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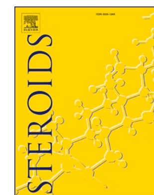

# Synthesis and preliminary biological evaluation of novel steroidal compounds as antibacterial agents

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ABSTRACT

Antimicrobial resistance is currently one of the most serious and alarming threats to human health; therefore, the identification of novel antimicrobial agents is a compelling need. Recently, we identified the heterocyclic steroid PYED-1 as a novel promising antibacterial and antibiofilm agent. In an effort to broaden the repertoire of active compounds and elucidate the structural features responsible for their antibacterial activity, two novel derivatives of PYED-1 have been conceived herein. The target compounds have been readily obtained in few steps and with very good yields. The antibacterial activity has been evaluated against *S. aureus* and *A. baumannii* strains, as examples of Gram-positive and -negative bacteria, by the broth microdilution method, while hemolysis assay has been used for the assessment of cytotoxicity. One of the two derivatives was able to inhibit the growth of *S. aureus* strains with lower MIC values (8 μg/mL) compared with those of PYED-1 (16 μg/mL) without showing hemolytic effect suggesting therefore a favorable safety profile. Overall, this study provides further indications on the functional groups required for the antibacterial activity of these novel steroidal derivatives.

## 1. Introduction

The improper and excessive use of antimicrobials, together with both the intrinsic and acquired ability of bacteria to form antibiotic-resistant biofilms leading to chronic infections, have increase the prevalence of multidrug-resistant and difficult-to-treat pathogens [1,2]. Nowadays antimicrobial resistance (AMR) is one among the top three major public health threats and has becoming a silent pandemic [3]. According to "The Review on Antimicrobial Resistance", AMR will cause the death of 10 million people per year by 2050, thus exceeding those who will have died for cancers [4,5]. Indeed, AMR is a worsening problem with very serious consequences on human health especially for the treatment of life-threatening infections in presence of immunosuppressive situations such as cancer chemotherapy [6], advanced and invasive surgical procedures and in presence of underlying health conditions or chronic diseases [7–9]. Resistance mechanisms have been seen to nearly all antibiotics that have been introduced. Nevertheless, AMR was under control up to 1980 as there was a continuous identification of novel drugs with approximately 200 new antimicrobials approved between 1950 and 1980 [10]. However, in the last decades, research and development in antibiotic fields were very limited, essentially for scientific, regulatory, and economic issues. As a result, very few new drugs have been licensed in the 21st century and the majority of compounds in the clinical development pipeline belongs to established antibiotic classes.[11–13]. Accordingly, the identification of innovative antibiotics, as well as of alternative strategies to face with the serious and global threat of the AMR is an urgent need [14,15].

In the frame of our studies aimed to the development of novel candidates of pharmaceutical interest, we focused our attention on the search for novel and innovative antimicrobial candidates [16–18]. In this context, we recently identified the heterocyclic steroid PYED-1 (pregnadiene-11-hydroxy-16,17-epoxy-3,20-dione-1, **1**, Fig. 1a), a synthetic derivative of the corticosteroid drug Deflazacort [19–21], as a promising broad-spectrum antibacterial and antibiofilm agent [22]. Particularly, PYED-1 exhibited bacteriostatic and bactericidal properties against Gram-positive and Gram-negative pathogens such as *Staphylococcus aureus* (*S. aureus*), *Enterococcus faecalis* (*E. faecalis*), *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*),

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*Escherichia coli* (*E. coli*), *Stenotrophomonas maltophilia* (*S. maltophilia*), *Candida* species and, in some cases, was also able to prevent biofilm formation, disrupt mature biofilms, and inhibit the expression of main virulence factors [23,24]. The observed activities were retained against clinical isolates, exhibiting different antibiotic-resistance profiles and, in some cases, synergistic and/or additive effects with conventional antibiotics were also detected, highlighting an interesting potential for its use against multidrug-resistant strains [25,26]. More recently other PYED-1 derivatives were also investigated for their ability to inhibit the growth of *S. aureus, E. faecalis, A. baumannii, P. aeruginosa, E. coli*. Among them, only compounds **2** and **3** (Fig. 1a) showed a weak antibacterial effect. Particularly, the replacement of the epoxy function at C16-C17 positions with a double bound led to a raising of both minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values compared to those of PYED-1 (compare **2** with **1**, Fig. 1a).

In addition, compound **3**, in which also the hydroxyl function at C11 position was missing, worked only at very high concentration (1000 μg/mL) against all the tested bacteria. Lastly, the antibacterial effect was completely lost in compound **4** where the acetyl group at C21 position was missing [16].

In our continuous efforts to identify novel antibacterial agents, this work describes the synthesis and the evaluation of the antibacterial activity of two PYED-1 derivatives **5** and **6** (Fig. 1b), with the aim to extend the pipeline of steroids acting against bacterial infections and to investigate which functionalities can modulate the therapeutic effect.

## 2. Results and Discussion

### 2.1. Chemistry

Our previous results suggested that C11 functionalization and the acetyl residue on the C21 position were required to observe any antibacterial effect. In addition, the presence of an epoxy function on the steroid scaffold seems to improve the antibacterial activity as highlighted by the MIC values of PYED-1 compared to those observed for derivative **2** (see Fig. 1a).

On these bases, two PYED-1 derivatives **5** and **6** (Fig. 1b) have been herein synthesized and evaluated for their activity against bacterial infections. For both compounds, acetyl function at C21 has been retained while the OH group of PYED-1 at C11 has been substituted by a keto function, as first approach, to verify whether the kind of functional group at C11 could influence the antibacterial properties of these compounds. On the other hand, compound **5** is characterized by a double bond at C16-C17, while derivative **6** retains the epoxy function to confirm whether the epoxy function is crucial for the antibacterial activity.

Steroids **5** and **6** can be useful intermediate in the synthesis of glucocorticoid based drugs [27–31]. However, to the best of our knowledge no data have ever been reported on their pharmacological properties as antibacterial agents. Herein, both **5** and **6** were prepared starting from **2** (Scheme 1), in turn obtained from the commercially available tetraene acetate (3TR) [32], another key intermediate used for the synthesis of bioactive corticoids [33], and already used by us for the synthesis of PYED-1 [16].

In our previous works, we tried various oxidating systems for the regioselective introduction of the epoxy function on the C16-C17 double bond. If in some cases, a mixture of overoxidation products was formed, none of them could be attributed to structure **6**, herein desired. To verify if **6** could be obtained in a single step by a contemporary oxidation of the secondary hydroxyl function and introduction of the epoxy function in the desired position, herein we have tried an oxidation system commonly used for both transformations. To this purpose, we selected TBHP (*tert*-butyl-hydroperoxide) as oxidant agent and $Ti(O^iPr)_4$ as a catalyst to be reacted with derivative **2** (Scheme 1) [34,35]. Unfortunately, the reaction yielded compound **6** in only modest yield (40 %) after purification. Therefore, a more practical two-step approach was undertaken, involving the oxidation of the hydroxyl group (**2** → **5**) followed by a stereoselective epoxidation of the double bond, ultimately affording compound **6**. Accordingly, the treatment of steroid **2** with pyridinium chlorochromate (PCC) in DCM for 3 h at room temperature (rt) led to our first target steroid **5** (95 % yield; Scheme 1), whose structure was confirmed by NMR analyses. In detail, the oxidation of the secondary alcohol group into a carbonyl function was immediately evident by the disappearance of the H-11 of **2** in $^1$H NMR spectrum as well as by the presence of a downfield carbon (207.9 ppm) relative to the C-11 carbonyl function in $^{13}$C NMR spectrum (see Supplementary Material). Eventually, the regioselective C16-C17 double bond oxidation [16] was achieved only by the use of an organic peroxy acid as the phthalic anhydride in the presence of 50 % aqueous $H_2O_2$ [36], to give after 24 h at reflux temperature epoxide **6** in 79 % yield. In this case, the installation of the epoxy function led to the up-field shift of H-16 from 6.81 ppm to 3.93 ppm in the $^1$H NMR spectrum. The regioselectivity of the epoxidation reaction at the C16–C17 double bond was further supported by $^1$H–$^{13}$C HMBC correlations (Table 1). Notably, the H-16 gave correlations with C-17 and C-20, while the H-18 methyl protons correlated with C-17 carbon.

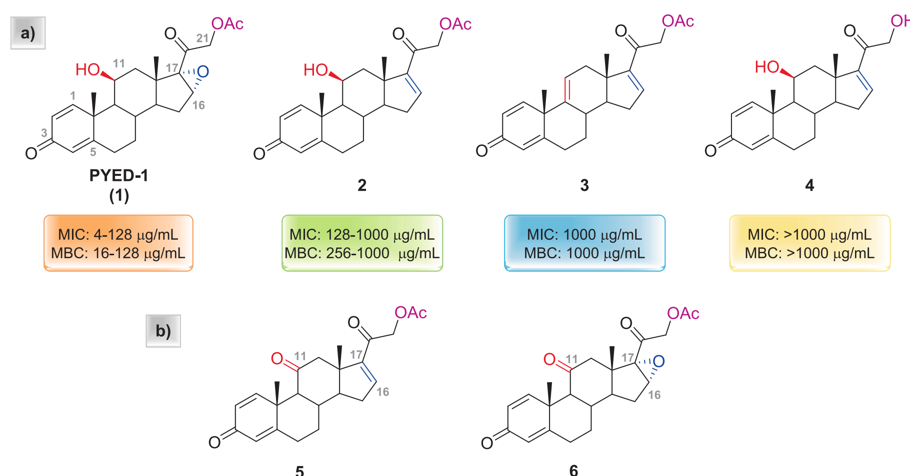
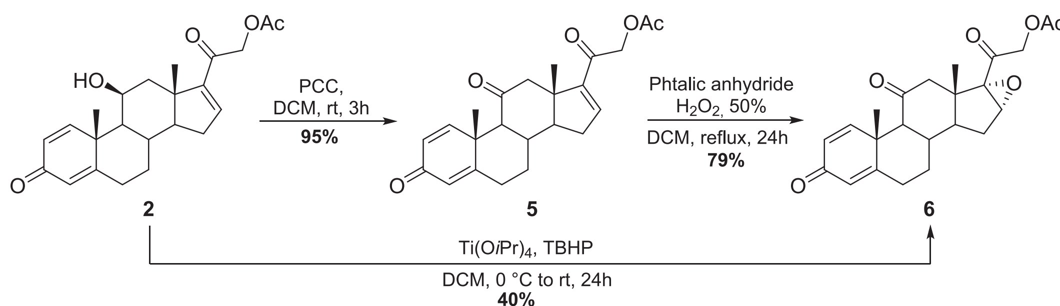
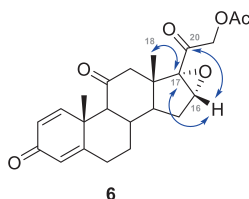



**Fig. 1.** A) Chemical structure, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranges against Gram-positive and -negative bacteria of PYED-1 (**1**) and its synthetic derivatives **2–4** (see ref. [16]); b) Novel PYED-1 derivatives **5** and **6**.




**Scheme 1.** Synthesis of derivatives **5** and **6.**

**Table 1**
Main correlations in $^1H$–$^{13}C$ HMBC and $^1H$–$^{13}C$ HSQC spectra of **6.**




| $^1$H, ppm | $^{13}$C, ppm HMBC | HSQC |
|---|---|---|
| 3.95 (H-16) | 68.4 (C-17) 197.9 (C-20) | 61.2 (C-16) |
| 1.13 (H-18) | 68.4 (C-17) | 15.9 (C-18) |

*2.2. In silico ADMET prediction*

To predict whether compounds **5** and **6** could exhibit physicochemical and pharmacokinetic features consistent with drug-likeness, a comparative ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) prediction was carried out. The selected descriptors—molecular weight, logP, TPSA, QED, SAscore, clearance, and toxicity alerts—were chosen for their recognized relevance in antibiotic development (Table 2) [37–40]. Both compounds comply with Lipinski's rule of five (MW $<$ 500, logP $<$ 5, HBA $\leq$ 10, HBD $\leq$ 5), further supporting their potential as orally bioavailable small-molecule antibiotics [41]. From the ADMET prediction, compound **5** emerges with moderate lipophilicity (logP = 2.23, *entry* 2) and balanced polarity (TPSA = 77.5 Å$^2$, *entry* 3), two favorable features that support solubility and membrane permeability, including potential penetration through bacterial porins—an essential requirement for activity against Gram-negative pathogens. In contrast, compound **6**, despite showing a similar lipophilicity (logP = 2.11, *entry* 2), displays a slightly higher polarity (TPSA = 90.0 Å$^2$, *entry* 3), which may limit intracellular accumulation. With respect to Gram-positive bacteria, the structural constraints are less stringent, since the absence of an outer membrane makes passive uptake less dependent on narrow physicochemical windows. Within this context, both compounds fall within a suitable range of lipophilicity and polarity to diffuse through the thick peptidoglycan layer. Nevertheless, the higher drug-likeness and lower clearance of compound **5** may again confer an advantage by supporting more sustained intracellular concentrations in Gram-positive pathogens. Specifically, compound **5** achieved a higher QED score (0.701 *vs* 0.535 for **6,** *entry* 4) and exhibited lower clearance (6.6 *vs* 10.3 mL/min/kg for **6,** *entry* 7), suggesting prolonged systemic exposure and a greater likelihood of maintaining effective tissue concentrations during infection. In addition, although both compounds fall within acceptable ranges for plasma protein binding (85.9 % for **5** and 78.3 % for **6,** *entry* 6), the slightly higher binding of **5** may further contribute to sustained drug exposure. Toxicological profiling provides an additional layer of distinction. Regarding toxicity, while compound **5** displayed only limited liabilities, compound **6** triggered multiple alerts, including potential genotoxic and non-genotoxic carcinogenicity, skin sensitization, and respiratory toxicity—factors that represent significant hurdles in the development of systemic antibiotics (*entry* 8). Taken together, these descriptors provide a rational framework for assessing the translational potential of steroid-based scaffolds as antibacterial agents, highlighting compound **5** as a particularly promising lead, since it combines properties aligned with key ADMET criteria for the antibiotic efficacy. Its physicochemical profile is compatible with activity against both Gram-negative and Gram-positive bacteria, with the stricter permeability barrier of Gram-negative organisms making compound **5** especially noteworthy. Conversely, compound **6** may require stereochemical

**Table 2**
Comparative ADMET profile of compounds **5** and **6** with relevance to antibacterial development. Physicochemical, pharmacokinetic, and toxicity parameters are reported together with their implications for antibiotic efficacy.

| *Entry* | Property | Compound 5 | Compound 6 | Antibiotic relevance |
|---|---|---|---|---|
| *1* | Molecular Weight (Da) | 382.18 | 398.17 | Both **5** and **6** within optimal range for antibiotic leads (<500 Da) |
| *2* | logP | 2.23 | 2.11 | Moderate lipophilicity of both **5** and **6** supports membrane penetration |
| *3* | TPSA (Å$^2$) | 77.5 | 90.0 | **5** in the optimal range for Gram–negative permeability; **6** at upper limit |
| *4* | QED | 0.701 | 0.535 | **5** has better drug-likeness, closer to ideal antibiotic profile |
| *5* | SAscore | 4.36 | 4.65 | Both **5** and **6** synthetically accessible; **6** slightly more complex to synthetize |
| *6* | Plasma Protein Binding (%) | 85.9 | 78.3 | Acceptable for both **5** and **6**; high values may prolong half-life |
| *7* | Clearance (mL/min/kg) | 6.6 | 10.3 | **5** shows lower clearance than **6** suggesting longer systemic exposure |
| *8* | Toxicity Alerts | Limited liabilities | Multiple alerts (carcinogenicity, skin sensitization) | **5** is safe for systemic antibiotic use; **6** may require optimization |

refinement or targeted structural modifications to mitigate its liabilities before advancing as an antibiotic lead.

### 2.3. Biological assays

#### 2.3.1. Antibacterial activity

The antibacterial activity of compounds **5** and **6** was evaluated against *S. aureus* and *A. baumannii* as first examples of Gram-positive and -negative bacteria, both responsible for nosocomial and difficult-to-treat infections [42,43]. Particularly, methicillin-sensitive (ATCC 29213) and methicillin-resistant (ATCC 43300 and 00717) *S. aureus* [44], as well as *A. baumannii* strains were considered and the antibacterial effect was compared to that of PYED-1. As shown in Table 3, compound **5** was more active than PYED-1 against *S. aureus* ATCC 29213 and ATCC 43300 strains, with MIC values of 8 μg/mL, compared to 16 μg/mL for PYED-1 (Table 2, entries 1–3). However, its bactericidal activity was markedly lower, with MBC values ranging from 128 to 256 μg/mL, whereas PYED-1 showed MBCs between 16 and 32 μg/mL. On the other hand, compound **6** exhibited weak activity, with MICs of ≥ 512 μg/mL and MBCs of > 1024 μg/mL for all *S. aureus* strains. When tested against *A. baumannii*, both compounds **5** and **6** were inactive (Table 3, entries 4–6), with MICs ≥ 256 μg/mL and no bactericidal effect even at the highest tested concentrations (MBC > 1024 μg/mL), while PYED-1 showed moderate activity (MICs 16–32 μg/mL). The results suggest that compound **5** has improved MICs against *S. aureus* strains compared to PYED-1 but lacks comparable bactericidal efficacy while it is not as effective against *A. baumannii* strains.

#### 2.3.2. Cytotoxicity assay

The potential cytotoxicity of compound **5** was evaluated using a hemolysis assay on red blood cells at concentrations ranging from 8 to 1024 μg/mL. As shown in Fig. 2, there was no significant hemolysis at any tested concentration of compound **5** in contrast to the positive control (1 % Triton X-100). The level of hemolysis was similar to the PBS-treated negative control, suggesting that there was no effect on the membranes of red blood cells. These results show that compound **5** is not toxic to cells, thus supporting its safety profile for potential therapeutic applications.

## 3. Conclusion

Inspired by our previous work highlighting the antibacterial properties of the steroid PYED-1, herein two novel derivatives **5** and **6** have been synthesized and a preliminary evaluation of their antibacterial activity has been carried out. Target compounds have been easily prepared in satisfactory yield starting from a common steroidal precursor. In silico ADMET analysis predicted a better profile for steroid **5** compared with **6**. Biological assays revealed that steroid **6** did not show activity exception made for a very slight effect on the growth of *S. aureus* strains. On the other hand, steroid **5** was able to inhibit *S. aureus* growth and to a little extent that of *A. baumannii*. Even if MIC values (*S. aureus*) of **5** were lower compared to those of PYED-1, no bactericidal effect

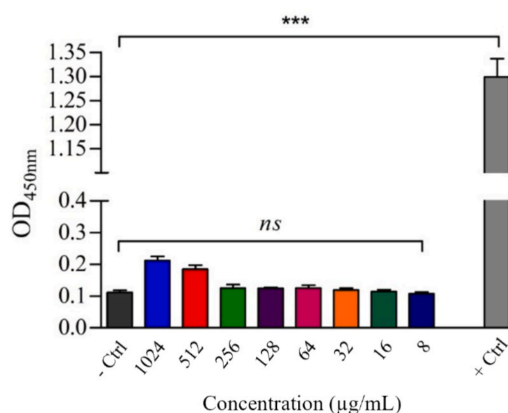



**Fig. 2.** Hemolysis assay for compound **5**. After incubating for 60 min at 37 °C of red blood cells with either 1 % (v/v) Triton X-100 (positive control), PBS (negative control), or different concentrations of compound **5**, the absorbance was measured at 450 nm. The data are expressed as the mean ± standard deviation (SD) of three replicates. ***$p < 0.001$, significance was analyzed using the one-way analysis of variance (ANOVA); ns, not statistically significant. – Ctrl: Negative Control; + Ctrl: Positive Control.

could be observed. Therefore, the carbonyl group at C11 seems to have a beneficial effect in terms of growth inhibition only when combined with the presence of a double bond in C16-C17 positions ($\mathbf{5}_{MIC}$ vs $\mathbf{2}_{MIC}$). Conversely, a detrimental action is shown if the C11 carbonyl and the epoxy C16-C17 functions coexist (PYED-$1_{MIC}$ vs $\mathbf{6}_{MIC}$). Overall, although the results indicate that PYED-1 remains the most promising candidate among the derivatives synthesized to date, this study provides further insights regarding the influence of specific functional groups on the steroidal scaffold in modulating the antibacterial activity.

## 4. Experimental Section

### 4.1. Chemistry

#### 4.1.1. General Information

Commercially available chemicals and solvents were purchased with the highest degree of purity from Merck Life Sciences or VWR and used without further purification. 9-Bromotriene acetate **7** was provided by Symbiotec Pharmalab (Pvt Ltd, Pithampur, Indore, India). All moisture-sensitive reactions were performed under argon atmosphere using oven-dried glassware. TLC (precoated silica gel plate F254, Merck Life Science S.r.l., Milan, Italy) were used for monitoring all reactions. Products were detected by exposure to ultraviolet radiation (254 nm), iodine vapor, and spraying with ethanol/sulfuric acid mixture. Compounds were purified by column chromatography (Merck Kieselgel 60, 70–230 mesh, Merck Life Science S.r.l., Milan, Italy). High-resolution ESI mass spectra (HR ESI MS) were recorded in positive mode on a Thermo Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). NMR spectra were recorded on NMR spectrometers operating at 400 and 600

**Table 3**
MIC (μg/mL) and MBC (μg/mL) values of compounds **5** and **6** on *S. aureus* and *A. baumannii*.[a]

| Entry | Strain | Compounds | | | | | |
|---|---|---|---|---|---|---|---|
| | | PYED-1[b] | | 5 | | 6 | |
| | | MIC | MBC | MIC | MBC | MIC | MBC |
| 1 | *S. aureus* ATCC 29213 | 16 | 16 | 8 | 256 | 512 | >1024 |
| 2 | *S. aureus* ATCC 43300 | 16 | 32 | 8 | 256 | 512 | >1024 |
| 3 | *S. aureus* 00717 | 16 | 16 | 16 | 128 | 1024 | >1024 |
| 4 | *A. baumannii* ATCC 17978 | 16 | 16 | 512 | >1024 | >1024 | >1024 |
| 5 | *A. baumannii* ATCC 19606 | 32 | 32 | 256 | >1024 | >1024 | >1024 |
| 6 | *A. baumannii* 62258 | 16 | 16 | 256 | >1024 | >1024 | >1024 |

[a] Experiments were performed in triplicate and were repeated independently at least three times.
[b] PYED-1 has been herein considered as reference compound [16].

MHz (Bruker AVANCE, equipped with TOPSPIN, Bruker Corp., Billerica, MA, USA) or 500 MHz (Varian Inova, equipped with a VnmrJ 4.0 software, Agilent Technologies, Santa Clara, CA, USA), using $CDCl_3$ solutions with residual non-deuterated solvent used as internal standard ($\delta$ 7.26 for $^1$H NMR and 77.0 for $^{13}$C NMR [45]). Coupling constant values ($J$) were reported in Hz.

#### 4.1.2. Synthesis and characterization of target compounds

**Ketone 5**. Debrominated derivative **2** (0.26 g, 0.68 mmol) was added in one portion to a magnetically stirred suspension of pyridinium chlorochromate (PCC) (0.22 g, 1.02 mmol) and celite 545 (0.26 g) in anhydrous DCM (1.5 mL) at rt and under argon atmosphere. The resulting dark-brown reaction mixture was kept at the same temperature for 3 h, then diluted with $Et_2O$, and filtered through a sintered-glass septum funnel. The solvents were evaporated under reduced pressure, and the crude residue was purified by column chromatography on silica gel (DCM:Acetone = 95:5) to give the steroid **5** (0.24 g, 95 % yield). $^1$H NMR (400 MHz): $\delta$ 7.74 (d, $J$ = 10.3, 1H), 6.81 (s, 1H), 6.20 (d, $J$ = 10.3, 1H), 6.08 (s, 1H), 5.00 (d, $J$ = 16.2, 1H), 4.88 (d, $J$ = 16.2, 1H), 3.09 (d, $J$ = 12.8, 1H), 2.63–2.46 (m, 2H), 2.59–2.47 (m, 2H), 2.47–2.33 (m, 2H), 2.31–2.14 (m, 5H), 2.13–1.97 (m, 2H), 1.93 (d, $J$ = 11.0, 1H), 1.44 (s, 3H), 1.39–1.19 (m, 2H), 0.92 (s, 3H) [31]. $^{13}$C NMR (100 MHz): $\delta$ 207.9, 189.8, 186.2, 170.4, 165.9, 155.1, 149.3, 143.7, 127.7, 125.0, 65.4, 61.8, 54.2, 53.4, 48.9, 42.5, 34.8, 33.3, 32.2, 20.5, 18.9, 17.5. HR MS (ESI, $m/z$): calcd 383.1853 ($[M + H]^+$); found 383.1853 ($[M + H]^+$).

**Epoxide 6**. Phthalic anhydride (0.38 g, 2.6 mmol) was added in one portion to a stirred solution of compound **5** (0.25 g, 0.65 mmol) in DCM (10 mL) at rt. 50 % aqueous $H_2O_2$ (0.54 mL) was then added dropwise in 0.5 h and the mixture was heated to reflux temperature for 24 h. Thereafter, water and solid sodium bicarbonate were added to the cooled reaction mixture until pH 7 was reached. The resulting mixture was extracted with DCM and the organic layer was washed with brine, dried ($Na_2SO_4$), concentrated under reduced pressure, and purified by column chromatography on silica gel (DCM:Acetone = 96:4) to give the pure epoxide **6** (0.20 g, 79 % yield). $^1$H NMR (400 MHz): $\delta$ 7.69 (d, $J$ = 10.1, 1H), 6.20 (d, $J$ = 10.1, 1H), 6.07 (s, 1H), 4.63 (d, $J$ = 17.9, 1H), 4.58 (d, $J$ = 17.9, 1H), 3.93 (bs, 1H), 2.67 (d, $J$ = 12.2, 1H), 2.56–2.33 (m, 3H), 2.15 (s, 3H), 2.09–1.93 (m, 2H), 1.88 (d, $J$ = 10.6, 1H), 1.79–1.69 (m, 1H), 1.68–1.58 (m, 1H), 1.59–1.48 (m, 1H), 1.43 (s, 3H), 1.34–1.17 (m, 1H), 1.12 (s, 3H) $^{13}$C NMR (100 MHz): $\delta$ 207.1, 198.1, 186.3, 170.5, 165.7, 154.9, 127.9, 125.1, 68.6, 65.6, 61.9, 61.4, 50.1, 44.9, 43.9, 42.5, 34.5, 33.0, 32.2, 27.6, 20.5, 19.1, 16.2. HR MS (ESI, $m/z$): calcd 399.1802 ($[M + H]^+$); found 399.1819 ($[M + H]^+$).

### 4.2. In silico ADMET prediction

The ADMET properties of the synthesized steroidal derivatives **5** and **6** were predicted using the freely available web server ADMETlab 2.0 tool (https://admetmesh.scbdd.com/). SMILES strings used for the compounds were:

O=C(COC(C)= (5O)C1=CCC([C@]1(C)C2)C3CCC4=CC(C=C[C@]4(C)C3C2=O)=O (**5**).

O=C(COC(C)=O)[C@@]12[C@H](O2)CC([C@]1(C)C3)C4CCC5=CC(C=C[C@]5(C)C4C3=O)=O (**6**)

### 4.3. Biological assays

#### 4.3.1. Bacterial strains and antibacterial activity

Clinical bacterial isolates were obtained from a collection previously established at the Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II [16,46]. Ethical approval was not required as no patient data were accessed. Bacteria were cultured on Luria–Bertani agar (Del-Tek, Pozzuoli, Italy) at 37 °C under aerobic conditions. Stock cultures were stored in 10 % glycerol at − 80 °C. For assays, bacteria were grown in cation-adjusted Mueller–Hinton broth (CA-MHB; Sigma-Aldrich, Milan, Italy). Minimum inhibitory concentration (MIC) values of compounds against planktonic bacteria were determined by broth microdilution [16]. Title compounds were dissolved in DMSO (50 mg/mL), serially diluted (ranging from 2 μg/mL to 1000 μg/mL) in triplicate and placed into a polystyrene 96-well plate. Bacterial suspensions were added to the microtiter plates to achieve a final inoculum of $5 \times 10^5$ CFU/well. CA-MHB served as a negative control and wells without the adding of compounds as growth controls. Plates were incubated at 37 °C, 300 rpm, for 18–24 h. Microbial growth was evaluated measuring the optical density at 595 nm using a microplate reader (Bio-Rad Laboratories S.r.l., Milan, Italy). The DMSO effects (0.1 %–1%) were also separately assessed. For the minimum bactericidal concentration (MBC) determination, bacterial suspensions from MIC assay wells were plated on TSA after PBS dilution and incubated at 37 °C for 18 h. MBC was defined as the lowest concentration yielding $\geq$ 99.9 % ($\geq$3 $\log_{10}$) reduction in CFU. All experiments were performed in triplicate and repeated three times.

#### 4.3.2. Haemolysis assay

The hemolytic activity of compound **5** was assessed as previously described [47]. Briefly, 190 µL of a 1:50 PBS-diluted red blood cell (RBC) suspension was incubated with 10 µL of compound **5** in a 96-well plate. PBS and 1 % Triton X-100 (Sigma-Aldrich, Milan, Italy) served as negative and positive controls, respectively. Plates were incubated at 37 °C for 1 h, then centrifuged (500 rpm, 5 min). Supernatants (150 µL) were transferred to a new plate, and absorbance was measured at 450 nm using a microplate reader. Hemolysis (%) was calculated as:100 $\times$ ($A_{sample} - A_{PBS}$)/($A_{Triton\ X\text{-}100} - A_{PBS}$), where $A_{sample}$ is the experimental absorbance of compound **5**, $A_{PBS}$ is the control absorbance of untreated erythrocytes, and $A_{Triton\ X\text{-}100}$ is the absorbance of 1 % Triton X-100-lysed cells. Data are reported as mean ± SD from three independent experiments, each in triplicate. Statistical significance was determined using one-way ANOVA followed by Dunnett's post hoc test (GraphPad Prism 8).

## CRediT authorship contribution statement

**Anna Esposito:** Writing – original draft, Methodology, Investigation, Conceptualization. **Maria Stabile:** Validation, Methodology, Investigation. **Antonella Migliaccio:** Investigation, Formal analysis. **Eliana De Gregorio:** Writing – review & editing, Methodology. **Stefano D'Errico:** Writing – original draft, Supervision, Conceptualization. **Annalisa Guaragna:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2025.109686.

## Data availability

Data will be made available on request.

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