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Short communication

Synthesis and cytotoxicity evaluation of A-ring derivatives of cycloartanone

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ABSTRACT

Sixteen derivatives, eight of which are new compounds, were prepared from cycloartenone (**1**) and cycloartanone (**2**), and the cytotoxic activity of 14 of these compounds, together with **1** and **2**, was evaluated against a panel of four cell lines. Compound **1** was obtained from the epiphyte plant *Tillandsia tenuifolia* collected at Salta, Argentina. Due to chemoselectivity and regioselectivity problems observed in the reactions of compound **1**, this substance was hydrogenated to compound **2**. The attempted transformations were focused on ring A of the cited triterpenes with the aim to verify previous structure-activity relationships obtained from related compounds. The cytotoxicity results of the derivatives showed that only the diosphenol **13** displayed significant activity against all the tested cell lines. These results show that an oxidized side chain, for example an ether bridge between the side chain and ring D, is necessary for the cytotoxic activity of cycloartane derivatives.

1. Introduction

Cycloartanes are one of the most widespread classes of triterpenoids, and the main secondary metabolites in plants of the genus *Tillandsia* (Bromelliaceae) (Cabrera, 2000). This genus comprises more than 500 species, all of which are native to North or South America (Luther, 2002). Most of *Tillandsia* species are epiphytes, growing not only on trees but also on fences, wires, and electricity cables, absorbing water and nutrients directly from the atmosphere, as well as heavy metals and other pollutants, and, for this reason they have been used as environmental biosensors (Figueiredo et al., 2004, 2007; Malm et al., 1998). Although many *Tillandsia* species are considered ornamental, other are treated as weeds since they are a threat to tree plantations and citric orchards. The two most common species of this genus, *T. usnoides* and *T. recurvata* produce large amounts of cycloartenol, (Cabrera and Seldes, 1995; Cabrera et al., 1996) together with several minor derivatives, while other species produce cycloartenone as a main component of the extracts. Some cycloartenoids from *Tillandsia* have shown interesting biological activities, which have been reviewed (Lowe et al., 2014a, 2014b). As part of a study of the secondary metabolites produced by Bromelliaceae of the province of Salta, Argentina, a considerable amount of cycloartenone (**1**) was isolated from *T. tenuifolia* L. This abundant compound gave the opportunity for a structural diversification project, aimed at the preparation of new bioactive substances, which would add value to this neglected biological resource.

Although the most common pentacyclic triterpenoids were discovered long ago, there has been a recent interest in the use of these compounds as starting materials for chemical diversification projects and structure-activity studies (Sun et al., 2006). For example, there have been reports on synthetic modifications of ring A in oleananes (Honda et al., 1997, 1998, 2000; Sporn et al., 2011), betulin (Pettit et al., 2014), allobetulin (Heller et al., 2015), betulinic acid (Li et al., 2016), and boswellic acid (Fan et al., 2016). Quite surprisingly, despite their relative abundance, there are only a few studies on the chemistry and structure-activity relationships of cycloartenoids, when compared to those of pentacyclic triterpenes. The groups of Parra-Delgado and Martínez-Vazquez have prepared a series of derivatives on ring A of argentatins A, B and D, major components of guayule (*Parthemium argentatum*) resin, studied their anti-inflammatory activity and cytotoxicity against several tumor cell lines, and have drawn some interesting preliminary structure-activity relationships (Parra-Delgado et al., 2005, 2006; Romero et al., 2014). However, since the argentatins have an

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ether bridge between the side chain and ring D, it was not clear if the modifications on ring A exerted a cytotoxic effect by themselves or just modulated the effect produced by the side chain, or if the observed bioactivity was a combination of both parts of the molecule. In order to clarify these effects, in the present work several modifications were attempted on ring A of cycloartenone. During the initial attempts of oxidation and halogenation reactions, the Δ24 double bond of cycloartenone (**1**) generated regio- and chemoselectivity problems, and, for this reason, compound **1** was quantitatively hydrogenated to cycloartanone (**2**). This compound gave an excellent opportunity to perform simple transformations on the A-ring without the interference of additional functional groups, and to study the structure-activity relationships obtained from these single modifications. In particular, based on the previous work of Martínez-Vazquez et al. it was interesting to study the individual and combined effects of a Δ1 double bond and the presence of halogens at C-2. In this work, 16 derivatives of compounds **1** and **2** were prepared, 8 of them new compounds, and their cytotoxic activity was evaluated against a panel of four cell lines (Denizot and Lang, 1986).

2. Results and discussion

Initial attempts to oxidize ring A of cycloartenone (**1**) to form a Δ1 double bond with PhSeCl and oxone produced a mixture of 25-hydroxy-Δ23 derivatives **3** and **4**, in which the Δ24 double bond of **1** was isomerized upon oxidation at C-25, a process which is common in nature (Smith-Kielland et al., 1996). On the other hand, chlorination of **1** also yielded a mixture of compounds (**5** and **6**) in which the Δ24 double bond was isomerized to a Δ25(26) double bond with chlorination at the C-24 allylic position. To make things worse, **5** and **6** were obtained as mixtures of epimers at C-24. In view of these problems, the Δ24 double bond was reduced by hydrogenation over Pd/C, in order to avoid regio- and stereochemical issues in further transformations. In this way, cycloartenone **1** yielded quantitatively cycloartanone **2** (Fig. 1).

The structural modifications performed on **2** were mainly focused on the introduction of a conjugated double bond on ring A, as well as substitutions with different groups (halogens, amino or azide) at C-2. Compound **2**, upon oxidation with PhSeCl and oxone® gave a (4:1) mixture of the 3-keto-Δ1 derivative **7** and the 2-Cl-3-keto-Δ1 compound **8**, and both compounds were purified by preparative TLC. The formation of compound **8** as a minor product may be explained by a nucleophilic attack of chloride ion on the initially formed phenyl selenide. A second enolization, in this case of the C-2 chloride, followed by a second electrophilic attack of PhSeCl should give a C-2 disubstituted (with Cl and PhSe) intermediate, which, by the expected oxidation and elimination under the reaction conditions would produce compound **8**. Chlorination of **2** at C-2 using mild conditions ($SOCl_2$ in dichloroethane) gave a mixture of the mono and dichlorinated cycloartanes **9** and **10**, which were also separated by TLC, although **10** could be isolated only in minor quantities and was not submitted to bioassays. Analysis of the NOESY spectrum of **9** confirmed that the chlorine at C-2 was pseudoequatorial as expected. The C-2 epimer of **9**, although initially detectable in the crude mixture by TLC and NMR, isomerized to the more stable conformation with a pseudoequatorial chlorine atom during purification. In the case of compound **10**, the disubstitution at C-2 was evident in ^{1}H NMR spectrum, in which both H-1's appeared as AB doublets at δ: 2.83 and 2.61. Bromination of **2** (Br_2/AcOH) yielded also a mixture of two products: the previously reported 2-α-Br-cycloartanone **11** and 2,2′-dibromocycloartanone **12**, and in this case the latter was the main product. In the ^{1}H NMR spectrum of **12**, both H-1′s showed the same AB doublets as in **10**, but in this case at higher δ: 3.19 and 2.79 ppm.

Treatment of **12** with NEt_3/DMF in the presence of traces of water and O_2, yielded the 2-OH-3-keto-Δ1-cycloartanone **13** by hydrolysis of the *gem*-dihaloketone. The diosphenol **13** was an interesting product since this functionality is usually present in bioactive compounds, a fact that was confirmed by the cytotoxicity assays. In order to achieve fluorination at C-2, compound **2** was converted (vinyl benzoate, pTsOH, microwave heating) to the enol benzoate **14**, which was in turn treated with selectfluor® to yield a mixture of epimeric C-2 fluoro derivatives **15** and **16**. In this case, due to the smaller size of the fluorine atom compared to chlorine, both epimeric fluoro derivatives could be purified by preparative TLC and completely characterized by NMR. In the ^{1}H NMR spectra of both compounds, H-2 was clearly identified as a *ddd*

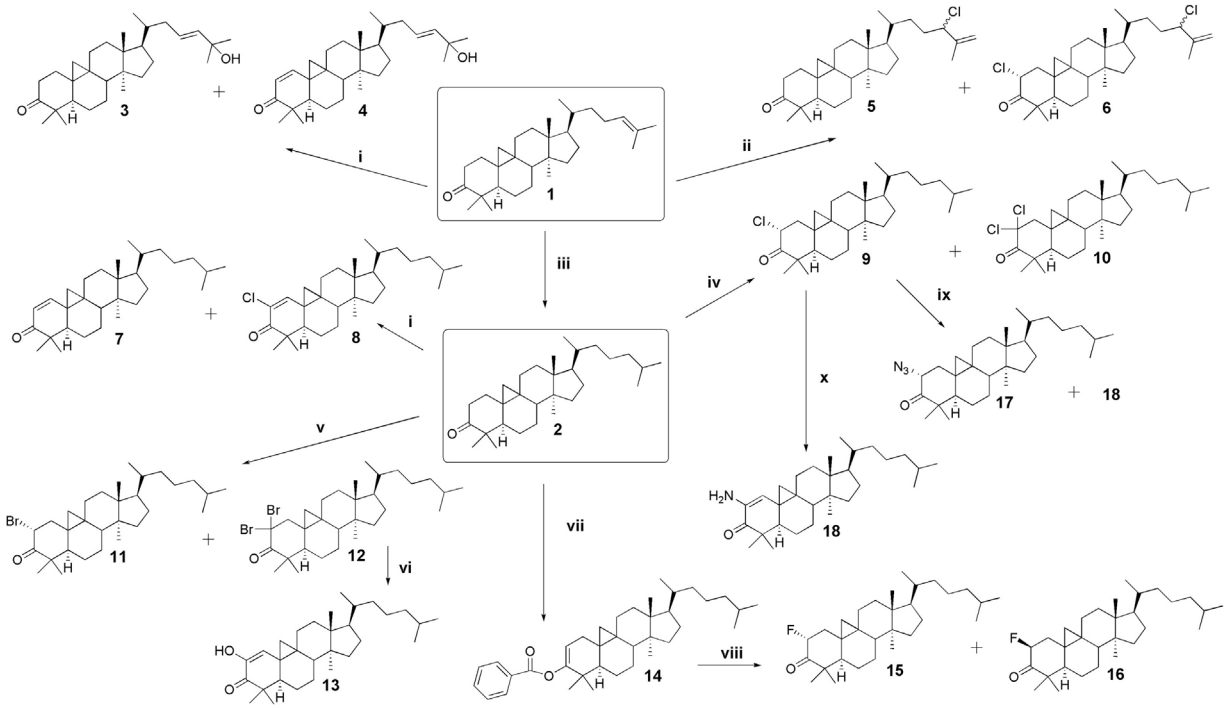

Fig. 1. Reaction conditions: i) PhSeCl, Oxone, THF; ii) Cl_2 (g), r.t., cyclohexane; iii) H_2, Pd/C, EtOAc; iv) SO_2Cl_2, DCE; v) Br_2, AcOH, r.t.; vi) NEt_3, DMF, 75 °C; vii) vinyl benzoate, p-TsOH, microwave, 15 min; viii) Selectfluor^R, CH_3CN, r.t.; ix) NaN_3, DMF, r.t.; x) NaN_3, DMF, 60 °C.

(49.0, 12.0, 7.5 Hz in **15**, 50.0, 7.7, 6.3 Hz in **16**) in which the large germinal ^{1}H-^{19}F coupling was dominant. Compound **15** was identified as the 2α-F derivative by the ax–ax (12 Hz) coupling H-1ax-H-2. NOE correlations were observed, of H-2 with Me-30, and also with one of the cyclopropyl protons at δ: 0.68, thus confirming that in **15**, H-2 was in the β-face and pseudoaxial. On the other hand, in the NOESY spectrum of **16**, correlations were observed between H-2 and Me-29, as well as with H-5, all of which point to the presence of a fluorine in a 2β configuration.

Encouraged by the previous results, additional functional group modifications for the A ring were sought. It is known that the nitrogen atom of an enaminone can act as a good hydrogen acceptor or donor, properties which usually translate into biological activity. In particular, some enaminones formed in rings A of tetracyclic triterpenoids, such as the cucurbitacins, are particularly stable, making an enaminone in the ring A of a cycloartane an interesting target. On the other hand, the azide group is a widely used warhead in medicinal chemistry, as well as an entry to further chemical transformations due to its ability as a leaving group, and also for its use in click chemistry for further structural diversification.

Treatment of the chlorinated cycloartanone **9** with NaN_3 in DMF at room temperature yielded the azide **17** as the main product together with the enaminone **18**, while, when the same reaction was carried out at 60 °C, only compound **18** was obtained. The formation of **18** proceeds in this case by base-promoted loss of the acidic H-2 in **17** with a subsequent irreversible nitrogen elimination. However, compound **18** was too unstable to become a useful derivative.

The cytotoxic activities of compounds **1–9**, **11–13**, and **15–18,** were evaluated against a panel of four cell lines: MCF7 (human mammary carcinoma), LM2 (murine mammary adenocarcinoma), HB4a (human normal mammary cells) and HaCaT (human normal keratinocytes), and the results are displayed in Table 1.

3. Conclusions

Based on the results shown in Table 1, it is evident that compounds **1** and **2** can be considered inactive, and that most of the derivatives had very low activity, with the exception of compound **13,** which is the only derivative with an IC50 lower than 30 μM. In the work on argentatins A and B, it was pointed out that a bromine atom, or another bulky substituent at C-2, or the presence of a Δ1 double bond, both enhanced the cytotoxicity of the compounds. In this work, similar transformations were performed on cycloartanone, not only by introduction of a Δ1 double bond, but also by substitution at C-2 by halogens (F, Cl, Br), disubstitution by bromine and also the combination of the double bond and substitution by chlorine, and in all cases, an increase in cytotoxicity was not observed. This is particularly evident in the case of the 2-Br derivatives: a ten-fold increase of cytotoxicity against certain cell lines was observed in 2-Br-argentatin A when compared to the parent triterpenoid (Parra-Delgado et al., 2005). However, in the case of cycloartanone, no effect was observed on the cytotoxicity upon bromination at C-2. These results show that the oxidized side chain of the argentatins, such as the ether bridge with ring D, is necessary for the cytotoxic activity, and that the modifications on ring A modulate the activity, but are not sufficient cause for the observed cytotoxicity. The efficient preparation of the azide and diosphenol derivatives of cycloartanone may allow the synthesis of new series of derivatives, in particular with heterocyclic rings, to further expand the chemistry and bioactivity studies of cycloartenoids. Derivatives **9–10** and **12–18** are new compounds, and their NMR spectra were fully assigned by 2D-NMR experiments (see Table 2, Supplementary material)

Table 1
Cytotoxic activity of cycloartane derivatives IC_{50} (μg/mL).

Cpd.	MCF-7	HB4a	HaCaT	LM2
1	92.6 ± 2.4	> 100	69.2 ± 8.5	80.9 ± 39
2	94.0 ± 11.2	96.3 ± 5.8	77.4 ± 11.9	79.3. ± 8.1
3	34.0 ± 8.4	43.6 ± 5.9	20.3 ± 3.2	18.8 ± 6.3
4	61.1 ± 16.5	44.1 ± 12.3	38.6 ± 4.5	36.5 ± 4.9
5	67.3 ± 9.1	87.7 ± 18.4	54.4 ± 6.3	59.1 ± 1.2
6	79.1 ± 9.8	> 100	45.8 ± 6.3	64.3 ± 1.6
7	80.2 ± 11.2	75.3 ± 7.8	41.9 ± 6.3	64.4 ± 10.3
8	74.8 ± 7.6	> 100	76.9 ± 8.2	> 100
9	60.5 ± 11.2	56.2 ± 2.6	58.6 ± 8.3	50 ± 5.2
11	65.3 ± 9.6	58.3 ± 11.7	66.7 ± 8.6	64.7 ± 9.7
12	70.3 ± 6.5	86.3 ± 10.6	> 100	> 100
13	28.0 ± 8.3	29.4 ± 7.1	20.8 ± 8.3	23.3 ± 2.1
15	74.3 ± 5.2	98.0 ± 6.9	88.7 ± 18.6	> 100
16	77.6 ± 8.0	65.9 ± 7.1	68.7 ± 18.3	> 100
17	> 100	> 100	92.4 ± 7.9	> 100
18	> 100	> 100	60.2 ± 10.4	57.8 ± 8.7
Doxo	2.4 ± 0.7	5.8 ± 0.8	8.8 ± 0.9	0.6 ± 0.1

4. Experimental

4.1. General experimental procedures

All solvents were distilled for chromatography; CH_2Cl_2 was distilled from phosphorous pentoxide. NMR spectra were recorded on Bruker AC-200 (200.13 MHz) and Bruker Avance II (500.13 MHz) spectrometers, using the signals of residual nondeuterated solvents as internal reference. All 2D NMR experiments (COSY, DEPT-HSQC, HMBC, and NOESY), were performed using standard pulse sequences. HRMS expeiments were acquired on a Bruker micrOTOF-Q II spectrometer. UV spectra were obtained on a Hewlett Packard 8453 spectrophotometer and IR spectra were obtained on an FT-IR Nicolet Magna 550 instrument. TLC was carried out on Merck Sílicagel 60 F254 plates. TLC plates were sprayed with 2% vainillin in concentrated H_2SO_4. Merck Silicagel (230–400 mesh) was used for column chromatography. All other reagents were purchased from Sigma-Aldrich. Analytical HPLC was carried out on a Gilson 506C instrument, equipped with a 322 pump and an UV detector (Gilson 170). A Phenomenex Synergi Fusion-RP (4 μm, 4.60 mm–150 mm) column was used with a 0.2 mL/min flow rate. Gradient elution was performed using A: MeOH and B: H_2O, starting with 70% A and raising to 100% MeOH within 20 min, followed by 5 min in isocratic mode.

4.2. Plant material

Specimens of *T. tenuifolia* were collected near Campo Alegre dam, La Caldera, Province of Salta, Argentina, in spring time. A voucher specimen (SI 139665) was identified by Dr. Sabina Donadío and stored at the herbarium of the Darwinion Botanical Institute (San Isidro, Buenos Aires, Argentina).

4.3. Extraction and purification of cycloartenone (1)

A typical procedure is as follows: ground fresh plant material (1000 g) was extracted exhaustively twice with ethanol and the twice with EtOAc at room temperature. The combined extracts were evaporated at reduced pressure to yield a syrupy residue (9 g). This residue was partitioned between MeOH: H_2O (9:1) and cyclohexane. The cyclohexane layer was taken to dryness to yield 1.5 g of lipophilic subextract, which was fractionated by VLC on silica using a cyclohexane-EtOAc gradient. The different eluates were monitored by TLC and combined to give three fractions: L1-L3. Fraction L1 (575 mg, eluted with cyclohexane-EtOAc 9:1) was purified by column chromatography on silica gel to yield 210 mg of pure cycloartenone (**1**).

4.4. Preparation of derivatives of cycloartenone (1)

4.4.1. Preparation of compounds 3–4

To 20 mg (0.048 mmol, 1eq) of cicloartenone (**1**) disolved in EtOAc, were added 13 mg (0.07 mmol, 1.5eq) of PhSeCl and an orange-red solution was obtained, which was stirred at 35C. When the solution turned yellow, it was washed with 1 mL of water. Then, 1 mL of THF was added, followed by a dropwise addition of 1 mL of an aqueous solution of 0.86 mg (0.14 mmol, 2.9eq) of Oxone®, with constant stirring. After 2 h, the reaction mixture was diluted with EtOAc and extracted with H_2O and 5% Na_2CO_3. The organic phase was dried with Na_2SO_4, filtered and taken to dryness. The crude mixture consisted of two main products, which were separated by prep. TLC (cyclohexane-EtOAc, 8:2) to yield 15% of compound **3** and 25% of **4** (purified yields).

4.4.2. Preparation of compounds 5–6

Chlorine gas (externally generated by dropwise addition of HCl (c) to 80 mg of $KMnO_4$) was bubbled for 5 min into a cyclohexane solution of 10 mg of compound **1**. When the reaction was completed, the crude product was taken to dryness and purified by prep. TLC (cyclohexane-EtOAc (95:5)) to yield 30% of compound **5** and 30% of compound **6**.

4.4.3. Hydrogenation of compound 1

An EtOAc solution of 200 mg of compound **1** was hydrogenated over Pd/C (10%) at 1 atm for 1 h. The solution was filtered, and then taken to dryness. The crude product was analysed by ^{1}H NMR, identified as pure cycloartanone (**2**), and used for the preparation of derivatives without further purification.

4.5. Preparation of derivatives of cycloartanone (2)

4.5.1. Preparation of compounds 7–8

To a solution of 22 mg (0.051 mmol, 1eq) of **2** in 2 mL of EtOAC were added 14 mg (0.073 mmol, 1.4 eq) of PhSeCl dissolved in 1 mL EtOAc. The reaction was stirred at room temperature until the orange-red solution turned yellow, and then washed with 1 mL of H_2O. Then, 2 mL of THF and 82 mg (0.13 mmol, 2.5eq) of Oxone® dissolved in 1 mL of H_2O were added. The reaction mixture was stirred at 35C for 3 h and controlled by TLC. After completion, the reaction mixture was diluted with EtOAc and washed first with H_2O and then repeatedly with Na_2CO_3 (satd. soln.) until neutralization. The organic layer was taken to dryness and the products were purified by prep.TLC (hexane-EtOAc (9:1)) to yield 40% of compound **7** and 10% of compound **8**.

4.5.1.1. 2-Chloro-cycloart-1-en-3-one (8). UV ($CHCl_3$): λ_{max} (log ε): 254 (3.71) nm. IR (film, cm^{-1}): 2947; 1690. $[\alpha]_D^{20}$ −16° (*c* 0.10, $CHCl_3$). ESI$^+$HRMS (*m/z*): 459.3391 $[M+H]^+$(calcd. for $C_{30}H_{48}ClO^+$: 459.3388); 476.3655 $[M+NH_4]^+$(calcd. for $C_{30}H_{51}ClNO^+$: 476.3654); 481.3211 $[M+Na]^+$(calcd. for $C_{30}H_{47}ClNaO^+$: 481.3208). ^{1}H NMR: see Table 2 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.5.2. Preparation of compounds 9–10

To a solution of 31 mg of **2** (0.073 mmol, 1eq) in 500 μL of bidistilled 1,2-dichloroethane cooled in an ice bath, were added 110 μL (0.14 mmol, 2eq) of SO_2Cl_2 in dichloroethane. Then, the reaction mixture was stirred for 1 h at room temperature. The reaction was quenched by addition of $NaHSO_3$ (satd. soln.), and the mixture was diluted with CH_2Cl_2. The organic layer was washed succesively with $NaHCO_3$ (satd. soln.) and H_2O, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The purification of the crude mixture by prep. TLC (cyclohexane-EtOAc (97:3)) yielded 50% of compound **9** and 5% of compound **10**.

4.5.2.1. 2α –Chloro-cycloartan-3-one (9). UV ($CHCl_3$): λ_{max} (log ε): 278 (3.71) nm. IR (film, cm^{-1}): 2987; 1731. $[\alpha]_D^{20}$ +26° (*c* 0.35, $CHCl_3$). ESI$^+$HRMS (*m/z*): 461.3552 $[M+H]^+$(calcd. for $C_{30}H_{50}ClO^+$: 461.3545); 478.3828 $[M+NH_4]^+$(calcd. for $C_{30}H_{53}ClNO^+$: 478.3810); 483.3371 $[M+Na]^+$(calcd. for $C_{30}H_{49}ClNaO^+$: 483.3364). ^{1}H NMR: see Table 2 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.5.2.2. 2,2-Dichloro-cycloartan-3-one (10). UV ($CHCl_3$): λ_{max} (log ε): 284 (3.40) nm. IR (film, cm^{-1}): 2947; 1729. ^{1}H NMR: see Table 2 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.5.3. Preparation of compounds 11–12

Compound **2** (48 mg, 0.11 mmol) was dissolved in 5 mL of glacial acetic acid with constant stirring in an ice bath. A 3.7 M solution of bromine in acetic acid was added dropwise until the solution turned orange-red, after which, the reaction mixture was stirred at room temperature for 1 h. To quench the reaction, the mixture was poured over crushed ice, then 50 mL of EtOAc were added, and the organic layer was washed succesively with H_2O, $NaHCO_3$ (satd. soln.), $NaHSO_3$ (satd. soln.), and finally once again with H_2O. The organic layer was taken to dryness, and the crude products were purified by prep. TLC (cyclohexane-EtOAc (95:5)) to yield compounds **11** (35% yield) and **12** (60% yield).

4.5.3.1. 2,2-Dibromo-cycloartan-3-one (12). UV ($CHCl_3$): λ_{max} (log ε): 294 (3.40) nm. IR (film, cm^{-1}): 2951; 1735. $[\alpha]_D^{20}$ +77° (*c* 0.85, $CHCl_3$). ESI$^+$HRMS (*m/z*): 600.2422 $[M+NH_4]^+$ (calcd. for $C_{30}H_{52}Br_2NO^+$: 600.2410); ^{1}H NMR: see Table 2 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.5.4. Preparation of compound 13

Compound **12** (12 mg, 0.02 mmol) was disolved in 4 mL of DMF and 3 mL (0.02 mmol) of NEt_3 were added dropwise with constant stirring. The mixture was then heated in a glycerine bath at 75C for 3 h. The reaction was quenched by dilution in EtOAc and addition of 2N HCl (15 mL). The organic layer was washed with H_2O and taken to dryness, and pure compound **13** was obtained in 83% yield without further purification.

4.5.4.1. 2-Hydroxy-cycloart-1-en-3-one (13). UV ($CHCl_3$): λ_{max} (log ε): 269 (3.32) nm. IR (film, cm^{-1}): 2930; 1726; 1673. $[\alpha]_D^{20}$ +48° (*c* 0.50, $CHCl_3$). ESI$^+$HRMS (*m/z*): 463.3546 $[M+Na]^+$ (calcd. for $C_{30}H_{48}NaO_2^+$: 463.3547); ^{1}H NMR: see Table 2 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.5.5. Preparation of compound 14

A sealed glass tube containing 65 mg of compound **2** (0.15 mmol), 18 mg of p-TsOH (0.1 mmol) and 0.2 mL of vinyl benzoate (1.5 mmol), was introduced in the cavity of a microwave reactor (CEM Co., Discover) and irradiated with stirring under maximum potency (300 W), The temperature was raised up to 170C in the lapse of 5 min, followed by an additional 10 min of heating. After cooling of the mixture to room temperature, the reaction vessel was opened and the crude product was purified by VLC on reversed Rp-18 phase, eluting first with MeOH-H_2O (9:1) to eliminate the excess of vinyl benzoate and then with MeOH to obtain compound **14** (95% yield), which was characterized only by NMR and used without further purification for the preparation of compounds **15** and **16**.

4.5.5.1. 3-Benzoyl-cycloart-2-ene (14). ^{1}H NMR: see Table 3 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

*4.5.6. Preparation of compounds **15–16***

Compound **14** (28 mg, 0.053 mmol), and selectfluor® (95 mg, 0.27 mmol) were disolved in 1 mL of CH_3CN, and the reaction mixture was stirred for 24 h at room temperature under inert atmosphere (N_2). The mixture was then diluted with EtOAc and washed several times with H_2O. The organic layer was taken to dryness to yield, after purification by prep. TLC (cyclohexane- EtOAc (95:5)), compounds **15** (17%) and **16** (20%).

*4.5.6.1. 2α −Fluor-cycloartan-3-one (**15**)*. UV ($CHCl_3$): λ_{max} (log ε): 289 (3.62) nm. IR (film, cm^{-1}): 2939, 2865, 1735, 1467, 1382. ESI^+HRMS (*m/z*): 445.3836 $[M+H]^+$(calcd. for $C_{30}H_{50}FO^+$: 445.3840); 462.4094 $[M+NH_4]^+$(calcd. for $C_{30}H_{53}FNO^+$: 462.4106); 483.3371 $[M+Na]^+$(calcd. for $C_{30}H_{49}FNaO^+$: 467.3660). ^1H NMR: see Table 3 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

*4.5.6.2. 2β −Fluor-cycloartan-3-one (**16**)*. UV ($CHCl_3$): λ_{max} (log ε): 287 (3.61) nm. IR (film, cm^{-1}): 2941, 2861, 1735, 1467, 1382. ESI^+HRMS (*m/z*): 462.4107 $[M+NH_4]^+$(calcd. for $C_{30}H_{53}FNO^+$: 462.4106); 467.3645 $[M+Na]^+$(calcd. for $C_{30}H_{49}FNaO^+$: 467.3660). ^1H NMR: see Table 3 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

*4.5.7. Preparation of compounds **17–18***

Compound **9** (40 mg, 0.087 mmol) was disolved in 300 μL of dry DMF, and then 96 mg (1.5 mmol) of NaN_3 were added. The reaction mixture was stirred at room temperature under inert atmosphere for 72 h. Then, Et_2O was added and the organic layer was washed five times with brine and then H_2O, taken to dryness and purified by prep. TLC (cyclohexane-EtOAc (95:5)), to yield 45% of compound **17**, and 15% of compound **18**. When the same reaction was carried out at 60C for two hours, and worked-up in a similar way, only compound **18** was obtained (40% yield). Due to its instability, compound **18** was only characterized by MS and NMR.

*4.5.7.1. 2α −Azido-cycloartan-3-one (**17**)*. IR (film, cm^{-1}): 2944, 2866, 2103, 1726. $[\alpha]_D^{20}$ +54° (*c* 0.40, $CHCl_3$). ^1H NMR: see Table 3 (Supplementary material).^{13}C NMR: see Table 4 (Supplementary material).

*4.5.7.2. 2-Amino-cycloart-1-en-3-one (**18**)*. ESI^+HRMS (*m/z*): 440.3897 $[M+H]^+$ (calcd. for $C_{30}H_{50}NO^+$: 440.3887); ^1H NMR: see Table 3 (Supplementary material). ^{13}C NMR: see Table 4 (Supplementary material).

4.6. Cytotoxicity assays

The human mammary carcinoma MCF7 cells were obtained from ATCC, the murine mammary adenocarcinoma LM2 cells were provided by Dr A. Eijan (Instituto Roffo, Argentina), the human mammary HB4a cells were provided by Prof M. O´Hare (University College London, UK), and the human normal keratinocytes HaCaT were obtained from Dr M. Quintanilla (Instituto de Investigaciones Biomedicas, Madrid, *Spain*). The cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 Lg/ml streptomycin and maintained at 37C in a 5% CO_2 atmosphere.

Cells were detached with 0.1% trypsin–EDTA and viable cells were counted using a hematocytometer. Cells (70,000 to 10,000 cells/ml) were seeded in 48-well microtiter plates and incubated at 37C. After 24 h, the cells were treated with different concentrations (1–100 μM) of the test compounds initially dissolved in dimethylsulfoxide (DMSO, 5 mM) and further diluted in medium to produce the desired concentrations. Final DMSO concentrations were lower than 2%. The plates were incubated for another 48 h at 37C. Doxorubicin (Sigma) was used at different concentrations (0.01–10 μM) as a positive control. Immediately after treatment, cell viability was measured employing the MTT method (Denizot and Lang, 1986). Briefly, 25 μL of MTT (2.5 mg/ml) was added to the wells and incubated for 1 h. After DMSO dissolution of the resulting formazan crystals, absorbance was recorded at 570 nm in a microplate reader.The concentrations required to inhibit cell growth by 50% (IC50) were calculated from the abscissa intercept from logistic curves constructed by plotting cell survival (%) versus drug concentration (μM). The purity of all tested compounds was evaluated by ^{13}C NMR and by HPLC and in all cases was > 95%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2017.06.021.

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