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New fluorescent bile acids: Synthesis, chemical characterization, and disastereoselective uptake by Caco-2 cells of 3-deoxy 3-NBD-amino deoxycholic and ursodeoxycholic acid

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ABSTRACT

Deoxycholic acid (DCA), a secondary bile acid (BA), and ursodeoxycholic acid (UDCA), a tertiary BA, cause opposing effects in vivo and in cell suspensions. Fluorescent analogues of DCA and UDCA could help investigate important questions about their cellular interactions and distribution.

We have prepared a set of isomeric 3α- and 3β-amino analogues of UDCA and DCA and derivatised these with the discrete fluorophore, 4-nitrobenzo-2-oxa-1,3-diazol (NBD), forming the corresponding four fluorescent adducts. These absorb in the range 465–470 nm and fluoresce at approx. 535 nm.

In order to determine the ability of the new fluorescent bile acids to mimic the parents, their uptake was studied using monolayers of Caco-2 cells, which are known to express multiple proteins of the organic anion-transporting peptide (OATP) subfamily of transporters. Cellular uptake was monitored over time at 4 and 37 °C to distinguish between passive and active transport.

All four BA analogues were taken up but in a strikingly stereo- and structure-specific manner, suggesting highly discriminatory interactions with transporter protein(s). The α-analogues of DCA and to a lesser extent UDCA were actively transported, whereas the β-analogues were not. The active transport process was saturable, with Michaelis–Menten constants for 3α-NBD DCA (**5**) being K_m = 42.27 ± 12.98 μM and V_{max} = 2.8 ± 0.4 nmol/(mg protein*min) and for 3α-NBD UDCA (**3**) K_m = 28.20 ± 7.45 μM and V_{max} = 1.8 ± 0.2 nmol/(mg protein*min). These fluorescent bile acids are promising agents for investigating questions of bile acid biology and for detection of bile acids and related organic anion transport processes.

1. Introduction

The secondary bile acid (BA), deoxycholic acid (DCA, **1** Fig. 1) is a putative promoter of intestinal carcinogenesis. DCA causes DNA fragmentation, oxidative stress, Golgi fragmentation and apoptosis.[1] Conversely, the clinically used ursodeoxycholic acid (UDCA, **2** Fig. 1) is cytoprotective, immunomodulatory, anti-inflammatory and anti-apoptotic.[2] In general, UDCA's effects oppose those of DCA.[3–5] For example, pre-treatment with UDCA can prevent DCA induced apoptosis and Golgi fragmentation in colon cancer cells.[6]

The divergent cellular effects of DCA and UDCA have been widely investigated and there has been progress in characterising some of the molecular mechanisms involved.[7–10] However, new tools and strategies are required to investigate direct cellular interactions of the compounds, their uptake and distribution. Fluorescent steroid analogues can prove useful for this kind of problem. Such compounds may also be used to probe interacting proteins and related cellular transport mechanisms.

The 4-nitrobenzo-2-oxa-1,3-diazole (NBD) group is a particularly useful reporter group in this context because of its relatively low steric impact, its sensitivity to its environment, satisfactory quantum yield and stability.[11,12] In the BA field, the synthesis and chemical characterisation of 3-,7- and 12-α- and β NBD analogues of cholic acid (CA, Fig. 1a) and their tauro conjugates have been reported, followed by comprehensive studies of their uptake and hepatic metabolism in vitro and in rats.[12–14] Recently, the 3- and 7-amino NBD analogues of CA were studied as uptake probes for flow cytometry in isolated rat hepatocytes.[15] NBD-labelled 24-lysyl conjugates of UDCA (Fig. 1a) and DCA have been studied as substrates for hepatic[16] and intestinal transport proteins.[17] Other investigations into (tauro)-UDCA have employed the UDCA 7-NBD analogue.[5,18,19]

Astonishingly, the 3-deoxy 3-NBD-amino derivatives of DCA and UDCA have not been reported to date. Placement of the fluorescent reporter group at C-3 appears to us to be optimal for exploring the disposition of UDCA and DCA because this preserves

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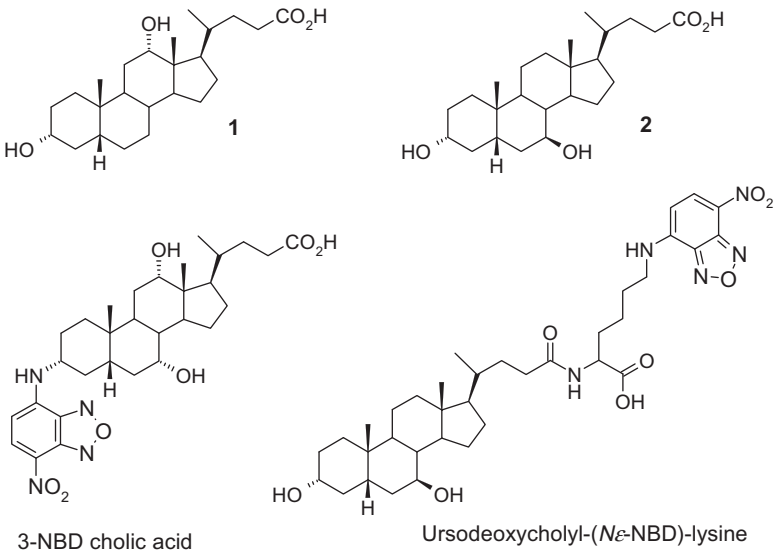


Figure 1a. Structures of bile acids UDCA (**1**) and DCA (**2**) along with fluorescent analogues of cholic acid and UDCA that have been studied previously.

the 7- and 12-OH groups that account for the differing chemical and biological properties of the parent compounds. Importantly, C-3 modification also preserves the characteristic free bile acid side chain. Structural studies probing the interaction of UDCA with cognate proteins would also support incorporation of a reporter group at the BA C3. In the 3.0 Å crystal structure of human type III 3α-hydroxysteroid dehydrogenase (HSD)/bile acid binding protein (AKR1C2) complexed with NADP(+) and UDCA anion, the carboxylate group of UDCA was modelled in the oxyanion hole, deep in the protein with the 3-OH solvent exposed (11513593, PDB 1 ihi). The liver cytosolic bile acid binding protein (BABP) binds BAs in a similar fashion, which led to the elegant design of diagnostic compounds elaborated at C-3 with gadolinium binding EDTA groups.[20] Additionally, various SAR surveys and QSAR models of the human apical sodium-dependent bile acid transporter (ASBT, SLC10A2) suggest that the C3-OH is tolerant of modification.[21,22] Finally, various bile acid carrier pro-drug conjugates have proven that intestinal bile acid transporters can tolerate payload incorporation at C-3.[23]

We have synthesised isomeric 3-deoxy 3-NBD-amino derivatives of UDCA and DCA (Fig. 1b) as probes for studying the distribution of these important steroids. A control compound lacking the steroidal nucleus (**7**) was also prepared to detect non-specific effects. In order to assess the suitability of the new analogues for investigating the disposition of the parent BAs, we have studied their uptake into polarised monolayers of intestinal epithelial Caco-2 cells. The Caco-2 cell line exhibits many of the features of the normal intestinal epithelium including expression of various BA transporter proteins, such as the sodium-dependent bile acid transporter (ASBT) and various members of the organic anion-transporting peptide (OATP) subfamily.[24,25] Caco-2 cells are therefore routinely used for assessing bile acid uptake and transport[26] and they are considered a suitable model for preliminary assessment of the NBD analogues as surrogates of DCA and UDCA.

The uptake of the DCA and UDCA 3-NBD compounds **3** and **5** was strikingly structure and stereo-specific. The DCA α-analogue was sufficiently rapidly taken up to suggest general applicability in the detection of active transport processes with significant practical advantages over experimental set-ups utilising radiolabelled substrates.

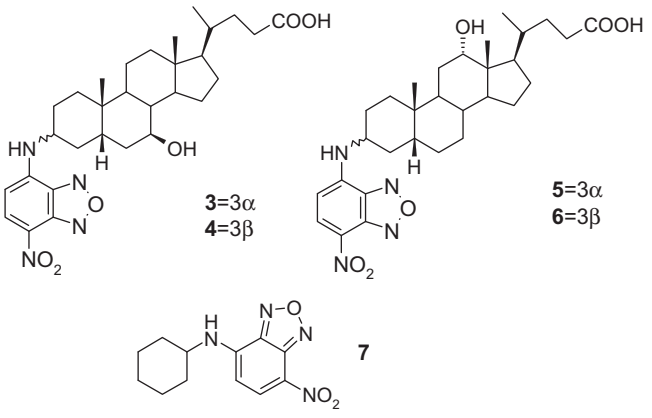


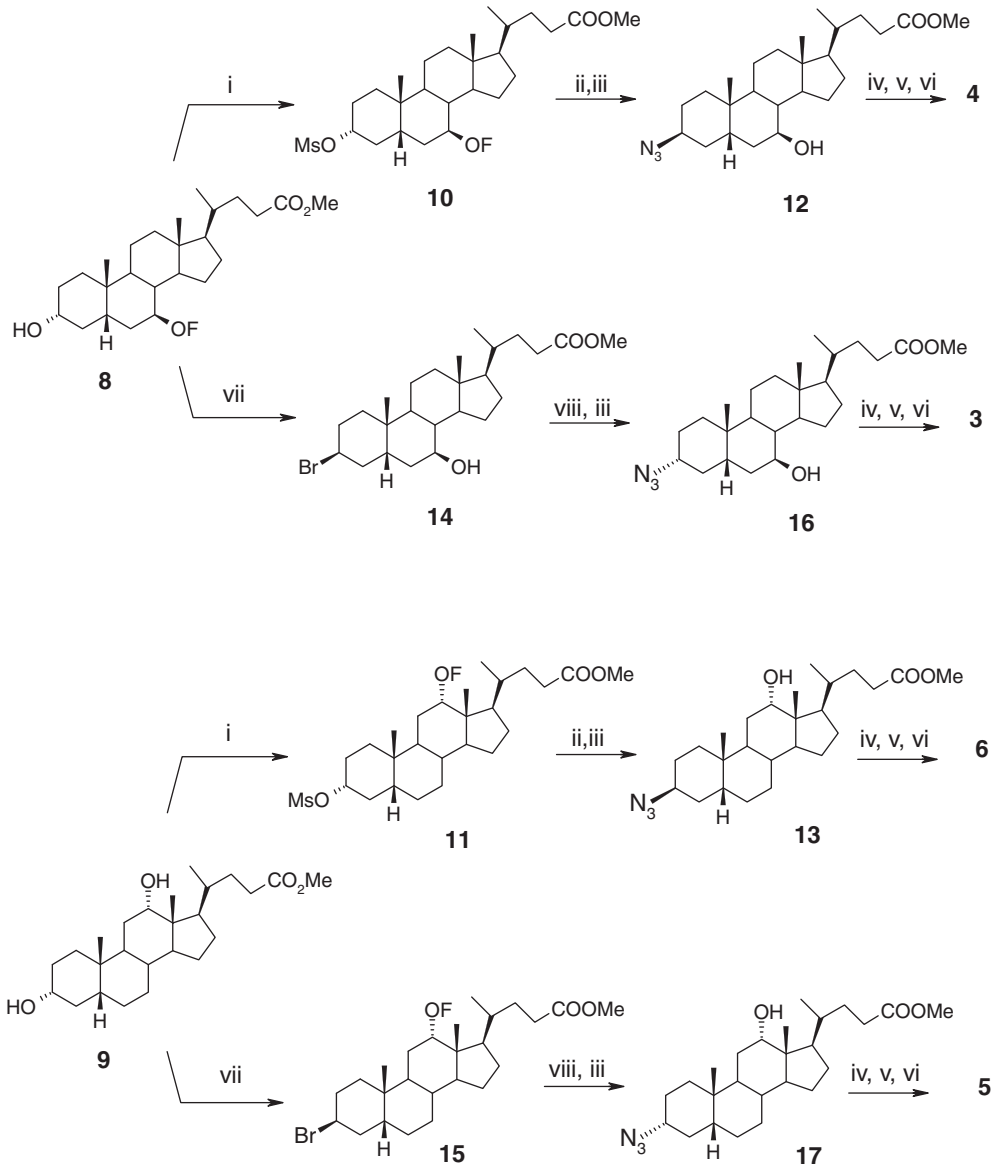
Figure 1b. New NBD analogues of UDCA and DCA **3–6**; cyclohexylamine-NBD **7** was produced as a non-bile acid fluorescent control.

2. Results/Discussion

2.1. Chemistry

The NBD compounds (**3–6**) were produced as shown in Scheme 1 by formation of the appropriate azides, reduction and displacement of NBD chloride. The orthogonally protected formate-methyl esters of UDCA and DCA (**8**, **9**) were obtained in three steps from the parent bile acids. In order to produce the β-NBD compounds, these were first converted to the corresponding 3-α mesylates (**10** and **11**), followed by SN2 substitution to give the β-azides. In early experiments the 3-amino-NBD group was found to be labile to conditions required to remove the formyl protection. Therefore, the formate esters were selectively removed (acetyl chloride in dry MeOH) at the azide stage to yield **12**, **13**. The α-azides were reduced and BOC-protected in situ followed by purification by flash chromatography. The BOC protection was removed, the NBD introduced and finally the 24-ester groups hydrolysed to give **4** and **6**.

The α-compounds **3**, **5** required amine introduction at C-3 with retention of configuration. After several unsuccessful attempts with introduction of methanesulfonate, we turned to bromide and generated 3-βbromides **14**, **15** using a mixture of $P(Ph_3)_3$ and *N*-bromosuccinimide in THF at low temperature. Azide displacement of the β-bromides producing **16**, **17** was faster than the displacement of the α-mesylates in the 3-β series (**10**, **11**). The



Scheme 1. Synthesis of UDCA and DCA NBD analogues **3**–**6**. Reagents and conditions: (i) MsCl, NEt_3, DCM, 0 °C, 20 min; (ii) NaN_3, DMPU, 50 °C, 6–8 d; (iii) AcCl, an. MeOH, 0 °C to rt, overnight; (iv) 10% Pd/C, $(BOC)_2O$, H_2, EtOAc, rt, overnight; (v) (a) 10% TFA/DCM, rt, overnight (b) NBD-Cl, $NaHCO_3$, MeOH, reflux, 5–14 d; (vi) 2 M NaOH/MeOH (pH∼14), 40 °C, 4 h; (vii) $P(Ph_3)_3$, NBS, THF, −18 °C to rt, 1.5 h; (viii) NaN_3, DMPU, rt, overnight.

route to the α-NBD compounds was otherwise similar to that used to generate the β-compounds with regioselective deformylation, reduction, BOC protection and purification, de-BOC, NBD formation and de-esterification. Amino analogues of cholic acid and their NBD derivatives have been produced by stereoselective reduction of the corresponding oximes. The current approach is longer but it is clean, high yielding and the SN2 substitution provides stereochemical control. The NBD derivative of cyclohexylamine was obtained by direct treatment of the amine with NBD chloride. The NBD compounds were bright orange crystalline solids, pure by HPLC (>98%) and their structure confirmed by HRMS/NMR.

2.2. Stability and physicochemical characterisation

The stability of 3β−NBD UDCA was assessed in pH 7.4 buffer (37 °C) using HPLC to monitor remaining compound as a function of time. There was no change in the LC-fluorescence peak area over 24 h under these conditions showing that the 3-NBD group was stable. The same HPLC method could also be used to assess lipophilicity. The β-compounds (calculated capacity factor (k') for 3β-NBD-DCA was 7.3 and for 3β-NBD-UDCA 5.1) were more polar than the corresponding α-compounds (k' for 3α-NBD-DCA was 10.8 and for 3α-NBD-UDCA 5.6). As expected the DCA analogues were more hydrophobic than the corresponding UDCA analogues.

2.3. Photo/physical characterization

The UV/Vis absorption spectra of **3**–**6** measured in ethanol solution exhibited maxima at 228, 335 and 470 nm (Table 1). The β-compounds were red-shifted relative to the α-compounds by around 5 nm (Fig. 2). The fluorescence emission spectra for **3**–**6** exhibited maxima at λ_{Em} 537 nm with a 2–3 nm blue shift for the α-isomers (λ_{Em} was recorded using an λ_{abs} of 470 nm). These values were not affected by oxygen exposure. They are broadly in agreement with previously reported fluorescence values for cholic acid NBD derivatives.[13,15]

Table 1
Excitation/emission characteristics of 3-amino-NBD derivatives of UDCA and DCA. Quantum yields (ΦF) were estimated relative to fluorescein in 0.01 KOH (ΦF_{ST} = 0.925)

	3 (3α-NBD-UDCA)	**4** (3β-NBD-UDCA)	**5** (3α-NBD-DCA)	**6** (3β-NBD-DCA)	**7** Cyclohexyl amine-NBD
λ_{abs} (nm)	231	227	228	227	225
	335	331	335	331	334
	470	465	470	466	469
λ_{Em} (nm)	534	537	535	537	536
ΦF	0.268	0.228	0.259	0.221	0.232

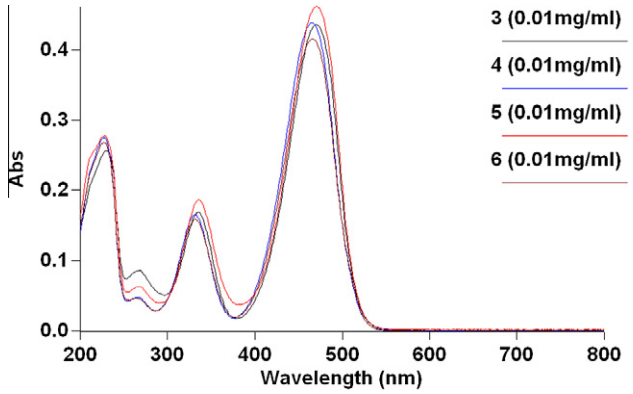


Figure 2. UV–Vis spectra overlaid of **3**–**6** acquired in EtOH at 0.01 mg/ml overlaid.

2.4. Cell viability

Bile acids can cause apoptosis through intrinsic and extrinsic pathways and necrosis through detergent effects.[8] The effect of derivatising the bile acids is not predictable though toxicity may be related to physicochemical properties such as hydrophobicity.[10] Since the NBD-bile acids were designed to investigate the disposition and uptake of the parent compounds, it was important to characterise their effect on cell viability. The cell viability effects of isomeric NBD-BAs (1–100 μM) was therefore measured using the MTT assay after incubating Caco-2 monolayers with the compounds for up to 100 min. The cytoxicity of the compounds was also assessed in other relevant cells lines including oesophageal adenocarcinoma (SKGT-4) and hepatic cancer cell lines (HUH-7). Compounds **3**–**6** did not affect cell viability in any of these cell lines up to 24 h up to 100 μM.

2.5. Caco-2 uptake

The time-course of uptake of **3**–**6** (10 μM) into Caco-2 cell monolayers was studied at 37 °C (which included contributions from active and passive uptake) and at 4 °C (which reflects only passive diffusion) (Fig. 2). In general, uptake increased in a time-dependent manner for up to 60 min. Differences in uptake of the β compounds (**4** and **6**) between 37 and 4 °C were not significant (P = 0.12 and P = 0.25 for **4** and **6**, respectively) (Fig. 2B and D). The β compounds were therefore taken up exclusively through passive diffusion at 37 °C. In contrast, uptake of the α -compounds **3** and **5** at 37 °C was significantly higher (P <0.01) to that at 4 °C (Fig. 2A and C). The passive component did not exceed 30% of the total uptake in either case.

The active transport of **3** and **5** was then kinetically characterised. Cells were incubated for 20 min with the compounds at concentrations ranging from 1 to 100 μM at 37 °C and 4 °C. The obtained values at 4 °C were subtracted from total uptake at 37 °C, before performing kinetic analysis. The concentration-dependent uptake of the bile acid compounds, **3** and **5** was clearly saturable (Fig. 3A and B). An Eadie-Hofstee transformation (i.e., a graphical representation of transport kinetics in which reaction velocity is plotted as a function of the velocity (v) versus substrate concentration ratio (v/[S]) obtained a single straight line for both substrates, suggesting that the uptake of **3** and **5** was mediated by a single transporter site in Caco-2 cells (Fig. 3C). The kinetic parameters were estimated to be K_m = 42.27 ± 12.98 μM and V_{max} = 2.8 ± 0.4 nmol/(mg protein∗min) for **3** and K_m = 28.20±7.45 μM and V_{max} = 1.8±0.2 nmol/(mg protein∗min) for **5**, respectively. We also characterised the uptake of the cyclohexyl-NBD adduct (**7**), a substance used previously to control for non-specific fluorescent effects in a similar context (Fig. 4). Compound **7** established rapid equilibrium with the Caco-2 cells (Fig. 5), however, there was no difference in uptake whether the experiments were carried out at 37 or 4 °C, indicating that only passive diffusion contributed to cellular uptake.

The time-dependent uptake studies allowed ranking the uptake efficiency of the NBD compounds in the order 3α-NBD-DCA (**5**) >3α-NBD-UDCA (**3**) >3β-NBD-DCA (**6**) >3β-NBD-UDCA (**4**). The DCA analogues in each pair were more effectively taken up than the corresponding UDCA compound, possibly due to their greater hydrophobicity. Remarkably, the β-isomers in each case were taken up exclusively through passive diffusion, whereas both α-compounds were mostly actively transported. This peculiar diastereoselective exclusion of a fluorescent BA from active transport has not been noted in the bile acid literature as far as we are aware. Possible reasons for the observed diastereoselectivity include: (i) a steric clash between the β-group and the boundary of the pocket accommodating the steroidal A ring or (ii) a high dependence on the equatorial H-bond donor in the natural bile acid mimicked by the α-NH in the corresponding NBD analogue. This seems less likely since a loss of a H-bond opportunity would reduce binding enthalpy as reflected in the pseudo K_m value, rather than exclusion as observed. Representative low energy conformers for the DCA isomers appear in Figure 5 along with a model of DCA. Whatever its cause, the exclusion of 3β-analogues is relevant to the design of bile acid pro-drug conjugates as well as inhibitors and substrates for bile acid transporters.

The Caco-2 cell line has been used to assess the permeability of various bile acid pro-drug conjugates and it exhibits active uptake of sterically bulky 24-side chain peptide conjugates.[27]

The high diastereoselectivity of the process as well as the Eadie-Hofstee transformation suggest that the active transport process was mediated by a single transporter site. Bile acids are transported via several transport proteins such as NTCP (SLC10A1), ASBT (SLC10A2), BSEP (ABCB11), OSTα/OSTβ and OATP.[28,29] Of these, NTCP, ASBT and BSEP are reported to be inconsistently expressed in the Caco-2 literature.[30,31] Moreover, the free bile acids were not substrates for hASBT-transfected MDCK cells, although they inhibited hASBT translocation of taurocholate with low μM potency.[32] The presence of the basolateral transporters OSTα and OSTβ in Caco-2 cells was recently demonstrated by Ming et al. on mRNA level.[33] Several groups have shown numerous OATP transporters to be expressed in Caco-2 cells particularly OATP2A1, OATP4A1 and OATP2B1.[24,31,34] OATP2B1 is highly expressed in the apical cell membrane, making the transporter the most likely candidate for the uptake observed in Caco-2 cells in the present study.

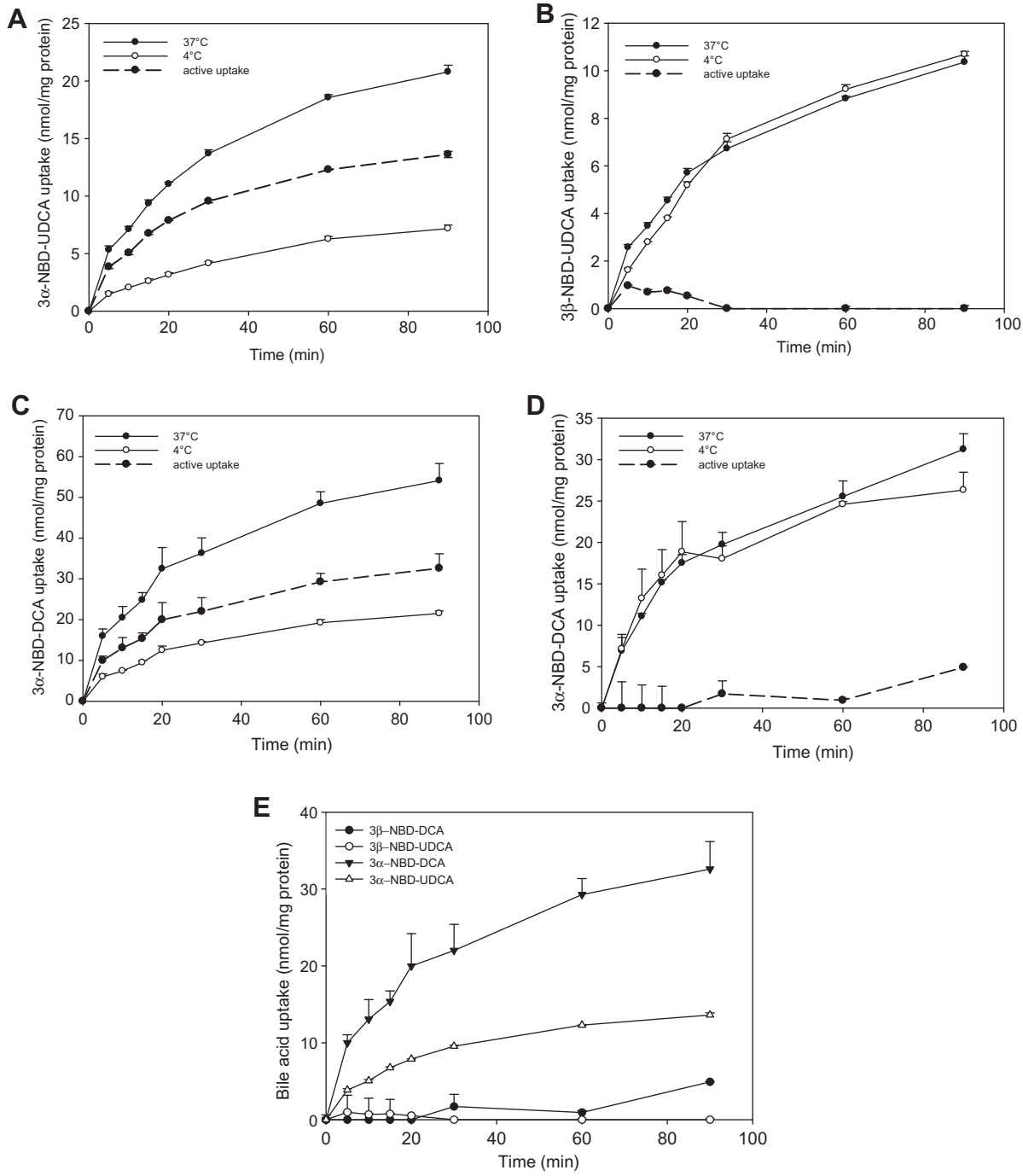


Figure 3. Time course of bile acid (**3**, **4**, **5** and **6**) uptake into Caco-2 cell monolayers. Uptake of **3** (**A**), **4** (**B**), **5** (**C**) and **6** (**D**) was studied for 90 min at 37 °C (●) and 4 °C (○). The active uptake rate of bile acid compounds (10 μM) was estimated from the difference between the fluorescence after exposure to **3**, **4**, **5** and **6** at 37 °C and 4 °C (**E**). Data represent mean ± SD (n = 3).

Previously, OATP2B1 was found to be expressed in the jejunal epithelium and has been described to mediate uptake of taurocholate with a K_m of 72 μM, which is in the same order of magnitude as observed here.[35]

The use of aminoNBD bile acids as fluorescent tools goes back almost 20 years to the synthesis, characterisation, distribution and metabolism of 3-, 7- and 12-aminoNBD analogues of CA.[12–14] In an interesting re-exploration of some of these compounds, Rohacova *et al.* reported that 3α, 3β, 7α and 7β NBD CA accumulate in freshly isolated rat hepatocytes in a manner that was transporter mediated, because uptake was markedly attenuated by troglitazone, an inhibitor of hepatic NCTP and OATP.[15] While 3α NBD CA was efficiently taken up, all of the isomers underwent active transport. The same group recently reported on some transport characteristics of a series of 3- and 7α/β CA dansyl analogues in hepatocytes.[36] In this series the 7α- and β compounds appeared to be taken up to similar extents, and, although there was a marked difference in extent of uptake of the 3-isomers, there was still significant active uptake of the 3β-analogue.

Analogues of chenodeoxycholic acid (CDCA), CA, UDCA, DCA and lithocholic acid incorporating a 24-(Nε-NBD)lysine were reported to be transported via OATP1B1 and OATP1B3-expressing

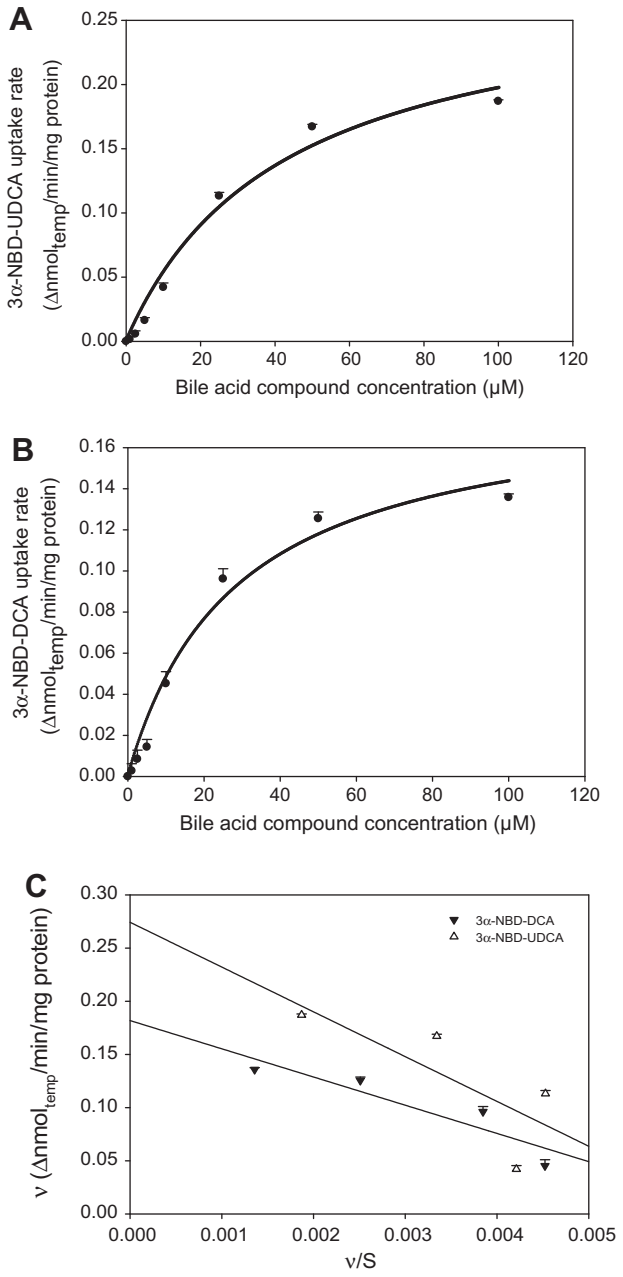


Figure 4. Concentration dependence of **3** and **5** uptake by Caco-2 cell monolayers. Uptake of bile acid compounds **3** (**A**) and **5** (**B**) was measured for 20 min at 37 °C and 4 °C. Active uptake of bile acid compounds was calculated as the difference between the fluorescence after exposure to **3** and **5** at 37 °C and 4 °C. Data represent mean ± SD (n = 3). Eadie-Hofstee transformations of the data (**C**): v, uptake rate; S, bile acid compound concentration. In Caco-2 monolayers, one single transporter site was identified for the uptake of both NBD bile acid compounds. Data are represented as mean ± SD, (n = 3).

HepG2 cells. The CDCA analogue was most efficiently transported by both OATP subtypes with K_m values one order of magnitude lower than observed in the present study for the 3α NBD compounds.[16] Fluorescent-lysyl analogues should properly be considered as reporters for the corresponding bile acid glycine conjugates since the terminal acid group is similarly placed with respect to the steroidal scaffold. In these compounds the pentyl-N(ε-NBD reporter group replaces one of the hydrogen atoms on the glycyl methylene carbon. This arrangement, which substantially increases mass and steric bulk, produces high affinity substrates for OATPs (and ASBTs) but this kind of reporter is not suitable for investigating mechanistic questions related to unconjugated bile acids. It is important to note in this context that free DCA and UDCA possess chemical properties such as size, polarity and pK_a that are distinct from their glycine and tauro conjugates and they cause different biological effects.[8]

3. Conclusions

3α- and 3β-deoxy NBD-amino derivatives of DCA and UDCA have been prepared as fluorescent probes for investigating the contrasting effects of the parent bile acid compounds for detection of organic anion-transporting peptide mediated processes. The compounds possess adequate aqueous stability and are non-toxic in several cell lines. They have similar fluorescent characteristics to previously reported NBD analogues. The uptake of the compounds was strikingly diastereospecific; whereas the 3α-compounds taken up by active transport, the 3β-compounds were not. Eadie-Hofstee analysis indicated that the active transport was mediated by a single protein. However, to identify the transporter site which is mainly mediating the uptake of fluorescent bile acids, further uptake studies would need to be carried out in relevant heterologous expression systems. 3α-NBD DCA warrants further study as a tool for functional activity studies associated with organic anion transporter proteins. The specificity of the interactions suggests that the α-compounds are able to mimic the natural bile acid parents. The 3α-UDCA/DCA pair will be employed to investigate mechanistic aspects of the functional antagonism exhibited by these compounds in intestinal biology.

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 Brucker 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 m/z 100 to 1000. 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. 24-Methyl 3α-(methylsulfonyl)oxy, 7β-formyloxy-5β-cholanoate (10)

A solution of triethylamine (0.1 ml) and methanesulfonyl chloride (0.08 ml) in anhydrous DCM (5 ml) was added dropwise to a stirred solution of **8** (0.286 g) in anhydrous DCM (20 ml) at 0 °C and the mixture allowed to warm to rt. After 20 min, when TLC analysis showed the reaction was complete, the mixture was poured into water (100 ml) and extracted with DCM (3 × 50 ml). The organic phase was dried over $MgSO_4$ then removed under reduced pressure to give the product as colourless semi-solid (0.330 g, 98%). ^{1}H

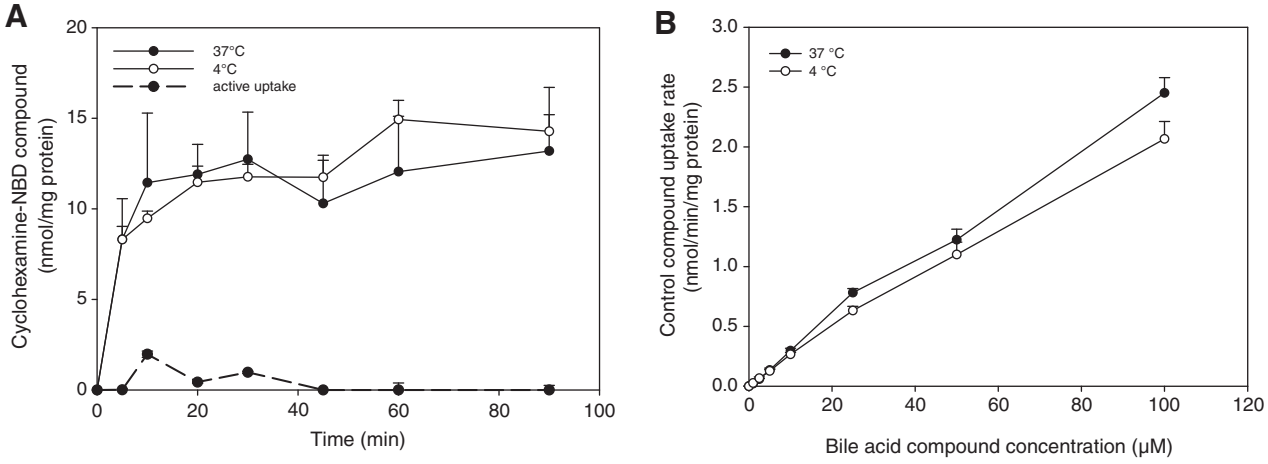


Figure 5. Uptake of the cyclohexamine NBD adduct (**7**) into Caco-2 cell monolayers was studied for 90 min at 37 °C (●) and 4 °C (○). The active component was estimated from the difference between fluorescence levels after exposure at 37 °C and 4 °C. Panel B shows concentration dependent uptake of **7** by Caco-2 cell monolayers. Uptake was measured for 20 min at 37 °C and 4 °C. Data are represented as mean ± SD, (n = 3).

NMR δ ($CDCl_3$): 7.99 (s, 1H, 7-OC═O*H*), 4.91 (6, 1H, J_1 = 5.02 Hz, J_2 = 5.52 Hz, 7α-H), 4.62 (m, 1H, 3β-H), 3.68 (s, 3H, –O–CH_3), 3.02 (s, 3H, –OSO_2CH_3), 1.00 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.02 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.12 (C═O, 24-C), 161.41 (C═O, 7-OC═OH), 81.89 (CH, 3-C), 73.83 (CH, 7-C), 53.00 (CH_3, OSO_2CH_3), 51.97 (CH_3, OCH_3). IR_{vmax} (KBr): 3438.55, 2952.63, 2874.54, 1718.71, 1354.77, 1174.23, 929.55 and 527.59 cm^{-1}. HRMS: Found: $(M-K)^+$ = 551.2451.

4.1.2. 24-Methyl 3β-azido, 7β-formyloxy-5β-cholanoate

Sodium azide (0.387 g) was added to a stirred solution of **10** (0.306 g) in DMPU (15 ml) at 50 °C and the reaction was monitored by TLC (hexane/ethyl acetate 5:1). After 8 d the mixture was partitioned between water and ethyl acetate. The organic phase was retrieved and dried over Na_2SO_4. The crude product was cleaned by column chromatography using 15% ethyl acetate in hexane as mobile phase to yield white solid as product (0.211 g, 77%). ^{1}H NMR δ ($CDCl_3$): 8.00 (s, 1H, 7-OC═O*H*), 4.89 (m, 1H, 7α-H), 3.96 (m, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 1.02 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.50 (C═O, 24-C), 160.90 (C═O, 7-OC═OH), 73.56 (CH, 7-C), 57.87 (CH, 3-C), 51.34 (CH_3, OCH_3). IR_{vmax} (KBr): 3422.62, 2946.08, 2884.65, 2111.22, 1737.16 and 1188.73 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 482.3002.

4.1.3. 24-Methyl 3β-azido, 7β-hydroxy-5β-cholanoate (12)

To a stirred solution of 24-methyl 3β-azido, 7β-formyloxy-5β-cholanoate (0.308 g) in anhydrous MeOH (30 ml) was added acetyl chloride (0.06 ml in 5 ml anhydrous MeOH) dropwise at 0 °C and allowed to warm to rt. The reaction was monitored by TLC until there was no starting material present. Then, the mixture was partitioned between saturated aq. $NaHCO_3$ and ethyl acetate. The organic phase was washed with water (2 × 100 ml) and brine (1 × 100 ml), dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give the product as light yellow semi-solid (0.273 g, 97%). ^{1}H NMR δ ($CDCl_3$): 3.93 (s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 3.55 (m, 1H, 7α-H), 0.99 (s, 3H, 19-CH_3), 0.95 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 17.86 (C═O, 24-C), 71.48 (CH, 7-C), 58.42 (CH, 3-C), 51.66 (CH_3, OCH_3). IR_{vmax} (DCM): 3431.06, 2935.49, 2866.03, 2103.17 and 1739.37 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3051.

4.1.4. 24-Methyl 3β-(*N*-BOC), 7β-hydroxy-5β-cholanoate

To a pre-reduced suspension of 10% Pd/C (~50 mg) in ethyl acetate (10 ml) was added a solution of **12** (0.273 g) and di-*tert*-butyl dicarbonate (0.166 g) in ethyl acetate (5 ml) under H_2 at rt and stirred overnight. Then the Pd/C was filtered out on a celite pad and the solvent was removed under reduced pressure. The crude product was cleaned on a flash column using 25% ethyl acetate in hexane as mobile phase to afford title compound as a white foam (0.276 g, 86%). ^{1}H NMR δ ($CDCl_3$): 4.83 (d, 1H, J = 7.03 Hz, 3-NH), 3.88 (br s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 3.56 (br s, 1H, 7α-H), 1.46 (s, 9H, –$OC(CH_3)_3$), 0.99 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.86 (C═O, 24-C), 71.41 (CH, 7-C), 51.66 (CH_3, OCH_3), 28.59 ($3CH_3$, -OC(CH_3)$_3$). IR_{vmax} (KBr): 3457.49, 2932.95, 2865.84, 1698.40, 1168.99 and 1021.60 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 528.3685.

4.1.5. 24-Methyl 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoate

24-Methyl 3β-(*N*-BOC), 7β-hydroxy-5β-cholanoate (0.269 g) was stirred in 10% TFA/DCM (20 ml) at rt until there was no BOC-protected compound evident by TLC. The solvent was removed and the residue dried under reduced pressure to give a light brown solid crude product. This was dissolved in MeOH (20 ml) then sodium bicarbonate (0.112 g) and NBD chloride (0.117 g) were added and the solution stirred at 70 °C for 5 d (monitored by TLC using hexane/ethyl acetate 1:1 as mobile phase). After this time the mixture was poured into brine (70 ml) and extracted with ethyl acetate (3 × 60 ml). The collected organic phase was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure then the product was separated by column chromatography (50% ethyl acetate in hexane as mobile phase) to give orange solid as product (0.202 g, 67%). ^{1}H NMR δ ($CDCl_3$): 8.51 (d, 1H, J = 9.03 Hz, aromatic-H), 6.42 (d, 1H, J = 7.03 Hz, 3-NH), 6.19 (d, 1H, J = 8.53 Hz, aromatic-H), 4.26 (s, 1H, 7β-OH), 4.10 (s, 1H, 3α-H), 3.69 (s, 3H, –O–CH_3), 3.61 (s, 1H, 7α-H), 1.10 (s, 3H, 19-CH_3), 0.96 (d, 3H, J = 6.52 Hz, 21-CH_3), 0.71 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.15 (C═O, 24-C), 144.86 (C, aromatic-C), 144.36 (C, aromatic-C), 143.21 (C, aromatic-C), 136.94 (CH, aromatic-C), 124.29 (C, aromatic-C), 99.30 (CH, aromatic-C), 71.58 (CH, 7-C) 52.00 (CH_3, OCH_3). IR_{vmax} (KBr): 3404.92, 2932.39, 2865.09, 1736.45, 1574.72, 1307.25 and 1265.61 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 591.3157.

4.1.6. 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoic acid (4)

24-Methyl 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoate (0.2 g) was dissolved in a mixture of MeOH

(20 ml) and 2 M NaOH (pH ~14) at 40 °C for 3 h. The mixture was poured into 2 M HCl (50 ml) and extracted with ethyl acetate (3 × 50 ml). The crude product was cleaned by flash chromatography using DCM/MeOH 9:1 as mobile phase to give orange solid as product (0.188 g, 96%). Mp: 208–210 °C. IR_{vmax} (KBr): 3413.89, 2931.04, 2865.99, 1574.58, 1307.39 and 1250.03 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 577.3031.

4.1.7. 24-Methyl 3β-bromo, 7β-formyloxy-5β-cholanoate (14)

Triphenylphosphine (2.097 g) was added to a solution of **8** (1.738 g) in anhydrous THF (80 ml) and *N*-bromosuccinimide (1.423 g) was added in 3 parts over 1 h at −18 °C then allowed to warm to rt and stirred overnight. The reaction mixture was poured into 1 M HCl solution (100 ml) and extracted with ethyl acetate (3 × 75 ml). The organic layer was washed with brine (1 × 100 ml) and after drying over $MgSO_4$ the solvent was removed under reduced pressure. The crude product was flash columned (hexane/ethyl acetate 5:1) to afford the product as white foam (1.694 g, 85%). ^1H NMR δ ($CDCl_3$): 7.99 (s, 1H, 7-OC=O*H*), 4.84 (m, 1H, 7α-H), 4.76 (s, 1H, 3α-H), 3.67 (s, 3H, –O–CH_3), 1.06 (s, 3H, 19-CH_3), 0.93 (d, 3H, *J* = 6.53 Hz, 21-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.12 (C=O, 24-C), 161.50 (C=O, 7-OC=OH), 74.41 (CH, 7-C), 51.97 (CH_3, OCH_3). IR_{vmax} (DCM): 2947.48, 2871.35, 1737.49, 1719.36 and 1178.56 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 519.2070.

4.1.8. 24-Methyl 3α-azido, 7β-formyloxy-5β-cholanoate

To a solution of **14** (1.693 g) in DMPU (50 ml) was added sodium azide (2.21 g) at rt and stirred overnight. Then the reaction mixture was poured into water (100 ml) and extracted with ethyl acetate (3 × 75 ml). The organic phase was washed with brine (1 × 100 ml), dried over Na_2SO_4, filtered and the solvent was evaporated in vacuum. The crude product was cleaned on a flash column using hexane/ethyl acetate 5:1 as mobile phase to obtain the product as colourless oil (1.464 g, 94%). ^1H NMR δ ($CDCl_3$): 8.00 (s, 1H, 7-OC=O*H*), 4.91 (6, 1H, J_1 = 5.27 Hz, J_2 = 5.53 Hz, 7α-H), 3.68 (s, 3H, –O–CH_3), 3.30 (m, 1H, 3β-H), 1.00 (s, 3H, 19-CH_3), 0.94 (d, 3H, *J* = 6.52 Hz, 21-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.81 (C=O, 24-C), 161.12 (C=O, 7-OC=OH), 73.76 (CH, 7-C), 60.81 (CH, 3-C), 51.64 (CH_3, OCH_3). IR_{vmax} (DCM): 2946.73, 2871.54, 2093.30, 1720.62, 1453.67, 1255.13 and 1187.30 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 482.2998.

4.1.9. 24-Methyl 3α-azido, 7β-hydroxy-5β-cholanoate (16)

Acetyl chloride (0.65 ml) was added dropwise to a stirred solution of 24-methyl 3α-azido, 7β-formyloxy-5β-cholanoate (1.411 g) in anhydrous MeOH (60 ml) at 0 °C and allowed to warm to rt. After overnight the reaction mixture was poured into saturated $NaHCO_3$ (100 ml) and extracted with ethyl acetate (3 × 100 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was cleaned on flash column (hexane/ethyl acetate 3:1) to give the product as yellow semi-solid (1.210 g, 91%). ^1H NMR δ ($CDCl_3$): 3.69 (s, 3H, –O–CH_3), 3.60 (m, 1H, 7α-H), 3.30 (m, 1H, 3β-H), 0.98 (s, 3H, 19-CH_3), 0.95 (d, 3H, *J* = 6.27 Hz, 21-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.85 (C=O, 24-C), 71.29 (CH, 7-C), 60.99 (CH, 3-C), 51.65 (CH_3, OCH_3). IR_{vmax} (DCM): 3424.26, 2938.77, 2866.74, 2092.43, 1739.29, 1452.41, 1254.58, 1167.83 and 1022.77 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3057.

4.1.10. 24-Methyl 3α-(*N*-BOC), 7β-hydroxy-5β-cholanoate

Pd/C (~100 mg) in ethyl acetate (30 ml) was pre-reduced under H_2 atmosphere over 30 min then a solution of **16** (1.182 g) and di-*tert*-butyl dicarbonate (0.717 g) in ethyl acetate (20 ml) was added via syringe and the mixture stirred at rt overnight. The reaction mixture was filtered through a celite pad and washed with ethyl acetate (3 × 10 ml). The solvent was removed under reduced pressure and the crude product was flash columned using 30% ethyl acetate in hexanes as mobile phase to yield the product as white foam (1.300 g, 94%). ^1H NMR δ ($CDCl_3$): 4.42 (br s, 1H, 3-NH), 3.68 (s, 3H, –O–CH_3), 3.56 (m, 1H, 7α-H), 3.39 (br s, 1H, 3β-H), 1.45 (s, 9H, -OC(CH_3)$_3$), 0.96 (s, 3H, 19-CH_3), 0.95 (d, 3H, *J* = 6.27 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.85 (C=O, 24-C), 71.48 (CH, 7-C), 51.65 (CH_3, OCH_3), 28.56 ($3CH_3$, –OC(CH_3)$_3$). IR_{vmax} (DCM): 3380.47, 2933.70, 1692.59 and 1170.19 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 528.3669.

4.1.11. 24-Methyl 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoate

24-Methyl 3α-(*N*-BOC), 7β-hydroxy-5β-cholanoate (1.265 g) was dissolved in 10% TFA/DCM (60 ml) and stirred at rt overnight (followed by TLC analysis using hexane/ethyl acetate 1:1 as mobile phase) then, the solvent was removed under vacuum. The residue was further dried by successive evaporation of toluene (3 × 100 ml). The yield foam was dissolved in MeOH (50 ml) then $NaHCO_3$ (2.102 g) and NBD chloride (0.549 g) were added to the solution. The reaction mixture was stirred vigorously for 2 weeks at reflux; reaction completion was checked by TLC using hexane:ethyl acetate (50:50) as mobile phase. It was poured into brine (150 ml) and extracted with ethyl acetate (3 × 100 ml). The organic phase was dried over Na_2SO_4, filtered and the solvent was removed in vacuum to afford the crude product as black semi-solid. This was purified with flash column using 40–70% ethyl acetate in hexanes as mobile phase to obtain the product as orange foam (0.622 g, 44%). ^1H NMR δ ($CDCl_3$): 8.49 (d, 1H, *J* = 8.54 Hz, aromatic-H), 6.88 (d, 1H, *J* = 7.53 Hz, 3-NH), 6.19 (d, 1H, *J* = 9.04 Hz, aromatic-H), 3.67 (s, 5H, –O–CH_3, 7α-H, 3β-H), 1.08 (s, 3H, 19-CH_3), 0.95 (d, 3H, *J* = 6.03 Hz, 21-CH_3), 0.72 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.44 (C=O, 24-C), 144.16 (C, aromatic-C), 143.87 (C, aromatic-C), 136.48 (CH, aromatic-C), 123.07 (C, aromatic-C), 71.14 (CH, 7-C), 51.35 (CH_3, OCH_3). IR_{vmax} (KBr): 3402.60, 2933.80, 2867.01, 1733.12, 1579.17 and 1304.77 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 591.3169.

4.1.12. 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoate (3)

NaOH (2 M) was added to a stirred methanolic solution of 24-methyl 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 7β-hydroxy-5β-cholanoate (0.589 g) and the mixture warmed to 40 °C. The reaction mixture was left at this temperature overnight then poured into 2 M HCl (100 ml) and extracted with ethyl acetate (3 × 75 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography using 2% acetic acid in ethyl acetate as mobile phase. The product was recrystallised from hexane to give orange solid as final product (0.465 g, 81%). Mp: 148–150 °C. ^1H NMR δ (acetone-d_6): 8.51 (d, 1H, *J* = 9.04 Hz, aromatic-H), 6.55 (d, 1H, *J* = 9.04 Hz, aromatic-H), 3.93 (br s, 1H, 7α-H), 3.51 (m, 1H, 3β-H), 1.05 (s, 3H, 19-CH_3), 0.97 (d, 3H, *J* = 6.53 Hz, 21-CH_3), 0.72 (s, 3H, 18-CH_3). ^{13}C NMR ppm (acetone-d_6): 175.03 (C=O, 24-C), 70.94 (CH, 7-C). IR_{vmax} (KBr): 3436.13, 2932.86, 2868.26, 1708.42, 1621.14, 1579.48 and 1304.52 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 577.3005.

4.1.13. 24-Methyl 3α-(methylsulfonyl)oxy, 12α-formyloxy-5β-cholanoate (11)

The solution of triethylamine (0.1 ml) and methanesulfonyl chloride (0.15 ml) in anhydrous DCM (5 ml) was added drop-wise to a stirred solution of **9** (0.279 g) in anhydrous DCM (20 ml) at 0 °C and allowed to warm to rt. After 20 min when TLC analysis showed the reaction was complete the mixture was poured into H_2O (50 ml) and extracted with DCM (3 × 50 ml). The organic phase

was dried over $MgSO_4$ then removed under reduced pressure to give the product as colourless oil (0.322 g, 98%). ^{1}H NMR δ ($CDCl_3$): 8.15 (s, 1H, 12-OC=O*H*), 5.26 (s, 1H, 12β-H), 4.63 (m, 1H, 3β-H), 3.67 (s, 3H, –O–CH_3), 3.01 (s, 3H, -OSO_2CH_3), 0.93 (s, 3H, 19-CH_3), 0.85 (d, 3H, J = 6.03 Hz, 21-CH_3), 0.75 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.69 (C=O, 24-C), 160.71 (C=O, 12-OC=OH), 82.79 (CH, 3-C), 75.97 (CH, 12-C), 52.67 (CH_3, OSO_2CH_3), 51.66 (CH_3, O*C*H_3). IR_{vmax} (DCM): 2947.01, 2871.16, 1717.89, 1353.88, 1174.09 and 932.87 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 535.2710.

4.1.14. 24-Methyl 3β-azido, 12α-formyloxy-5β-cholanoate

Sodium azide (0.408 g) was added to a stirred solution of **11** (0.322 g) in DMPU (20 ml) at 50 °C and the reaction was monitored by TLC (hexane:ethyl acetate 5:1). After 6 d the mixture was partitioned between water and ethyl acetate. The organic phase was retrieved and dried over Na_2SO_4. The crude product was cleaned by column chromatography using 20% ethyl acetate in hexane as mobile phase to yield colourless oil as product (0.253 g, 87%). ^{1}H NMR δ ($CDCl_3$): 8.13 (s, 1H, 12-OC=O*H*), 5.26 (s, 1H, 12β-H), 3.95 (s, 1H, 3α-H), 3.67 (s, 3H, –O–CH_3), 0.95 (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 ($CDCl_3$): 174.39 (C=O, 24-C), 160.37 (C=O, 12-OC=OH), 76.00 (CH, 12-C), 58.35 (CH, 3-C), 51.35 (CH_3, O*C*H_3). IR_{vmax} (DCM): 2938.73, 2872.92, 2101.83, 1738.92, 1720.86 and 1171.43 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 482.2974.

4.1.15. 24-Methyl 3β-azido, 12α-hydroxy-5β-cholanoate (13)

To a stirred solution of 24-methyl 3β-azido, 12α-formyloxy-5β-cholanoate (0.322 g) in anhydrous MeOH (30 ml) was added acetyl chloride (0.07 ml in 5 ml anhydrous MeOH) dropwise at 0 °C and allowed to warm to rt. The reaction was monitored by TLC until there was no starting material present. Then the mixture was partitioned between saturated $NaHCO_3$ and ethyl acetate. 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 reduced pressure to give the product as yellow semi-solid (0.294 g, 97%). ^{1}H NMR δ ($CDCl_3$): 4.01 (s, 1H, 12β-H), 3.97 (s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 0.99 (d, 3H, J = 5.87 Hz, 21-CH_3), 0.96 (s, 3H, 19-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.51 (C=O, 24-C), 73.00 (CH, 12-C), 58.52 (CH, 3-C), 51.36 (CH_3, O*C*H_3). IR_{vmax} (KBr): 3528.85, 2932.82, 2093.51, 1725.62, 1449.91, 1312.94 and 1196.78 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3052.

4.1.16. 24-Methyl 3β-(*N*-BOC), 12α-hydroxy-5β-cholanoate

To a pre-reduced suspension of 10% Pd/C (~50 mg) in ethyl acetate (10 ml) was added a solution of **13** (0.245 g) and di-*tert*-butyl dicarbonate (0.149 g) in ethyl acetate (5 ml) and stirred under H_2 at rt. The reaction mixture was stirred overnight (TLC: hexane:ethyl acetate 3:1) then it was filtered on a celite pad. The celite was washed with ethyl acetate (3 × 10 ml) and the organic phase was removed in vacuum. The product was cleaned by flash chromatography (mobile phase: 30% ethyl acetate in hexane) to yield a white foam as product (0.260 g, 90%). ^{1}H NMR δ ($CDCl_3$): 4.84 (br s, 1H, 3-NH), 4.00 (s, 1H, 12β-H), 3.92 (br s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 1.46 (s, 9H, –OC(CH_3)$_3$), 0.99 (d, 3H, J = 6.02 Hz, 21-CH_3), 0.96 (s, 3H, 19-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.54 (C=O, 24-C), 72.97 (CH, 12-C), 51.34 (CH_3, OCH_3), 28.29 (3CH_3, –OC(*C*H_3)$_3$). IR_{vmax} (KBr): 3459.32, 2937.54, 2865.92, 1698.37 and 1171.03 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 528.3654.

4.1.17. 24-Methyl 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate

24-Methyl 3β-(*N*-BOC), 12α-hydroxy-5β-cholanoate (0.259 g) was stirred in 10% TFA/DCM (20 ml) at rt until there was no BOC-protected compound present. Then the solvent was removed and the residue dried under reduced pressure to give light brown solid as crude product. This was dissolved in MeOH (20 ml) then $NaHCO_3$ (0.130 g) and NBD chloride (0.112 g) were added to the solution and stirred at 70 °C for 10 d (monitored by TLC using hexane/ethyl acetate 1:1 as mobile phase). After this time the mixture was poured into brine (50 ml) and extracted with ethyl acetate (3 × 50 ml). The collected organic phase was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure then the product was separated by column chromatography (50% ethyl acetate in hexane as mobile phase) to give orange solid as product (0.213 g, 73%). ^{1}H NMR δ ($CDCl_3$): 8.49 (d, 1H, J = 9.04 Hz, aromatic-H), 6.47 (d, 1H, J = 7.03 Hz, 3-NH), 6.19 (d, 1H, J = 9.04 Hz, aromatic-H), 4.26 (s, 1H, 12α-OH), 4.12 (s, 1H, 3α-H), 4.05 (s, 1H, 12β-H), 3.69 (s, 4H, –O–CH_3), 1.03 (s, 3H, 19-CH_3), 1.00 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.72 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.10 (C=O, 24-C), 144.88 (C, aromatic-C), 143.30 (C, aromatic-C), 137.07 (CH, aromatic-C), 134.40 (C, aromatic-C), 124.07 (C, aromatic-C), 104.33 (CH, aromatic-C), 73.53 (CH, 12-C), 52.01 (CH_3, OCH_3). IR_{vmax} (KBr): 3538.32, 3408.93, 2943.25, 2864.97, 1736.80, 1574.32, 1336.49 and 1314.61 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 591.3163.

4.1.18. 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate (6)

The hydrolysis of 24-methyl 3β-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate (0.205 g) was carried out in MeOH (20 ml) with 2 M NaOH at pH ~14 at 40 °C for 4 h. The mixture was poured into 2 M HCl (50 ml) and extracted with ethyl acetate (3 × 50 ml). The crude product was purified by column chromatography using ethyl acetate as mobile phase to afford the product as orange solid (0.152 g, 76%). Mp: 216–218 °C. ^{1}H NMR δ (DMSO-d_6): 8.31 (d, 1H, J = 9.03 Hz, aromatic-H), 6.29 (s, 1H, aromatic-H), 4.10 (br s, 1H, 3α-OH), 3.81 (s, 1H, 12β-H), 0.93 (s, 6H, 21-CH_3 and 19-CH_3), 0.61 (s, 3H, 18-CH_3). ^{13}C NMR ppm (DMSO-d_6): 175.27 (C=O, 24-C), 145.65 (C, aromatic-C), 144.87 (C, aromatic-C), 135.39 (CH, aromatic-C), 131.60 (C, aromatic-C), 128.66 (C, aromatic-C), 100.02 (CH, aromatic-C), 71.10 (CH, 12-C). IR_{vmax} (KBr): 3410.69, 2927.61, 2863.42, 1703.83, 1575.98 and 1310.90 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 577.3019.

4.1.19. 24-Methyl 3β-bromo, 12α-formyloxy-5β-cholanoate (15)

Triphenylphosphine (2.135 g) was added to a solution of **9** (1.769 g) in anhydrous THF (80 ml) and *N*-bromosuccinimide (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 ethyl acetate (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:ethyl acetate 5:1) to afford the product as pale white solid (1.919 g, 95%). ^{1}H NMR δ ($CDCl_3$): 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 ($CDCl_3$): 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.

4.1.20. 24-Methyl 3α-azido, 12α-formyloxy-5β-cholanoate

To a solution of **15** (1.852 g) in DMPU (50 ml) was added sodium azide (2.420 g) at rt and stirred overnight. Then the reaction mixture was poured into water (100 ml) and extracted with ethyl acetate (3 × 50 ml). The organic phase was washed with brine (100 ml), dried over Na_2SO_4, filtered and the solvent was evaporated in vacuum. The crude product was cleaned on a flash column using hexane:ethyl acetate 5:1 as mobile phase to obtain the product as colourless semi-solid (1.652 g, 97%). ^{1}H NMR δ ($CDCl_3$): 8.14

(s, 1H, 12-OC=O*H*), 5.26 (s, 1H, 12β-H), 3.67 (s, 3H, –O–CH_3), 3.34 (m, 1H, 3β-H), 0.93 (s, 3H, 19-CH_3), 0.85 (d, 3H, J = 6.03 Hz, 21-CH_3), 0.75 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.40 (C=O, 24-C), 160.42 (C=O, 12-OC=OH), 75.70 (CH, 12-C), 60.98 (CH, 3-C), 51.34 (CH_3, OCH_3). IR_{vmax} (DCM): 2941.27, 2867.58, 2092.01, 1738.62, 1720.31, 1448.60, 1251.73 and 1177.39 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 482.2995.

4.1.21. 24-Methyl 3α-azido, 12α-hydroxy-5β-cholanoate (17)

Acetyl chloride (0.51 ml) was added drop-wise to a stirred solution of 24-methyl 3α-azido, 12α-formyloxy-5β-cholanoate (1.645 g) in anhydrous MeOH (60 ml) at 0 °C and allowed to warm to rt. After overnight stirring, the reaction mixture was poured into saturated $NaHCO_3$ (100 ml) and extracted with ethyl acetate (3 × 100 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The product was recrystallised from diethyl ether to obtain light-yellow crystals (1.457 g, 94%). ^{1}H NMR δ ($CDCl_3$): 4.00 (s, 1H, 12β-H), 3.68 (s, 3H, –O–CH_3), 3.35 (m, 1H, 3β-H), 0.99 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.94 (s, 3H, 19-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.15 (C=O, 24-C), 73.50 (CH, 12-C), 61.69 (CH, 3-C), 51.97 (CH_3, OCH_3). IR_{vmax} (DCM): 3524.40, 2939.82, 2866.15, 2091.49, 1739.60, 1448.24 and 1252.74 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 454.3038.

4.1.22. 24-Methyl 3α-(*N*-BOC), 12α-hydroxy-5β-cholanoate

A suspension of 10% Pd/C (~100 mg) in ethyl acetate (30 ml) was pre-reduced under hydrogen atmosphere over 30 min then the solution of **17** (1.424 g) and di-*tert*-butyl dicarbonate (0.864 g) in ethyl acetate (20 ml) was added via syringe and stirred at rt overnight. The reaction mixture was filtered through a celite pad and washed with ethyl acetate (3 × 10 ml). The solvent was removed under reduced pressure and the crude product was flash columned using 30% ethyl acetate in hexanes as mobile phase to yield the product as white foam (1.549 g, 93%). ^{1}H NMR δ ($CDCl_3$): 4.45 (br s, 1H, 3-NH), 4.00 (s, 1H, 12β-H), 3.68 (s, 3H, –O–CH_3), 3.43 (br s, 1H, 3β-H), 1.45 (s, 9H, -OC(CH_3)$_3$), 0.99 (d, 3H, J = 6.27 Hz, 21-CH_3), 0.93 (s, 3H, 19-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.83 (C=O, 24-C), 73.30 (CH, 12-C), 51.65 (CH_3, OCH_3), 28.57 (3CH_3, -OC(CH_3)$_3$). IR_{vmax} (DCM): 3380.58, 2935.81, 1691.31 and 1171.11 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 528.3666.

4.1.23. 24-Methyl 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate

24-Methyl 3α-(*N*-BOC), 12α-hydroxy-5β-cholanoate (1.524 g) was dissolved in 10% TFA/DCM (60 ml) and stirred at rt overnight (followed by TLC analysis using hexane:ethyl acetate 3:1 as mobile phase) then the solvent was removed in vacuum. The residue was dried under reduced pressure using toluene (3 × 100 ml). The yield foam was dissolved in MeOH (60 ml) then $NaHCO_3$ (2.533 g) and NBD chloride (0.862 g) were added to the solution. The reaction mixture was stirred vigorously for 10 d at reflux; reaction completion was checked by TLC using hexane:ethyl acetate (50:50) as mobile phase. It was poured into brine (100 ml) and extracted with ethyl acetate (3 × 100 ml). The organic phase was dried over Na_2SO_4, filtered and the solvent was removed in vacuum to afford the crude product as black semi-solid. This was purified with flash column using 30–40% ethyl acetate in hexane as mobile phase to obtain the product as orange foam (0.679 g, 98%). ^{1}H NMR δ ($CDCl_3$): 8.46 (d, 1H, J = 8.53 Hz, aromatic-H), 6.51 (s, 1H, 3-NH), 6.17 (d, 1H, J = 8.53 Hz, aromatic-H), 4.26 (s, 1H, 12α-OH), 4.07 (s, 1H, 12β-H), 3.67 (s, 4H, 3β-H, –O–CH_3), 1.02 (s, 3H, 19-CH_3), 0.97 (d, 3H, J = 6.03 Hz, 21-CH_3), 0.71 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.48 (C=O, 24-C), 144.09 (C, aromatic-C), 143.78 (C, aromatic-C), 136.50 (CH, aromatic-C), 133.78 (C, aromatic-C), 122.93 (C, aromatic-C), 103.71 (CH, aromatic-C), 72.95 (CH, 12-C), 51.36 (CH_3, OCH_3). IR_{vmax} (DCM): 3529.91, 3294.51, 2938.41, 2867.28, 1735.15, 1582.76 and 1304.42 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 591.3142.

4.1.24. 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate (5)

Methyl 3α-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino, 12α-hydroxy-5β-cholanoate (1.621 g) was dissolved in a mixture of methanol and aq. NaOH (2 M) and the mixture warmed to 40 °C. The reaction mixture was left at this temperature overnight then poured into 2 M HCl (100 ml) and extracted with ethyl acetate (3 × 100 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was flash columned using first 20% hexane then 2% acetic acid in ethyl acetate as mobile phase to yield orange solid as product (0.997 g, 63%). Mp: 225 °C. ^{1}H NMR δ (DMSO): 11.93 (s, 1H, 24-COOH), 9.52 (s, 1H, 3-NH), 8.47 (d, 1H, J = 8.54 Hz, aromatic-H), 6.46 (d, 1H, J = 9.04 Hz, aromatic-H), 4.25 (s, 1H, 12α-OH), 3.82 (s, 2H, 12β-H and 3β-H), 0.93 (d, 6H, J = 4.52 Hz, 21-CH_3 and 19-CH_3), 0.61 (s, 3H, 18-CH_3). ^{13}C NMR ppm (DMSO): 174.98 (C=O, 24-C), 144.40 (C, aromatic-C), 144.27 (C, aromatic-C), 138.01 (CH, aromatic-C), 136.18 (C, aromatic-C), 120.06 (C, aromatic-C), 105.85 (CH, aromatic-C), 70.89 (CH, 12-C). IR_{vmax} (DCM): 3309.77, 2937.30, 2859.90, 1707.17, 1582.18 and 1304.06 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 577.2999.

4.2. Stability and physiochemical characterisation

For the HPLC measurements, a WATERS Alliance system was used with 2475 Multi λ Fluorescence Detector and 2487 Dual λ Absorbance Detector controlled by EMPOWER Software. The applied column symmetry was C18 5 μm 4.6 × 250 mm from Xbridge™. For the stability study of **4**, a stock solution was prepared in 0.01 mg/ml concentration with 2% MeCN/phosphate buffer (pH = 7.4). The stability study was carried out at 37 °C over 24 h with injection every h. HPLC conditions were flow rate: 1.0 ml/min; wavelength of detection: 346 nm; injection volume: 20 μl; temperature of column and sample: 37 °C; runtime: 25 min; gradient 0 min 50% mobile phases A and B, 0–10 min increase to 80% mobile phase B, 10–20 min 80% mobile phase B, 20–21 min decrease to 50% mobile phase B, 21–25 min 50% mobile phases A and B; mobile phase A: pH = 2.5 phosphate buffer; mobile phase B: MeCN.

To assess lipophilicity of **3**–**6** the samples were prepared (1 μM) in MeCN/water 75:15. HPLC conditions were flow rate: 1.5 ml/min; wavelength of detection: 335 nm (absorbance detector); wavelength of excitation: 470 nm, wavelength of detection: 535 nm (fluorescence detector); injection volume: 20 μl; temperature of column and sample: rt; runtime: 10 min; mobile phase: isocratic–water/1% pH = 3.0 ammonium formate buffer/MeCH (15:10:75).

4.3. Photophysical characterisation

A Cary 300 Scan UV–Visible Spectrophotometer was used to determine the absorption maxima of **3**–**6**. The stock solutions were prepared at 0.01 mg/ml in EtOH. Samples were scanned from 200–800 nm. Cuvette type was Hella Blue quartz 10 mm. To obtain the fluorescence maxima the stock solutions were prepared in 0.001 mg/ml concentration in ethanol and measured with RF-1501 Spectrofluorophotometer (SHIMADZU). The emission wavelength range was chosen between 220–900 nm, while the excitation wavelength was 470 nm. Fluorescein and rhodamine 6G, were cross calibrated by calculating the quantum yield of each relative to the other. The results matched the literature ±10%: fluorescein Φ*F* 0.922, rhodamine 6G Φ*F* 0.953. The absorbance and

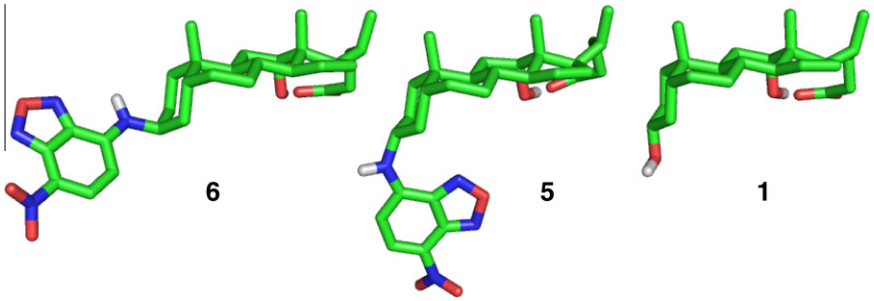


Figure 6. Minimum energy conformations for DCA-3-α and β analogues **5** and **6** and DCA. These were generated by modifying a model of CA (pdb code 2QO4) in MOE and energy minimising with MMFF94x (this approach predicts a H-bond between the acid chain and 12α-OH). The picture shows the different orientations available to the 3-NH in the two configurations. In the α-orientation, the NH may mimic the OH group of the parent bile acid in H-bond donation capacity.

emission levels in the concentration range 10–50 μM for five solutions of each sample and standard were obtained. (Fluorescein in ethanolic KOH (0.01 M), all other solutions in EtOH.) The slope of a plot of fluorescence intensity versus absorbance was used to calculate the quantum yield (ΦF) for each sample using the equation: $\Phi F = \Phi_{ST}(S_x/S_{ST})$.

4.4. Uptake studies

Caco-2 cells were grown to polarised monolayers on 24-well plates for 21 d, before uptake studies were performed in bicarbonated Krebs-Ringer buffer (KRB: 15 mM HEPES, 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$ and 5.55 mM glucose; pH 7.4). Uptake of the fluorescent bile acid compounds (**3–6**) was studied at different time points, concentrations and temperatures. Solutions of bile acid compounds were prepared using KRB containing 0.1% DMSO at all times.

Time-dependence of bile acid uptake was studied by incubating cell monolayers (at 10 μM) for up to 90 min at either 37 or 4 °C. After 5, 10, 15, 30, 45, 60 and 90 min, monolayers were washed twice with ice-cold KRB, solubilised in 1% (w/v) Triton X-100 solution and bile acid activity was measured in the cell lysate (see below). Concentration-dependence of bile acid uptake was studied at a compound concentration range of 1–100 μM for 20 min at 37 °C and 4 °C. Active uptake of bile acid compounds was calculated as the difference between the fluorescence, after exposure to compounds **3–6** at 37 °C and 4 °C.

The fluorescence activity of the bile acids compounds was analysed in 24-well plates using an automated plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively. The samples were diluted with KRB, where appropriate. For standardization, the total protein amount of cell layers was determined by bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, USA).

4.5. Uptake kinetics

The saturable uptake of the bile acid compounds was analysed by assuming Michaelis–Menten type carrier-mediated transport represented by the equation:

$$v = V_{max} \times (S)/[(K_m + (S)]$$

where (S) was [bile acid compound].

Half-saturation constant (K_m) and maximum uptake rates (V_{max}) of the bile acid compounds was calculated by this equation to the experimental profile of the uptake rate (v) versus the substrate concentration (S) using a non-linear least squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA, USA).

4.6. Molecular modelling

The structures of DCA and its 3-α and -β NBD analogues were generated by in silico modification in MOE (Chemical Computing Group, Montreal, Canada) of cholic acid extracted from pdb code 2QO4[37] The bile acid models were energy minimised using Merck Molecular Force Field 94x (MMFF94x) interfaced to MOE with gradient 0.5. Then structures were further manipulated in MOE and the picture in Figure 6 generated using the programme PyMOL.

4.7. Statistical analysis

All uptake studies were performed in triplicate. Results were expressed as mean ± SD, were compared using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test. P <0.05 was considered as significant.

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References and notes

1. Payne, C. M.; Crowley-Skillicorn, C.; Bernstein, C.; Holubec, H.; Moyer, M. P.; Bernstein, H. *Nutr. Cancer* **2011**, *62*, 825.
2. Zhou, Y.; Doyen, R.; Lichtenberger, L. M. *Biochim. Biophys. Acta.* **2009**, *1788*, 507.
3. Amaral, J. D.; Viana, R. J.; Ramalho, R. M.; Steer, C. J.; Rodrigues, C. M. *J. Lipid Res.* **2009**, *50*, 1721.
4. Rodrigues, C. M.; Steer, C. J. *Expert Opin. Investig. Drugs.* **2001**, *10*, 1243.
5. Sola, S.; Aranha, M. M.; Steer, C. J.; Rodrigues, C. M. *Curr. Issues Mol. Biol.* **2007**, *9*, 123–138.
6. Byrne, A. M.; Foran, E.; Sharma, R.; Davies, A.; Mahon, C.; O'Sullivan, J.; O'Donoghue, D.; Kelleher, D.; Long, A. *Carcinogenesis* **2010**, *31*, 737.
7. Hofmann, A. F. *Arch. Intern. Med.* **1999**, *159*, 2647.
8. Hofmann, A. F.; Hagey, L. R. *Cell Mol. Life Sci.* **2008**, *65*, 2461.
9. Sharma, R.; Long, A.; Gilmer, J. F. *Curr. Med. Chem.* **2011**, *18*, 4029.
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. Ghosh, P. B.; Whitehouse, M. W. *Biochem. J.* **1968**, *108*, 155.
12. Schramm, U.; Dietrich, A.; Schneider, S.; Buscher, H. P.; Gerok, W.; Kurz, G. *J. Lipid Res.* **1991**, *32*, 1769.
13. Schneider, S.; Schramm, U.; Schreyer, A.; Buscher, H. P.; Gerok, W.; Kurz, G. *J. Lipid Res.* **1991**, *32*, 1755.
14. Schramm, U.; Fricker, G.; Buscher, H. P.; Gerok, W.; Kurz, G. *J. Lipid Res.* **1993**, *34*, 741.
15. Rohacova, J.; Marin, M. L.; Martinez-Romero, A.; Diaz, L.; O'Connor, J. E.; Gomez-Lechon, M. J.; Donato, M. T.; Castell, J. V.; Miranda, M. A. *ChemMedChem* **2009**, *4*, 466.
16. Yamaguchi, H.; Okada, M.; Akitaya, S.; Ohara, H.; Mikkaichi, T.; Ishikawa, H.; Sato, M.; Matsuura, M.; Saga, T.; Unno, M.; Abe, T.; Mano, N.; Hishinuma, T.; Goto, J. *J. Lipid Res.* **2006**, *47*, 1196.
17. Holzinger, F.; Schteingart, C. D.; Ton-Nu, H. T.; Eming, S. A.; Monte, M. J.; Hagey, L. R.; Hofmann, A. F. *Hepatology* **1997**, *26*, 1263.
18. Sola, S.; Amaral, J. D.; Castro, R. E.; Ramalho, R. M.; Borralho, P. M.; Kren, B. T.; Tanaka, H.; Steer, C. J.; Rodrigues, C. M. *Hepatology* **2005**, *42*, 925.

19. Sola, S.; Castro, R. E.; Kren, B. T.; Steer, C. J.; Rodrigues, C. M. *Biochemistry* **2004**, *43*, 8429.
20. Assfalg, M.; Gianolio, E.; Zanzoni, S.; Tomaselli, S.; Russo, V. L.; Cabella, C.; Ragona, L.; Aime, S.; Molinari, H. *J. Med. Chem.* **2007**, *50*, 5257.
21. Zheng, X.; Ekins, S.; Raufman, J. P.; Polli, J. E. *Mol. Pharm.* **2009**, *6*, 1591.
22. Hu, N. J.; Iwata, S.; Cameron, A. D.; Drew, D. *Nature* **2011**, *478*, 408.
23. Balakrishnan, A.; Polli, J. E. *Mol. Pharm.* **2006**, *3*, 223.
24. Endter, S.; Francombe, D.; Ehrhardt, C.; Gumbleton, M. *J. Pharm. Pharmacol.* **2009**, *61*, 583.
25. Alrefai, W. A.; Sarwar, Z.; Tyagi, S.; Saksena, S.; Dudeja, P. K.; Gill, R. K. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2005**, *288*, G978.
26. Geyer, J.; Wilke, T.; Petzinger, E. *Naunyn. Schmiedebergs Arch. Pharmacol.* **2006**, *372*, 413.
27. Swaan, P. W.; Hillgren, K. M.; Szoka, F. C., Jr.; Oie, S. *Bioconjug. Chem.* **1997**, *8*, 520.
28. Klaassen, C. D.; Aleksunes, L. M. *Pharmacol. Rev.* **2010**, *62*, 1.
29. Hagenbuch, B.; Gui, C. *Xenobiotica* **2008**, *38*, 778.
30. Hilgendorf, C.; Ahlin, G.; Seithel, A.; Artursson, P.; Ungell, A. L.; Karlsson, J. *Drug Metab. Dispos.* **2007**, *35*, 1333.
31. Sai, Y.; Kaneko, Y.; Ito, S.; Mitsuoka, K.; Kato, Y.; Tamai, I.; Artursson, P.; Tsuji, A. *Drug Metab. Dispos.* **2006**, *34*, 1423.
32. Balakrishnan, A.; Wring, S. A.; Polli, J. E. *Pharm. Res.* **2006**, *23*, 1451.
33. Ming, X.; Knight, B. M.; Thakker, D. R. *Mol. Pharm.* **2011**, *8*, 1677.
34. Tamai, I.; Nezu, J.; Uchino, H.; Sai, Y.; Oku, A.; Shimane, M.; Tsuji, A. *Biochem Biophys. Res. Commun.* **2000**, *273*, 251.
35. Nozawa, T.; Kobayashi, D.; Imai, K.; Nezu, J.; Tsuji, A.; Tamai, I. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 703.
36. Rohacova, J.; Marin, M. L.; Martinez-Romero, A.; O'Connor, J. E.; Gomez-Lechon, M. J.; Donato, M. T.; Castell, J. V.; Miranda, M. A. *Org. Biomol. Chem.* **2009**, *7*, 4973.
37. Capaldi, S.; Guariento, M.; Saccomani, G.; Fessas, D.; Perduca, M.; Monaco, H. L. *J. Biol. Chem.* **2007**, *282*, 31008.