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Preliminary communication

Antibacterial activities of a few prepared derivatives of oleanolic acid and of other natural triterpenic compounds

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Abstract

Oxidizing, phosphorus, ester and amide derivatives of oleanolic acid **1** have been prepared. The antibacterial activity of compound **1**, isolated from the fruit barks of *Periploca laevigata* (Asclepiadaceae), and its derivatives have been tested using Tween-80 as complex agent to form a water-soluble triterpenes. The same activity of maslinic acid acetate **2**, β-amyrin **3**, and its acetylated derivative **3a** (Fig. 1), isolated from the same source as that of oleanolic acid **1**, have also been investigated. ***To cite this article : F. Hichri et al., C.R. Chimie 6 (2003).***

Résumé

Des esters, des amides, des dérivés d'oxydation et des dérivés phosphorés de l'acide oléanolique **1** ont été préparés. L'activité antibactérienne du composé **1**, isolé des écorces de fruits de la plante *Periploca laevigata* (Asclépiadacées), ainsi que celles de ses dérivés ont été testées en utilisant le Twee-80 en tant qu'agent complexant pour solubiliser dans l'eau les triterpènes isolés et préparés. L'activité antibactérienne de l'acide maslinique acétylé **2**, de la β-amyrine **3** et de son dérivé acétylé **3a**, isolés de la même source que l'acide oléanolique **1**, a aussi été testée. ***Pour citer cet article : F. Hichri et al., C.R. Chimie 6 (2003).***

Keywords: Periploca laevigata; oleanolic acid; structural analogous; antibacterial activity

Mots clés : Periploca laevigata ; acide oléanolique ; analogues structuraux ; activité antibactérienne

1. Introduction

A review of the literature showed that some triterpenes and their derivatives (Fig. 1) have been associated with various pharmacological activities. They have shown good anti-inflammatory [1–2], antitumoral [3], anticancer [4], hypoglycaemic [5], antiviral [6–8], antibacterial [9–12] and antifungal [13–14] activities. These properties as well as the important amount of oleanolic acid **1**, isolated in our laboratory from the fruit barks of *Periploca laevigata* (Asclepiadaceae)

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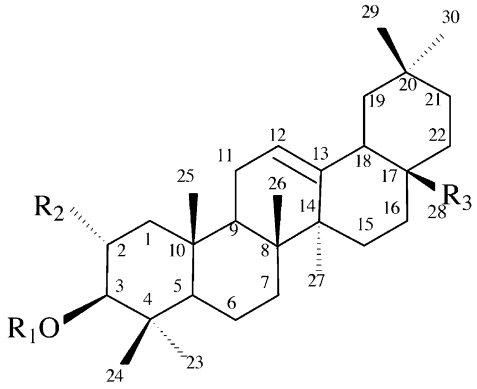

1. R_1=H, R_2=H, R_3=COOH
1a. R_1=$COCH_3$, R_2=H, R_3=COOH
2. R_1=$COCH_3$, R_2=$OCOCH_3$, R_3=COOH
3. R_1=H, R_2=H, R_3=CH_3
3a. R_1=$COCH_3$, R_2=H, R_3=CH_3

Fig. 1. Structures of the natural triterpenic compounds **1**, **2** and **3** and of the acetylated derivatives **1a** and **3a**.

[15], motivated us to prepare some derivatives (oxidation derivatives, phosphorized derivatives, esters and amides) in order to study their antibacterial activities and structure–activity relationships.

2. Results and discussion

2.1. Chemistry

The treatment of oleanolic acid **1** with acetic anhydride in pyridine gives the acetyloleanolic acid **1a** in 98% yield. Its IR spectrum showed absorptions attributable to acetyloxy (1734 cm^{-1}) and carboxylic (1711 cm^{-1}) carbonyl functions. Its structure was confirmed by the appearance of signals at 2.06 and 4.42 ppm attributable to CH_3 of the acetyloxy group and H_3, respectively, in the ^{1}H-NMR spectrum. The ^{13}C-NMR spectrum showed signals at 80.9 ppm and 171.0 ppm corresponding to C_3 and the carbonyl of the acetyloxy group, respectively. **1a** has been used as a starting material for the preparation of most derivatives. The specific epoxidation of Δ^{12-13} using a mixture $KMnO_4$–$CuSO_4$ in refluxing CH_2Cl_2 for 4 h, in the presence of a small amount of water and tertiobutanol, did not give the desired product (3-acetyl-12,13-epoxioleanolic acid), but provided the 12α-hydroxy-δ-lactone **4** in 82% yield along with an other secondary product 12-oxo-δ-lactone **5** in 11% yield (Fig. 2). We think that the approach and the stereochemistry of the carboxy group at C_{17} in **1a** allowed the lactonisation via the epoxidation of the C_{12}–C_{13} double bound. The

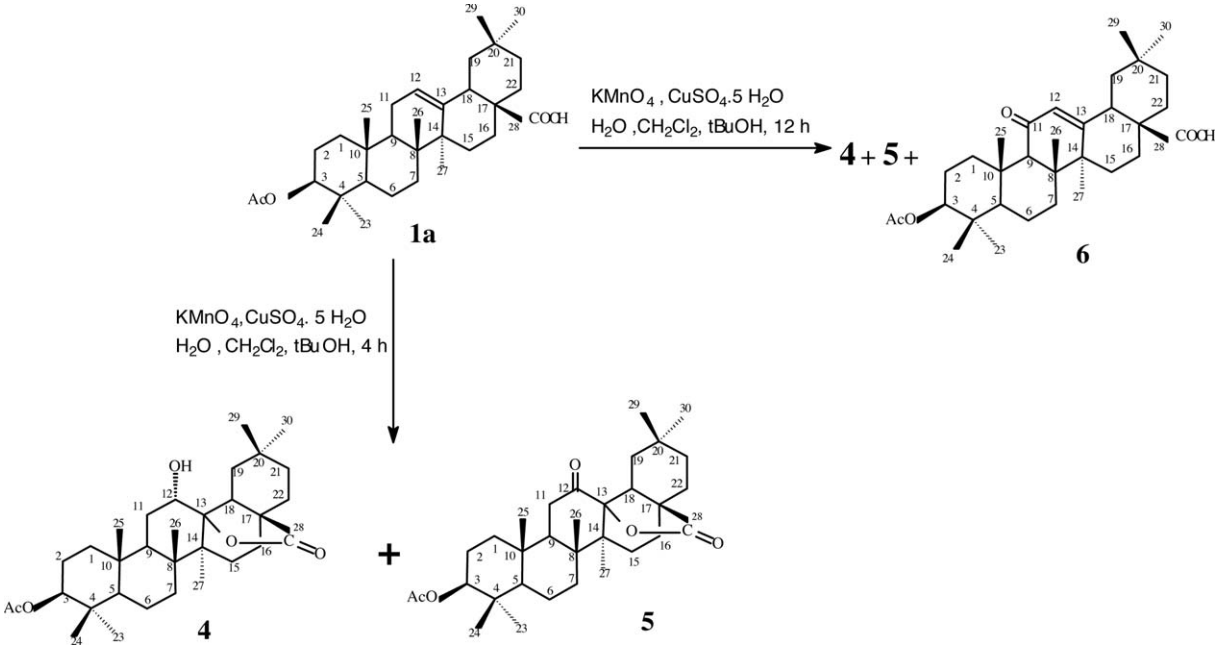

Fig. 2. Structures of the prepared oxidizing derivatives **4**, **5** and **6**.

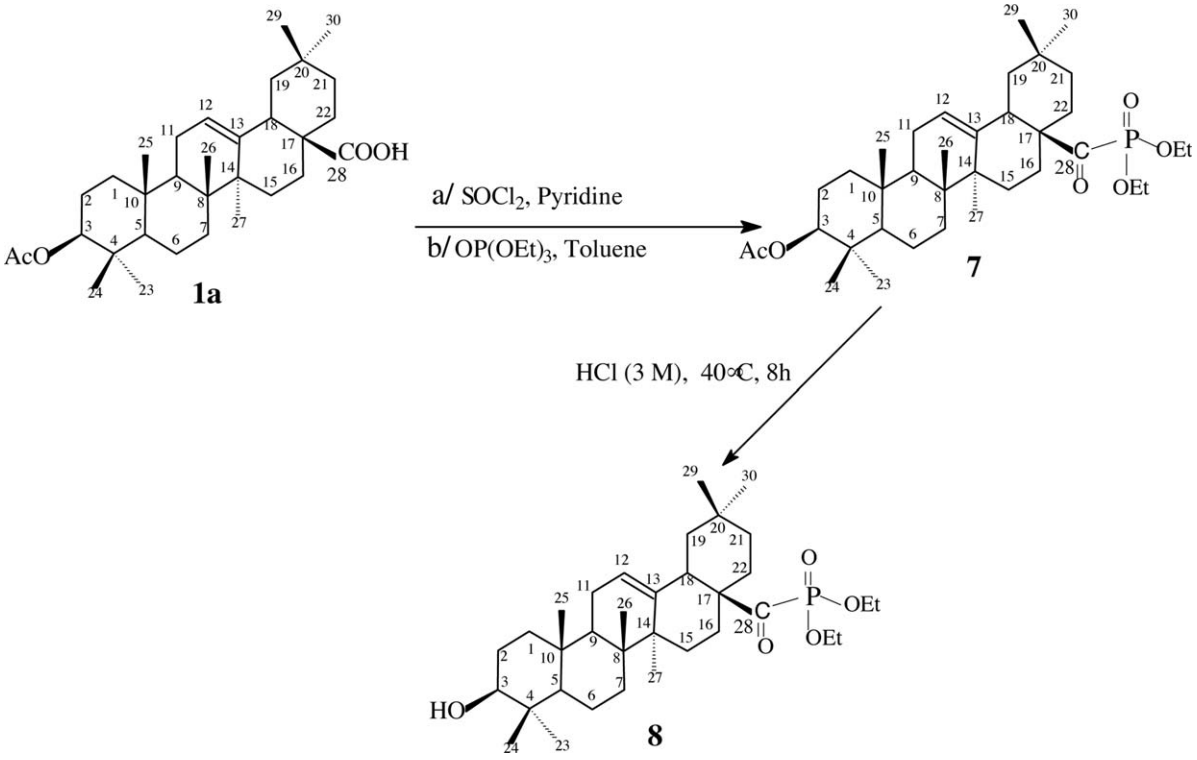

Fig. 3. Structures of the prepared phosphorus derivatives **7** and **8**.

same reaction, carried out again under the same conditions but for 12 h, gave besides **4** and **5** another secondary product, the 3-acetyl-11-oxo-oleanoic acid **6** in 5%, 60% and 27% yield, respectively (Fig. 3).

The compound **4** was obtained as a white powder. The ESI+ mass spectrum showed ion peak at *m*/*z* 515 $[M + H]^+$. The IR spectrum revealed absorptions attributable to a free hydroxyl group (3529 cm^{-1}), δ-lactonic and acetyloxy (1733 cm^{-1}) carbonyl functions. The structure was evidenced by the disappearance in the ^{1}H-NMR spectrum of the signal at 5.19 ppm relative to the ethylenic proton H_{12} in **1a** and the appearance of a signal at 3.80 ppm attributable to the same proton H_{12}, but attached to a carbon-bearing hydroxyl group. The ^{13}C-NMR spectrum reinforced this structure by the disappearance of the two ethylenic carbon signals C_{12} and C_{13} in **1a** and the appearance of a signal at 90.5 ppm attributed to an oxycarbonyl-lacton-group-bearing quaternary carbon atom, as well as signals at 76.1 and 179.8 ppm attributable to a tertiary carbon bearing a hydroxyl group and a lactone carbonyl function, respectively. The position of the hydroxyl group at C_{12} was deduced from the COSY spectrum showing H_{12}–H_{11a} and H_{12}–H_{11b} correlations. The HMBC spectrum confirmed the location of this hydroxyl group in C_{12} by observation of the ^2J heteronuclear correlation between H_{12} and the quaternary carbon C_{13}. The relative stereochemistry of C_{12} was determined on the basis of the NOESY spectrum, which proved the α-orientation of the hydroxyl group at C_{12} by the absence of the dipolar coupling between H_{12} and the β-oriented methyl group CH_3-27. This result was also confirmed by the multiplicity (triplet-like) and the weak coupling constant $J_{12–13}$ deduced from the 1D TOCSY NMR spectrum.

Compound **5** was obtained as a white powder. The ESI+ mass spectrum exhibited a protoned $[M + H]^+$ and a sodiated $[M + Na]^+$ molecular ion at *m*/*z* 513 and 535, respectively. The $[2 M + Na]^+$ at *m*/*z* 1048 was also observed in the same spectrum. All these data are compatible with the molecular formula $C_{32}H_{48}O_5$ (M = 512). The IR spectrum of **5** showed absorptions attributable to ketonic (1724 cm^{-1}), δ-lactonic and acetyloxy (1734 cm^{-1}) carbonyl functions. On the

other hand, the structure of **5** was confirmed by the disappearance in the ^{1}H-NMR spectrum of the signal at 5.19 ppm relative to the ethylenic proton H_{12} in **1a** and the appearance of two signals at 2.31 ppm and 2.48 ppm, attributable to H_{11} protons. The ^{13}C-NMR spectrum reinforced this structure by the disappearance of the two ethylenic carbon signals C_{12} and C_{13} in **1a**, and the appearance of signals at 178.9 ppm corresponding to a lactonic carbonyl function, and at 206.4 ppm relative to the ketonic carbonyl function.

The compound **6** was obtained as a white powder. Its ESI+ mass spectrum was recorded and showed a protoned molecular ion $[M + H]^+$ at *m*/*z* 513. The IR spectrum exhibited absorptions attributable to an ethylenic system (1667 cm^{-1}), ketonic (1684 cm^{-1}), carboxylic (1708 cm^{-1}) and acetyloxy (1736 cm^{-1}) carbonyl functions. The α,β-unsaturated ketonic moiety was immediately recognized from the following ^{1}H and ^{13}C-NMR data H_{12}(5.56; s), H_{18}(2.86; m), H_9(2.30; s), C-11 (200.4), C-28 (182.5), C-13 (163.2) and C-12 (131.2).

Mechanistically, the oxidation of C_{11} could be initiated by potassium permanganate, which abstracts a hydrogen atom from the activated carbon. The formed radical continues to react with O_2 to give a peroxide, which is converted into a ketonic function at C_{11} with the formation of a water molecule [16].

Acetyl oleanoic acid chloride previously prepared, readily reacts with triethylphosphate to give the Michaelis–Arbusov product: diethyl 3-acetylolean-12-en-28-oxyphosphonate **7** in 93% yield (Fig. 3). It was obtained as a white powder. Its ESI+ mass spectrum showed a protoned $[M + H]^+$ and a sodiated $[M + Na]^+$ molecular ion at *m*/*z* 619 and 641, respectively. The IR spectrum of **7** revealed absorptions attributable to ethylenic (1674 cm^{-1}), ketonic (1718 cm^{-1}), acetyloxy (1728 cm^{-1}), P=O (1240 cm^{-1}) and P–O–C (1020 cm^{-1}) systems. The presence of the $OCPO(OEt)_2$ system in compound **7** was ascertained by the observation of a new multiplet (q-like) at 4.20 ppm due to methylenic protons of the two ethyloxy moiety fixed at the phosphorus atom as well as new signals in the high-field region of the ^{1}H-NMR spectrum relative to the methyl groups of the same system. Furthermore, the presence of the ketonic carbonyl function in compound **7** was deduced from its ^{13}C–NMR spectrum (C_{28}: 214.4 ppm). The ^{31}P–NMR spectrum revealed two signals at 0.27 and –0.01 ppm, showing the presence of two conformers anti and syn at the $COPO(OEt)_2$ moiety. The hydrolysis of the compound **7** was carried out with aqueous HCl and gave the diethyl 3-hydroxyolean-28-oxyphosphonate **8** in 92% yield (Fig. 3). The structure elucidation of **8** was made by comparison of its ^{1}H and ^{13}C-NMR spectra with those of compound **7**. This comparison let us note the disappearance of the H-3 signal at 4.51 ppm in **7** and the appearance of a new signal at 3.15 ppm attributable to the same proton in **8**. Moreover, we have noted the absence of the singlet at 2.05 ppm relative to the methyl group of the acetyloxy system in compound **7**. The absence in the compound **8** ^{13}C-NMR spectrum of the ester carbonyl signal at 170.9 ppm (present in the case of **7**) proved the hydrolysis. This result was reinforced by the absorption revealed from the IR spectrum of **8** at 3420 cm^{-1} relative to the free hydroxyl group. The ^{31}P-NMR spectrum of **8** showed signals at 0.26 and –0.01 ppm showing the presence, as in the case of **7**, of two conformers anti and syn at the $COPO(OEt)_2$ moiety. The absorptions relative to P=O (1242 cm^{-1}) and P–O–C (1012 cm^{-1}) groups deduced from the IR spectrum of **8** confirmed the presence of the phosphorus moiety in the molecule.

The three esters **9**, **10** and **11** were prepared by reaction of oleanolic acid **1** with benzoylchloride, 2-hydroxybenzoylchloride and 2-acetyloxybenzoylchloride in pyridine, in 32%, 19% and 15% yield, respectively (Fig. 4).

The compound **9** was obtained as a white crystalline powder. Its EI mass spectrum showed a molecular ion peak at *m*/*z* 560 $M^{+\bullet}$. The ^{1}H-NMR spectrum showed a new triplet at 4.69 ppm (J = 6.7 Hz) attributable to H-3 and new other signals at 7.40– 8.00 ppm corresponding to the aromatic protons. The ^{13}C-NMR spectrum of **9** showed a signal at 81.4 ppm relative to C_3, signals at 128.2, 129.4, 130.8 and 132.6 ppm attributable to the aromatic carbons. The same spectrum showed signals at 122.4 and 143.4 ppm corresponding to C_{12} and C_{13}, respectively. The signals at 166.1 and 183.3 ppm attributable to the ester and carboxylic acid carbonyl functions, respectively, were also observed.

The compound **10** was obtained as a white crystalline powder. Its EI mass spectrum showed an ion peak at *m*/*z* 439 $[M–C_7H_5O_3]^+$ and 438 $[M–C_7H_6O_3]^{+\bullet}$ relative to the loss of the benzoyloxy moiety fixed at C-3. The ^{1}H-NMR spectrum exhibited, in particular, signals at 6.80–8.20 ppm attributable to the aromatic protons. It showed also a multiplet at 4.17 ppm relative to H-3.

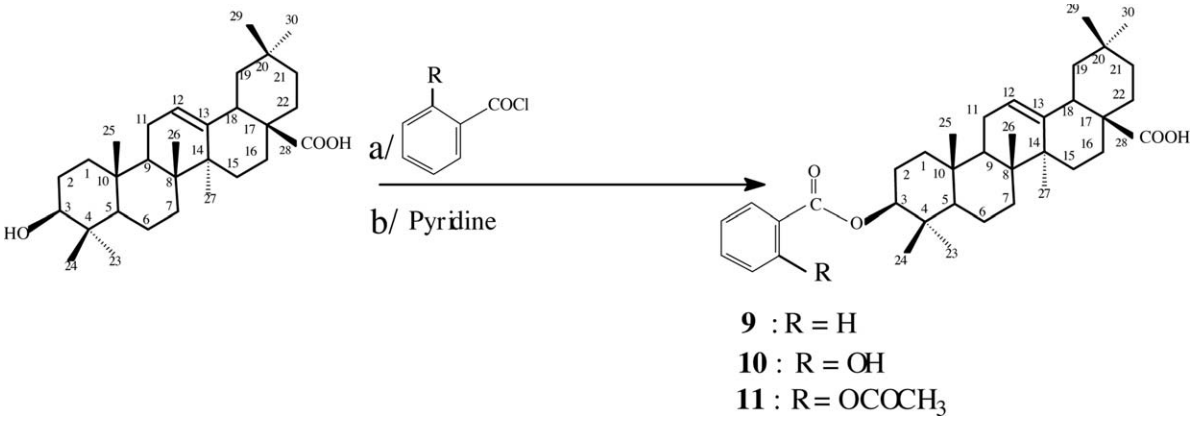

Fig. 4. Structures of the prepared esters **9**, **10** and **11**.

Compound **11** was obtained as a white crystalline powder. The recorded APCI+ mass spectrum revealed ion peak at *m*/*z* 439 $[M–C^9H_7O_4]^+$ corresponding to the loss of the aryloxy system attached to C-3. The ^{1}H-NMR spectrum showed a singlet at 2.07 ppm attributable to the methyl of the acetyloxy group fixed to C-2' of the aromatic ring. The multiplet observed in the same spectrum at 4.54 ppm was attributed to H-3. The aromatic protons were also identified by the observation of the corresponding signals at 7.00-8.10 ppm.

The reaction of oleanolic acid chloride in refluxing chloroform with 3-aminopyridine, heptylamine, hexylamine, 1-methylpropylamine and decylamine afforded the corresponding *N*-3-pyridinacetyloleanolic amide **12**, *N*-heptylacetyloleanolic amide **13**, *N*-hexyacetyloleanolic amide **14**, *N*-l-methylpropylacetyloleanolic

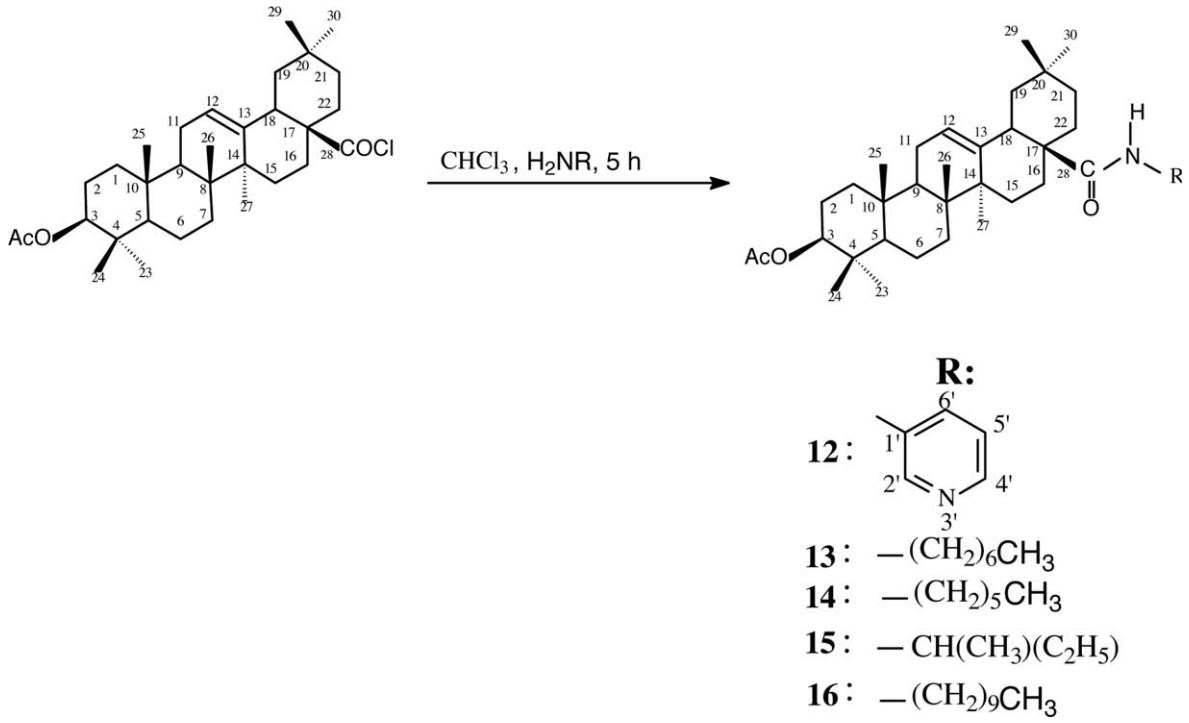

Fig. 5. Structures of the prepared amides **12**, **13**, **14**, **15** and **16**.

Table 1
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of triterpenic compounds (expressed as μg ml^{-1}).

Compounds	*Staphylococcus aureus*		*Escherichia coli*		*Pseudomonas aeruginosa*		*Salmonella typhi*	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
1	700	95	900	95	—	> 97	300	65
1a	900	90	900	90	900	> 97	900	> 97
2a	700	90	900	90	900	90	900	90
3	—	> 97	900	97	900	97	300	95
3a	700	90	700	90	500	78	500	95
4	—	> 97	—	> 97	—	> 97	300	65
5	900	90	900	90	—	> 97	300	97
6	500	90	900	90	900	97	900	90
7	—	> 97	—	> 97	—	> 97	—	> 97
8	700	85	900	85	900	90	900	90
12	900	90	900	90	300	90	700	90
15	—	> 97	—	> 97	—	> 97	—	> 97
16	—	> 97	—	> 97	—	> 97	—	> 97

amide **15** and *N*-decylacetyloleanolic amide **16**, in 65%, 74%, 80,%85% and 84% yield, respectively (Fig. 5).

Compound **12** was obtained as a white powder. Its FABMS exhibited $[M + H]^+$ at *m/z* 575. Its IR spectrum showed absorptions attributable to ketonic (1690 cm^{-1}), acetyloxy (1734 cm^{-1}) carbonyl functions, and C=N aromatic double bounds (1518 cm^{-1}). The ^{1}H–NMR spectrum showed a singlet at 7.75 ppm attributable to H-2' of the pyridinic system. Three multiplets were also observed at 7.16, 8.15 and 8.22 ppm relative to H-5', H-6' and H-4' of the pyridinic moiety, respectively. The ^{13}C–NMR spectrum confirmed the above spectral data by the observation of five signals at 123.7, 127.1, 134.9, 140.1 and 140.9 ppm relative to C-5', C-2', C-4', C-1' and C-6' of the pyridinic system, respectively. The same spectrum showed signals at 171.0 and 177.2 ppm attributable to the carbonyls of the acetyloxy group at C-3 and the amide function at C-17, respectively.

The compounds **13**, **14**, **15** and **16** were obtained as a white powder. Their structures were evidenced by the appearance in the corresponding ^{1}H-NMR spectra signals at 2.80–3.80 ppm relative to $–NCH_2–$ and –NCH– protons, at 5.0–6.0 ppm corresponding to HN–CO protons. Moreover, new signals were observed in the high-field region of each spectrum of the above compounds relative to the hydrocarbon chain attached to the corresponding amine. The ^{13}C-NMR spectra of these compounds showed in particular HN–CO signal at 178.1 ppm (**13**), 178.2 ppm (**14**), 177.3 ppm (**15**) and 178.0 ppm (**16**).

2.2. Antibacterial activity

General analysis of the data from the antibacterial effects of the tested compounds (Table 1) shows that compounds **1**, **3**, **4** and **5** exhibited interesting activities against *Salmonella typhimurium*, the most susceptible bacteria, with a minimum bactericidal concentration (MBC) value of 300 μg ml^{-1} and with minimum inhibitory concentrations (MIC) values of 65, 95, 65 and 97 μg ml^{-1}, respectively. Compounds **1**, **1a**, **2a**, **3**, **3a**, **5**, **6**, **8** and **12** showed moderate activities against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* bacteria, whereas compounds **4**, **7**, **15** and **16** can be considered inactive against these bacteria.

The comparison of the MBC of **1** (300 μg ml^{-1}) with **1a** (900 μg ml^{-1}) and that of **3** (300 μg ml^{-1}) with **3a** (500 μg ml^{-1}) showed that the free β-hydroxyl group at C_3 contributes to the antibacterial activity of **1** and **3**. On the other hand, the MBC of **1a** (900 μg ml^{-1}) and **3a** (500 μg ml^{-1}) let us conclude that the β-oriented carboxyl function at C_{17} in compound **1** decreases the antibacterial activity, but when it was converted into a C_{13}–C_{17} lactone function or when the formation of the α-hydroxyl and the ketonic function at C_{12} in compounds **4** and **5**, respectively, occurred, the activity against *Salmonella typhimurium* was recovered. It is

interesting to note the activity observed for *N*-3-pyridinacetyloleanolic amide **12** against *Pseudomonas aeruginosa*, one of the most resistant bacteria to antibiotics and antiseptics, with MBC value of 300 μg ml^{-1} and MIC value of 90 μg ml^{-1}. Comparison of these data with those of the other inactive amides **15** and **16** indicated that the activity is related to the structure of the system attached to the amine.

3. Experimental section

3.1. General experimental procedures

FTIR spectra were measured on a Perkin-Elmer 157G infrared spectrophotometer. Shimadzu QP-1000EX and MS-80RF spectrometers were used in the EI, FAB, ESI+ and APCI+ experiments, respectively. ^{1}H (200, 300 and 400 MHz) and ^{13}C (50, 75 and 100 MHz) one- and two-dimensional NMR spectra were recorded on a Bruker AM-200, AM-300, ARX-400 and WM-400 spectrometers, using $CDCl_3$ and $DMSOd_6$ as solvents and TMS as internal standard. Melting points were determined on a Büchi 510 apparatus using capillary tubes.

3.2. Preparation of compounds *4* and *5*

A mixture of $KMnO_4$ (0.8 g) and $CuSO_4{\cdot}5\ H_2O$ (0.4 g) was ground to fine powder. Water (0.04 ml) was added, and the slightly wet mixture transferred to the reaction flask. To a stirred suspension of this mixture in CH_2Cl_2 (2 ml), 200 mg of the acetylated oleanolic acid (0.4 mmol) are added, followed by the addition of tert-butanol (0.2 ml) [17]. After stirring for 4 h, the resulting solution was filtered, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The resulting residue was separated by silica gel column chromatography (petroleum ether: EtOAc 9:1 then 8:2) to give **4** (0.166 g, 82%) and **5** (0.022 g, 11%).

3.3. Preparation of compound *6*

3-acetyl-11-oxo-oleanoic acid **6** was prepared as indicated above (see compounds **4** and **5**). In this case, the stirring time of the reaction mixture was extended to 12 h. The separation of the resulting residue by column chromatography on silica gel (petroleum ether: EtOAc 9:1 then 8:2) gave **4** (0.01 g, 5%), **5** (0.123 g, 60%) and **6** (0.055 g, 27%).

3.4. Preparation of compound *7*

To a solution of acetyloleanolic acid chloride (0.130 g, 0.252 mmol), previously prepared by the general procedure [18], in toluene (2.5 ml) was added 2 equiv of triethyphosphate (0.09 ml, 0.504 mmol) and the mixture was stirred at 80–90 °C for 1 h [19]. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (petroleum ether: EtOAc 7.5: 2.5) to afford **7** (0.145 g, 93%).

3.5. Preparation of compound *8*

To a solution of **7** (0.07 g, 1.2 mmol) in $(CH_3)_2CO$–H_2O 1:1, was added 1 ml of aqueous HCl (3 M). The resulting mixture was stirred at 40 °C for 8 h. After removal of $(CH_3)_2CO$ in vacuo, the reaction mixture was extracted twice with $CHCl_3$. The organic layer was washed with an excess of 10% $NaHCO_3$ then with water and dried over anhydrous Na_2SO_4. The resulting product precipitated from MeOH to give **8** (0.064 g, 92%).

3.6. Preparation of esters *9*, *10* and *11*

The acid chlorides were prepared according to the general procedure [18], using excess of thionylchloride in refluxing dry pyridine.

To a mixture of oleanolic acid (0.05 g, 0.11 mmol) in refluxing pyridine, the appropriate acid chloride (1 equiv) was added and the mixture was refluxed for 4 h. The solvent was then removed under reduced pressure. The resulting mixture was washed with water to remove salts, then extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4. $CHCl_3$ was removed in vacuo and the resulting product precipitated from EtOH to give the corresponding esters: **9** (0.02 g, 32%), **10** (0.012 g, 19%) and **11** (0.01 g, 15%).

3.7. Preparation of amides *12*, *13*, *14*, *15* and *16*

A mixture of acetyloleanolic acid chloride and the appropriate amine (2.5 equiv mol) in dry $CHCl_3$ (4 ml) was refluxed for 5 h. The reaction mixture was diluted with ice and water, then extracted with $CHCl_3$. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column

chromatography (petroleum ether: EtOAc 9:1) to afford the corresponding amides **12** (0.09 g, 65%), **13** (0.106 g, 74%), **14** (0.121 g, 80%), **15** (0.124 g, 85%) and **16** (0.061 g, 84%).

*3.7.1. Oleanolic acid **1***

White powder; mp 310 °C; $[\alpha]^{20}_{D}$ +60.0 (c = 0.01, $CHCl_3$); IR (cm^{-1}); ν_{O-H} = 3500, ν_{CH_3} = 2950, ν_{CH_2} = 2850, $\nu_{C=O}$ = 1715; EIMS *m/z* (relative intensity) 456 ($M^{+\bullet}$) (5), 412 (3), 248 (100), 203 (50), 167 (25), 44 (51). ^{1}H–NMR ($DMSOd_6$, 250 MHz) δ 0.75 (3H, s, CH_3-26), 0.87 (3H, s, CH_3-24), 0.89 (3H, s, CH_3-23), 0.91 (3H, s, CH_3-25), 0.96 (3H, s, CH_3-30), 1.12 (3H, s, CH_3-27), 2.87 (1H, m, H-18), 3.23 (1H, m, H-3), 5.28 (1H, m, H-12); ^{13}C-NMR ($DMSOd_6$, 62.5 MHz) δ 15.1 (C-25), 15.9 (C-24), 16.8 (C-26), 18.0 (C-6), 22.6 (C-16), 22.9 (C-11), 23.4 (C-30), 26.9 (C-15), 27.2 (C-2), 28.2 (C-23), 30.4 (C-20), 32.4 (C-21), 32.9 (C-29), 33.4 (C-7), 36.6 (C-10), 38.1 (C-1), 38.4 (C-4), 41.3 (C-14), 45.5 (C-8 and C-17), 47.1 (C-9), 54.9 (C-5), 76.9 (C-3), 121.5 (C-12), 143.7 (C-13), 178.6 (C-28).

*3.7.2. Oleanolic acid acetate **1a***

White powder; mp 220–222 °C; IR (cm^{-1}); ν_{CH_3} = 2924, ν_{CH_2} = 2860, $\nu_{C=O}$ = 1734, $\nu_{C=O\ (COOH)}$ = 1711, ν_{C-O-C} = 1242; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.67 (3H, s, Me-26), 0.79 (3H, s, Me-29), 0.87 (3H, s, Me-24), 0.88 (3H, s, Me-23), 1.00 (3H, s, Me-30), 1.05 (3H, s, Me-25), 1.18 (3H, s, Me-27), 2.06 (3H, s,OAc-31), 2.78 (1H, m, H-18), 4.42 (1H, m, H-3), 5.19 (1H, m, H-12); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 15.5 (C-25), 16.7 (C-24), 17.2 (C-26), 18.1 (C-6), 22.8 ($\underline{CH_3}COO$), 23.4 (C-16), 23.6 (C-30), 24.0 (C-11), 27.7 (C-2), 28.0 (C-15), 29.7 (C-23), 30.7 (C-20), 32.8 (C-7), 33.1 (C-29), 33.8 (C-21), 37.7 (C-10), 38.0 (C-1), 39.0 (C-4), 41.5 (C-14), 46.6 (C-17), 48.0 (C-9), 55.3 (C-5), 80.9 (C-3), 122.5 (C-12), 143.6 (C-13), 171.0 ($CH_3\underline{COO}$), 178.6 (C-28).

*3.7.3. 12-hydroxy-δ-lactone **4***

White powder from (EtOAc-petroleum ether); mp = 285–287 °C ; IR (cm^{-1}): ν_{O-H} = 3529, $\nu_{C=O(\delta\text{-lactone) and (OAc)}}$ = 1733, ν_{C-O-C} = 1246; ESI + *m/z* (relative intensity) 1052 [2 M + Na]$^+$ (13), 537 [M + Na]$^+$ (54), 515 [M + H]$^+$ (100), 469 (58), 455 (22), 437 (19), 409 (13), 301 (8), 247 (14), 220 (11), 205 (22), 189 (24), 135 (16); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.76 (3H, s, CH_3-25), 0.79 (3H, s, CH_3-23), 0.85 (3H, s, CH_3-30), 0.86 (3H, s, CH_3-24), 0.93 (3H, s, CH_3-29), 1.08 (3H, s, CH_3-26), 1.07 (1H, m, H_{2b}), 1.25 (3H, s, CH_3-27), 1.45 (1H, m, H_{11b}), 1.63 (1H, m, H_{2a}), 2.02 (3H, s, CH_3COO), 2.00 (1H, m, H_{11a}), 2.04 (1H, m, H_{18}), 3.80 (1H, m, H_{12}), 4.41 (1H, m, H_3); ^{13}C-NMR ($CDC1_3$, 50 MHz) δ 16.2 (C-24), 16.3 (C-25), 17.5 (C-6), 18.4 (C-27 and C-26), 21.0 (C-16), 21.2 ($\underline{CH_3}COO$), 23.4 (C-2), 23.7 (C-30), 27.3 (C-22), 27.8 (C-23), 27.9 (C-15), 28.7 (C-11), 29.5 (C-20), 33.1 (C-29), 33.7 (C-7), 34.0 (C-21), 36.2 (C-10), 37.6 (C-1), 39.2 (C-4), 41.9 (C-14), 42.1 (C-8), 44.3 (C-9), 44.5 (C-17), 50.9 (C-18), 55.1 (C-5), 76.1 (C-12), 80.6 (C-3), 90.5 (C-13), 170.9 ($O\underline{CO}CH_3$), 179.8 (C-28).

*3.7.4. 12-oxo-δ-lactone **5***

White powder from (EtOAc-petroleum ether); mp = 288 °C; IR (cm^{-1}): ν_{CH_3} = 2932, ν_{CH_2} = 2868, $\nu_{C=O(\delta\text{-lactone) and (OAc)}}$ = 1734, $\nu_{C=O(ketone)}$ = 1724, ν_{C-O-C} = 1242; ESI + *m/z* (relative intensity) 1048 [2M + Na]$^+$ (23), 535 [M + Na]$^+$ (100), 513 [M + H]$^+$ (36), 398 (18), 301 (18), 279 (12), 220 (12), 150 (24); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.60 (9H, s, CH_3-25, CH_3-24 and CH_3-23), 0.61 (3H, s, CH_3-29), 0.70 (3H, s, CH_3-26), 0.75 (3H, s, CH_3-30), 1.12 (3H, s, CH_3-27), 1.83 (3H, s, CH_3COO), 2.14 (1H, dd, J_1 = 14.4 Hz and J_2 = 3 Hz, H_{18}), 2.31 (1H, t-like, H_{11a}), 2.48 (1H, t-like, H_{11b}), 4.25 (1H, m, H_3); ^{13}C-NMR ($CDC1_3$, 75 MHz) δ 51.4 (C-9), 55.4 (C-5), 80.6 (C-3), 91.4 (C-13), 171.4 ($O\underline{CO}CH_3$), 178.9 (C-28), 206.4 (C-12).

*3.7.5. Acetyl-11-oxooleanolic acid **6***

White powder from (EtOAc-petroleum ether); mp = 335 °C ; IR (cm^{-1}): ν_{CH_3} = 2938, ν_{CH_2} = 2862, $\nu_{C=O(OAc)}$ = 1736, $\nu_{C=O(COOH)}$ = 1708, $\nu_{C=O(ketone)}$ = 1684, $\nu_{C=C}$ = 1667, ν_{C-O-C} = 1234; ESI + *m/z* (relative intensity) 513 [M + H]$^+$ (10), 512 ($M^{+\bullet}$) (35), 511 [M–H]$^+$ (100), 467 (6), 424 (4), 351 (34), 301 (6), 270 (6), 250 (4), 239 (6), 185 (67), 169 (6), 135 (12), 127 (20), 119 (9), 111 (12); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.70 (6H, s, CH_3-24 and CH-25), 0.80 (3H, s, CH_3-23), 0.86 (3H, s, CH_3-29), 0.95 (3H, s, CH_3-30), 1.15 (3H, s, CH_3-27), 2.05 (3H, s, CH_3COO), 2.15 (1H, m, H_{19b}), 2.30 (1H, s, H_9), 2.38 (1H, m, H_{19a}), 2.86 (1H, m, H_{18}), 4.51 (1H, dd, J_1 = 11.4 Hz and J_2 = 5.6 Hz, H_3), 5.56 (1H, s, H_{12}); ^{13}C-NMR ($CDC1_3$, 75 MHz) δ 200.0 (C-11), 182.5 (C-28), 171.4 ($O\underline{CO}CH_3$), 163.2 (C-13), 131.2 (C-12), 81.0 (C-3).

*3.7.6. Compound **7***

White powder from (EtOAc-petroleum ether); mp = 124–128 °C; IR (cm^{-1}): ν_{CH_3} = 2926, ν_{CH_2} = 2856, $\nu_{C=O(OAc)}$ = 1728, $\nu_{C=O}$ = 1718, $\nu_{C=C}$ = 1674, $\nu_{P=O}$ = 1240, ν_{P-O-C} = 1020; ESI + *m/z* (relative intensity) 641 $[M + Na]^+$ (52), 619 $[M + H]^+$ (100), 559 $[MH^+–(CH_3$ and $OCH_2CH_3)]$ (44); ^{1}H–NMR ($CDCl_3$, 400 MHz) δ 0.75 (3H, s, CH_3-25), 0.87 (3H, s, CH_3-24), 0.88 (3H, s, CH_3-23), 0.92 (3H, s, CH_3-29), 0.94 (3H, s, CH_3-26), 0.95 (3H, s, CH_3-30), 1.15 (3H, s, CH_3-27), 1.36 (6H, m, $H_{2'}$), 2.05 (3H, s, CH_3COO), 3.05 (1H, m, H_{18}), 4.20 (4H, q-like, $H_{1'}$), 4.51 (1H, m, H_3), 5.35 (1H, m, H_{12}); ^{13}C-NMR ($CDC1_3$, 75 MHz) δ. 15.4 (C-26), 16.3 (C-2'), 16.4 (C-24), 16.9 (C-25), 18.1 (C-6), 21.2 (C-16 and $\underline{CH_3}COO$), 23.4 (C-2), 23.5 (C-30), 23.5 (C-11), 25.6 (C-27), 28.0 (C-23), 32.6 (C-21 and C-29), 37.6 (C-10), 41.7 (C-8), 47.4 (C-9), 55.2 (C-5), 63.3 (C-1'), 80.8 (C-3), 122.6 (C-12), 143.2 (C-13), 170.9 ($O\underline{CO}CH_3$), 215 and 214.4 (C-28); ^{31}P-NMR ($CDCl_3$, 121.48 MHz) δ (0.27, 75.9%) and (–0.01, 24.1%).

*3.7.7. Compound **8***

White powder from (EtOAc-petroleum ether); mp = 153 °C; IR (cm^{-1}): ν_{O-H} = 3420, ν_{CH_3} = 2918, ν_{CH_2} = 2860, $\nu_{C=O}$ = 1716, $\nu_{P=O}$ = 1242, ν_{P-O-C} = 1012; FABMS *m/z* (relative intensity) $[MH]^+$ 577 (4), 559 (3), 411 (100), 393 (21), 269 (5), 255 (11), 241 (12), 215 (26), 203 (49), 189 (59), 187 (44); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.62 (3H, s, CH-25), 0.68 (3H, s, CH_3-24), 0.71 (3H, s, CH_3-23), 0.82 (3H, s, CH_3-29), 0.85 (3H, s, CH_3-26), 0.90 (3H, s, CH_3-30), 0.95 (3H, s, CH_3-27), 1.31 (6H, m, $H_{2'}$), 2.97 (1H, m, H_{18}), 3.15 (1H, m, H_3), 4.15 (4H, q-like, $H_{1'}$), 5.27 (1H, m, H_{12}); ^{13}C-NMR ($CDC1_3$, 75 MHz) δ 63.8 (C-1'), 79.3 (C-3), 123.0 (C-12), 143.6 (C-13), 215.0 (C-28); ^{31}P-NMR ($CDCl_3$, 121.48 MHz) δ (0.26, 75%) and (–0.01, 25%).

*3.7.8. Product **9***

White crystalline powder from (EtOAc-petroleum ether); EI *m/z* (relative intensity) 560 ($M^{+\bullet}$) (6), 514 $[M–HCOOH]^{+\bullet}$ (21), 438 $[M\text{-}C_7H_6O_2]^{+\bullet}$ (6), 248 (100), 203 (43), 190 (28), 105 $[C_7H_5O]^{+\bullet}$ (33); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.70 (3H, s, CH_3-25), 0.85 (3H, s, CH_3-30), 0.87 (3H, s, CH_3-23), 0.89 (3H, s, CH_3-29), 1.02 (3H, s, CH_3-26), 1.04 (3H, s, CH_3-24), 1.17 (3H, s, CH_3-27), 2.76 (1H, m, H_{18}), 4.69 (1H, m, H_3), 5.23 (1H, m, H_{12}), 7.40 (2H, m, $H_{4'}$ and $H_{6'}$), 7.49 (1H, m, $H_{5'}$), 8.00 (2H, m, $H_{3'}$ and $H_{7'}$); ^{13}C–NMR ($CDC1_3$, 100 MHz) δ 55.2 (C-5), 81.4 (C-3), 122.4 (C-12), 128.2 (C-4' and C-6'), 129.4 (C-3' and C-7'), 130.8 (C-2'), 132.6 (C-5'), 143.4 (C-13), 166.1 (C-1'), 183.3 (C-28).

*3.7.9. Product **10***

White crystalline powder from (EtOAc-petroleum ether); EI *m/z* (relative intensity) 439 $[M–C_7H_5O_3]^+$ (8), 438 $[M\text{-}C_7H_6O_3]^{+\bullet}$ (7), 248 (100), 203 (53), 121 $[C_7H_5O_2]^{+\bullet}$ (67); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 2.78 (1H, m, H_{18}), 4.17 (1H, m, H_3), 5.21(1H, m, H_{12}), 6.80–8.20 (4H, m, $4H_{arom}$).

*3.7.10. Product **11***

White crystalline powder from (EtOAc–petroleum ether); APCI + *m/z* (relative intensity) 439 $[M\text{-}C_9H_7O_4]^+$ (100), 440 $[M–C_9H_7O_4+H]^+$ (28), 281 (25), 149 (92); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.77 (3H, s, CH_3-25), 0.85 (6H, s, CH_3-30 and CH_3-23), 0.94 (3H, s, CH_3-29), 0.96 (3H, s, CH_3-26), 1.15 (3H, s, CH_3-24), 1.26 (3H, s, CH_3-27), 2.07 (3H, s, CH_3COO), 2.86 (1H, m, H_{18}), 4.54 (1H, m, H_3), 5.33 (1H, m, H_{12}), 7.00–8.10 (4H, m, $4H_{arom.}$).

*3.7.11. Compound **12***

White powder from (EtOAc-petroleum ether); mp = 122 °C ; IR (cm^{-1}): ν_{CH_3} = 2922, ν_{CH_2} = 2860, $\nu_{C=O(OAc)}$ = 1734, $\nu_{C=O(ketone)}$ = 1690, $\nu_{C=C\ arom}$ = 1634, $\nu_{C=N\ arom}$ = 1518, ν_{C-O-C} = 1242; FABMS *m/z* (relative intensity) 575 $[MH]^+$ (79), 562 (68), 515 (12), 484 (14), 470 (12), 396 (15), 394 (14), 350 (71), 313 (24), 279 (23), 236 (100); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.60 (3H, s, CH_3-25), 0.74 (3H, s, CH_3-24), 0.76 (3H, s, CH_3-23), 0.81 (3H, s, CH_3-29), 0.85 (3H, s, CH_3-26), 0.89 (3H, s, CH_3-30), 1.11 (3H, s, CH_3-27), 1.97 (3H, s, CH_3COO), 2.62 (1H, m, H_{18}), 4.41 (1H, m, H_3), 5.49 (1H, m, H_{12}), 7.16 (1H, m, $H_{5'}$), 7.75 (1H, s, $H_{2'}$), 8.15 (1H, m, $H_{6'}$), 8.22 (1H, m, $H_{4'}$), 8.32 (1H, m, $\underline{H}NCO$); ^{13}C–NMR ($CDC1_3$, 50 MHz) δ 55.2 (C-5), 80.8 (C-3), 123.6 (C-12), 123.7 (C-5'), 127.1 (C-2'), 134.9 (C-4'), 140.1 (C-1'), 140.9 (C-6'), 144.9 (C-13), 171.0 ($O\underline{CO}CH_3$), 177.2 (CONH).

*3.7.12. Compound **13***

White powder from (EtOAc-petroleum ether); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.70 (3H, s, CH_3-25), 0.79 (3H, s, CH_3-24), 0.83 (3H, s, CH_3-23), 0.87 (3H, s,

CH_3-29), 1.01 (3H, s, CH_3-26), 1.08 (3H, s, CH_3-30), 1.20 (3H, s, CH_3-27), 2.01 (3H, s, CH_3COO), 2.42 (1H, m, H_{18}), 2.96 (1H, m, $H_{l'b}$), 3.26 (1H, m, $H_{l'a}$), 4.42 (1H, m, H_3), 5.25 (1H, m, H_{12}), 5.83 (1H, m, HNCO); ^{13}C-NMR ($CDC1_3$, 50 MHz) δ 55.2 (C-5), 80.8 (C-3), 122.6 (C-12), 145.2 (C-13), 171.0 ($OCOCH_3$), 178.1 (CONH).

*3.7.13. Compound **14***

White powder from (EtOAc-petroleum ether); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.69 (3H, s, CH_3-25), 0.78 (3H, s, CH_3-24), 0.82 (3H, s, CH_3-23), 0.86 (3H, s, CH_3-29), 1.01 (3H, s, CH_3-26), 1.04 (3H, s, CH_3-30), 1.20 (3H, s, CH_3-27), 1.98 (3H, s, CH_3COO), 2.70 (1H, m, H_{18}), 2.92 (1H, m, $H_{l'b}$), 3.28 (1H, m, $H_{1'a}$), 4.40 (1H, m, H_3), 5.19 (1H, m, H_{12}), 5.86 (1H, m, HNCO); ^{13}C-NMR ($CDC1_3$, 50 MHz) δ 55.3 (C-5), 80.9 (C-3), 122.5 (C-12), 145.2 (C-13), 171.0 ($OCOCH_3$), 178.2 (CONH).

*3.7.14. Compound **15***

White powder from (EtOAc-petroleum ether); ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 0.72 (3H, s, CH_3-25), 0.76 (3H, s, CH_3-24), 0.80 (3H, s, CH_3-23), 0.84 (3H, s, CH_3-29), 0.95 (3H, s, CH_3-26), 1.05 (3H, s, CH_3-30), 1.18 (3H, s, CH_3-27), 2.02 (3H, s, CH_3COO), 2.50 (1H, m, H_{18}), 3.76 (1H, m, $H_{l'}$), 4.39 (1H, m, H_3), 5.20 (1H, m, H_{12}), 5.52 (1H, m, HNCO); ^{13}C-NMR ($CDC1_3$, 50 MHz) δ 55.2 (C-5), 80.8 (C-3), 122.4 (C-12), 144.8 (C-13), 171.0 ($OCOCH_3$), 177.3 (CONH).

*3.7.15. Compound **16***

White powder from (EtOAc-petroleum ether); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.77 (3H, s, CH_3-25), 0.85 (3H, s, CH_3-24), 0.87 (3H, s, CH_3-23), 0.89 (3H, s, CH_3-29), 0.94 (3H, s, CH_3-26), 1.15 (3H, s, CH_3-30), 1.25 (3H, s, CH_3-27), 2.01 (3H, s, CH_3COO), 2.27 (1H, m, H_{18}), 3.00 (1H, m, $H_{l'b}$), 3.34 (1H, m, $H_{l'a}$), 4.42 (1H, m, H_3), 5.38 (1H, m, H_{12}), 5.90 (1H, m, HNCO); ^{13}C-NMR ($CDC1_3$, 75 MHz) δ 55.2 (C-5), 80.8 (C-3), 122.5 (C-12), 145.2 (C-13), 171.0 ($OCOCH_3$), 178.0 (CONH).

3.8. Antibacterial activity

3.8.1. Microbial cultures growth conditions

Test microorganisms included the following Gram negative bacteria such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* and Gram-positive strain *Staphylococcus aureus* (ATCC 25923). Cultures of these bacteria were done on Nutrient Mueller–Hinton medium produced by 'Institut Pasteur (Tunis)' and were incubated at 37 °C.

3.8.2. Experimental

All tested compounds are not water-soluble. To overcome this problem, we have included an emulsifier, Tween 80 at the concentration of 5% (v/v) [20].

The compounds were dissolved in sterile physiological water with 5% Tween 80 and were tested for antimicrobial activity using the microdilution method on liquid media to determine the minimal inhibitory concentration by using the following concentrations mentioned in Table 1. The MIC was considered as the lowest concentration of the sample that prevented visible growth.

The test of antibacterial activity is done by using the decimal dilution method in both Mueller–Hinton media. The minimal bactericidal concentrations were determined by streaking the Mueller–Hinton nutrients that were incubated for 24 h at 37 °C, in the nutritive agar plates. The MBC was defined as the lowest concentration of the tested compound at which 99.99% of bacteria have been killed.

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