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Synthesis of a new cytotoxic cephalostatin/ritterazine analogue from hecogenin and 22-*epi*-hippuristanol

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ABSTRACT

A new cephalostatin/ritterazine analogue was prepared from the commercially available hecogenin acetate and the natural cytotoxic steroid 22-*epi*-hippuristanol. The method involved the reductive dimerization of enaminoketones (condensation of α-aminoketones) and condensation between an enaminoketone and an α-hydroxyketone. The new analogue showed higher cytotoxic activity than the cytotoxic 22-*epi*-hippuristanol against MDA-MB-231, A-549 and HT-29 cultured tumor cell lines.

1. Introduction

The role of natural products in drug discovery has enjoyed a renaissance in recent years.[1] After a decade in the wilderness, natural products have made a comeback, especially in oncology.[2] Three new drugs, ixabepilone (Ixempra®), trabectedin (ET-743, Yondelis®), and temsirolimus (CCI-779, Torisel®), derived from natural products were very recently approved by the FDA for the treatment of different cancers.[3]

The cephalostatins—along with the structurally related ritterazines—form a unique class of trisdecacyclic compounds that consist of two steroidal units linked through a pyrazine ring.[4] The first cephalostatins were isolated by Pettit et al.[5] from the Indian Ocean tube worm *Cephalodiscus gilchristi*. Cephalostatin 1 (**1**) exhibits extraordinarily high cytostatic activity against a broad spectrum of cancer cell lines and proved to be one of the most powerful cell growth inhibitors ever tested in the NCI. Compound **1** is considerably more active in vitro than paclitaxel and has an unprecedented mechanism of action.[6] More than 18 other cephalostatins were characterized and they showed the same unique cytotoxicity profile in the NCI-60 cell line panel.[7] Closely related ritterazines were isolated by Fusetani and co-workers from the Japanese marine tunicate *Ritterella tokioka*[8] and they showed a similar pattern of cytotoxic activity.

The availability of the cephalostatins and ritterazines from their natural sources is extremely limited. For example, only 139 mg of cephalostatin 1 (**1**) could be isolated from 166 kg of 'crude' marine worms. This low accessibility, along with their exceptional cytotoxic activity, has led to interest in the synthesis of these compounds and their analogues as potential antitumor agents. Many different routes for the construction of these pyrazine-based dimeric steroids have been reported and reviewed.[9] The synthesis of new analogues and the evaluation of their cytotoxic activity could provide new insights into the structure–activity relationships and will help to identify the structural fragment(s) responsible for the high biological activity of these heterodimers.

Some time ago we reported the isolation of novel polyoxygenated steroids from the Indonesian gorgonian *Isis hippuris*. The main component of the steroid mixture was the known compound 22-*epi*-hippuristanol (**2**), which has a spiroketal ring in the side chain and showed significant cytotoxic activities against several cultured cell tumor lines.[10] As a continuation of our studies on cytotoxic steroids form marine organisms,[11] and due to the structural similarities between **1** and the monomer unit of the aforementioned pyrazine-based bis-steroids, we report here the synthesis of a new unsymmetrical bis-steroidal pyrazine analogue, compound **4**, from the commercially available hecogenin acetate (**3**) and 22-*epi*-hippuristanol (**2**) (Chart 1).

2. Results and discussion

2.1. Chemistry

In the synthesis of the bis-steroidal pyrazine **4**—a cephalostatin analogue—we employed the symmetrical and non-symmetrical routes developed by Winterfeldt.[12] The natural cytotoxic spiroketal 22-*epi*-hippuristanol (**2**) was first transformed into its enaminoke-

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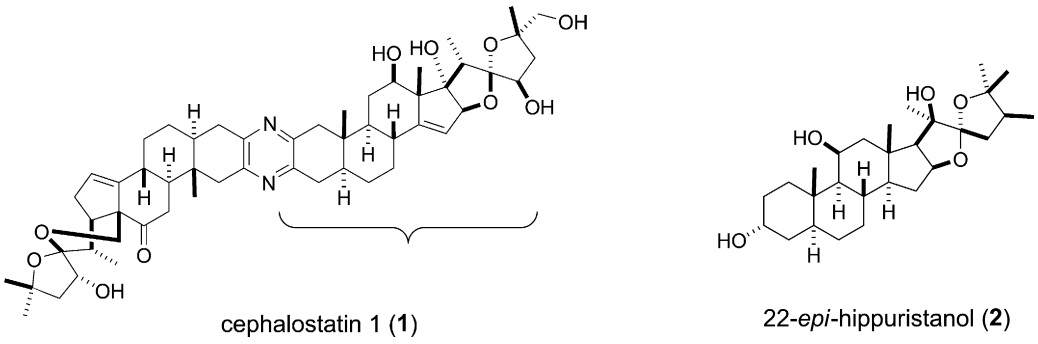


Chart 1.

tone derivative **7** and this was then coupled to enaminoketone **11** (symmetrical route) or the α-hydroxyketone **12** (non-symmetrical route) obtained from the commercially available low cost starting material hecogenin acetate (**3**).

The preparation of enaminoketone **7** began with the oxidation of the natural product **2** with PDC in DMF to give the diketone **5** in almost quantitative yield (96%).[13] The structure of **5** was confirmed from its (+)-HRESIMS and its NMR spectra. Thus, the absence of signals corresponding to H3β (4.32 ppm) and H11α (4.05 ppm) of **2** in the ^{1}H NMR spectrum of **5** and the presence of carbon signals at 211.4 and 210.4 ppm, corresponding to two carbonyl groups, in the ^{13}C NMR spectrum confirmed this transformation. The chemo-, regio- and stereoselective bromination of **5** with PTAP afforded the 2α-bromo-3,11-dione **6** in 68% yield. The bromination of **5** was confirmed by the chemical shift of H2β at 4.78 (1H, dd, J = 13.2 and 6.4 Hz) in the ^{1}H NMR spectrum of **6** along with its (+)-HRESIMS. Finally, treatment of 2α-bromo-3,11-dione **6** with sodium azide in DMF and a catalytic amount of NaI, afforded the enaminoketone **7** in 97% yield, with the structure confirmed by its spectroscopic data (Scheme 1).

The 2α-bromo-3,11-dione **10** was synthesized from hecogenin acetate (**3**) by saponification of the C-3 acetate and subsequent PDC oxidation and bromination. Treatment of the bromo ketone **10** with sodium azide afforded the enaminoketone **11**,[12] whereas treatment with K_2CO_3, acetone and water[14] gave the α-hydroxyketone **12** (Scheme 2).

Reductive dimerization of the enaminoketones **7** and **11** with palladium on charcoal/hydrogen (5%) afforded the C_2-unsymmetrical diketone **4** in 22% yield along with the corresponding autocondensation products, compounds **13**[15] and **14**[16] (Scheme 3). Compounds **4** and **13** were purified by normal phase HPLC and their structural characterization was supported by (+)-HRESIMS and by 1D and 2D NMR experiments. For example, the absence of proton signals corresponding to H1 of the enaminoketones **7** and **11** in the ^{1}H NMR spectrum of **4**, along with the quaternary carbon signals at 148.5 and 148.3 ppm in the ^{13}C NMR spectrum, indicated the formation of the non-symmetrical bis-steroidal pyrazine. Condensation between the α-hydroxyketone **12** and the enaminoketone **7** in the presence of ammonium acetate at elevated temperature using the unsymmetrical route[17] afforded **4** in 16% yield after purification by HPLC (Scheme 3).

2.2. Biological evaluations

The cytotoxic activity of the bis-steroid pyrazine analogues **4** and **13** were evaluated in vitro against MDA-MB-231, A-549 and HT-29 tumor cells. The results, expressed as GI_{50} values in μM, are reported in Table 1 and show an increase in biological activity for **4** in comparison to **2**. Curiously, the other new synthetic derivatives of 22-*epi*-hippuristanol, compounds **5**–**7**, were inactive to these tumor cells.

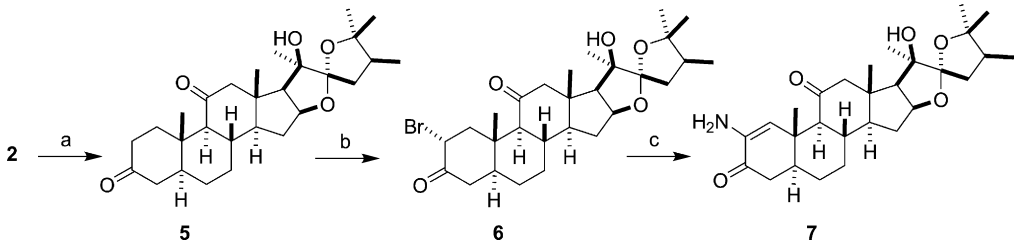
Compound **4** shares a similar structural framework to ritterazines B, F–I, Y and cephalostatin **7**, differing in the presence of an additional methyl group at C24, the absence of $\Delta^{14,15}$ in the left-hand part of the molecule, and the oxidation positions. Although compound **4** shows molecular dissymmetry, which was considered to be a prerequisite for tumor-inhibition, its moderate cytotoxic activity (at μM level) in relation to the former ritterazines (at nM level) indicates that the appropriate oxidation positions are essential for exceptional potency.

3. Conclusions

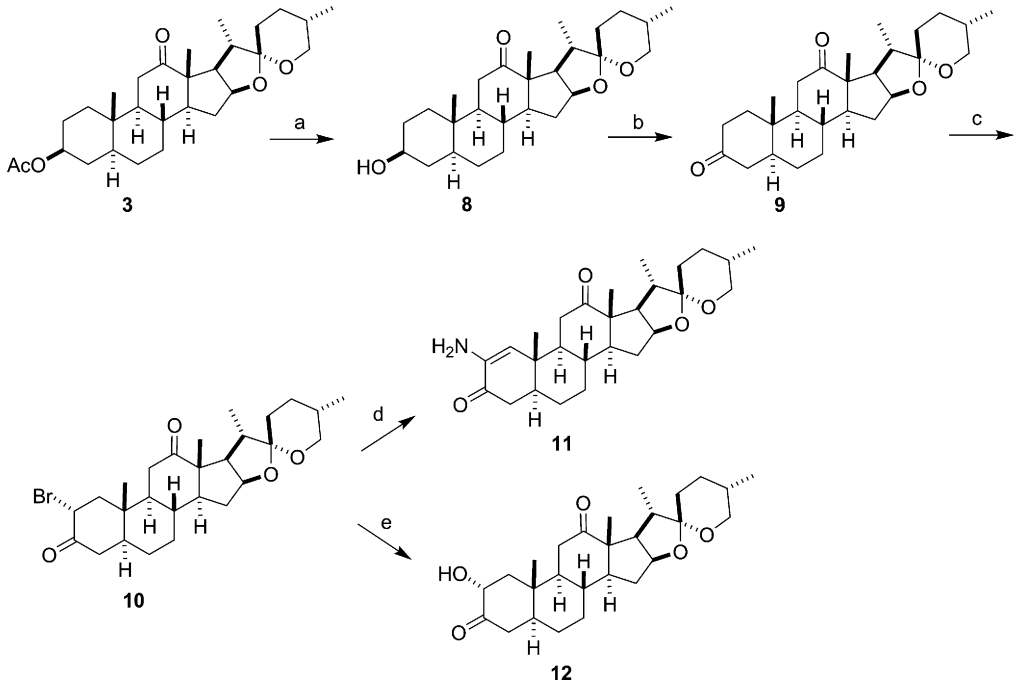
In summary, a new cephalostatin/ritterazine analogue was synthesized from the natural cytotoxic steroid 22-*epi*-hippuristanol and the commercially available hecogenin acetate using the symmetrical and unsymmetrical routes designed by Winterfeldt. Although the new analogue **4** showed a higher cytotoxic activity (at the μM level) than its natural precursor **2**, it was far less cytotoxic than the natural cephalostatin/ritterazine.

4. Experimental

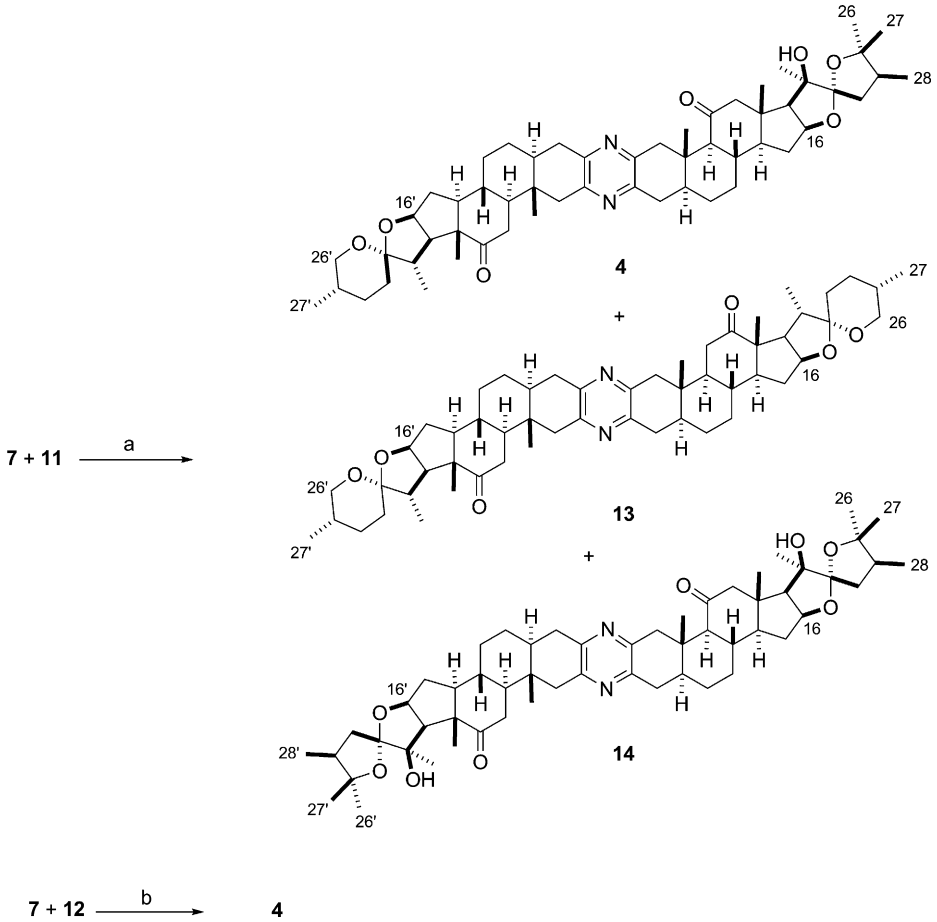
Melting points (mp) were determined on electrothermal digital melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra (proton and carbon) were recorded on Bruker



Scheme 1. Reagents and conditions: (a) PDC, DMF, 96%; (b) PTAP, THF, 0 °C, 68%; (c) NaI, NaN_3, DMF, 50 °C, 97%.



Scheme 2. Reagents and conditions: (a) KOH, MeOH, 99%; (b) PDC, DMF, 99%; (c) PTAP, THF, 0 °C, 63%; (d) NaI, NaN_3, DMF, 50 °C, 92%; (e) K_2CO_3, Ac_2O:H_2O (1:1), 45 °C, 80%.



Scheme 3. Reagents and conditions: (a) H_2, EtOAc, MeOH, HOAc, Pd/C, 22%; (b) NH_4OAc, MeOH, CH_2Cl_2, 50 °C, 16%.

AC200 F, 300 or 500 Advance spectrometers at the University of A Coruña, using $CDCl_3$ and CD_3OD as the solvents and internal standards. Multiplicities of ^{13}C signals were obtained by DEPT. Medium-pressure chromatographic separations were carried out on Silica Gel 60 (230–400 mesh). FT-IR spectra were recorded on a Bruker VECTOR22 spectrophotometer. LREIMS and LRFABMS were

Table 1
The in vitro activities (GI_{50} values in μM) of 22-*epi*-hippuristanol **2**, analogue **4**, and dimer **13**

Compound	MDA-MB-231	A-549	HT-29
2	5.6	3.2	4.5
4	2.0	1.6	1.6
13	2.6	1.7	2.1

recorded on a Thermo MAT-95XP spectrometer, while (+)–(−) HRESIMS were measured on Applied Biosystems QSTAR Elite.

4.1. 22-*epi*-Hippuristan-3,11-dione (5)

22-*epi*-Hippuristanol (**2**) (0.1 g, 0.22 mmol) was treated with PDC (0.2 g, 0.5 mmol) in anhydrous DMF (2 ml) in the presence of activated molecular sieves and stirred at room temperature for 6 h. Insoluble materials were filtered off through a Celite pad and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (silica gel, hexanes/ethyl acetate, 8:2) to afford the 3,11-dione **5** (95 mg, 96%) as a white semi-solid: mp 220 °C, lit. 229.5–232 °C;[18] IR (neat) ν_{max} 3434, 2924, 1701, 1050, 1031, 1009, 970, 923, 874 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.48 (H16α, 1H, m); 1.30 (H18, 3H, s); 1.27 (H27, 3H, s); 1.21 (H21, 3H, s); 1.07 (H19, 3H, s); 0.97 (H26, 3H, s); 0.94 (H28, 3H, d, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 211.4 (C11, s); 210.4 (C3, s); 118.5 (C22, s); 84.3 (C25, s); 81.8 (C20, s); 79.0 (C16, d); 64.0 (C17, d); 63.3 (C9, d); 58.1 (C12, t); 55.7 (C14, d); 46.9 (C5, d); 45.8 (C13, s); 44.3 (C23, t); 40.9 (C24, d); 39.6 (C4, t); 37.9 (C1, t); 37.1 (C2, t); 35.7 (C8, d); 35.2 (C10, s); 32.3 (C7, t); 31.5 (C15, t); 29.0 (C26, q); 28.2 (C6, t); 26.0 (C18, q); 22.9 (C27, q); 17.5 (C21, q); 14.0 (C28, q); 11.0 (C19, q). LREIMS (70 eV, m/z%): 458 (M^+, 4); 315 (100). (+)-LRFABMS m/z (%): 459 ($[M+H]^+$, 37); 154 (100). (+)-HRESIMS: m/z 459.3104 $[M+H]^+$ (calcd for $C_{28}H_{43}O_5$, 459.3105).

4.2. 2α-Bromo-22-*epi*-hippuristan-3,11-dione (6)

A cold (0 °C) solution of trimethyl(phenyl)ammonium perbromide (80 mg, 0.2 mmol) in dry THF (2 ml) was added dropwise over a period of 3 h to a solution of the 3,11-dione **5** (95 mg, 0.21 mmol) in dry THF (2 ml). After a further 45 min, the reaction was quenched by the addition of saturated aqueous sodium hydrogen carbonate (5 ml). The product was extracted with EtOAc and the organic layer was washed with brine and dried over $MgSO_4$. The crude product was subjected to chromatography (silica gel, hexanes/ethyl acetate, 9:1) to give 2α-bromo-3,11-diketone **6** (76 mg, 68%) as a white, crystalline product: mp 115–121 °C; IR (neat) ν_{max} 3494, 2925, 1702, 1025, 970, 923, 871 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.78 (H2β,1H, dd, J = 13.2, 6.4 Hz); 4.48 (H16α, 1H, m); 1.30 (H18, 3H, s); 1.28 (H27, 3H, s); 1.24 (H21, 3H, s); 1.07 (H19, 3H, s); 0.98 (H26, 3H, s); 0.94 (H28, 3H, d, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 211.4 (C11, s); 210.4 (C3, s); 118.5 (C22, s); 84.3 (C25, s); 81.8 (C20, s); 79.0 (C16, d); 64.0 (C17, d); 63.3 (C9, d); 57.1 (C12, t); 56.1 (C2, d); 54.7 (C14, d); 45.7 (C5, d); 45.3 (C13, s); 44.8 (C23, t); 40.6 (C24, d); 39.9 (C4, t); 37.4 (C1, t); 35.8 (C8, d); 35.6 (C10, s); 32.6 (C7, t); 31.8 (C15, t); 28.7 (C26, q); 28.2 (C6, t); 25.9 (C18, q); 22.5 (C27, q); 17.4 (C21, q); 14.2 (C28, q); 11.4 (C19, q). (+)-LRFABMS m/z (%): 539 ($[M+H]^+$, 5) (^{81}Br)/537 ($[M+H]^+$, 2) (^{79}Br); 154 (100). (+)-HRESIMS: m/z 537.2212 $[M+H]^+$ (^{79}Br) (calcd for $C_{28}H_{42}O_5{}^{79}Br$, 537.2210).

4.3. 2-Amino-22-*epi*-hippurist-1-en-3,11-dione (7)

2α-Bromo-3,11-diketone **6** (76 mg, 0.14 mmol) was dissolved in DMF (5 ml) and NaN_3 (0.1 g, 8.0 mmol) and a catalytic amount of NaI was added. The suspension was stirred for 1 h under argon at 50 °C. The reaction was quenched by the addition of water (50 ml) and the mixture was extracted with ether. The combined extracts were washed with brine and dried over $MgSO_4$. The crude product was subjected to chromatography (silica gel, hexanes/ethyl acetate, 7:3) to give enaminoketone **7** (65 mg, 97%) as a white semi-solid. ^1H NMR (300 MHz, $CDCl_3$) δ_H: 6.57 (H1, 1H, br); 4.53 (H16α, 1H, m); 1.36 (H18, 3H, s); 1.31 (H27, 3H, s); 1.26 (H21, 3H, s); 1.12 (H19, 3H, s); 1.04 (H26, 3H, s); 0.99 (H28, 3H, d, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 210.2 (C11, s); 195.3 (C3, s); 137.8 (C2, s); 127.5 (C1, d); 118.6 (C22, s); 84.5 (C25, s); 81.3 (C20, s); 79.1 (C16, d); 63.2 (C17, d); 61.4 (C9, d); 57.1 (C12, t); 54.7 (C14, d); 45.8 (C5, t); 45.2 (C13, s); 44.5 (C23, t); 40.4 (C24, d); 39.5 (C4, t); 35.7 (C8, d); 35.5 (C10, s); 32.1 (C7, t); 31.0 (C15, t); 29.0 (C26, q); 28.2 (C6, t); 25.9 (C18, q); 23.0 (C27, q); 17.7 (C21, q); 14.7 (C28, q); 11.4 (C19, q). LREIMS (70 eV, m/z%): 471 (M^+, 19); 148 (100). (+)-LRFABMS m/z (%): 472 ($[M+H]^+$, 13); 109 (100). (+)-HRESIMS: m/z 472.3063 $[M+H]^+$ (calcd for $C_{27}H_{42}NO_5$, 472.3057).

4.4. Hecogenin-3-one (9)

Hecogenin (1.0 g, 2.3 mmol) was treated with PDC (2.0 g, 5.0 mmol) in anhydrous DMF (20 ml) in a similar way to **2**. The product was purified by column chromatography (silica gel, hexanes/ethyl acetate, 8:2) to give the 3,12-dione **9** (0.99 g, 99%) as a white semi-solid. ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.34 (H16α, 1H, m); 3.49 (H26b, 1H, ddd, J = 10.9, 4.4, 2.0 Hz); 3.34 (H26a, 1H, t, J = 10.9 Hz); 2.54 (H17, 1H, dd, J = 9.0, 8.0 Hz); 2.48 (H11b, 1H, dd, J = 10.5, 13.5 Hz); 2.38 (H8, 1H, br s); 2.43–2.32 (H2, 2H, m); 2.17 (H11a, 1H, dd, J = 10.5, 5.0 Hz); 2.20–1.90 (H4, 2H, m); 1.25 (H18, 3H, s); 1.09 (H21, 3H, d, J = 7.0 Hz); 1.06 (H19, 3H, s); 0.79 (H27, 3H, d, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 212.8 (C12, s); 210.7 (C3, s); 109.2 (C22, s); 79.1 (C16, d); 66.8 (C26, t); 55.5; 55.1 (C13, s); 54.8; 53.5; 46.2; 44.4; 42.2; 37.8; 37.7; 36.2 (C10, s); 34.2; 31.5; 31.4; 31.2; 31.1; 30.2; 28.5; 28.7; 17.1 (C27, q); 16.0 (C21, q); 13.2 (C19, q); 11.2 (C18, q). LREIMS (70 eV, m/z%): 428 (M^+, 33); 139 (100). (+)-LRFABMS m/z (%): 429 ($[M+H]^+$, 57); 133 (100). (+)-HRESIMS: m/z 429.2998 $[M+H]^+$ (calcd for $C_{27}H_{41}O_4$, 429.2999).

4.5. 2α-Bromohecogenin-3-one (10)

A solution of 3,12-dione **9** (6.0 g, 14.0 mmol) in dry THF (20 ml) was reacted with trimethyl(phenyl)ammonium perbromide (5.0 g, 12.5 mmol) in dry THF (20 ml) in a similar way to **5**. The product was purified by column chromatography (silica gel, hexanes/ethyl acetate, 9:1) to give 2α-bromo-3,12-diketone (**10**) (4.5 g, 63%) as a white semi-solid. ^1H NMR (300 MHz, $CDCl_3$) δ_H: 4.70 (H2β, 1H, dd, J = 13.2, 6.4 Hz); 4.32 (H16α, 1H, m); 3.46 (H26b, 1H, m); 3.32 (H26a, 1H, t, J = 11.0 Hz); 2.23 (H11a, 1H, dd, J = 10.5, 5.0 Hz); 2.20–1.90 (H4, 2H, m); 1.22 (H18, 3H, s); 1.05 (H21, 3H, d, J = 6.8 Hz); 1.05 (H19, 3H, s); 0.78 (H27, 3H, d, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 211.8 (C12, s); 200.2 (C3, s); 109.2 (C22, s); 78.9 (C16, d); 66.8 (C26, t); 61.8; 55.2; 54.4; 53.5 (C2, d); 53.4 (C13, s); 50.8; 46.9; 43.5; 42.1; 39.2 (C10, s); 37.6; 33.7; 31.3; 31.0; 30.8; 30.1; 28.7; 27.9; 17.0 (C27, q); 15.9 (C21, q); 13.2 (C19, q); 11.8 (C18, q). LREIMS (70 eV, m/z%): 508 (M^+, 15) (^{81}Br)/506 (M^+, 8) (^{79}Br); 139 (100). (+)-LRFABMS m/z (%): 509 ($[M+H]^+$, 36) (^{81}Br)/507 ($[M+H]^+$, 28) (^{79}Br); 132 (100). (+)-HRESIMS: m/z 507.2114 $[M+H]^+$ (^{79}Br) (calcd for $C_{27}H_{40}O_4{}^{79}Br$, 507.2104).

4.6. 2-Aminohecogenin-1-en-3-one (11)

2α-Bromo-3,12-diketone **10** (4.0 g, 7.9 mmol) was dissolved in DMF (100 ml) and was reacted with NaN_3 (5.2 g, 80 mmol) and a catalytic amount of NaI in a similar way as **6**. The product was puri-

fied by column chromatography (silica gel, hexanes/ethyl acetate, 7:3) to give the enaminoketone **11** (3.2 g, 92%) as a yellow solid which was crystallized from diethyl ether/ethyl acetate: mp 235–240 °C; IR (neat) ν_{max} 3447, 3349, 2929, 1703, 1667, 1633, 1038, 1007, 954, 868 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H: 5.87 (H1, 1H, br s); 4.34 (H16α, 1H, m); 3.47 (H26b, 1H, m); 3.34 (H26a, 1H, t, *J* = 10.8 Hz); 1.25 (H18, 3H, s); 1.08 (H19, 3H, s); 1.07 (H21, 3H, d, *J* = 6.8 Hz); 0.79 (H27, 3H, d, *J* = 6.6 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 211.9 (C12, s); 194.7 (C3, s); 137.7 (C2, s); 123.5 (C1, d); 108.8 (C22, s); 78.6 (C16, d); 66.4 (C26, t); 55.1 (C13, s); 54.8; 53.2; 51.8; 44.1; 41.8; 39.6 (C10, s); 38.0; 37.3; 34.0; 31.0; 30.6; 30.5; 29.7; 28.3; 26.6; 16.6 (C27, q); 15.6 (C21, q); 13.4 (C19, q); 12.8 (C18, q). LREIMS (70 eV, *m*/*z*%): 441 (M^+, 19); 118 (100). (+)-LRFABMS *m*/*z* (%): 442 ($[M+H]^+$, 91); 133 (100). (+)-HRESIMS: *m*/*z* 442.2959 $[M+H]^+$ (calcd for $C_{27}H_{40}NO_4$, 442.2951).

4.7. 2α-Hydroxyhecogenin-3-one (12)

A mixture of 2α-bromo-3,12-diketone **10** (0.20 g, 0.39 mmol), K_2CO_3 (1.0 g, 7.2 mmol), acetone (15 ml), and water (5 ml) was heated at 45 °C for 15 h. The mixture was diluted with water and then partly evaporated in vacuo. The residue was extracted with $CHCl_3$. The extract was dried over $MgSO_4$ and evaporated to give the 2α-hydroxy-3,12-diketone (**8**) (0.14 g, 80%) as a white semi-solid. ^1H NMR (300 MHz, $CDCl_3$) δ_H: 5.80 (OH, 1H, br s); 4.39 (H16α, 1H, m); 4.28 (H2β, 1H, dd, *J* = 12.0, 7.0 Hz); 3.53 (H26b, 1H, m); 3.38 (H26a, 1H, t, *J* = 10.8 Hz); 1.28 (H18, 3H, s); 1.11 (H21, 3H, d, *J* = 6.8 Hz); 1.10 (H19, 3H, s); 0.83 (H27, 3H, d, *J* = 6.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 212.4 (C12, s); 210.2 (C3, s); 109.3 (C22, s); 79.1 (C16, d); 72.5 (C2, d); 66.9 (C26, t); 55.2; 55.1; 54.6; 48.0; 47.7; 42.2; 42.3; 37.9; 37.5; 33.6; 31.4; 31.2; 31.1; 30.2; 29.7; 28.8; 28.2; 17.7 (C27, q); 16.0 (C21, q); 13.3 (C19, q); 12.6 (C18, q). LREIMS (70 eV, *m*/*z*%): 444 (M^+, 23); 139 (100). (+)-LRFABMS *m*/*z* (%): 445 ($[M+H]^+$, 18); 154 (100). (+)-HRESIMS: *m*/*z* 445.2961 $[M+H]^+$ (calcd for $C_{27}H_{41}O_5$, 445.2948).

4.8. Compounds 4 and 13

4.8.1. Method A

A mixture of enaminoketone **7** (12 mg, 0.025 mmol) and enaminoketone **12** (12 mg, 0.025 mmol) was dissolved in EtOAc (3 ml). The solution was mixed with MeOH (0.1 ml) and two drops of acetic acid. The catalyst [65 mg of Pd/C (5%)] was added and the compound was hydrogenated at room temperature overnight. After the reaction was complete the mixture was filtered and the filtrate was washed with saturated aqueous sodium bicarbonate and saturated aqueous sodium chloride, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by HPLC (Scharlau C18, flow rate 1.5 ml/min, EtOAc/Hex, 1:1) to give 5 mg of **4** (retention time 68 min) as a white semi-solid in 22% yield and 3 mg of compound **13**.

4.8.2. Method B

A solution of enaminoketone **7** (10 mg, 0.021 mmol) and NH_4OAc (9 mg) in moist MeOH (5 ml < 0.1% H_2O) was heated under reflux for 30 min. A solution of 2α-hydroxy-3,12-diketone **12** (10 mg, 0.022 mmol) in CH_2Cl_2 (0.6 ml) was added and the resulting suspension was heated under reflux for 3 h. The mixture was quenched with H_2O, extracted with CH_2Cl_2 and the organic layer was washed with brine and dried over $MgSO_4$. The solvent was removed and the residue was purified by HPLC as described above to give 3 mg of **4** as a white semi-solid in 16% yield.

Spectral data for **4**: ^1H NMR (500 MHz, $CDCl_3$) δ_H: 4.48 (H16α, 1H, m); 4.40 (H16′α, 1H, m); 3.54 (H26′b, 1H, m); 3.41 (H26′a, 1H, m); 2.60 (H15′, 2H, m); 2.19 (H15, 2H, m); 1.65 (H25′, 1H, m); 1.30 (H18, H27, 6H, s); 1.11 (H18′, 3H, s); 1.26 (H21, 3H, s); 1.13 (H21′, 3H, d, *J* = 6.5 Hz); 1.12 (H19, 3H, s); 1.07 (H19′, 3H, s); 1.03 (H26, 3H, s); 0.99 (H28, 3H, d, *J* = 6.8 Hz); 0.84 (H27′, 3H, d, *J* = 6.4 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: 213.0 (C12′, s); 204.5 (C11, s); 148.5/148.5/148.3/148.3 (C2, C2′, C3, C3′, s); 117.2 (C22, s); 109.4 (C22′, s); 84.5 (C25, s); 81.9 (C20, s); 79.8 (C16, d); 79.2 (C16′, d); 66.9 (C26′, t); 65.6; 63.6; 57.0; 55.7; 55.0; 54.8; 53.5; 45.3; 45.4; 42.5; 41.6; 41.0; 37.7; 37.1; 36.1; 35.3; 34.2; 33.8; 32.8; 32.2; 31.9; 31.4; 31.2; 30.6; 30.3; 30.1; 29.8; 29.7; 29.6; 29.4; 28.9; 28.1; 27.1; 26.4; 22.7 (C26, q); 19.8 (C21, q); 17.2 (C27′, q); 16.0 (C21′, q); 14.2 (C28, q); 13.1 (C19, q); 13.8 (C18′, q); 11.7 (C19′, q). (+)-LRFABMS *m*/*z* (%): 879 ($[M+H]^+$, 18); 133 (100). HREIMS (70 eV, *m*/*z*%): 878.5771 [M^+] (calcd for $C_{55}H_{78}N_2O_7$, 878.5804). (+)-HRESIMS: *m*/*z* 879.5888 [M+H]+ (calcd for $C_{55}H_{79}N_2O_7$, 879.5882).

Spectral data for **13**: ^1H NMR (500 MHz, $CDCl_3$) δ_H: 4.40 (H16α, 1H, m); 3.54 (H26b, 1H, m); 3.39 (H26a, *J* = 11.0 Hz); 1.29 (H18, 3H, s); 1.10 (H21, 3H, d, *J* = 6.3 Hz); 1.08 (H19, 3H, s); 0.84 (H27, 3H, d, *J* = 6.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: 213.3 (C12, s); 148.4/148.4 (C2, C3, s); 109.2 (C22, s); 78.8 (C16, d); 66.9 (C26, t); 55.4; 54.9; 51.8; 49.7; 45.4; 43.1; 42.8; 37.6; 36.2; 35.3; 34.0; 31.5; 31.2; 30.5; 29.7; 28.8; 28.2; 17.5 (C27, q); 16.2 (C21, q); 13.3 (C18, q); 11.8 (C19, q). LREIMS (70 eV, *m*/*z*%): (+)-848 (M^+, 14); 139 (100). LRFABMS *m*/*z* (%): 849 ($[M+H]^+$, 9); 133 (100). (+)-HRESIMS: *m*/*z* 849.5775 $[M+H]^+$ (calcd for $C_{54}H_{77}N_2O_6$, 849.5776).

4.9. Biological activity

A-549 (ATCC CCL-185), lung carcinoma; HT-29 (ATCC HTB-38), colorectal carcinoma, and MDA-MB-231 (ATCC HTB-26), breast adenocarcinoma cell lines were obtained from the ATCC. Cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml penicillin and streptomycin, at 37 °C and 5% CO_2. Triplicate cultures were incubated for 72 h in the presence or absence of test compounds (at ten concentrations ranging from 40 to 0.01 μg/ml). For quantitative estimation of cytotoxicity, the colorimetric sulforhodamine B (SRB) method was used essentially performed as described previously.[19] Briefly, cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% SRB solution for 30 min at room temperature. Cells were then rinsed several times with 1% acetic acid solution and air-dried. Sulforhodamine B was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Results are expressed as GI_{50}, the concentration that causes 50% inhibition in cell growth after correction for cell count at the start of the experiment (NCI algorithm).

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