behave differently towards PDE5. These isomers interact with the protein through a set of residues (mostly by hydrophobic interactions) that are not similar to those described for icarisid II. Docking experiments were also carried out on the 2H44 PDE5 model showing superimposing results (see Supplementary material).

To support the reliability of this prediction, the same screening routine was applied to a small set of natural flavones and isoflavones that were previously tested *in vitro* for their inhibitory activity on PDE5 (Ko et al. 2004). The selected compounds include weak (quercetin, myricetin, apigenin; IC_{50} > 100 μM), modest (genistein, IC_{50} 73.9) and fairly strong (luteolin, diosmetin and icariin; IC_{50} 19.3, 15.3 and 5.9 μM respectively) natural PDE5 inhibitors (Ko et al. 2004; Dell'Agli et al. 2008). The screening of this training set confirmed that all the small molecules bind to the same pocket, partially sharing the interaction pattern. The experiment also confirmed that the number of hydroxyl groups influences the binding orientation (see Supplementary material for the chemical structures of the compounds, docked poses and

docking results). On the other hand, icariin, which is the strongest *in vitro* inhibitor of the set, does not appear to interact with the same region of the protein and does not bind to the pocket. This is probably due to non-compatible steric features (i.e. bulky sugar groups bound to the phenols) as the comparison with the crystal structure of the related aglycone icarisid II would suggest. Considering that icariin may anyway exploit its action as a pro-drug *in vitro* and *in vivo*, the results of this screening confirm the relevance of some steric requirements for an efficient interaction with the target.

3. Conclusions

Osajin, pomiferin, scandenone and auriculasin were extracted from *M. pomifera* and their structures were elucidated and compared. The *in silico* screening of the compounds towards PDE5 highlighted how small structural differences (e.g. the presence of a cathecol instead of a phenol) may heavily influence the binding mode and the interaction pattern with PDE5, also in comparison with that of icarisid II. Concluding, binding motif and predicted binding energy suggest that scandenone has a good potential to interact with PDE5 and could be investigated as a novel inhibitor.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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