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Benzophenones as xanthone-open model CYP11B1 inhibitors potentially useful for promoting wound healing

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ABSTRACT

The inhibition of steroidogenic cytochrome P450 enzymes has been shown to play a central role in the management of life-threatening diseases such as cancer, and indeed potent inhibitors of CYP19 (aromatase) and CYP17 (17α hydroxylase/17,20 lyase) are currently used for the treatment of breast, ovarian and prostate cancer. In the last few decades CYP11B1 (11-β-hydroxylase) and CYP11B2 (aldosterone synthase), key enzymes in the biosynthesis of cortisol and aldosterone, respectively, have been also investigated as targets for the identification of new potent and selective agents for the treatment of Cushing's syndrome, impaired wound healing and cardiovascular diseases.

In an effort to improve activity and synthetic feasibility of our different series of xanthone-based CYP11B1 and CYP11B2 inhibitors, a small series of imidazolylmethylbenzophenone-based compounds, previously reported as CYP19 inhibitors, was also tested on these new targets, in order to explore the role of a more flexible scaffold for the inhibition of CYP11B1 and -B2 isoforms. Compound **3** proved to be very potent and selective towards CYP11B1, and was thus selected for further optimization via appropriate decoration of the scaffold, leading to new potent 4′-substituted derivatives. In this second series, **4** and **8**, carrying a methoxy group and a phenyl ring, respectively, proved to be low-nanomolar inhibitors of CYP11B1, despite a slight decrease in selectivity against CYP11B2. Moreover, unlike the benzophenones of the first series, the 4′-substituted derivatives also proved to be selective for CYP11B enzymes, showing very weak inhibition of CYP19 and CYP17.

Notably, the promising result of a preliminary scratch test performed on compound **8** confirmed the potential of this compound as a wound-healing promoter.

1. Introduction

Cortisol, a steroidal glucocorticoid hormone biosynthesized in the adrenal gland under the control of the hypothalamus-pituitaryadrenal axis, plays a crucial role in regulating many essential physiological functions, including stress response, suppression of immune system, various metabolic processes and neurogenesis. Its production and release are regulated by two main signals, corticotropin-releasing hormone (CRH)[a] and adrenocorticotropic hormone (ACTH), and a dysregulation of this refined control mechanism leads to hypercortisolism and to the onset of pathologies such as Cushing's Syndrome (CS) and impaired wound healing [1]. CS is a life-threatening disease characterized by a vast array of symptoms, including hypertension, obesity and diabetes, which could ultimately lead to fatal complications. The currently applied therapeutic strategy includes the use of glucocorticoid receptor antagonists, nonselective inhibitors of adrenal steroidogenesis or drugs able to reduce ACTH secretion, often showing limited efficacy and unacceptable incidence of side effects [2]. On the other hand, the occurrence of chronic wounds, affecting around 1–2% of the population, is generally a comorbid condition, often arising in combination

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[a] CRH (corticotropin-releasing hormone); ACTH (adrenocorticotropic hormone); CS (Cushing's Syndrome); CYP11B1 (11-beta hydroxylase); CYP11B2 (aldosterone synthase); CYP (cytochrome P450); CYP19 (aromatase); CYP17 (17α hydroxylase/17,20 lyase); 1-ImiXs (1-imidazolylmethylxanthones); 3-ImiXs (3-imidazolylmethylxanthones); NBS (*N*-bromosuccinimide); BPO (Benzoylperoxyde).

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with diabetes and obesity. Due to the increased incidence of these diseases and to the aging of population, the number of patients is expected to progressively increase. The onset of chronic wounds is probably linked to a cutaneous overexpression of cortisol, leading to an impaired inflammatory response, blocking fibroblast proliferation, and collagen synthesis, and finally to an increased risk of infection [3]. Innovative treatments for this unpleasant disease involve stem cell applications and skin transplantation, techniques that proved to be expensive and often unsatisfactory. In this framework, drugs able to reduce cortisol concentration at skin level by reducing its biosynthesis could represent a valuable therapeutic option.

CYP11B1 (11-β-hydroxylase) and CYP11B2 (aldosterone synthase) are cytochrome P450 (CYP) isoforms mainly expressed in the adrenal cortex, CYP11B1 in the zona fasciculata and CYP11B2 in the zona glomerulosa, where they catalyse key steps in the biosynthesis of cortisol and aldosterone, respectively. Like CYP19 (aromatase), responsible for the production of estrogens and CYP17 (17α hydroxylase/17,20 lyase), involved in the biosynthesis of androgens, CYP11B1 and B2 represent validated drug targets for treating disorders related to abnormally increased levels of adrenal steroidal hormones. These isoforms share a high degree of sequence homology (up to 94%), differing by only 30 residues, all located away from the substrate recognition site. Therefore, the identification of specific features allowing the design of selective inhibitors becomes particularly challenging. Despite some exciting improvements in the search for selective compounds able to inhibit CYP11B1 [4–6], no specific inhibitor has been approved to treat hypercortisolism. Yet, some compounds have been reported [4], for which the ability to inhibit CYP11B1 with high potency could be exploited to promote chronic wound healing, overcoming the drawback of low selectivity *vs* CYP11B2. Indeed, the biosynthesis of CYP11B1 was found to be up-regulated in injured tissues [7], so that an excess of cortisol impairing wound healing is produced, while CYP11B2 isoform has not been observed in skin cells [8]. On the other hand, in the last years a number of potent and selective inhibitors of CYP11B2 have been reported [9–12], highlighting the crucial role of a nitrogen-containing heterocyclic function (pyridine or imidazole) for a proper inhibition. It is indeed well known that CYPs inhibition takes advantage of the presence of a nitrogenated heterocycle, able to coordinate the heme iron establishing the primary interaction with the enzyme, while a properly decorated suitable scaffold could discriminate between different related cytochromes.

In our long lasting experience in the field of steroidogenic cytochromes inhibitors, we investigated the ability of appropriately substituted oxaheterocyclic cores, structurally related to natural compounds (xanthones, chromones and flavones), to inhibit different CYPs [13–15]. Key feature for inhibition appeared to be an imidazolylmethyl moiety, able to interact with the heme-iron in the active site of these enzymes, while appropriate and correctly positioned substituents could account for fine-tuning of activity. In particular, selective modulation of the potency towards different cytochromes was obtained with a suitable pattern of substitution on the xanthone scaffold, whose large conjugated π system would conveniently form π-π interactions with several aromatic aminoacid residues in the active sites and contribute, together with the selected substituent, to the inhibitory activity [15]. This heterocycle again confirmed to be a highly versatile “privileged structure”, whose activity can be directed to different targets when appropriately functionalized.

In recent papers, the design and synthesis of series of 1- and 3-imidazolylmethylxanthones (**1-ImiXs** and **3-ImiXs**, Fig. 1) carrying different substituents in positions 4 or 6 of the central core, as potent inhibitors of CYP11B1-2 enzymes, was reported [15–17]. Notably, moving the imidazolylmethyl moiety from position 1 to position 3 led to a remarkable shift in activity towards CYP11B1. Considering the primary interaction of the sp^2 hybridized imidazole nitrogen of the ligands via coordination of the heme iron, which normally shows a perpendicular setting and serves as an anchor to binding, modification of the substitution pattern was proved to change the orientation of the molecule, leading to different inhibitory activities towards the two highly homologue enzymes. Some derivatives endowed with low-nanomolar potency for the inhibition of CYP11B1 were obtained and noteworthy, in the 6-substituted **3-ImiXs** series, more than in previously reported ones, the nature of substituents seemed negligible for activity on this isoform.

In this study, with the aim to evaluate whether the planar and conformationally constrained xanthone moiety is a critical feature for optimal interaction with these enzymes, its ether oxygen was removed and a small series of flexible imidazolylmethylbenzophenones (Fig. 1) was designed and synthesized. A similar strategy had been successfully applied in the search for aromatase inhibitors, enabling us to obtain potent compounds [18], and offered the advantage of a large synthetic accessibility, which also allowed for an easy identification of the best positioning of the imidazole nitrogen on the benzophenone scaffold. Indeed, in that series the position of the imidazole ring on the benzophenone was varied, and substituents were also introduced in different positions.

For the new series reported here, the unsubstituted 2-, 3- and 4-imidazolylmethylbenzophenone already tested for aromatase inhibition [18] (**1–3**, Fig. 2) were screened for activity towards CYP11B enzymes and were found to be quite potent inhibitors of CYP11B1, while showing lower efficacy towards CYP11B2 (Table 1, see also Biological evaluation section). Among them **3**, carrying the imidazolylmethyl chain in position 4, proved to be the most potent and selective for CYP11B1 over CYP11B2, while appreciable but not extremely high potency on aromatase was previously reported. Taking into account that the corresponding xanthone derivative **3-ImiX** (Fig. 2) had been the most promising of its series as regards selectivity for CYP11B1 (Table 1) [17], **3** was selected for further modifications. Therefore, this compound was decorated with different substituents in position 4′, mimicking the 6-substituted parent **3-ImiXs** (Fig. 1). In particular, methoxy and phenyl moieties were selected to evaluate the roles of different size and lipophilicity on activity; hydroxyl groups were also introduced to study the effect of H-bond donors. Finally, a fluorine atom was appended, due to its known ability to influence pharmacokinetic and physicochemical properties such as metabolic stability, membrane permeation and intrinsic potency, when properly introduced into potential drugs, since it could affect the metabolism of the adjacent hydroxyl group. The new compounds, whose structures are reported in Fig. 3, were tested for inhibition of CYP11B1/2 and for inhibition of the related steroidogenic enzymes CYP19 and 17, to establish their selectivity.

2. Results and discussion

2.1. Chemistry

The compounds were prepared according to Scheme 1. The synthesis of the benzophenone nucleus was performed *via* Friedel-Craft acylation of biphenyl or the selected anisole with *p*-toluylchloride in dry methylene chloride, using $AlCl_3$ as Lewis acid. The 4-methylbenzophenone intermediates **9–12** were then brominated with NBS in the presence of benzoyl peroxide (BPO) and the bromomethyl derivatives, without purification, were then reacted with imidazole in refluxing acetonitrile under nitrogen to provide the intermediates **13** and **14** and the final compounds **4** and **8**. The hydroxy derivatives **5–7** were obtained by demethylation of **4**, **13** and **14** by refluxing in HBr 48% solution.

2.2. Biological evaluation

The testing results for CYP11B1-2 inhibition of the previously reported unsubstituted imidazolylmethylbenzophenones **1–3** are collected in Table 1, together with earlier data obtained for CYP19 and

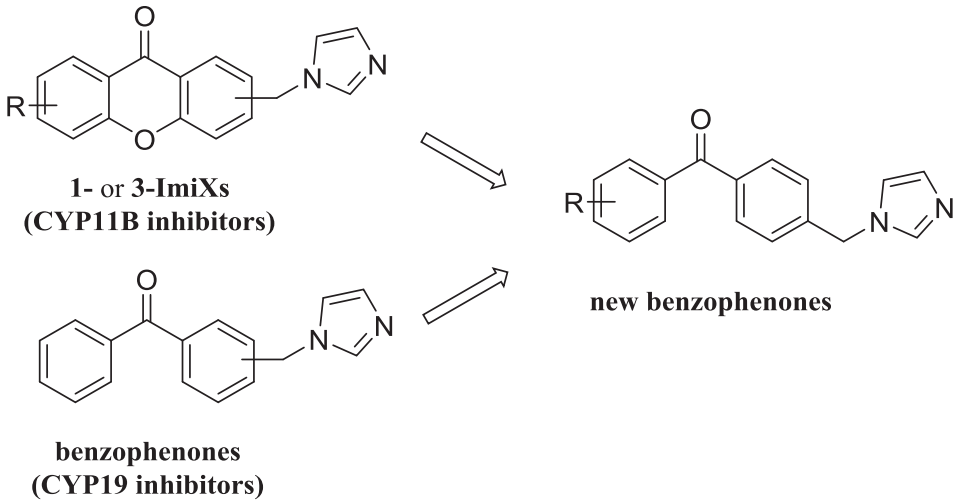


Fig. 1. Design strategy.

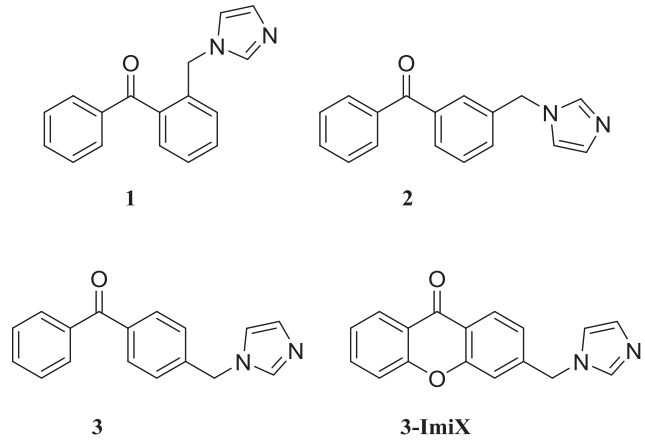


Fig. 2. Benzophenones **1–3** previously tested on aromatase and **3-ImiX** as reference compound.

Table 1
Inhibition of CYP11B, CYP19 and CYP17 for previously reported unsubstituted benzophenones and **3-ImiX**.

Compound	CYP11B1[a] IC_{50} (nM)[b]	CYP11B2[a] IC_{50} (nM)[b]	CYP19[c] IC_{50} (nM)[b]	CYP17[d] % inhib. 2.5 μM	SF[e] B2/B1
1	66.2	105.9	560[f]	NA[f]	1.6
2	69.5	178.7	7.3[f]	NA[f]	2.6
3	17.3	103.5	252[f]	NA[f]	6.0
3-ImiX[g]	70.8	344.1	390	33%	4.9

[a] The deviations were within 25%. Hamster fibroblasts expressing human CYP11B1/B2; substrate: deoxycorticosterone 100 nM.
[b] The given values are mean values of at least three experiments.
[c] The deviations were within 5%. Human placental CYP19; substrate androstenedione, 500 nM.
[d] *Escherichia coli* expressing human CYP17; substrate progesterone, 25 μM.
[e] Selectivity factor: IC_{50}CYP11B2/ IC_{50}CYP11B1.
[f] See Ref. [18].
[g] See Ref. [17]. NA = no activity detected.

CYP17 inhibition. In this small series, while the compounds showed the same range of activity on CYP11B2, a small difference could be seen for inhibition of CYP11B1. Compound **3**, in which the nitrogen-containing chain is located in position 4, proved to be the most potent and selective inhibitor of this isoform (IC_{50} = 17.3 nM and $SF_{B2/B1}$ = 6.0), showing that this site is the most appropriate for the positioning of the imidazolylmethyl group on this scaffold. This finding is in line with the results obtained when the position of the chain was varied on the xanthone nucleus [17], where **3-ImiX** proved to be one of the most active compounds and the most selective for CYP11B1. In fact, the removal of the ether oxygen and the consequent increase in flexibility of the molecule proved to enhance activity on both CYP11B enzymes. Compound **3** also showed appreciable selectivity towards CYP19 and CYP17, making it a suitable compound for further modifications.

The results obtained with the introduction of substituents on the 4′-position of the scaffold of **3**, collected in Table 2, show that hydrophilic groups (as in compounds **5**, **6** and **7**) led to a general decrease in inhibitory activity and selectivity with respect to the unsubstituted **3**. In contrast, compounds **4** and **8**, bearing more lipophilic substituents (namely methoxy and phenyl, respectively), were endowed with low-nanomolar potency towards both CYP11B1 (IC_{50} values of 16.7 nM and 8.6 nM, respectively) and CYP11B2 (29.9 nM and 17.2 nM, respectively). In particular, these substituents proved to positively influence the inhibition of CYP11B2 to a higher extent with respect to CYP11B1, for which an appreciable increase was observed only with the introduction of a phenyl group (compound **8**). As a consequence, selectivity between the CYP11B isoforms was somewhat lost compared to the parent compound **3**. Remarkably, while comparing the unsubstituted **3** to the 4′-methoxy derivative **4** only an increase in activity towards CYP11B2 could be seen (IC_{50} values of 103.5 nM *vs* 29.9 nM, respectively), the presence of a phenyl group in compound **8** was able to affect the activities on different CYPs, leading to an increase in potency for inhibition of CYP11B1 and –B2 and a 10-fold decrease for CYP19. This behavior could represent a favorable feature for the development of agents to be used for the promotion of wound healing, for which the selectivity between the two CYP11B isoforms does not seem to be a crucial issue, while the inhibition of CYP19 could be detrimental for activity. Indeed, the expression of CYP19 and CYP17 in epidermis tissue has been reported, and estrogens in the skin could have a moisturizing effect, thus preventing aging and favorably influencing wound healing [19].

From a structural point of view, these data seem to indicate that an increase in flexibility allowed the compounds to properly fit in the enzyme active site and to maintain a relevant activity, compared to the more rigid xanthone-based compounds. However, the potency seemed to be related to the lipophilicity and/or size of the substituent in position 4′, as demonstrated by the progressive reduction in activity noticed by shifting from the 4′-phenyl (**8**) to the 4′-methoxy (**4**), the unsubstituted (**3**) and the 4′-hydroxy (**5**) derivative. Moreover, the simultaneous introduction of two substituents (3′ and 4′-positions) led to a marked decrease in activity on both enzymes (**6** and **7** with respect to **5**), which was more significant with the introduction of an additional

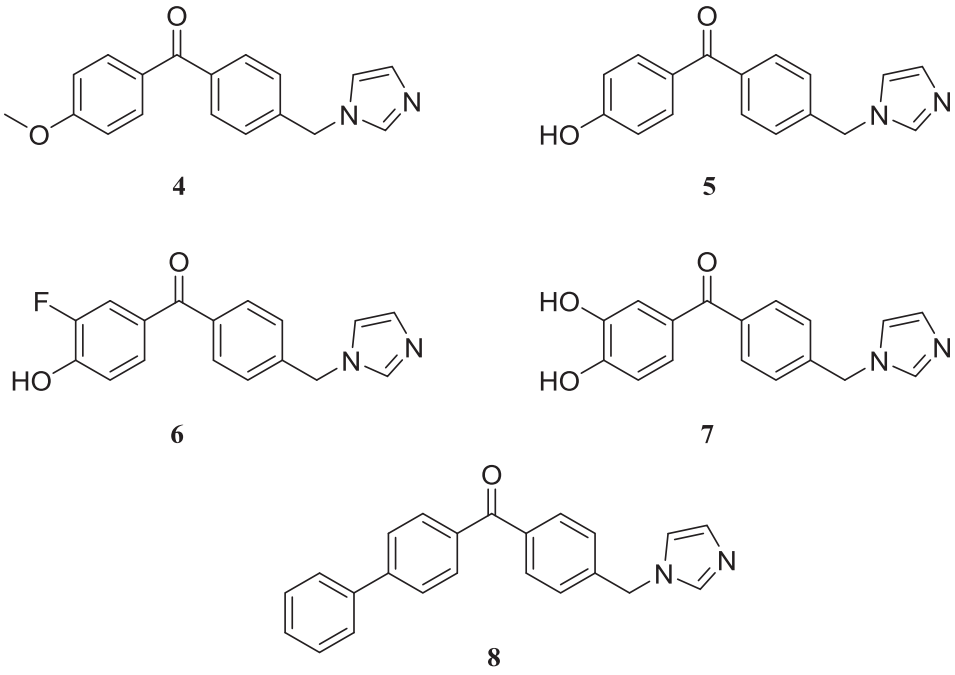
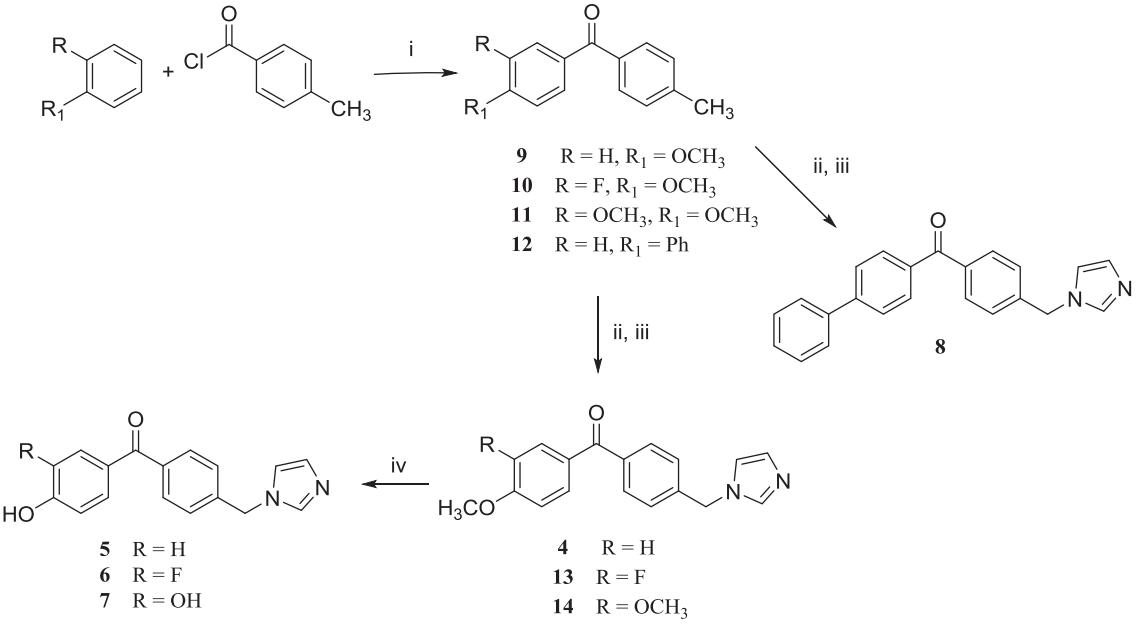


Fig. 3. Newly synthetized benzophenones **4–8**.

hydroxyl group than with a fluorine atom, very similar in size to the hydrogen. Finally, while selectivity between CYP11B1 and –B2 was somewhat decreased by the introduction of substituents with respect to **3**, some of the new compounds proved to be considerably selective towards related steroidogenic enzymes, showing a micromolar potency on CYP19 and being devoid of CYP17 inhibiting activity.

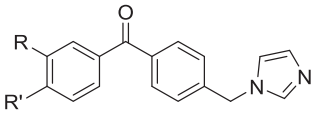
To further investigate the binding of the synthesized inhibitors in CYP11B1, compounds **3** and **8** were docked into a human CYP11B1 homology model [20]. In the most favorable binding poses, free binding energy values of −9.13 and −9.39 Kcal/mol were observed for compounds **3** and **8**, respectively, indicating high binding affinities. Not surprisingly, the compounds coordinate to the heme iron in perpendicular manners (Fig. 4). With such an anchor, both molecules lean against the β2-sheet, however, in different modes: the benzophenone core of compound **3** stretches along the β2-sheet, in contrast to the cross position that compound **8** forms with its additional phenyl moiety. The binding of both compounds is riveted by the π-π interactions between their conjugating body and Phe130, Phe381 and Phe487, while an



Reagents and conditions: i) $AlCl_3$, dry CH_2Cl_2, rt; ii) NBS, CCl_4, hν, BPO; iii) imidazole, CH_3CN, N_2; iv) HBr 48%.

Scheme 1. Synthesis of the studied compounds.

Table 2
Inhibition of CYP11B, CYP19 and CYP17 for the studied benzophenones.



Compound	R	R′	CYP11B1[a] IC_{50} (nM)[b]	CYP11B2[a] IC_{50} (nM)[b]	CYP19[c] IC_{50} (nM)[b]	CYP17[d] % inhib. 10 μM	SF[e] B2/B1
3	H	H	17.3	103.5	252[f]	NA[f]	6.0
4	H	OCH_3	16.7	29.9	237.8	17.5%	1.8
5	H	OH	44.7	113.5	1100	34.4%	2.5
6	F	OH	232.4	256.9	1237.5	31%	1.1
7	OH	OH	808.1	1028.1	5275	3.4%	1.3
8	H	Ph	8.6	17.2	2531	16.6%	2

[a] The deviations were within 25%. Hamster fibroblasts expressing human CYP11B1/B2; substrate: deoxycorticosterone 100 nM.
[b] The given values are mean values of at least three experiments.
[c] The deviations were within 15%. Human placental CYP19; substrate androstenedione, 500 nM.
[d] *Escherichia coli* expressing human CYP17; substrate progesterone, 25 μM.
[e] Selectivity factor (SF): IC_{50}CYP11B2/ IC_{50}CYP11B1.
[f] See Ref. [18].

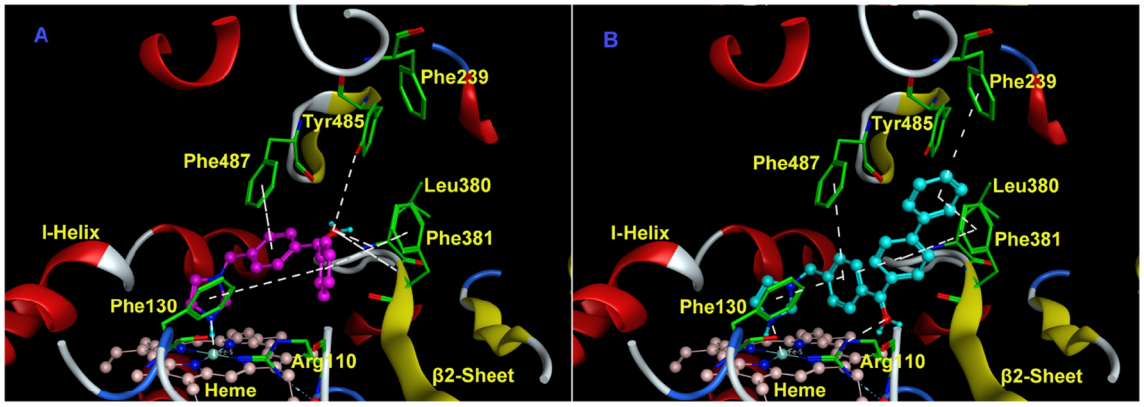


Fig. 4. Docking of compounds **3** (A, magenta) and **8** (B, cyan) in a CYP11B1 homology model.

additional π-π interaction between the far-end phenyl group and Phe239 is observed for compound **8**. Furthermore, the ketones point into distinct directions: in compound **3**, it forms a net of hydrogen-bonds with the phenol and amide H of Tyr485 as well as Phe381 and Leu380, respectively, whereas only the interaction with Arg110 is present for compound **8**. These hydrogen bonds contribute significantly to the stability of this binding mode and thus to the affinity for the target enzyme.

Finally, in the light of the obtained results and aiming at evaluating the wound healing potential of the newly synthetized molecules, an in vitro scratch test was performed on the most active compound **8** employing the HT-29 cell line, a human colon cancer cell line expressing relatively high levels of CYP11B1 [21]. This inexpensive and straightforward assay allows obtaining a first insight on the ability of compounds to positively influence new tissue formation, promoting cell migration following a purposely induced injury in a cell monolayer, mimicking what happens during wound healing in vivo [22]. First, the cytotoxicity of the compound was measured with the 4-methylumbelliferyl heptanoate (MUH) assay to select the concentrations to be used in the scratch test. The compound exhibited a cytotoxic effect only at the highest tested concentration (100 μM) (data not shown). Thus, 4 and 40 nM treatments were chosen to perform the scratch test.

As reported in Fig. 5, the compound stimulated the filling of the scratch with healthy cells starting after 4 days of treatment. A more pronounced effect was recorded after 8 days. By then, at 4 and 40 nM treatments, the compound provoked 47.71 ± 1.20% and 63.62 ± 1.99%, respectively, of wound healing compared to 31.52 ± 0.95% of untreated cells. These preliminary data seem to validate the ability of this compound to act as a wound-healing promoter, making this class of derivatives worth of further in-depth studies.

3. Conclusions

In this study, taking advantage of the positive results obtained in the field of CYP19 inhibitors, the role of the increase in flexibility obtained by moving from the xanthone to the benzophenone scaffold was also evaluated for the homologues enzymes CYP11B1 and –B2. The best positioning of the key imidazolylmethyl chain for interaction with CYP11B1 was identified and an optimization process was attempted with the synthesis of a purposely designed small series of 4′-substituted derivatives. An evident preference for lipophilic groups was noticed, as activity progressively increased shifting from hydroxyl to hydrogen, methoxy and phenyl substituents on the benzophenone core.

Compound **8**, bearing an additional phenyl ring and thus embodying a biphenyl moiety, emerged as the most promising derivative of the series, being endowed with low nanomolar potency on CYP11B1 and a remarkable selectivity towards CYP19 and CYP17. The potential use of this compound for topical application, supported by the results of a preliminary in vitro assay, would circumvent the low selectivity for the highly omologue isoform CYP11B2, making it a suitable drug candidate for the acceleration of chronic wound healing.

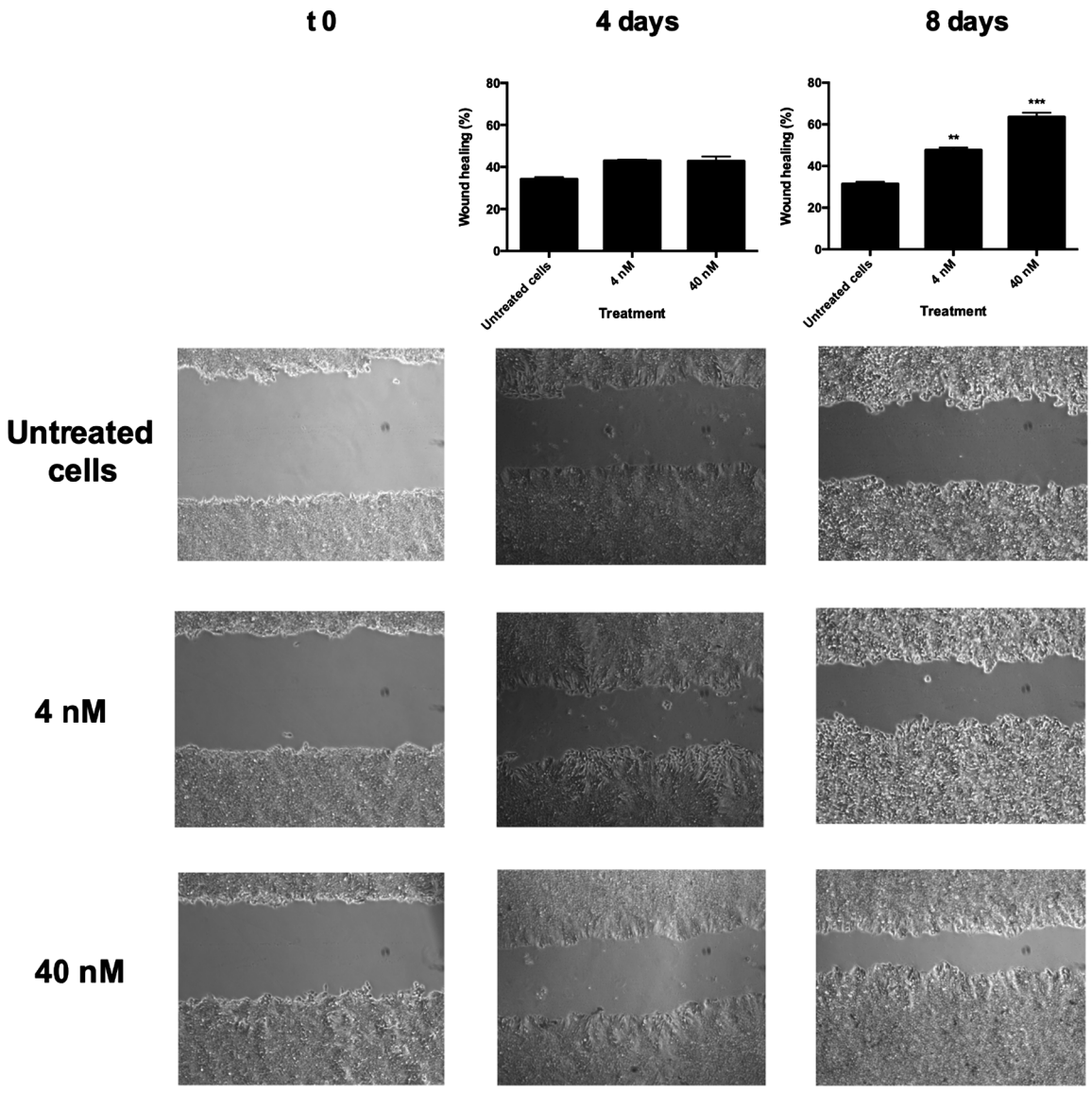


Fig. 5. Percentage of wound healing and representative images of the progression of wound closure after 4 and 8 days of treatment with compound **8**. Original magnification ×10. Data shown are means ± SEM, $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Experimental

4.1. Chemistry

4.1.1. General methods

All melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, unless otherwise indicated, on a Varian VXR Gemini spectrometer 400 MHz and 101 MHz, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as internal standard and spin multiplicities are given as s (singlet), d (doublet), t (triplet), m (multiplet) or br (broad). Direct infusion ES-MS spectra were recorded on a Waters Micromass ZQ 4000 apparatus. Chromatographic separations were performed by flash chromatography on silica gel columns (Kieselgel 40, 0.040–0.063 mm; Merck). Organic solutions were dried over anhydrous sodium sulfate. Elemental analysis was used to determine purity of the described compounds. Analyses indicated by the symbols of the elements were within ± 0.4% of the theoretical values. All chemicals were purchased from Aldrich Chemistry, Milan (Italy), or from Alfa Aesar, Milan (Italy), and were of the highest purity. Compounds were named relying on the naming algorithm developed by CambridgeSoft Corporation and used in ChemDraw Professional 15.0.

4.1.2. General method for the preparation of benzophenones **9–12**

To a solution of the selected anisole or biphenyl (13.0 mmol) in 30 mL of dry CH_2Cl_2 at 0° C, *p*-toluoylchloride (2 g, 13.0 mmol), and $AlCl_3$ (3 g, 22.05 mmol) were added and the mixture was stirred at rt overnight. After addition of ice, the mixture was diluted with CH_2Cl_2, washed with $NaHCO_3$ saturated solution, dried and evaporated to dryness.

4.1.2.1. (4-methoxyphenyl)(p-tolyl)methanone ***9***. Using the previously reported procedure and starting from 1.4 g of anisole, 2.41 g of **9** were obtained (82%), mp 91–92 °C (lit. [23] mp 94–95 °C). ^{1}H NMR: δ 2.46 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 7.09 (d, J = 8.8 Hz, 2H arom.), 7.37 (d, J = 8.4 Hz, 2H arom.), 7.66 (d, J = 8.0 Hz, 2H arom.), 7.75 (d, J = 8.4 Hz, 2H arom.).

4.1.2.2. (3-fluoro-4-methoxyphenyl)(p-tolyl)methanone ***10***. Using the previously reported procedure and starting from 1.64 g (1.46 mL) of 2-fluoroanisole, 2.47 g of **10** were obtained (78%), mp 78–80 °C (lit.

[23] mp 79–81 °C). ^{1}H NMR: δ 2.45 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), 7.11 (s, 1H arom.), 7.24 (d, J = 8.4 Hz, 2H arom) 7.46 (d, J = 7.2 Hz, 2H arom.), 7.75 (d, J = 8.0 Hz, 2H arom.).

4.1.2.3. (3,4-dimethoxyphenyl)(p-tolyl)methanone ***11***. Using the previously reported procedure and starting from 1.8 g of 1,2-dimethoxybenzene, 2.65 g of **11** were obtained (80%), mp 105–106 °C (lit. [23] mp 105–107 °C). ^{1}H NMR: δ 2.42 (s, 3H, CH_3), 3.95 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 7.18 (s, 1H arom.), 7.28 (d, J = 8.4 Hz, 2H arom) 7.53 (d, J = 7.2 Hz, 2H arom.), 7.81 (d, J = 8.0 Hz, 2H arom.).

4.1.2.4. [1,1′-biphenyl]-4-yl(p-tolyl)methanone ***12***. Using the previously reported procedure and starting from 2.0 g of biphenyl, 2.54 g of **12** were obtained (72%), mp 127–129 °C (lit. [24] mp 121–122 °C). ^{1}H NMR: δ 2.47 (s, 3H, CH_3), 7.31 (d, J = 8.0 Hz, 2H arom.), 7.42–7.51 (m, 3H arom), 7.66 (d, J = 7.2 Hz, 2H arom.), 7.71 (d, J = 8.0 Hz, 2H arom.), 7.77 (d, J = 8.0 Hz, 2H arom.), 7.88 (d, J = 8.4 Hz, 2H arom).

4.1.3. General method for the preparation of imidazole derivatives ***4, 8, 13, 14***

A mixture of the selected benzophenone (5.0 mmol) and *N*-bromosuccinimide (0.85 g, 5.0 mmol) in CCl_4 (50 mL), in the presence of a catalytic amount of benzoyl peroxide, was refluxed for 5 h and then hot filtered. The solvent was evaporated to dryness to give the corresponding bromomethyl derivative, which was used without further purification. A mixture of this derivative and imidazole (0.9 g, 14 mmol) in CH_3CN (40 mL) was refluxed under N_2 for 6 h. The solvent was evaporated to dryness and the residue was purified by flash chromatography (toluene/acetone 4:1) to give the desired compound (yield 15–21%).

4.1.3.1. (4-((1H-imidazol-1-yl)methyl)phenyl)(4-methoxyphenyl) methanone ***4***. Using the previously reported procedure and starting from 1.13 g of **9**, **4** was obtained, mp 103–106 °C (ligroin). ^{1}H NMR (DMSO): δ 3.85 (s, 3H, OCH_3), 5.32 (s, 2H, CH_2-imi), 6.94 (s, 1H, imi), 7.08 (d, J = 8.8 Hz, 2H arom.), 7.24 (s, 1H, imi), 7.37 (d, J = 8.4 Hz, 2H arom.), 7.67 (d, J = 8.0 Hz, 2H arom.), 7.73 (d, J = 8.4 Hz, 2H arom.), 7.80 (s, 1H, imi). ^{13}C NMR δ: 49.14, 55.63, 113.98, 119.78, 127.34, 128.90, 129.37, 129.77, 132.23, 137.16, 137.65, 141.94, 163.06, 194.05. MS (ES) *m/z*: 293 (M + H), 315 (M + Na). Anal. $C_{18}H_{16}N_2O_2$ (C, H, N).

4.1.3.2. (4-((1H-imidazol-1-yl)methyl)phenyl)(3-fluoro-4-methoxyphenyl)methanone ***13***. Using the previously reported procedure and starting from 1.22 g of **10**, **13** was obtained as oily compound. ^{1}H NMR: δ 4.01 (s, 3H, OCH_3), 5.25 (s, 2H, CH_2-imi), 6.95–7.18 (m, 2H arom + imi), 7.28 (d, J = 8.4 Hz, 2H arom) 7.53–7.55 (m, 3H, arom + imi), 7.62 (s, 1H, imi), 7.81 (d, J = 8.0 Hz, 2H arom.).

4.1.3.3. (4-((1H-imidazol-1-yl)methyl)phenyl)(3,4-dimethoxyphenyl) methanone ***14***. Using the previously reported procedure and starting from 1.28 g of **11**, **14** was obtained as oily compound. ^{1}H NMR: δ 3.95 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 5.31 (s, 2H, CH_2-imi), 6.96–7.18 (m, 2H, arom + imi), 7.28–7.31 (m, 3H, arom + imi), 7.53 (d, J = 7.2 Hz, 2H arom.), 7.62 (s, 1H, imi), 7.81 (d, J = 8.0 Hz, 2H arom.).

4.1.3.4. (4-((1H-imidazol-1-yl)methyl)phenyl)([1,1′-biphenyl]-4-yl) methanone ***8***. Using the previously reported procedure and starting from 1.36 g of **12**, **8** was obtained, mp 123–125 °C. ^{1}H NMR: δ 5.24 (s, 2H, CH_2-imi), 6.96 (s, 1H, imi), 7.16 (s, 1H, imi),7.26 (d, J = 8.0 Hz, 2H arom.), 7.42–7.51 (m, 3H arom), 7.62 (s, 1H, imi), 7.65 (d, J = 7.2 Hz, 2H arom.), 7.71 (d, J = 8.0 Hz, 2H arom.), 7.83 (d, J = 8.0 Hz, 2H arom.), 7.88 (d, J = 8.4 Hz, 2H arom). ^{13}C NMR δ: 50.61, 119.50, 127.15, 127.20, 127.45, 128.42, 129.14, 130.19, 130.82, 136.03, 137.67, 137.90, 140.01, 140.67, 145.66, 195.68. MS (ES) *m/z*: 339 (M + H), 361 (M + Na). Anal. $C_{23}H_{18}N_2O$ (C, H, N).

4.1.4. General method for demethylation

To the selected methoxy derivative (2.0 mmol) an excess of HBr 48% (20 mL) was added, and the mixture was heated under reflux for 8 h. After dilution with H_2O, the solution was basified with NaOH pellets, washed with CH_2Cl_2, acidified to pH 6 with HCl 37%. The solid was collected by filtration.

4.1.4.1. (4-((1H-imidazol-1-yl)methyl)phenyl)(4-hydroxyphenyl) methanone ***5***. Using the previously reported procedure and starting from 0.58 g of **4**, **5** was obtained, yield 58%, mp 120 °C (d). ^{1}H NMR (DMSO): δ 5.32 (s, 2H, CH_2-imi), 6.88 (d, J = 8.8 Hz, 2H arom.), 6.95 (s, 1H, imi), 7.25 (s, 1H, imi), 7.36 (d, J = 8.0 Hz, 2H arom.), 7.62–7.76 (m, 4H arom), 7.81 (s, 1H, imi), 10.43 (s, 1H, OH). ^{13}C NMR δ: 49.99, 115.35, 127.65, 127.79, 129.66, 132.54, 137.81, 162.14, 193.89. MS (ES) *m/z*: 279 (M + H), 301 (M + Na). Anal. $C_{17}H_{14}N_2O_2$ (C, H, N).

4.1.4.2. (4-((1H-imidazol-1-yl)methyl)phenyl)(3-fluoro-4-hydroxyphenyl) methanone ***6***. Using the previously reported procedure and starting from 0.62 g of **13**, **6** was obtained, yield 52%, mp 232–235 °C. ^{1}H NMR (DMSO): δ 5.56 (s, 2H, CH_2-imi), 7.09 (t, J = 8.4 Hz, 1H arom.), 7.43–7.45 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H arom.), 7.51–7.56 (m, 3H arom + imi), 7.72–7.74 (m, 3H arom + imi), 7.84 (s, 1H, imi), 9.28 (s, 1H, arom), 10.99 (s, 1H, OH). ^{13}C NMR δ: 51.22, 117.44, 117.67, 120.69, 122.23, 128.04, 128.21 129.87, 135.82, 137.67, 139.00, 149.37, 150.04, 150.16, 151.79, 193.06. MS (ES) *m/z*: 297 (M + H). Anal. $C_{17}H_{13}FN_2O_2$ (C, H, N).

4.1.4.3. (4-((1H-imidazol-1-yl)methyl)phenyl)(3,4-dihydroxyphenyl) methanone ***7***. Using the previously reported procedure and starting from 0.64 g of **14**, **7** was obtained, yield 48%, mp 250 °C (d). ^{1}H NMR (DMSO): δ 5.38 (s, 2H, CH_2-imi), 6.84 (d, J = 7.6 Hz, 1H arom.), 7.03–7.08 (m, 2H arom), 7.22 (s, 1H, imi), 7.31 (s, 1H, imi), 7.37 (d, J = 7.6 Hz, 2H, arom), 7.63 (d, J = 7.2 Hz, 2H, arom), 7.96 (s, 1H, imi), 9.42 (s, 1H, OH), 9.92 (s, 1H, OH). ^{13}C NMR δ: 50.04, 116.04, 116.68, 125.65, 127.82, 129.66, 132.54, 133.42, 137.81, 145.70, 152.14, 193.98. MS (ES) *m/z*: 295 (M + H). Anal. $C_{17}H_{14}N_2O_3$ (C, H, N).

4.2. Biological methods

4.2.1. Inhibition of CYP11B1 and CYP11B2

V79 MZh cells expressing human CYP11B1 or CYP11B2 were grown on six-well cell culture plates with 9.6 cm^2 culture area per well until confluence. The reaction was subsequently started by the addition of [1,2-^{3}H]-11-deoxycorticosterone as substrate and corresponding inhibitor at different concentrations. After incubations of 6 h for V79MZh11B1 and 30 min for V79MZh11B2 cells, respectively, enzyme reactions were stopped by extracting the supernatant with chloroform. Samples were centrifuged (10,000*g*, 10 min) and the solvent was pipetted into fresh cups. The solvent was evaporated, the steroids were redissolved in chloroform and analysed by HPTLC [25,26].

4.2.2. CYP17 preparation and assay

Human CYP17 was co-expressed with rat NADPH-P450 reductase in *E. coli*, which was subsequently treated with lysozyme, incubated on ice with continuous shaking for 30 min and sonicated (50,000*g*, 20 min) at 4 °C to break cell wall and obtain membrane pellet preparations. A solution of progesterone as substrate, NADPH generating system (10 mM $NADP^+$, 100 mM glucose-6-phosphate, and 2.5 units of glucose-6-phosphate dehydrogenase) and inhibitors of various concentrations were pre-incubated at 37 °C for 5 min before a diluted membrane suspension was added to start the reaction. After an incubation of 30 min at 37 °C, it was quenched with 50 mL 1 N HCl and steroids were extracted with ethyl acetate. The 17α-hydroxylase activity of CYP 17 was determined by measuring the conversion of progesterone into 17α-hydroxyprogesterone and by-product 16α-hydroxyprogesterone [27,28].

4.2.3. CYP19 preparation and assay

Human CYP19 was obtained from the microsomal fraction of freshly delivered human term placental tissue. The incubation tubes containing [7] androstenedione as the substrate, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and inhibitors of various concentrations in phosphate buffer (0.05 M, pH 7.4) were pre-incubated at 30 °C in a shaking water bath for 5 min before microsomal protein was added to start the reaction. After further incubation for 3 h, the reaction was terminated with ethyl acetate and aliquots were pipetted into cold HgC1 solution (1 mM). After addition of aqueous dextran-coated charcoal (DCC) suspension (2%), the vials were shaken for 20 min and centrifuged at 1500*g* for 5 min to separate the charcoal-adsorbed steroids. Inhibition of enzyme activity was determined by measuring the formation of ^3H_2O [29].

4.2.4. Cell culture

HT-29 cells were cultured at 37 °C and 5% CO_2 in McCoy's 5A culture medium supplemented with 10% fetal bovine serum, 1% glutamine (final concentration 2 mM) and 1% penicillin/streptomycin solution 100 U/mL. Two to three times per week, cells were sub-cultivated with a ratio of 1:5 or 1:10 in order to avoid complete confluence.

4.2.5. Cytotoxicity assay

Cytotoxicity was measured using the MUH assay. MUH is a compound that becomes highly fluorescent after hydrolysis of the ester linkage and, thus, measures cellular lipase and esterase activity [30]. 10,000 cells were plated in each well of a 96 wells plate. After cultivation overnight, cells were treated with increasing concentrations of **8** (from 1 nM to 100 μM) for different times (0–192 h). Then, cells were washed with PBS and incubated with MUH 1 mg/ml. After 30 min of incubation at 37 °C and 5% CO_2, fluorescence was measured (330 nm excitation; 450 nm emission) using the microplate reader Victor X3 Perkin Elmer. Mean fluorescence values were determined by averaging data of at least 6 replicates for each condition. Three separate experiments were performed.

4.2.6. Wound healing assay (scratch assay)

HT-29 cells were seeded in 45-mm-diameter plates and cultured until they reached 90% confluence. Cell cultures were then scratched with a yellow 200-μl sterile tip (time 0), washed with PBS in order to remove floating cells, and then treated with **8** 0.4, 40 or 400 nM. The wound healing process was monitored by microscopy using Nikon Eclipse Ti equipped with Digital Sight camera DS U3 Nikon and measuring the "healing area" at different time points (time 0, 96 and 192 h). Images were digitally acquired and processed using the analysis software Image J. Data were expressed as percentage of reduction of the scratch surface area: $100 * (area_{(t0)} - area_{(tx)})/area_{(t0)}$ [31]. Mean area values were determined by averaging data of at least 10 images for each condition. Three separate experiments were performed.

4.3. Molecular docking

Compounds **3** and **8** were built and prepared in Amber12 force field within MOE, which were subsequently imported together with the CYP11B1 homology model into GOLD. The active-site was designated around the heme iron with a radius 19 Å, and the ligands were docked into this pocket in 50 iterations using genetic algorithm with default parameters. The resulting binding poses were subsequently ranked using the goldscore function with p450_pdb parameters, and illustrated in MOE.

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Appendix A. Supplementary material

Representative NMR spectra (compounds **4** and **8**). Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.066. These data include MOL files and InChiKeys of the most important compounds described in this article.

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