Bioorganic & Medicinal Chemistry 22 (2014) 256–268

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

New highly toxic bile acids derived from deoxycholic acid, chenodeoxycholic acid and lithocholic acid

Ferenc Májer [a], Ruchika Sharma [a], Claire Mullins [a], Luke Keogh [a], Sinead Phipps [b], Shane Duggan [b], Dermot Kelleher [b], Stephen Keely [c], Aideen Long [b], Gábor Radics [a], Jun Wang [a], John F. Gilmer [a,*]

[a] School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland
[b] Cell and Molecular Biology, Institute of Molecular Medicine, Trinity Centre for Health Science, St James's Hospital, Dublin 8, Ireland
[c] Molecular Medicine Lab, RCSI, Smurfit Building, Beaumont Hospital, Beaumont, Dublin 9, Ireland

ARTICLE INFO

Article history:
Received 12 July 2013
Revised 27 September 2013
Accepted 16 November 2013
Available online 23 November 2013

Keywords:
Bile acid
Cytotoxicity
Lipophilicity
Deoxycholic acid
Ursodeoxycholic acid

ABSTRACT

We have prepared a new panel of 23 BA derivatives of DCA, chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) in order to study the effect of dual substitution with 3-azido and 24-amidation, features zindividually associated with cytotoxicity in our previous work. The effect of the compounds on cell viability of HT-1080 and Caco-2 was studied using the 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Compounds with high potency towards reduction of cell viability were further studied using flow cytometry in order to understand the mechanism of cell death. Several compounds were identified with low micromolar IC_{50} values for reducing cell viability in the Caco-2 and HT1080 cell lines, making them among the most potent BA apoptotic agents reported to date. There was no evidence of relationship between overall hydrophobicity and cytotoxicity supporting the idea that cell death induction by BAs may be structure–specific. Compounds derived from DCA caused cell death through apoptosis. There was some evidence of selectivity between the two cell lines studied which may be due to differing expression of CD95/FAS. The more toxic compounds increased ROS production in Caco-2 cells, and co-incubation with the antioxidant *N*-acetyl cysteine blunted pro-apoptotic effects. The properties these compounds suggest that there may be specific mechanism(s) mediating BA induced cell death. Compound **8** could be useful for investigating this phenomenon.

1. Introductionz

Bile acids (BAs) are oxidative metabolites of cholesterol whose principal biological function is the solubilisation of enteral nutrients, facilitating their absorption.[1] BAs modulate their own biosynthesis, transport and distribution through interactions with cell surface and nuclear receptors.[2] These interactions have long range influence on metabolism but their evolution was probably a response to BA cytotoxicity. Particularly when in their unconjugated from, BAs can induce cell death in a wide range of mammalian cell types.[3] This phenomenon is relevant to the pathophysiology of hepatobiliary diseases and to BA promoter effects in colorectal and esophageal cancer.[1,4]

BAs cause cell lysis and necrosis–an effect attributed to a non-specific capacity to disrupt cell membranes. However BAs can also induce cell death at sublytic concentrations (a property they share with detergents such Triton-X).[5] Sublytic effects are due in part to ligand independent activation of the extrinsic or death receptor pathway (CD95/Fas).[6] These effects may be mediated inside-out by membrane activation of PLA_2, NAD(P)H oxidase, which causes intracellular ROS increases.[6] DCA causes ligand independent activation of EGFR and MAP kinases in some cell types, opposing the apoptotic effects due to Fas activation.[7] DCA effects on cell viability are therefore complex and sometimes self-attenuating. Increases in ROS can follow mitochondrial damage which can be direct effect (intrinsic pathway) or an amplification of death receptor activation. ER stress is also increasingly being investigated as a mechanism of BA induced cell death.[6]

A primary chemical trigger for these events continues to evade elucidation but they are widely believed to be non-specific in origin and are they are usually attributed to membrane perturbations, consistent with observations about the importance of lipophilicity to BA toxicity. However it is likely that there are specific effects at work too. Katona et al. reported the synthesis of enantiomeric BA pairs of LCA, DCA and CDCA.[8] LCA, DCA and CDCA had the same experimental critical micellar concentration values as their enantiomeric partners (*ent* LCA, *ent* DCA, *ent* CDCA) but the naturally occurring stereoisomers were more cytotoxic.[9] This indicates that at least some of the pro-apoptotic features of BAs are due to stereospecific interactions, most likely with protein receptors. We recently reported on the properties of extended library of BAs

* Corresponding author. Tel.: +353 1 896 2795; fax: +353 1 896 2793.
E-mail address: gilmerjf@tcd.ie (J.F. Gilmer).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.11.029

deliberately enriched with new members of widely differing physicochemical properties.[10] A correlation was observed between cytotoxicity and lipophilicity (r^2 >0.6) in this group, which was particularly strong in homologous subsets (r^2 >0.95). However, the toxicity of LCA, CDCA and DCA was not well predicted by these correlations. The study concluded that lipophilicity is a necessary but not sufficient property for BAs to exhibit cytotoxicity.

Much of the focus on the cellular effects of BAs has been concerned with explaining and preventing their contribution to hepatobiliary diseases. What if the pro-apoptotic properties of BAs could be amplified, producing compounds with useful cytocidal activity? Some of the azide analogs in our previous work exhibited unexpectedly high cytotoxic activity towards esophageal cells. We also identified simple amides of UDCA and DCA that were more toxic than the parent BAs. In the present study we have prepared and studied 23 new BA analogs integrating features that were individually associated with inhibition of cell viability in our previous work (Fig. 1). The effects of the compounds on cell viability was characterised using two cell lines with different intrinsic/extrinsic pathway susceptibility Caco-2 derived from colorectal adenocarcinoma and HT1080 from a lung fibrosarcoma.

2. Synthetic chemistry

A new panel of 23 BA (Fig. 2) derived from DCA, CDCA and LCA was prepared using synthetic approaches that we described previously for incorporating 3-azido and 24-amido functionality.[10,11] Some elaboration of the BA 7- and 12- positions was also explored. In order to obtain the 3β-azido, 7α-acetyloxy derivatives of CDCA (**1**–**3**), first the CDCA was converted to7α-hydroxyl acetate in two steps (Scheme 1). The 3-OH group on **24** was mesylated using methanesulfonyl chloride in DCM in the presence of Et_3N. The SN2 substitution on the mesylate using sodium azide afforded the 3β-azido intermediate, which gave **1** following treatment with aqueous base. The carboxylic acid was activated using N-OH-Su or HOBt monohydrate, treated with either ammonia or cyclopropyl amine to obtain 3β-azido, 7α-acetyloxy-24-amido CDCA derivatives **2**-**3**, respectively. We found the OAc group in **1**–**3** to be highly resistant to hydrolysis under basic conditions. In order to produce **4**–**6**, CDCA methyl ester was selectively acetylated on position 3, followed by mesylation of the 7-OH in the presence of tertiary base. This spontaneously eliminated when the reaction mixture in pyridine was allowed to stand at rt producing the 7,8-ene. Alkene **4** was activated at C24 and amidated to give **5** and the 24-cyclopropylamido compound **6**.

A panel of five amido analogs of DCA was synthesized with 3-β-azido group on the A-ring (**7**–**11**) (Scheme 2). The key intermediate **26** was obtained in three steps form DCA, mesylated on position 3 and azide introduced.[10,11] Using standard coupling procedures, this was reacted with each of five amines to afford 3β-azido, 24-amido (**7**), 24-cyclopropylamido (**8**), 24-benzylamido (**9**), 24-cyclohexylamido (**10**) and 24-propylamido (**11**) analogs.

Preparation of LCA derivatives (**12**–**17**) was carried out using similar approaches to those used to obtain the DCA derivatives (Scheme 3). The alpha azido DCA derivatives **18**, **19**, which are epimers of **7** and **8**, required introduction of the 3-azide with retention of configuration (Scheme 4). This was achieved using a modified Mitsunobu on the selectively protected DCA to produce the β-bromide **28**. Azide substitution and deprotection gave **29** which was amidated using HOBt/EDC and the appropriate amine.

We produced some CDCA analogs with 3α- or 3β-azido orientation without 7-acetoxy protection which had proven resistant to removal in **1**–**3** (Scheme 5). To synthesise the 3-β-azido CDCA, the 3-hydroxyl group of the methyl ester protected CDCA was regioselectively mesylated in cold DCM using stoichiometric amount of methanesulfonyl chloride in the presence of two equiv of triethylamine. The mixture was worked up immediately after completing the addition of the reagent then the mesylate was converted to azide in the next step. The methyl ester was hydrolysed, and compounds **20** and **21** obtained through mixed anhydride (ehtylchloroformate) which was reacted with the appropriate amine.

Synthesis of **23**, required introduction of the azide group at position-3 with retention of configuration (Scheme 6). Mitsunobu conditions on **30** using methanesulfonic acid were used to afford the 3-β-mesylate (**31**). This was reacted with sodium azide yielding. The methyl ester group was hydrolysed to obtain the free carboxylic acid **23**.

3. Effects on viability of Caco-2 and HT1080 cell lines

Caco-2, a cell line derived from a human colorectal epithelial tumour or HT1080 lung fibrosarcoma cells at 70% confluence were treated with the test compounds in serum free media for 1 h and

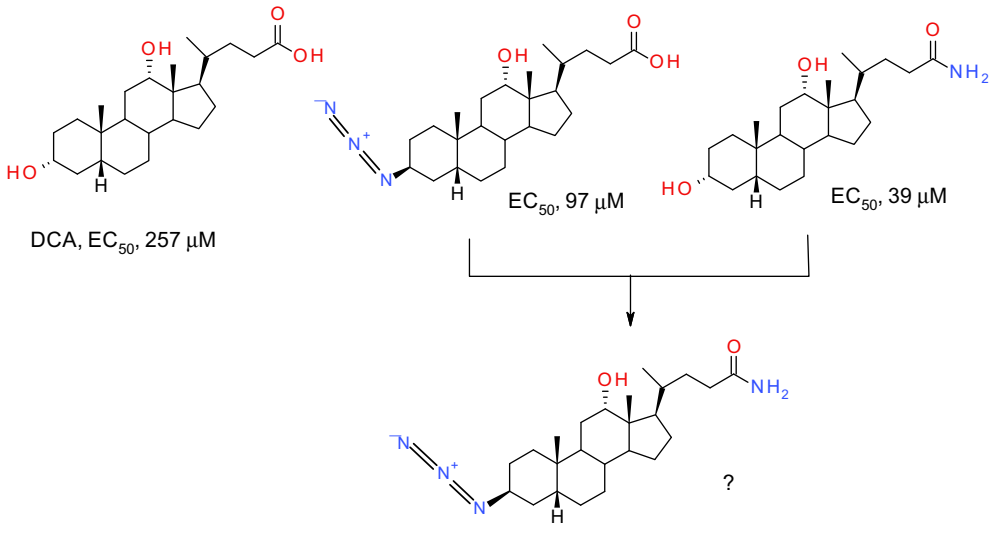


Figure 1. EC_{50} values for the inhibition of HET-1A (normal esophageal) cell viability by DCA and its amido and 3-azido analogues. The present study sought to characterise the effects of incorporating both types of modifications into a single novel BA analogue.

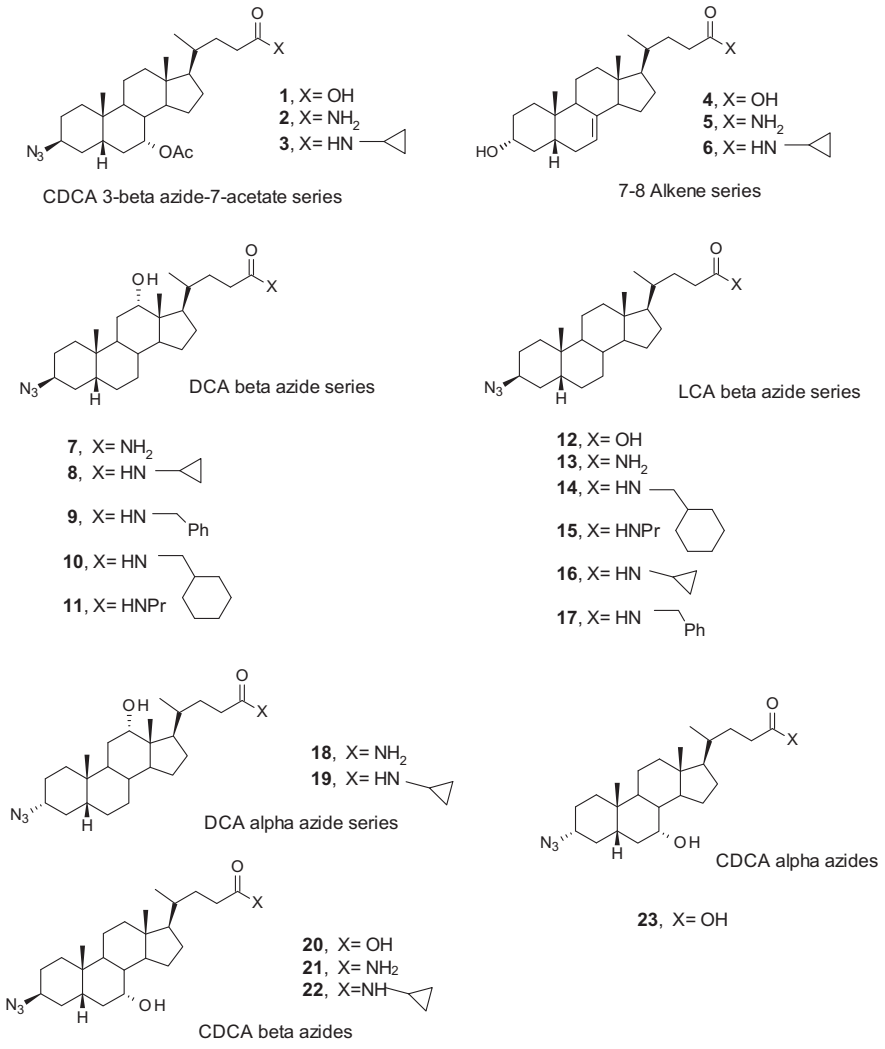
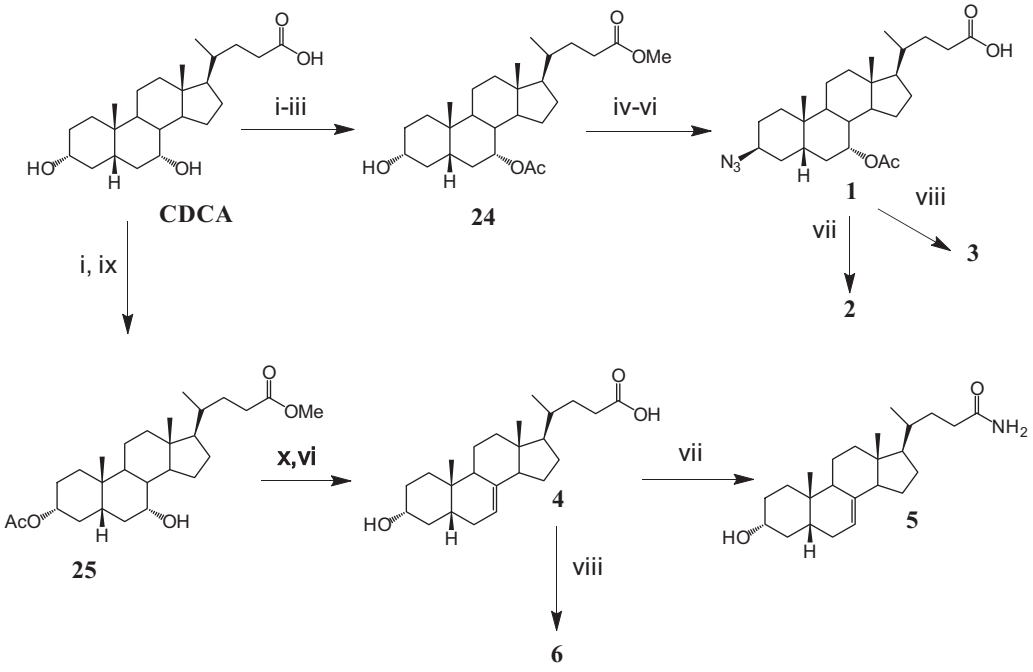


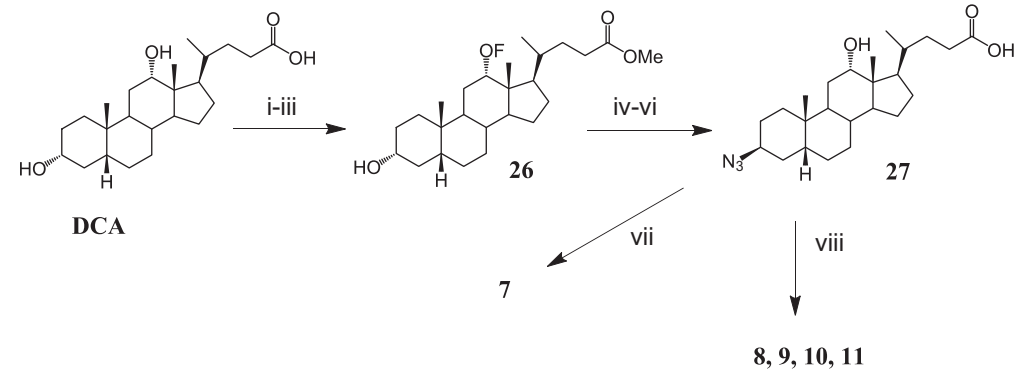
Figure 2. Structural formulae of the BA analogs studied.

24 h. Initial measurements of effects on cell viability were performed at 50 μM using the 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. IC_{50} (CC_{50}) values were subsequently estimated by determining the effect on cell viability over 24 h ($n = 6$) over eight concentration levels (Fig. 3, Table 1). IC_{50} values were also estimated for LCA, DCA and CDCA. BA induced cell death is attributed to necrotic effects at high concentration and to apoptotic events at lower concentration. We used flow cytometry to determine the relative amounts of necrosis and apoptosis induced by selected compounds from the new panel of BA derivatives. Apoptosis was detected by the signature presence of phosphatidylserine (PS) on the cell surface, necrotic cells by the permeability to the ionic propidium iodide (PI).[12] Early apoptotic cells are therefore positive for annexin V which binds PS but not permeable to PI; late apoptotic effects are positive for annexin V and PI permeable. Meanwhile, necrotic cells become permeable to PI without being positive to annexin V. Live/healthy cells are positive for neither annexin V nor PI. The protocol provided an indication of the relative contributions of apoptotic and necrotic effects to the reduced viability observed in the MTT assay.[12] Compounds **7**–**11**, **13**, **14**, **19** were assessed in this way at seven concentration levels along with LCA, DCA and CDCA (Fig. 4).

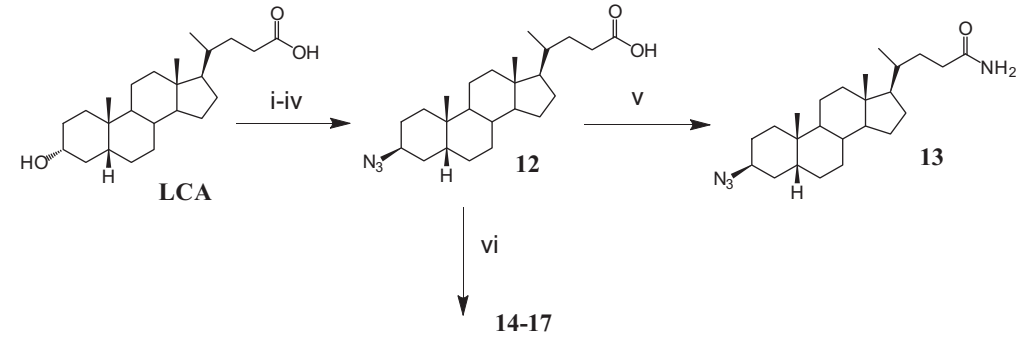
Replacement of the BA C3 α-OH with azide functionality significantly increased cytotoxicity in the case of LCA and CDCA-we have reported similar effects of replacement in the case of DCA.[8] Replacement of the 3-OH group with β-azide in the case of LCA decreased the IC_{50} for inhibition of Caco-2 cell viability from 56 to 6.3 μM; that of CDCA from 106 to 43.7 μM. In all three BA cases, the effect of replacing hydroxyl with azide was significantly amplified by primary amido or cyclpropylamido (CPA) substitution at C24. The most toxic compounds were the 3β- and α-azides of the DCA cyclopropyl amide **8** and **19** (IC_{50} 1.9 μM in HT1080 and **19**, 2.3 μM in Caco-2). These are among the most potently cytotoxic BAs to be reported to date. Results from the two epimeric pairs **8**/**19** and **20**/**22** indicate little difference in toxicity potential between alpha and beta orientations of the 3-azide in the DCA and CDCA series. In all three series (CDCA, LCA and DCA), the most cytotoxic compounds feature a primary amide or CPA. Interestingly, we reported that CPA substitution in UDCA produced a glucocorticoid receptor agonist which inhibits NFkB signalling whereas the corresponding CDCA and DCA analogues were without activity.[13] The *n*-propyl amide of DCA azide (**11**) was toxic but its LCA analogue (**15**) was not. Surprisingly, the more liphophilic but bulkier cyclohexyl and benzyl amides in the DCA and LCA series (**9**, **10**, **14**, **17**) were not toxic. The LCA analogues (**12**–**17**) were less toxic than their DCA analogues (**7**–**11**) which is not consistent with the relative toxicities of the parents. Furthermore, the alkenyl compounds **5** and **6** were not more toxic than their more hydrophilic CDCA analogues (**20**–**21**).



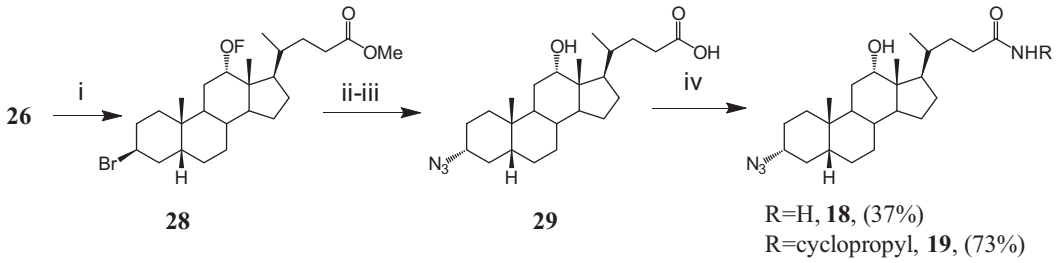
Scheme 1. Synthetic approaches to **1**–**6** from CDCA. Reagents and conditions: (i) MeOH, HCl, reflux, 8 h, 98%; (ii) Ac_2O, DMAP, pyridine, rt, 8 h, 67%; (iii), AcCl, MeOH, 0 °C to rt, 20 h, 91%; (iv) Et_3N, MsCl, DCM, 0 °C, 30 min, 96%; (v) NaN_3, DMPU, 50 °C, 5 d, 85%; (vi) 2 M NaOH, MeOH, reflux, 2 d, 95%; (vii) N-OH-Su, DCC, THF/MeCN, rt, overnight, then aq. NH_3, DMF, rt, 30 min, 93–98%; (viii) HOBt, EDC, DMF, N_2, 0 °C, 10 min, then cyclopropylamine, rt, 24 h, 43–90%; (ix) Ac_2O, pyridine, 16 h, 76%; (x) MsCl, pyridine, DCM, RT, 8 h, 93%.



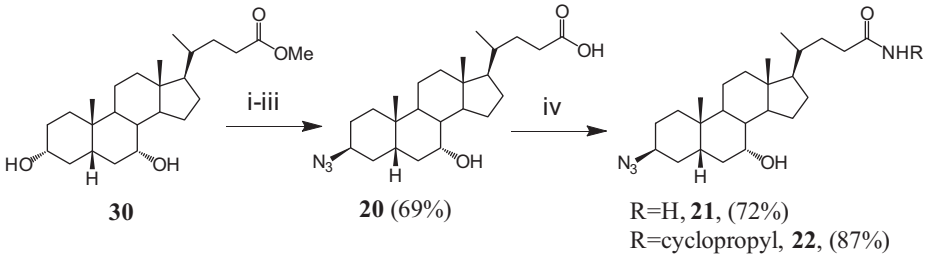
Scheme 2. Synthetic approaches to **7**–**11** from DCA. Reagents and conditions: (i) MeOH, HCl, reflux, 8 h, 98%; (ii) Formic acid, perchloric acid (cat), 60 °C, 20 min, 88%; (iii) $NaHCO_3$, H_2O/MeOH, 0 °C, 3 h, 94%; (iv) Et_3N, MsCl, DCM, 0 °C, 30 min, 92%; (v) NaN_3, DMPU, 50 °C, 5 d, 91%; (vi) 2 M NaOH, MeOH, reflux, 2 d, 95%; (vii) ClCOOEt, Et_3N, DCM, 0 °C, 30 min, rt, 8 h, then NH_3 (2 equiv), 67%; (viii) HOBt, EDC, DMF, N_2, 0 °C, 10 min, then appropriate amine, rt, 24 h, 37–47%.



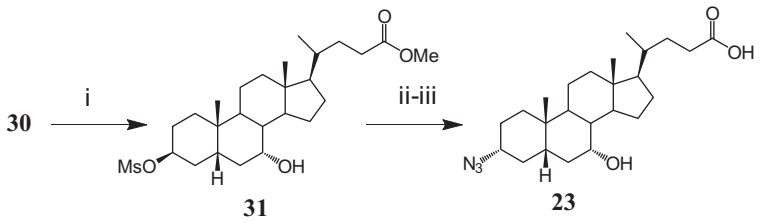
Scheme 3. Synthetic approaches to **12**–**17** from LCA. Reagents and conditions: (i) MeOH, HCl, reflux, 8 h, 98%; (ii) Et_3N, MsCl, DCM, 0 °C, 1.5 h, 80%; (iii) NaN_3, DMPU, 50 °C, 5 d, 96%; (iv) 2 M NaOH, MeOH, reflux, 2 d, 96%; (v) ClCOOEt, Et_3N, DCM, 0 °C, 30 min, rt, 8 h, then NH_3 (2 equiv), 72%; (vi) HOBt, EDC, DMF, N_2, 0 °C, 10 min, then appropriate amine, rt, 24 h, 56–83%.



Scheme 4. Synthetic approaches to DCA 3α-azido amides **18**, **19** from **26**. Reagents and conditions: (i) NBS, PPh_3, THF, −18 °C, >rt over 8 h, 95%; (ii) NaN_3, DMPU, rt, 4 d, 89%; (iv) 2 M NaOH, MeOH, reflux, 8 h, 82%; (iv) HOBt, EDC, DMF, N_2, 0 °C, 10 min, then, cyclopropyl amine, rt, 24 h, 37–73%.



Scheme 5. Synthesis of 3-beta azido derivatives of CDCA **20**, **21**. Reagents and conditions: (i) Et_3N MsCl, DCM, 0 °C, 93%; (ii) NaN_3, DMPU, 65 °C, 6 d, 76%; (iii) NaOH, MeOH, rt, 1 d, 69%; (iv) ClCOOEt, Et_3N in dioxane for 10 min at 0 °C then appropriate amine 8 h at rt, 72–87%.



Scheme 6. Reagents and conditions: Synthesis of **23**: PPh_3, Et_3N, MsOH, DIAD, anh THF, 0 °C, 2 d, 45 °C, 15%; (ii) NaN_3, DMPU, 50 °C, 4 d, 79%, (iii) aq. 2 M NaOH/MeOH (1:1), rt, 24 h, 88%.

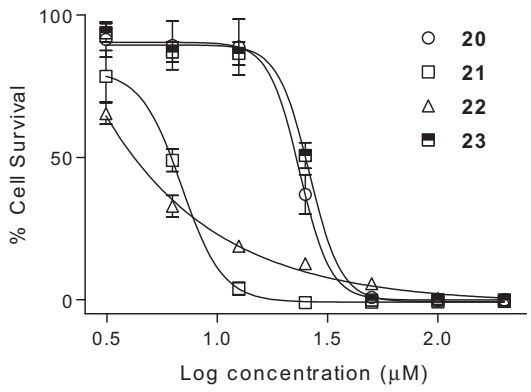


Figure 3. Example MTT concentration response curves for HT1080 cells incubated with **20**–**23** for 24 h. (n = 6 at 8 concentration levels).

Necrosis was not a prominent feature of the cytometric analysis of cell populations treated with high concentrations of DCA or CDCA (up to 500 μM), though there were some signs of necrosis in populations treated with LCA (50–100 μM). Compound **8** induced cell death almost exclusively through apoptosis (Fig. 4). This is significant because apoptosis is anti-inflammatory and cancer chemopreventative whereas necrosis is cancer promoting. Necrosis is less likely to selectively affect malignant populations. By contrast **13**, which is related to LCA caused significant amounts of necrosis. The data, which were collected for a range of the new derivatives, indicated that the cell viability reductions observed previously with the MTT assay were generally due to apoptosis. Figure 4 also shows the analysis of the relative effects of **12** on Caco-2 and HT1080 cell lines consistent with the difference observed using the MTT approach (IC_{50} Caco-2 >250 μM, for HT1080 21.3 μM). In general, the fibrosarcoma cell line (HT1080) was more susceptible to apoptosis induction to the BA analogues than the Caco-2 cell line. The differences were striking for **11**–**13** (e.g., **13**, IC_{50} 13.1 μM HT1080, >500 μM Caco-2).An important difference between the two cell lines that could be relevant to this is that FAS receptor/CD95 is not functionally active in the colorectal cell line (Caco-2), whereas it is in HT1080 cells.[6,14,15] A possible explanation for the greater effects in the colorectal cell line of the LCA-derived compounds (in particular) is that their apoptotic actions in HT1080 cells are mediated through the extrinsic pathway. By implication, the remaining compounds act through other mechanisms such as direct mitochondrial (intrinsic) or ER stress. Furthermore the specific discrepancy in the case of **12** and **13** suggests that these compounds have a different mechanism of action to those where potency across the two cell lines was similar. Compounds capable of inducing cell death specifically through the extrinsic pathway are promising for drug development because tumour cells tend not to become desensitised to death receptor signals.[16] Increased ROS is a prominent BA effect on various cell lines and this

Table 1
IC_{50} values for analogs on cell viability of Caco-2 and HT1080 cells at 24 h or % remaining at the given concentration (μM)

	IC_{50} (95% confidence interval, μM)	
Compound	Caco-2	HT1080
1	15.6 (13.4–17.6)	nd[a]
2	5.5 (5.2–5.8)	nd
3	6.2 (2.1–13.2)	nd
4	>100	nd
5	>100	nd
6	9.5 (4.8–18.9)	nd
7	10.7 (3.5–27)	3.6 (1.6–8.3)
8	6.3 (2.5–15)	1.9 (0.8–4.5)
9	>1 mM	>500
10	>1 mM	>100
11	60% @ 50	6.3 (2.5–16)
12	>250	21.3 (10.5–45)
13	>500	13.1 (12.4–13.8)
14	>1 mM	>1 mM
15	>1 mM	>200
16	>100	>100
17	>100	>100
18	12.6 (11.2–14.2)	10.0 (8.0–12.1)
19	2.3 (0.75–7.3)	5.7 (4.4–7.4)
20	40.3 (28.3–57)	23.6 (20.5–27)
21	9.8 (8.1–11.9)	6.9 (6.2–7.6)
22	7.6 (5.6–10.8)	3.2 (2.3–4.3)
23	43.7 (34–56)	26.2 (24.5–27.8)
DCA	80.3 (49.2–131)	110.0 (70.2–171)
CDCA	106.0 (98.3–122.6)	130.1 (80.7–210)
LCA	56.0 (37.3–84.3)	23.0 (15–35.5)

[a] nd, not determined.

is thought to contribute substantially to the pro-apoptotic effects of the BAs.[17] Therefore we measured ROS at 30 min and at 24 h after exposure to selected BA analogues. There was evidence of increased ROS at 30 min and this was significant at 24 h in the cases of **9**, **11**, **18**, **21** and **22** (Fig. 5A). The effect of the compounds on cell viability was furthermore attenuated when the compounds were incubated in the presence of the anti-oxidant *N*-acetyl cysteine (NAC) (Fig. 5B). The general trend was significant in the case of LCA and **18**. These data indicate that ROS plays a significant role in the apoptotic processes initiated by the new BAs, although it is not clear at which stage.

There have been several previous attempts to design BA-based cytotoxic/anti-microbial agents featuring extensive biochemical characterization of a relatively small number of compounds.[18] The effect of some amino acid and amino acid ester conjugates of UDCA and CDCA was studied using an extensive range of cancer cell types including hepatic, breast cancer, leukaemia, prostate cancer, stomach cancer, cervical and colon cancer cell lines.[19–21] CDCA amino acid conjugates have exhibited an anti-cancer effect on malignant glioblastoma cell lines U-118MG, U-87MG, T98G, and U-373M. Some of these conjugates exhibited promising effects in subsequent in vivo models of malignancy and in murine models of obesity. More recently Kihel et al. studied the proapoptotic effects of piperazinyl analogues of LCA and CDCA. These were reported to cause cell death in a multiple myeloma cell line with the most compound exhibiting LD_{50} in the μM range.[22]

These drug design efforts described above, which target pro-apoptotic pathways, can be distinguished from BA analogues that have been deliberately designed to exhibit increased amphilicity and tendency to self-organization in solution. Such compounds cause cell death mainly through necrosis. Although these have

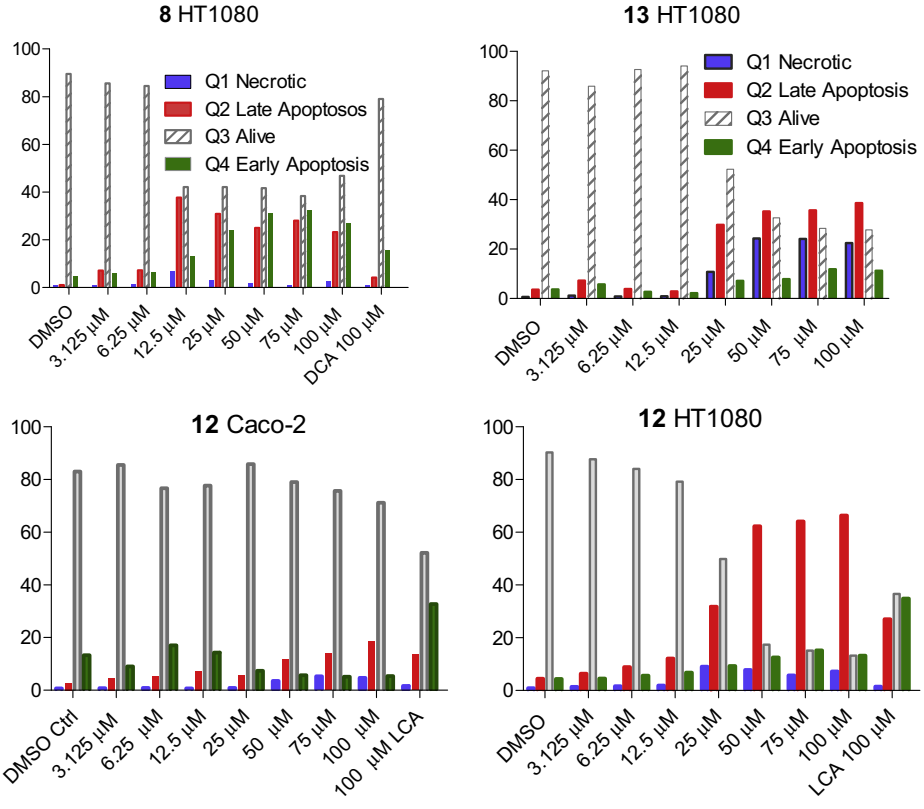


Figure 4. Flow cytometry analysis showing percentage cells (*y* axis) in each of the indicated stages following treatment of HT1080 cells with **8** and **13** for 24 h. The lower graphs show a comparison between the effects of **12** on Caco-2 and HT1080 cells.

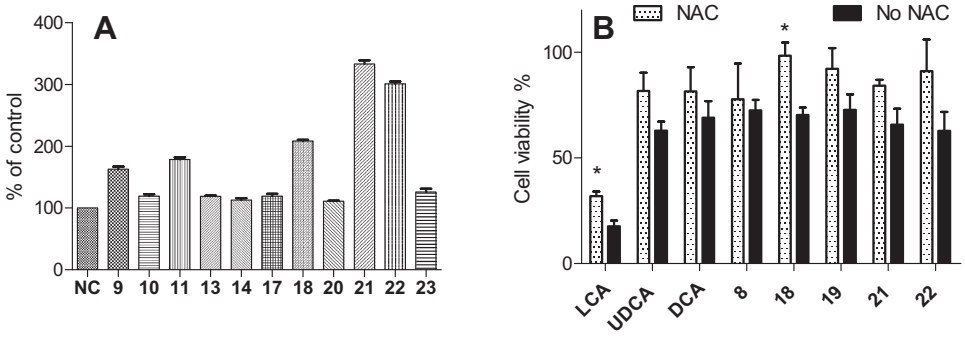


Figure 5. Plots showing **A**: Reactive oxygen species production at 24 h in Caco-2 cells treated with BA analogs. Data are shown relative to vehicle control and corrected for cell viability (MTT data). **B**: The effect of selected compounds on cell viability (MTT) is shown in the presence and absence of *N*-acetyl cysteine (NAC), an anti-oxidant Compounds were tested at 10 μM in both experiments ($n = 3$). Natural BAs were evaluated at 100 μM. (*p <0.05 Anova)

limited scope for therapeutically relevant selectivity or effective distribution in vivo, they can make effective anti-microbial agents. Examples in this more extensive line of research include analogues featuring hydrophobic side chains that act through membrane disruption causing fungal and bacterial cell death. A separate line of research associated with Bellini's lab identified simpler non-amphiphatic BA analogues with interesting antimicrobial effects.[23,24] 3-Amino and 24-amido DCA analogues caused pronounced effects on gram negative and positive bacteria as well as fungi. The molecular mechanism of action of these compounds is unclear; however they appear to be designed to act through receptor mediated rather than membrane dependent effects.

In summary, by integrating azido and amido functionality into DCA, we have produced interesting new BA analogues capable of inducing apoptosis in cancer cells exposed in the low micromolar range. It will be interesting to explore the SAR of the amido group further, since our initial work suggests quite tight structural requirements. The relatively high potency of **8** in this context suggests that its interactions are specific, and although its binding partner is unidentified, it would appear to be an interesting ligand for studies into the mechanism of BA cytotoxicity.

4. Experimental

4.1. General synthetic methods

All chemicals were purchased from Sigma–Aldrich (Dublin, Ireland), except where stated. All the reactions were monitored using TLC. Uncorrected melting points were measured on a Stuart Apparatus. Infra-red (IR) spectra were performed on a Perkin Elmer FT–IR Paragon 1000 spectrometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 27 °C on a Bruker DPX 400 spectrometer (400.13 MHz, ^{1}H; 100.61 MHz, ^{13}C). Coupling constants are reported in Hertz. For ^{1}H NMR assignments, chemical shifts are reported: shift value (number of protons, description of absorption, coupling constant(s) where applicable). Electrospray ionization mass spectrometry (ESI–MS) was performed in the positive ion mode on a liquid chromatography time-of-flight mass spectrometer (Micromass LCT, Waters Ltd, Manchester, UK). The samples were introduced into the ion source by an LC system (Waters Alliance 2795, Waters Corporation, USA) in MeCN:water (60:40% v/v) at 200 μl/min. The capillary voltage of the mass spectrometer was at 3 kV. The sample cone (de-clustering) voltage was set at 40 V. For exact mass determination, the instrument was externally calibrated for the mass range 100 –1000 m/z. A lock (reference) mass (m/z 556.2771) was used. Mass measurement accuracies of <±5 ppm were obtained. Compound purity/homogeneity was confirmed using a combination of NMR, TLC and HPLC.

4.1.1. (4*R*)-Methyl 4-((3*R*,5*S*,7*R*,10*S*,13*R*,17*R*)-7-acetoxy-3-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoate (24-methyl, 3α-hydroxy, 7α-acetoxy-5β-cholanoate) (24)

To a solution of CDCA (5 g, 12.7 mmol) dissolved in MeOH (94 ml), was added dropwise concentrated HCl (37%, 0.53 ml, 6.35 mmol). The mixture was stirred under reflux for 8 h. The solvent was removed under reduced pressure giving the methyl ester (**30**) as a white solid (5.07 g, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.88 (m, 1H, 7β-H), 3.67 (s, 3H, –OCH_3), 3.5 (m, 1H, 3β-H,), 0.96 (d, 3H, 21-CH_3), 0.92 (s, 3H, 19-CH_3), 0.67 (s, 3H 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.36 (C=O, 24-C), 71.76 (CH, 3-C), 68.26 (CH, 7-C), 51.09 (CH_3, OCH_3). IR_{vmax} (KBr): 3424.28, 2932.36, 2866.53, 1741.25, 1447.62, 1375.42 cm^{-1}. HRMS: Found: $(M\text{-}Na)^+$ = 429.2987, calculated for $C_{25}H_{42}O_4Na$ = 429.2981. CDCA methyl ester (2 g, 4.92 mmol) and 4-DMAP (120 mg, 0.98 mmol) were dissolved in anhydrous pyridine (30 ml). Ac_2O (14 ml, 148 mmol) was added and the reaction mixture allowed to stir for 8 h at rt. The mixture was poured into water (200 ml) and the compound extracted with EtOAc:hexane (1:1) (3 × 100 ml). The organic phases were combined and washed with 1 M HCl (100 ml), water (100 ml) and brine (100 ml). The organic layer was dried over $MgSO_4$ and filtered. The solvent was removed and the semi-solid residue was purified by flash column chromatography using hexane: EtOAc (3:1) as mobile phase. White foam (67%, 1.617 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.12 (m, 1H, 7β-H), 4.83 (sex, 1H, J_1 = 4.02 Hz, J_2 = 7.02 Hz, J_3 = 4.52 Hz, 3β-H), 3.91 (s, 3H, –O-CH_3), 2.48 (s, 3H, 3 –C=OCH_3), 2.43 (s, 3H, 7 –C=OCH_3), 1.31 (s, 3H, 19-CH_3), 1.28 (d, 3H, J = 6.53 Hz, 21-CH_3) 0.89 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): 173.54 (C=O, 24-C), 169.49 and 169.28 (2C=O, 3-*C*=OCH_3 and 7-*C*=OCH_3), 73.08 and 70.15 (2CH, 3-C and 7-C), 51.08 (CH_3, OCH_3), 21.17 (CH_3, 7-OC=OCH_3), 21.06 (CH_3, 3-OC=OCH_3). IR_{vmax} (KBr): 2942.84, 2872.20, 2848.09, 1734.83, 1466.86, 1438.18, 1378.44, 1364.49, 1248.94 cm^{-1}. HRMS: Found: $(M\text{-}Na)^+$ = 513.3208, calculated for $C_{29}H_{46}O_6Na$ = 513.3192. The CDCA diacetate methyl ester (1.55 g, 3.17 mmol) was dissolved in anhydrous MeOH (22 ml) then AcCl (0.29 ml, 4.078 mmol), dissolved in MeOH (3 ml) was added dropwise to the reaction mixture at 0 °C. The mixture was allowed to reach rt and left stirring for 20 h when it was quenched by the addition of saturated $NaHCO_3$ (70 ml) and water. The compound was extracted using EtOAc (3 × 50 ml) the organic phase was dried over $MgSO_4$. The organic solvent was removed under reduced pressure and the product formed as a white solid (1.294 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.86 (m, 1H, 7β-H), 4.12 (m, 1H, 3β-H), 3.65 (s, 3H, –O-CH_3), 1.97 (s, 3H, 7 –C=OCH_3), 0.91 (s, 3H, 19-CH_3), 0.9 (d, 3H, 21-CH_3) 0.63 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.30 (C=O, 24-C), 170.23 (C=O, 7-*C*=OCH_3),

71.32 and 70.91 (2CH, 3-C and 7-C), 51.08 (CH_3, OCH_3), 21.21 (CH_3, 7-OC=OCH_3). HRMS: Found: $(M\text{-}Na)^+$ = 471.3084, calculated for $C_{27}H_{44}O_5Na$ = 471.3086.

4.1.2. (4*R*)-4-((3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-7-Acetoxy-3-azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoic acid (3β-azido, 7α-acetoxy-5β-cholanoate) (1)

To a stirred solution of the **24** (1.273 g, 2.84 mmol) in anhydrous DCM (38 ml) was added Et_3N (0.43 ml, 3.105 mmol). Methanesulfonyl chloride (0.331 ml, 4.276 mmol) dissolved in anhydrous DCM (12 ml) was added dropwise at 0 °C. The mixture was stirred for 30 min when it was quenched using cooled water (50 ml). The compound was extracted using DCM (2 × 40 ml). The organic phase was washed with brine (100 ml), dried over $MgSO_4$ and filtered. The solvent was removed using the rotary evaporator, ensuring the bath temperature was no higher than 40 °C. The flask was placed under high pressure, giving semi-solid oil as the product (1.43 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.90 (m, 1H, 7β-H), 4.52 (m, 1H, 3β-H), 3.68(s, 3H, –O-CH_3), 3.02(s, 3H, –OSO_2CH_3), 2.08 (s, 3H, 7-C=OCH_3), 0.96 (s, 3H, 19-CH_3), 0.94 (d, 3H, 21-CH_3), 0.67 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.69 (C=O, 24-C), 170.43 (C=O, 7-O*C*=OCH_3), 81.39 (CH, 3-C), 71.01 (CH, 7-C), 52.57 (CH_3, –OSO_2-*C*H_3), 51.52 (CH_3, O*C*H_3), 21.59 (CH_3, 7-C=O*C*H_3). HRMS: Found: $(M\text{-}Na)^+$ = 549.2874, calculated for $C_{28}H_{46}O_7SNa$ = 549.2862. To the mesylate (1.791 g, 3.4 mmol) in DMPU, was added NaN_3 (2.21 g, 34 mmol) at 50 °C. The reaction mixture was left stirring for 5 d and then poured into distilled water (100 ml). EtOAc (3 × 100 ml) was used to extract the compound. The organic phase was washed with brine (100 ml), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure, the residue purified by flash column chromatography, using 25% EtOAc in hexane as mobile phase. The product azide was isolated as a white foam (1.369 g, 85%). The 3β-azido CDCA methyl ester (0.78 g, 1.647 mmol) was dissolved in MeOH (10 ml) and 1 M NaOH (2 ml) was added dropwise to the solution, until pH ~ 14 was attained. The mixture was stirred at 65 °C. The reaction was monitored using TLC analysis and once complete, the mixture was poured into 2 M HCl (50 ml) and extracted using EtOAc (3 × 50 ml). The organic phase was washed with water (2 × 100 ml) and brine (100 ml), dried over Na_2SO_4, filtered and the solvent removed under the high vacuum to afford the product as white foam (0.719 g, 95%). White foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.98 (m, 1H, 7β-H), 4.55 (m, 1H, 3β-H), 3.67 (s, 3H, –O-CH_3), 2.04 (s, 3H, 7-C=OCH_3), 0.93 (s, 3H, 19-CH_3), 0.92 (d, 3H, 21-CH_3), 0.65 (s, 3H, 18-CH_3). IR_{vmax} (KBr): 3329.05, 2927.43, 2101.15, 1732.77 and 1247.18 cm^{-1}. HRMS: Found: $[MH]^-$ = 458.3015, calculated for $C_{26}H_{40}N_3O_4$ = 458.3019.

4.1.3. (3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-17-((*R*)-5-Amino-5-oxopentan-2-yl)-3-azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-7-yl acetate (3β-azido, 7α-acetoxy-5β-cholan-24-amide) (2)

Compound **1** (0.15 g, 0.326 mmol) was dissolved in anhydrous THF (15 ml) and acetonitrile (1.5 ml), then N-OH-Su (0.124 g, 1.07 mmol) and DCC (0.22 g, 1.06 mmol) were added to the mixture and the reaction was left stirring overnight. The white solid formed was filtered off and the filtrate was concentrated using the rotary evaporator and placed under high vacuum. The compound was then dissolved in DMF and ammonia solution (37%, 0.054 ml) was added. The white solid precipitated from the mixture when brine (70 ml) was added. The mixture was filtered using suction filtration. The filtrate was washed with water (2 × 30 ml) and allowed to dry in the air. Column chromatography was carried out using 50–100% EtOAc in hexane as the mobile phase. The organic solvent was removed under reduced pressure leaving behind 0.147 g of white foam (98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.90 (m, 1H, 7β-H), 3.92 (m, 1H, 3β-H), 2.05 (s, 3H, 7-C=OCH_3), 0.97 (s, 3H, 19-CH_3), 0.93 (d, 3H, 21-CH_3), 0.66 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.67, 18.21, 20.80, 21.51, 23.04, 23.48, 27.95, 30.45, 30.64, 30.89, 32.44, 33.68, 35.68, 35.12, 36.38, 37.82, 39.46, 42.69, 50.38, 55.65, 58.50, 65.81, 71.47, 170.39, 180.28. IR_{vmax} (KBr): 3329.05, 2927.43, 2101.15, 1732.77 and 1247.18 cm^{-1}. HRMS: Found: $[M\text{-}H]^-$ = 458.3015, calculated for $C_{26}H_{40}N_3O_4$ = 458.3019.

4.1.4. (3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-3-Azido-17-((*R*)-5-(cyclopropylamino)-5-oxopentan-2-yl)-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-7-yl acetate (*N*-cyclopropyl 3β-azido, 7α-acetoxy-5β-cholan-24-amide) (3)

To a solution of **1** (0.1 g, 0.217 mmol) in anhydrous DMF (10 ml) was added HOBt monohydrate (0.044 g, 0.325 mmol) and EDC (0.047 ml, 0.26 mmol) at 0 °C under N_2 atmosphere. After 10 min, a solution of cyclopropylamine (0.023 ml, 0.322 mmol) dissolved in anhydrous DMF (0.5 ml) was added slowly to the mixture and left stirring for 24 h. Once complete, the reaction mixture was poured into brine (70 ml) and extracted using EtOAc (3 × 50 ml). The organic phase was dried over Na_2SO_4, filtered and the solvent was removed using the rotary evaporator. The crude product was purified by flash column chromatography using gradient elution (25-100% EtOAc in hexane). The solvent was removed under reduced pressure giving white foam (0.047 g, 43%) as product. ^{1}H NMR δ ($CDCl_3$): 5.64 (s, 1H, N-H), 4.89 (m, 1H, 7β-H), 3.92 (m, 1H, 3α-H), 2.04 (s, 3H, 7-OC=OCH_3), 0.95 (s, 3H, 19-CH_3), 0.92 (d, 3H, 21-CH_3), 0.64 (s, 3H, 18-CH_3), 0.68 (m, 2H, cyclopropyl-CH_2). ^{13}C NMR ppm ($CDCl_3$): 6.86, 11.91, 18.58, 21.03, 21.80, 22.82, 23.29, 23.73, 24.88, 28.30, 30.67, 31.15, 31.89, 32.66, 33.59, 33.90, 35.35, 35.65, 35.89, 36.61, 38.03, 39.69, 42.91, 50.61, 55.97, 58.74, 71.65, 170.55, 175.17. IR_{vmax} (KBr): 3429.95, 2938.13, 2100.42, 1734.02, 1651.02 and 1248.42 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 521.3463, calculated for $C_{29}H_{46}N_4O_3Na$ = 521.3468.

4.1.5. (4*R*)-Methyl 4-((3*R*,5*R*,7*R*,10*S*,13*R*,17*R*)-3-acetoxy-7-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoate (24-methyl, 3α-acetoxy, 7α-hydroxy-5β-cholanoate) (25)

To a solution of compound **30** (2 g, 4.9 mmol) in DCM (30 ml), was added Ac_2O (3.25 ml, 34.3 mmol) and pyridine (5.97 ml, 73.8 mmol). After 16 h at rt, TLC analysis showed the formation of several acetate products with consumption of starting material. EtOAc (100 ml) was added to the mixture and washed with 1 M HCl (100 ml) and water (2 × 100 ml). The organic layer was collected, dried over $MgSO_4$ and filtered. The crude product was purified by flash column chromatography (hexane: EtOAc, 3:1) and the solvent was removed under high vacuum to produce a white solid (1.668 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.82 (m, 1H, 3β-H), 4.10 (1H, 7β-H), 3.91 (s, 3H, –O-CH_3), 2.26 (3H, 3-C=O-CH_3), 1.18 (d, 3H, 21-CH_3), 1.16 (s, 3H, 19-CH_3), 0.91 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.33 (C=O, 24-C), 170.38 (C=O, 3-C=OCH_3) 74.28 (CH, 7-C), 68.39 (CH, 3-C), 51.44 (CH_3, O*C*H_3). IR_{vmax} (KBr): 3531.43, 2942.98, 2867.01, 1737.24, 1440.12, 1379.84, 1364.52, 1250.45 cm^{-1}. HRMS: Found: $(M\text{-}Na)^+$ = 471.3086, calculated for $C_{27}H_{44}O_5Na$ = 471.3086.

4.1.6. (4*R*)-4-((3*R*,5*R*,10*S*,13*R*,17*R*)-3-Hydroxy-10,13-dimethyl-2,3,4,5,6,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoic acid (3α-hydroxy, 7-ene-5β-cholanoate) (4)

Compound **25** (0.5 g, 1.114 mmol) was dissolved in anhydrous DCM (15 ml) at 0–4 °C then anhydrous pyridine (0.448 ml, 5.56 mmol) and methanesulfonyl chloride (0.434 ml, 4.4 mmol) were added to the mixture and stirred for 15 min. The mixture

was allowed to reach rt and held at that for 8 h. 0.1 M aq. HCl (80 ml) was added to the mixture and the alkene product was extracted using DCM (2 × 60 ml). The DCM layer was washed with brine (160 ml), dried using $MgSO_4$ and filtered. The solvent was removed with the rotary evaporator and the product 7-alkene-3-acetate methyl ester isolated by flash chromatography (0.450 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.12 (m, 1H, 7-H), 4.75 (m, 1H, 3β-H), 3.68 (s, 3H, –O-CH_3), 2.04 (s, 3H, 3-C=OCH_3), 0.96 (s, 3H, 19-CH_3), 0.94 (d, 3H, 21-CH_3), 0.71 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.31 (C=O, 24-C), 170.22 (C=O, 3-OC=OCH_3), 136.74 (quaternary C, 8-C), 114.92 (CH, 7-C), 73.23 (CH, 3-C), 51.085 (CH_3, –OCH_3), 21.02 (CH_3, 3-C=OCH_3). IR_{vmax} (KBr): 2952.47, 2871.89, 1730.01, 1451.58, 1437.29, 1379.29, 1362.42, 1326.88, 1311.19 cm^{-1}. HRMS: Found: $(M\text{-}Na)^+$ = 453.2959, calculated for $C_{27}H_{42}O_4Na$ = 453.2981. To a solution of the alkene methyl ester (0.7 g, 1.63 mmol) in MeOH (40 ml) was added 2 M NaOH (2 ml) dropwise to pH ~ 14. The mixture was stirred at 60 °C for 1 d then it was poured into 2 M HCl (50 ml) and extracted with EtOAc (3 × 50 ml). The organic phase was washed with water (2 × 100 ml) and brine (100 ml), dried over Na_2SO_4, filtered and solvent removed in vacuo, leaving a white solid (0.43 g, 71%). ^{1}H NMR δ (CD_3OD): 5.14 (m, 1H, 7-H), 3.54 (m, 1H, 3β-H), 3.31 (s, 3H, –O-CH_3), 0.98 (d, 3H, 21-CH_3), 0.88 (s, 3H, 19-CH_3), 0.59 (s, 3H, 18-CH_3). ^{13}C NMR ppm (CD_3OD): 12.49, 19.08, 22.90, 24.10, 25.24, 29.10, 29.92, 32.18, 32.40, 34.77, 35.92, 37.25, 38.17, 38.43, 41.34, 42.46, 45.02, 56.45, 57.36, 71.98, 116.78, 138.82, 178.31. IR_{vmax} (KBr): 3342.10, 2934.10, 2859.74, 1708.32, 1541.26, 1468.23, 1447.14, 1416.48, 1367.02, 1333.67, 1286.98 cm^{-1}. HRMS: Found: $[M]^-$ = 373.2757, calculated for $C_{24}H_{37}O_3$ = 373.2743.

4.1.7. (4*R*)-4-((3*R*,5*R*,10*S*,13*R*,17*R*)-3-Hydroxy-10,13-dimethyl-2,3,4,5,6,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (3α-hydroxy, 7-ene-5β-cholan-24-amide) (5)

To **4** (0.15 g, 0.4 mmol) in anhydrous THF (15 ml) and acetonitrile (1.5 ml) was added DCC (0.248 g, 1.2 mmol) and N-OH-Su (0.138 g, 1.19 mmol). The mixture was left stirring overnight. The solid was filtered off and the filtrate was concentrated. The product was dissolved in DMF (5 ml) and ammonia solution (37%, 0.044 ml) was added. The reaction mixture was stirred at 50 °C and left overnight, then the mixture was poured into brine (70 ml) and the precipitate collected by suction filtration. The solid collected on the filter paper was washed with distilled water (2 × 30 ml) and the solid (0.14 g, 93%) was allowed to dry under reduced pressure as a foam. ^{1}H NMR δ (CD_3OD): 5.14 (m, 1H, 7-H), 3.45 (m, 1H, 3β-H), 1.00 (d, 3H, 21-CH_3), 0.88 (s, 3H, 19-CH_3), 0.59 (s, 3H, 18-CH_3). ^{13}C NMR ppm (CD_3OD): 12.48, 19.15, 22.90, 24.10, 25.23, 29.13, 29.92, 32.19, 33.29, 33.66, 34.77, 35.92, 37.42, 38.19, 38.44, 41.36, 42.47, 45.03, 56.47, 57.35, 71.98, 116.78, 138.83, 179.95. IR_{vmax} (KBr): 3327.85, 2929.36, 1626.36 and 1576.41 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 396.2874, calculated for $C_{24}H_{39}NO_2Na$ = 396.2878.

4.1.8. (4*R*)-*N*-Cyclopropyl-4-((3*R*,5*R*,10*S*,13*R*,17*R*)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl) pentanamide (*N*-cyclopropyl 3α-hydroxy, 7-ene-5β-cholan-24-amide) (6)

Compound **6** was produced from **4** using the HOBt/EDC procedure described in 4.1.4 to give a white foam (0.053 g, 90%). ^{1}H NMR δ ($CDCl_3$): 5.66 (d, 1H, N-H), 5.10 (m, 1H, 7-H), 3.62 (1H, 3β-H), 0.92 (d, 3H, 21-CH_3), 0.85 (s, 3H, 19-CH_3), 0.76 (m, 2H, cyclopropyl-CH_2), 0.53 (s, 3H, 18-CH_3), 0.48 (2H, cyclopropyl-CH_2). ^{13}C NMR ppm ($CDCl_3$): 6.83, 12.09, 18.75, 21.83, 22.77, 23.03, 24.69, 28.18, 28.84, 31.58, 31.86, 33.64, 33.69, 34.86, 36.07, 36.86, 37.80, 40.07, 41.06, 43.92, 45.41, 55.23, 55.97, 71.44, 115.62, 137.43, 175.18. ^{13}C NMR ppm ($CDCl_3$): 175.41 (C=O, C=ONH_2), 137.67 (quaternary C, 8-C), 115.86 (CH, 7-C), 71.68 (CH, 3-C), 7.06 (CH_2, cyclopropyl-*C*H_2). IR_{vmax} (KBr): 3444.44, 2931.55 and 1663.29 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 436.3178, calculated for $C_{27}H_{43}NO_2Na$ = 436.3191.

4.1.9. (4*R*)-4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (3β-azido, 12α-hydroxy-5β-cholan-24-amide) (7)

3β-Azido DCA (**27**) (0.150 g, 0.36 mmol) was dissolved in anhydrous DCM (13 ml) and Et_3N (3 equiv, 1.1 mmol) was added at 0 °C, followed by the slow addition of ethyl chloroformate (1.2 equiv, 0.43 mmol). After 30 min at 0 °C, aqueous ammonia (35%, 1.2 equiv, 0.43 mmol) was added. The reaction was let warm up to rt for 8 h. The mixture was poured onto 1 M HCl and extracted with EtOAc (3 × 100 ml). The organic phase was collected and washed with water (100 ml) and brine (100 ml). The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to form a white foam (0.101 g, 67%). ^{1}H NMR δ (CD_3OD): 3.97 (s, 2H, 12β-H and 3α-H), 1.03 (d, 3H, J = 6.04 Hz, 21-CH_3), 0.96 (s, 3H, 19-CH_3), 0.71 (s, 3H, 18-CH_3). ^{13}C NMR ppm (CD_3OD): 13.33, 17.78, 24.22, 24.99, 25.59, 27.38, 27.82, 28.81, 30.13, 31.38, 31.92, 33.37, 33.70, 34.43, 35.83, 37.07, 37.37, 39.04, 47.74, 48.27, 49.50, 60.37 (CH, 3-C), 74.19 (CH, 12-C), 180.05 (C=O, 24-C). IR_{vmax} (KBr): 3400.40, 2937.50, 2102.52 and 1666.29 cm^{-1}. HRMS: Found: $(M+Na)^+$ = 439.3027. HRMS: Found: $[M+Na]^+$ = 439.3027, calculated for $C_{24}H_{40}N_4O_2Na$ = 439.3049.

4.1.10. (4*R*)-4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclopropylpentanamide (*N*-cyclopropyl 3β-azido, 12α-hydroxy-5β-cholan-24-amide) (8)

To a stirred solution of **27** (0.15 g, 0.359 mmol) in DMF (10 ml) was added HOBt monohydrate (0.243 g, 1.796 mmol) and EDC (0.32 ml, 1.796 mmol) at 0 °C and monitored by TLC. When the intermediate was formed the amine (1.2 equiv) was added at 0 °C and stirred at rt. When the reaction was complete the mixture was poured into brine (50 ml) and extracted with EtOAc (3 × 50 ml). The organic phase was collected and dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography using 50–100 EtOAc in hexane as gradient mobile phase to give the product. A similar general method was used to produce **9**–**11**. Compound **8** was isolated as a light yellow foam (40 mg, 37%). ^{1}H NMR δ (CD_3OD): 4.02 and 3.96 (d, 2H, 12β- and 3α-H), 2.64 (m, 1H, CPA-CH), 1.02 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.98 (s, 3H, 19-CH_3), 0.72 (m, 2H, CPA-CH_2), 0.71 (s, 3H, 18-CH_3), 0.49 (m, 2H, CPA-CH_2). ^{13}C NMR ppm (CD_3OD): 6.58 (2 × CH_2, CPA), 13.32, 15.13, 17.80, 23.44, 24.22, 24.98, 25.60, 27.39, 27.82, 28.83, 30.13, 31.38, 31.92, 33.43, 34.06, 34.43, 35.84, 37.04, 37.37, 39.04, 47.74, 48.25, 49.50, 60.38 (CH, 3-C), 74.18 (CH, 12-C), 178.60 (C=O, 24-C). IR_{vmax} (KBr): 3302.59, 2937.69, 2102.59 and 1648.38 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 479.3353, calculated for $C_{27}H_{44}N_4O_2Na$ = 479.3362.

4.1.11. (4*R*)-4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-benzylpentanamide (*N*-benzyl 3β-azido, 12α-hydroxy-5β-cholane-24-amide) (9)

Compound **9** was produced from **27** as described in 4.1.10. White foam (70 mg, 39%). ^{1}H NMR δ ($CDCl_3$): 7.30 (m, 5H, aromatic-H), 5.79 (br s, 1H, NH), 4.45 (m, 2H, benzyl-CH_2), 4.01 and 3.97 (d, 2H, 12β- and 3α-H), 1.00 (d, 3H, J = 6.00 Hz, 21-CH_3), 0.96 (s, 3H, 19-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$):

12.96, 17.66, 23.73, 23.77, 24.78, 26.15, 26.63, 27.69, 28.96, 30.30, 30.71, 31.85, 33.45, 33.77, 34.68, 35.35, 36.00, 37.47, 43.83, 46.71, 47.56, 48.54, 58.91 (CH, 3-C), 73.39 (CH, 12-C), 127.73, 128.06, 128.92, 138.54, 173.52 (C=O, 24-C). $IR_{\nu max}$ (KBr): 3414.05, 2927.53, 2107.71, 1646.85 and 698.15 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 529.3544, calculated for $C_{31}H_{46}N_4O_2Na$ = 529.3518.

4.1.12. (4*R*)-4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclohexylpentanamide (*N*-cyclohexyl 3β-azido, 12α-hydroxy-5β-cholane-24-amide) (10)

Compound **10** was produced from **27** as described in 4.1.10. Light yellow foam (85 mg, 47%). ^1H NMR δ ($CDCl_3$): 5.32 (br s, 1H, NH), 3.99 and 3.95 (d, 2H, 12β- and 3α-H), 3.77 (m, 1H, cyclohexyl-CH), 0.99 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.95 (s, 3H, 19-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 12.97, 17.69, 23.72, 23.77, 24.77, 25.07, 25.73, 26.16, 26.63, 27.69, 28.93, 30.29, 30.71, 31.93, 33.45, 34.03, 34.68, 35.34, 36.00, 37.47, 46.73, 47.59, 48.23, 48.53, 58.92 (CH, 3-C), 73.38 (CH, 12-C), 172.66 (C=O, 24-C). $IR_{\nu max}$ (KBr): 3434.18, 2932.36, 2102.19 and 1643.62 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 521.3864, calculated for $C_{30}H_{50}N_4O_2Na$ = 521.3831.

4.1.13. (4*R*)-4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-propylpentanamide (*N*-propyl 3β-azido, 12α-hydroxy-5β-cholane-24-amide) (11)

Compound **11** was produced from **27** as described in 4.1.10. White foam (0.065 g, 40%). ^1H NMR δ ($CDCl_3$): 5.47 (br s, 1H, NH), 3.99 and 3.95 (d, 2H, 12β- and 3α-H), 3.22 (m, 2H, propyl-CH_2), 1.01–0.92 (m, 9H, 21-CH_3+propyl-CH_3+21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.57 (CH3, propyl), 12.98, 17.69, 23.11, 23.73, 23.78, 24.80, 26.17, 26.64, 27.68, 28.98, 29.47, 30.32, 30.73, 31.94, 33.48, 33.86, 34.69, 35.37, 36.02, 37.49, 41.42 (CH2, propyl), 46.73, 47.61, 48.57, 58.93 (CH, 3-C), 73.41 (CH, 12-C), 173.65 (C=O, 24-C). $IR_{\nu max}$ (KBr): 3425.12, 2935.22, 2102.60 and 1638.55 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 481.3507, calculated for $C_{27}H_{46}N_4O_2Na$ = 481.3518.

4.1.14. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl) pentanoic acid (3β-azido -LCA) (12)

LCA (3 g, 7.966 mmol) was dissolved in MeOH and cc. HCl (0.34 ml, 3.983 mmol) added and the mixture was stirred at reflux. After the completion of the reaction the solvent was removed and the residue was dissolved in EtOAc (50 ml) then washed with water (50 ml) and brine (50 ml). The organic phase was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure giving LCA methyl ester as a white solid as product (3.049 g, 98%). To a solution of the LCA methyl ester (3.049 g, 7.807 mmol) and Et_3N (1.24 ml, 8.871 mmol) in anhydrous DCM (50 ml) was added methanesulfonyl chloride (0.94 ml, 12. 097 mmol) in anh. DCM (10 ml) dropwise at 0 °C and stirred at rt for 1.5 h. Then the reaction mixture was washed with 1 M HCl (2 × 100 ml), water (100 ml) and brine (100 ml), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure giving white solid as product (3.01 g, 80%). ^1H NMR (400 MHz, $CDCl_3$): 4.66 (m, 1H, 3β-H), 3.68 (s, 3H, –O-CH_3), 3.02 (s, 3H, –SO_2-CH_3), 0.94 (s, 3H, 19-CH_3), 0.93 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.65 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): 174.59 (C=O, 24-C), 82.81 (CH, 3-C), 51.32 (CH_3, –O-CH_3). The 3-α mesylate (2.88 g, 6.145 mmol) was converted to the 3-β azide by treating with NaN_3 in DMPU as already described. The yield product was white solid (2.44 g, 96%). ^1H NMR (400 MHz, $CDCl_3$): δ 3.97 (s, 1H, 3α-H), 3.68 (s, 3H, –O-CH_3), 0.96 (s, 3H, 19-CH_3), 0.93 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.66 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 175.24 (C=O, 24-C), 58.85 (CH, 3-C). $IR_{\nu max}$ (KBr): 2937.19, 2089.38 and 1734.37 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 438.3078, calculated for $C_{25}H_{41}N_3O_2Na$ = 438.3096. Thehe 3-β azido LCA methyl ester (2.35 g, 5.654 mmol) was subjected to hydrolysis using NaOH in aqueous methanol as already described and the product isolated as white foam (2.18 g, 96%).%). ^1H NMR δ ($CDCl_3$): 3.93 (s, 1H, 3α-H), 3.66 (s, 1H, 7β-H), 0.96 (d, 6H, 19-CH_3 and 21-CH_3), 0.72 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 12.49, 18.76, 22.13, 24.39, 25.24, 25.61, 27.43, 27.69, 29.23, 31.25, 31.83, 32.00, (32.20), 32.30, 36.08, 36.70, 37.06, 38.83, 41.33, 41.45, 43.90, (57.37), 57.44, 57.81, 60.16 (CH, 7-C), 178.21 (C=O, 24-C $IR_{\nu max}$ (KBr): 3450.29, 2927.53, 2101.43 and 1709.14 cm^{-1}. Found: $[M-H]^-$ = 400.2966, calculated for $C_{24}H_{38}N_3O_2$ = 400.2964.

4.1.15. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl) pentanamide (3β-azido, 7α-hydroxy-5β-cholane-24-amide) (13)

Compound **12** was amidated producing **13** as already described for the conversion of **27** to **7**. Compound **13** was isolated as a white foam (0.071 g, 72%). ^1H NMR δ ($CDCl_3$): 3.98 (s, 1H, 3α-H), 3.66 (s, 1H, 7β-H), 0.96 (d, 6H, 21-CH_3 and 19-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 12.27, 18.45, 21.20, 24.00, 24.37, 24.91, 26.49, 26.73, 28.38, 30.40, 30.88, 30.95, 31.15, 35.18, 35.51, 35.85, 37.52, 40.33, 40.38, 42.97, 56.16, 56.78, 59.00, 180.32 (C=O, 24-C). $IR_{\nu max}$ (KBr): 3436.57, 2927.53, 2101.06 and 1648.37 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 423.3086, calculated for $C_{24}H_{40}N_4ONa$ = 423.3100.

4.1.16. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclohexylpentanamide (*N*-cyclohexyl 3β-azido-5β-cholane-24-amide) (14)

Compound **14** was produced from **12** using HOBt/EDC in DMF as already described for compounds **8**–**11**. It was obtained as a light yellow foam (0.149 g, 83%). ^1H NMR δ ($CDCl_3$): 5.48 (br s, 1H, NH), 3.95 (s, 1H, 3α-H), 3.78 (m, 1H, cyclohexyl-CH), 0.95 (s, 3H, 19-CH_3), 0.92 (d, 3H, J = 6.04 Hz, 21-CH_3), 0.64 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 12.25, 18.59, 21.19, 23.98, 24.36, 24.89, 25.08, 25.74, 26.47, 26.72, 28.46, 30.37, 30.86, 32.08, 33.44, 33.47, 34.14, 35.16, 35.70, 35.83, 37.50, 40.31, 40.38, 42.95, 48.21, 56.28, 56.77, 58.98, 172.80 (C=O, 24-C). $IR_{\nu max}$ (KBr) 3318.76, 2930.15, 2100.76 and 1639.16 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 505.3865, calculated for $C_{30}H_{50}N_4ONa$ = 505.3882.

4.1.17. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-propylpentanamide (*N*-propyl 3β-azido-5β-cholane-24-amide) (15)

Compound **15** was produced from **12** using HOBt/EDC in DMF as already described for compounds **8**–**11**. It was obtained as a white foam (0.123 g, 74%). ^1H NMR δ ($CDCl_3$): 5.48 (br s, 1H, NH), 3.95 (s, 1H, 3α-H), 3.22 (m, 2H, propyl-CH_2), 0.95–0.91 (m, 9H, 19-CH_3+-propyl-CH_3+21-CH_3), 0.64 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.58, 12.26, 18.58, 21.19, 23.11, 23.98, 24.37, 24.89, 26.47, 26.72, 28.45, 30.38, 30.86, 32.08, 33.98, 35.16, 35.73, 35.83, 37.50, 40.31, 40.38, 41.39, 42.94, 56.28, 56.77, 58.98, 173.74 (C=O, 24-C). $IR_{\nu max}$ (KBr): 3278.17, 2935.82, 2101.57 and 1645.32 cm^{-1}. HRMS: Found: $[M-2H+Na]^+$ = 463.3401, calculated for $C_{27}H_{44}N_4ONa$ = 463.3413.

4.1.18. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclopropylpentanamide (16 *N*-cyclopropyl 3β-azido-5β-cholane-24-amide) (16)

Compound **16** was produced from **12** using HOBt/EDC in DMF as already described for compounds **8**–**11**. It was obtained as a white

foam (0.093 g, 56%). ^{1}H NMR δ ($CDCl_3$): 5.66 (br s, 1H, NH), 3.97 (s, 1H, 3α-H), 2.71 (m, 1H, CPA-CH), 0.97 (s, 3H, 19-CH_3), 0.93 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.79 (m, 2H, CPA-CH_2), 0.66 (s, 3H, 18-CH_3), 0.50 (m, 2H, CPA-CH_2). ^{13}C NMR ppm ($CDCl_3$): 6.84 (2 × CH2, CPA), 12.25, 18.57, 21.19, 22.81, 23.99, 24.36, 24.89, 26.47, 26.72, 28.45, 30.38, 30.86, 31.94, 33.65, 35.16, 35.72, 35.83, 37.51, 40.31, 40.38, 42.95, 56.25, 56.77, 58.98 (CH, 3-C), 175.24 (C=O, 24-C). IR_{vmax} (KBr): 2932.36, 2102.08 and 1645.39 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 463.3399, calculated for $C_{27}H_{44}N_4ONa$ = 463.3413.

4.1.19. (4*R*)-4-((3*S*,5*R*,10*S*,13*R*,17*R*)-3-Azido-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-benzylpentanamide (*N*-benzyl 3β-azido-5β-cholane-24-amide) (17)

Compound **17** was produced from **12** using HOBt/EDC in DMF as already described for compounds **8**–**11**. It was obtained as a light yellow foam (0.123 g, 67%). ^{1}H NMR δ ($CDCl_3$): 7.38–7.29 (m, 5H, aromatic-H), 5.77 (br s, 1H, NH), 4.47 (d, 2H, J = 5.52 Hz, benzyl-CH_2), 3.97 (s, 1H, 3α-H), 0.97 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.04 Hz, 21-CH_3), 0.66 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 12.26, 18.57, 21.19, 23.99, 24.37, 24.89, 26.47, 26.72, 28.47, 30.37, 30.86, 32.00, 33.86, 35.16, 35.71, 35.83, 37.50, 40.30, 40.37, 42.95, 43,81, 56.26, 56.77, 58.98 (CH, 3-C), 127.71, 128.05, 128.91, 138.59, 173.58 (C=O, 24-C). IR_{vmax} (KBr): 3293.28, 3065.29, 3030.69, 2932.36, 2101.90, 1644.72, 731.74 and 697.67 cm^{-1}. HRMS: Found: $[M+H]^+$ = 491.3751, calculated for $C_{31}H_{47}N_4O$ = 491.3750.

4.1.20. (4*R*)-Methyl 4-((3*S*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-bromo-12-(formyloxy)-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoate (24-methyl 3β-bromo, 12α-formyloxy-5β-cholanoate) (28)

Triphenylphosphine (2.135 g) was added to a solution of **26** (1.769 g) in anhydrous THF (80 ml) and NBS (1.449 g) was added in 3 parts over 1 h at −18 °C then allowed to warm to rt and stirred for 1.5 h. The reaction mixture was poured into 1 M HCl solution (100 ml) and extracted with EtOAc (3 × 75 ml). The organic layer was washed with brine (2 × 100 ml) and after drying over $MgSO_4$ the solvent was removed under reduced pressure. The crude product was flash columned (hexane/EtOAc 5:1) to afford the product as pale white solid (1.919 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (s, 1H, 12-OC=O*H*), 5.26 (s, 1H, 12β-H), 4.79 (s, 1H, 3α-H), 3.68 (s, 3H, –O-CH_3), 1.01 (s, 3H, 19-CH_3), 0.85 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.76 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): 174.70 (C=O, 24-C), 160.68 (C=O, 12-OC=OH), 76.29 (CH, 12-C), 51.67 (CH_3, OCH_3). IR_{vmax} (KBr): 3426.56, 2939.12, 2873.47, 1739.75, 1720.29 and 1180.89 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 519.2073, calculated for $C_{26}H_{41}BrO_3Na$ = 519.2086.

4.1.21. (4*R*)-4-((3*R*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoic acid (3α-azido, 12α–hydroxy-5β-cholanoate) (29)

To a stirred solution of **28** (0.712 g) in DMPU (20 ml) was added 10 equiv of NaN_3 (0.904 g) at rt. The mixture was stirred for 4 d then poured into water (100 ml) and extracted with EtOAc (3 × 50 ml). The organic phase was washed with brine (100 ml), dried over $MgSO_4$, filtered and the solvent was evaporated in vacuum. The product was separated on a flash column using 0–12% EtOAc in hexane as mobile phase to yield light yellow oil as product (0.589 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.09 (s, 1H, 12β-H), 3.68 (s, 3H, –O-CH_3), 3.29 (m, 1H, 3β-H), 2.11 (s, 3H, 12-C=OCH_3), 0.92 (s, 3H, 19-CH_3), 0.83 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.74 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 175.07 (C=O, 24-C), 171.04 (C=O, 12-OC=OCH_3), 76.27 (CH, 12-C), 61.41 (CH, 3-C), 51.98 (CH_3, OCH_3), 21.79 (CH_3, 12-C=OCH_3). IR_{vmax} (DCM): 2941.42, 2867.23, 2091.28, 1738.46 and 1245.13 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 496.3168, calculated for $C_{27}H_{43}N_3O_4Na$ = 496.3151. The 3-azido formate ester of DCA methyl ester (0.101 g) was subjected to hydrolysis using NaOH in aqueous methanol as described (0.074 g, 82%). mp: 88 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.01 (s, 1H, 12β-H), 3.35 (m, 1H, 3β-H), 1.01 (d, 3H, J = 6.03 Hz, 21-CH_3), 0.94 (s, 3H, 19-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 179.80 (C=O, 24-C), 73.29 (CH, 12-C), 61.40 (CH, 3-C). IR_{vmax} (KBr): 3440.00, 2941.49, 2866.14, 2090.46, 1709.85, 1679.00 and 1246.00 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 440.2878, calculated for $C_{24}H_{39}N_3O_3Na$ = 440.2889.

4.1.22. (4*R*)-4-((3*R*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-Azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanamide (3α–azido, 12α-hydroxy-5β-cholane-24-amide) (18)

The 3-α azido analogue of DCA **29** (0.15 g, 0.359 mmol) was converted to the corresponding primary amide using the HOBt approach with ammonia solution. The product was isolated as a white foam (0.055 g, 37%). ^{1}H NMR δ (acetone-d_6): 4.02 (s, 1H, 12β-H), 3.35 (m, 1H, 3β-H), 1.01 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.94 (s, 3H, 19-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm (acetone-d_6): 13.12, 17.68, 23.65, 24.55, 27.06, 27.28, 27.87, 28.32, 32.63, 33.08, 33.22, 34.29, 34.98, 36.17, 36.39, 36.96, 43.20, 47.32, 47.66, 48.78, 61.97 (CH, 3-C), 72.85 (CH, 12-C), 175.71 (C=O, 24-C). IR_{vmax} (KBr): 3382.75, 2939.74, 2091.78 and 1655.26 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 439.3039, calculated for $C_{24}H_{40}N_4O_2Na$ = 439.3049.

4.1.23. (4*R*)-4-((3*R*,5*R*,10*S*,12*S*,13*R*,17*R*)-3-azido-12-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclopropylpentanamide (24-cyclopropyl-3α–azido, 12α-hydroxy-5β-cholanamide) (19)

Compound **19** was produced from **29** using the HOBt coupling approach already described (0.119 g, 73%).^{1}H NMR δ ($CDCl_3$): 5.66 (s, 1H, NH), 3.99 (m, 1H, 12β-H), 3.35 (m, 1H, 3β-H), 2.71 (m, 1H, CPA-CH), 0.99 (d, 3H, J = 6.00 Hz, 21-CH_3), 0.94 (s, 3H, 19-CH_3), 0.78 (m, 2H, CPA), 0.69 (s, 3H, 18-CH_3), 0.49 (m, 2H, CPA). ^{13}C NMR ppm ($CDCl_3$): 6.83 (2 × CH2, CPA), 12.95, 17.63, 22.78, 23.43, 23.78, 26.21, 26.87, 27.20, 27.63, 28.86, 31.74, 32.62, 33.45, 33.85, 34.37, 35.30, 35.56, 36.15, 42.51, 46.65, 47.43, 48.36, 61.44 (CH, 3-C), 73.24 (CH, 12-C), 175.11 (C=O, 24-C). IR_{vmax} (KBr): 3434.92, 2938.91, 2086.95 and 1650.61 cm^{-1}. HRMS: Found: $[M+H]^+$ = 457.3542, calculated for $C_{27}H_{45}N_4O_2$ = 457.3543.

4.1.24. (4*R*)-4-((3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-3-Azido-7-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl) pentanoic acid (3β-azido, 7α-hydroxy-5β-cholane) (20)

Compound **30** was selectively mesylated at position 3 using a similar procedure to that already described in 4.1.2. The mesylate was obtained as a white foam (1.299 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.54 (m, 1H, 3β-H), 3.87 (s, 1H, 7β-H), 3.68 (s, 3H, –O-CH_3), 3.01 (s, 3H, –SO_2-CH_3), 0.95–0.93 (d, 6H, 21-CH_3 and 19-CH_3), 0.67 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.55 (C=O, 24-C), 82.57 (CH, 3-C), 68.73 (CH, 7-C). IR_{vmax} (KBr): 3552.50, 2942.04, 1737.27, 1348.89, 1170.76 and 926.45 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 507.2758, calculated for $C_{26}H_{44}O_6SNa$ = 507.2756. The mesylate was converted to azide using the NaN3/DMPU approach at 65 °C as already described. The product was isolated following chromatography using hexane: EtOAc 3:1 as mobile phase to afford colourless semi-solid as product (0.86 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.92 (s, 1H, 3α-H), 3.88 (s, 1H, 7β-H), 3.68 (s, 3H, –O-CH_3), 0.95–0.93 (d, 6H, 19-CH_3 and 21-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 175.20 (C=O, 24-C), 69.04 (CH, 7-C). IR_{vmax} (KBr): 3494.84, 2930.37, 2091.78 and 1739.85 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3029, calculated for $C_{25}H_{41}N_3O_3Na$ = 454.3046. The

methyl ester (0.84 g, 1.946 mmol) was subjected to hydrolysis in methanolic solution of aq. NaOH affording product as a white foam (0.277 g, 69%). ^{1}H NMR δ ($CDCl_3$): 3.93 (s, 1H, 3α-H), 3.89 (s, 1H, 7β-H), 0.96 (d, 6H, 19-CH_3 and 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.98, 18.43, 20.98, 23.35, 23.90, 24.85, 28.34, 30.79, 30.95, 31.18, 32.63, 33.31, 34.23, 35.54, 35.65, 36.97, 39.50, 39.80, 42.94, 50.64, 55.98, 58.95, 68.89 (CH, 7-C), 180.27(C═O, 24-C). IR_{vmax} (KBr): 3450.29, 2927.53, 2101.43 and 1709.14 cm^{-1}. HRMS: Found: $[M]^-$ = 416.2901, calculated for $C_{24}H_{38}N_3O_3$ = 416.2913.

4.1.25. (4*R*)-4-((3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-3-Azido-7-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl) pentanamide (3β-azido, 7α-hydroxy-5β-cholane-24-amide) (21)

Compound **21** was prepared from **20** using the ethyl chloroformate procedure. White foam (0.071 g, 72%). ^{1}H NMR δ ($CDCl_3$): 5.89 (br s, 2H, NH_2), 3.92 (s, 1H, 3α-H), 3.88 (s, 1H, 7β-H), 0.96 (d, 6H, 21-CH_3 and 19-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.97, 18.53, 20.97, 23.34, 23.90, 24.84, 28.42, 30.79, 31.77, 32.61, 32.79, 33.30, 34.27, 35.65, 36.98, 39.49, 39.80, 42.91, 50.64 (CH, 3-C), 55.95, 58.94, 68.78 (CH, 7-C), 176.86 (C═O, 24-C). IR_{vmax} (KBr): 3436.57, 2927.53, 2101.06 and 1648.37 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 439.3061, calculated for $C_{24}H_{40}N_4O_2Na$ = 439.3049

4.1.26. (4*R*)-4-((3*S*,5*S*,7*R*,10*S*,13*R*,17*R*)-3-Azido-7-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-cyclopropylpentanamide (24-cyclopropyl-3β-azido, 7α-hydroxy-5β-cholanamide) (22)

Compound **22** was produced from **20** using the ethyl chloroformate coupling procedure with cyclopropyl amine. White foam (0.095 g, 87%). ^{1}H NMR δ ($CDCl_3$): 6.12 (s, 1H, NH), 3.92 (s, 1H, 3α-H), 3.88 (s, 1H, 7β-H), 2.74 (m, 1H, CPA-CH), 0.96 (d, 6H, 21-CH_3 and 19-CH_3), 0.80 (m, 2H, CPA), 0.67 (s, 3H, 18-CH_3), 0.56 (m, 2H, CPA). ^{13}C NMR ppm ($CDCl_3$): 6.80 (2 × CH_2, CPA), 11.98, 18.56, 20.98, 23.11, 23.35, 23.92, 24.85, 28.41, 30.80, 32.03, 32.61, 33.30, 34.23, 35.65, 35.69, 36.99, 39.50, 39.79, 42.92, 50.62 (CH, 3-C), 55.93, 58.96, 67.28, 68.82 (CH, 7-C), 176.09 (C═O, 24-C). IR_{vmax} (KBr): 3429.43, 2929.05, 2096.61 and 1665.91 cm^{-1}. HRMS: Found: $[M+Na]^+$ = 479.3349, calculated for $C_{27}H_{44}N_4O_2Na$ = 479.3362.

4.1.27. (4*R*)-Methyl 4-((3*S*,5*R*,7*R*,10*S*,13*R*,17*R*)-7-hydroxy-10,13-dimethyl-3-(methylsulfonyloxy)hexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoate (24-Methyl 3β-(methanesulfonyl)oxy, 7α-hydroxy-5β-cholanoate) (31)

Compound **30** (0.8 g, 1.967 mmol) and PPh_3 (1.703 g, 6.492 mmol) were dissolved in anhydrous THF (40 ml) and cooled to 0 °C. Et_3N (0.55 ml, 3.935 mmol) and methanesulfonic acid (0.27 ml, 4.132 mmol) were added to the mixture at this temperature. The solution was warmed to 45 °C and DIAD (1.2 ml, 6.099 mmol) was added dropwise and the mixture was stirred until TLC analysis showed the reaction was complete. Then the solvent was removed and the residue was dissolved in EtOAc (100 ml) which was washed with 1 M HCl (2 × 100 ml), water (100 ml) and brine (100 ml). After drying the organic phase over $MgSO_4$ the solvent was removed and the product was separated by column chromatography using hexane: EtOAc 3:1, 1:1 and 1:3 as mobile phase. The yield product was odourless semi-solid (0.139 g, 15%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.00 (m, 1H, 3α-H), 3.88 (s, 1H, 7β-H), 3.68 (s, 3H, –O-CH_3), 3.01 (s, 3H, –SO_2-CH_3), 0.98 (s, 3H, 19-CH_3), 0.95 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 175.19 (C═O, 24-C), 81.22 (CH, 3-C), 68.98 (CH, 7-C). IR_{vmax} (KBr): 3448.15, 2939.07, 1737.73, 1351.28, 1173.17 and 901.12 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 507.2690, calculated for $C_{26}H_{44}O_6SNa$ = 507.2756.

4.1.28. (4*R*)-4-((3*R*,5*S*,7*R*,10*S*,13*R*,17*R*)-3-Azido-7-hydroxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoic acid (3α-azido, 7α-hydroxy-5β-cholane) (23)

Compound **23** was produced from **31** using the NaN_3/DMPU procedure already described above. The product was separated by column chromatography using hexane: EtOAc 3:1 as mobile phase to afford yellow semi-solid (0.098 g, 79%). ^{1}H NMR δ (400 MHz, $CDCl_3$): 3.87 (s, 1H, 7β-H), 3.68 (s, 3H, –O-CH_3), 3.16 (s, 1H, 3β-H), 0.95 (d, 6H, 21-CH_3 and 19-CH_3), 0.67 (s, 3H, 18-CH_3). ^{13}C NMR ppm (100 MHz, $CDCl_3$): δ 174.88 (C═O, 24-C), 68.44 (CH, 7-C). IR_{vmax} (KBr): 3516.06, 2934.95, 2086.95 and 1721.20 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3040, calculated for $C_{25}H_{41}N_3O_3Na$ = 454.3046. The methyl ester was hydrolysed in a methanolic NaOH solution as already described to give product as white foam (0.084 g, 88%). ^{1}H NMR δ ($CDCl_3$): 3.89 (s, 1H, 7β-H), 3.18 (s, 1H, 3β-H), 0.96 (d, 3H, J = 6.56 Hz, 21-CH_3), 0.93 (s, 3H, 19-CH_3), 0.68 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 11.98, 18.43, 20.76, 23.06, 23.88, 27.01, 28.34, 30.94, 31.15, 32.95, 34.59, 35.32, 35.54, 35.64, 35.75, 39.56, 39.69, 41.98, 42.90, 50.52, 55.93, 61.55, 68.60 (CH, 7-C), 180.01 (C═O, 24-C). HRMS: Found: $[M-H]^-$ = 416.2914. HRMS: Found: $(M)^-$ = 416.2914, calculated for $C_{24}H_{38}N_3O_3$ = 416.2913.

4.2. Biochemical methods

4.2.1. MTT assay

Cell viability was determined by MTT assay. Cells were seeded (Caco-2 at 8 × 10^3 cells/well and HT1080 at 12 × 10^4 cells/well) into 96-well plates and grown until 70% confluent. Cells were serum starved for 1 h and then treated with test compounds at 0.1–1000 μM, under serum free conditions for 24 h. Test articles were introduced in DMSO at maximum 0.5% at which concentration no effects on cell viability could be observed. At the end of this incubation, MTT (0.5 mg/mL) was added and incubated for 3 h at 37 °C. After 3 h, supernatants were removed, 100 μL of DMSO was added, and the plates were incubated with shaking for 10 min. The formazan crystals formed inside the viable cells were solubilized in DMSO and the optical density was read on a plate reader at 540 nm. The % cell viability was determined as follows: (optical density of treated cells/optical density of non-treated cells)∗100. The experiments were performed a minimum of three times with three replicates over eight concentration levels.

Resultant concentration effect curves were transformed and analyzed using GraphPad Prism 5 (San Diego, CA, USA). In order to evaluate the role of ROS in these observations, MTT assays were repeated but with co-incubation with NAC (1 mM).

4.2.2. Flow cytometry

Cells were grown to 80% confluence in T25 flasks and treated with test compound for 24 h in serum free medium, control cells were treated only with vehicle in serum free media for 24 h. Following 24 h treatment cells were removed from T25 flasks and washed with 1X Binding Buffer, before being re-suspended in 100 ml of 1X binding buffer. A 20 ml aliquot of these cells was stained using 5 ml annexin V-FITC and 5 ml propidium iodide and incubated at rt for 15 min in the dark. Following incubation, samples were diluted in 1X binding buffer and analyzed within 5 min using a BD FACSArray (BD Biosciences, Oxford, UK). Flow cytometry was performed on double stained cell samples (Caco-2 and HT1080 cells). The instrument was set up to measure the size (forward scatter), granularity (side scatter) and cell fluorescence. Antibody binding was measured by analyzing individual cells for fluorescence. Data were analyzed using BD FACS Array software and expressed as a percentage of control fluorescence in arbitrary units. Cells in late stages of apoptosis or those already dead have lost PM integrity and are permeable to PI whereas annexin V binds

to cells in early apoptosis and continues to be bound through cell death. Thus cells in early stage of apoptosis will be annexin V positive and PI negative (in Q4), cells in late stage of apoptosis (in Q2) or annexin V Positive and PI positive and finally already dead (in Q1). Healthy cells are annexin V and PI negative (in Q3).

4.2.3. Measurement of ROS

Human colonic epithelial cancer cells Caco-2 cells at 70–80% confluence were treated in serum free media with test articles for 30 min and 24 h. The medium was removed and replaced with 100 μM 2′,7′-dichlorfluorescein-diacetate for 30 min at 37 °C. The solutions were removed and the cells were washed with PBS. The DCF fluorescence was monitored with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

4.2.4. Statistical analysis

Data are presented as group of means with standard error of the mean of $n \geqslant 3$. Statistical analysis of the mean difference between multiple groups was determined by one-way ANOVA followed by Tukey–Kramer multiple comparison post-test. A P value <0.05 was considered to be statistically significant. All statistical analyses were performed using Prism version 4 for Windows, GraphPad Software, La Jolla California USA.

References and notes

1. Hofmann, A. F.; Hagey, L. R. *Cell. Mol. Life Sci.* **2008**, *65*, 2461.
2. Porez, G.; Prawitt, J.; Gross, B.; Staels, B. *J. Lipid Res.* **2012**, *53*, 1723.
3. Amaral, J. D.; Viana, R. J.; Ramalho, R. M.; Steer, C. J.; Rodrigues, C. M. *J. Lipid Res.* **2009**, *50*, 1721.
4. McQuaid, K. R.; Laine, L.; Fennerty, M. B.; Souza, R.; Spechler, S. J. *Aliment. Pharmacol. Ther.* **2011**, *34*, 146.
5. Powell, A. A.; LaRue, J. M.; Batta, A. K.; Martinez, J. D. *Biochem. J.* **2001**, *356*, 481.
6. Barrasa, J. I.; Olmo, N.; Lizarbe, M. A.; Turnay, J. *Toxicology in vitro: an international journal published in association with BIBRA* **2013**, *27*, 964.
7. Qiao, L.; Studer, E.; Leach, K.; McKinstry, R.; Gupta, S.; Decker, R.; Kukreja, R.; Valerie, K.; Nagarkatti, P.; El Deiry, W.; Molkentin, J.; Schmidt-Ullrich, R.; Fisher, P. B.; Grant, S.; Hylemon, P. B.; Dent, P. *Mol. Biol. Cell* **2001**, *12*, 2629.
8. Katona, B. W.; Cummins, C. L.; Ferguson, A. D.; Li, T.; Schmidt, D. R.; Mangelsdorf, D. J.; Covey, D. F. *J. Med. Chem.* **2007**, *50*, 6048.
9. Katona, B. W.; Anant, S.; Covey, D. F.; Stenson, W. F. *J. Biol. Chem.* **2009**, *284*, 3354.
10. Sharma, R.; Majer, F.; Peta, V. K.; Wang, J.; Keaveney, R.; Kelleher, D.; Long, A.; Gilmer, J. F. *Bioorg. Med. Chem.* **2010**, *18*, 6886.
11. Majer, F.; Salomon, J. J.; Sharma, R.; Etzbach, S. V.; Najib, M. N.; Keaveny, R.; Long, A.; Wang, J.; Ehrhardt, C.; Gilmer, J. F. *Bioorg. Med. Chem.* **2012**, *20*, 1767.
12. Walsh, G. M.; Dewson, G.; Wardlaw, A. J.; Levi-Schaffer, F.; Moqbel, R. *J. Immunol. Methods* **1998**, *217*, 153.
13. Sharma, R.; Prichard, D.; Majer, F.; Byrne, A. M.; Kelleher, D.; Long, A.; Gilmer, J. F. *J. Med. Chem.* **2011**, *54*, 122.
14. Favre-Felix, N.; Fromentin, A.; Hammann, A.; Solary, E.; Martin, F.; Bonnotte, B. *J. Immunol. (Baltimore, Md.: 1950)* **2000**, *164*, 5023.
15. Oikonomou, E.; Kothonidis, K.; Taoufik, E.; Probert, E.; Zografos, G.; Nasioulas, G.; Andera, L.; Pintzas, A. *Br. J. Cancer* **2007**, *97*, 73.
16. Villa-Morales, M.; Fernandez-Piqueras, J. *Expert Opin. Ther. Targets* **2012**, *16*, 85.
17. Ignacio Barrasa, J.; Olmo, N.; Perez-Ramos, P.; Santiago-Gomez, A.; Lecona, E.; Turnay, J.; Antonia Lizarbe, M. *Apoptosis* **2011**, *16*, 1054.
18. Sharma, R.; Long, A.; Gilmer, J. F. *Curr. Med. Chem.* **2011**, *18*, 4029.
19. Kim, N. D.; Im, E.; Yoo, Y. H.; Choi, Y. H. *Curr. Cancer Drug Targets* **2006**, *6*, 681.
20. Liu, H.; Qin, C. K.; Han, G. Q.; Xu, H. W.; Ren, W. H.; Qin, C. Y. *Cancer Lett.* **2008**, *270*, 242.
21. Park, S. E.; Lee, S. W.; Hossain, M. A.; Kim, M. Y.; Kim, M. N.; Ahn, E. Y.; Park, Y. C.; Suh, H.; Kim, G. Y.; Choi, Y. H.; Kim, N. D. *Cancer Lett.* **2008**, *270*, 77.
22. Brossard, D.; El Kihel, L.; Clement, M.; Sebbahi, W.; Khalid, M.; Roussakis, C.; Rault, S. *Eur. J. Med. Chem.* **2010**, *45*, 2912.
23. Bellini, A. M.; Mencini, E.; Quaglio, M. P.; Guarneri, M.; Fini, A. *Steroids* **1991**, *56*, 395.
24. Bellini, A. M.; Quaglio, M. P.; Cavazzini, G.; Cecchenini, R. *Farmaco Sci.* **1984**, *39*, 305.