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A fluorescent analogue of tauroursodeoxycholic acid reduces ER stress and is cytoprotective

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ABSTRACT

Tauroursodeoxycholic acid (TUDCA) is a cytoprotective ER stress inhibitor and chemical chaperone. It has therapeutic potential in a wide array of diseases but a specific macromolecular target or molecular mechanism of action remains obscure. This Letter describes an effective new synthetic approach to taurine conjugation of bile acids which we used to prepare 3α-dansyl TUDCA (**4**) as a probe for TUDCA actions.

As a model of ER stress we used the hepatocarcinoma cell line HUH7 and stimulation with either deoxycholic acid (DCA, 200 μM) or tunicamycin (5 μg/ml) and measured levels of Bip/GRP78, ATF4, CHOP and XBP1s/XBP1u. Compound **4** was more effective than UDCA at inhibiting ER stress markers and had similar effects to TUDCA. In a model of cholestasis using the cytotoxic DCA to induce apoptosis, pretreatment with **4** prevented cell death similarly to TUDCA whereas the unconjugated clinically used UDCA had no effect.

3α-Dansyl TUDCA (**4**) appears to be a suitable reporter for TUDCA effects on ER stress and related cytoprotective activity.

Tauroursodeoxycholic acid (TUDCA, Fig. 1) is polar bile acid present in low concentration in man but it is an important metabolite of ursodeoxycholic acid (UDCA), a substance used in the treatment of cholestatic liver disease especially primary biliary cirrhosis (PBC).[1] TUDCA is cytoprotective and it is believed to play a significant role in the clinical effects of UDCA.[2]

TUDCA promotes protein folding and it may exert therapeutic effects associated with UDCA through reduction of endoplasmic reticulum (ER) stress.[3] ER stress is a phenomenon caused by accumulation of misfolded proteins which can provoke the so-called unfolded protein response or UPR.[4] The UPR leads to reduction in protein synthesis, increased chaperone expression and protein folding capacity and degradation of misfolded proteins.[5] Persistent unresolved UPR triggers a step-wise apoptosis response.[6] ER stress is a component of diverse pathophysiological states and it is an emerging target for therapeutic modulation.[7] TUDCA is one of only a few substances that have been shown to reduce ER stress and/or promote protein folding.[8] Such substances are referred to as chemical or pharmacological chaperones. TUDCA has shown promising activity in diverse disease states with a significant ER stress component including diabetes, inflammatory bowel disease, Parkinsons', Alzheimer's disease, ALS and Huntington's disease, heart disease and heart failure, bipolar disorder, liver diseases and liver failure.[9–12] Although it is widely used in ER stress studies, and it has a role in treating hepatic disease, little is known about the mechanism of action of TUDCA in promoting cell survival or in attenuating ER stress and modulating the UPR. Fluorescent bile acids have proven useful in studying aspects of bile acid metabolism and distribution.[13–15] Fluorescent derivatives of TUDCA have not been reported to our knowledge. The purpose of this study was to prepare and validate 3α-dansyl-TUDCA as a fluorescent reporter analogue for use in mechanistic studies into ER stress modulation and cytoprotection.

Our synthetic approach to 3α-Dns-TUDCA (**4**) was similar to that reported in our previous work on NBD and other derivatives of UDCA and deoxycholic acid (DCA).[16,17] The key intermediate in this was the 7-formyl protected UDCA methyl ester **5** (Scheme 1). Treatment of **5** with NBS in the presence of PPh_3 produced the corresponding 3β-bromide in high yield. This was converted to the 3α-azide by SN_2 substitution with NaN_3 in DMPU at 50 °C. The 3α-azide was converted to BOC amine **6** by reduction over Pd/C in the presence of H_2 and BOC anhydride. Synthesis of taurine conjugates of bile acids can be challenging. The yields are usually poor using standard coupling technologies due to either low conversion

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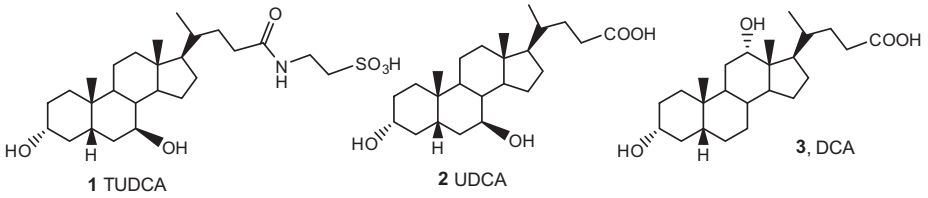
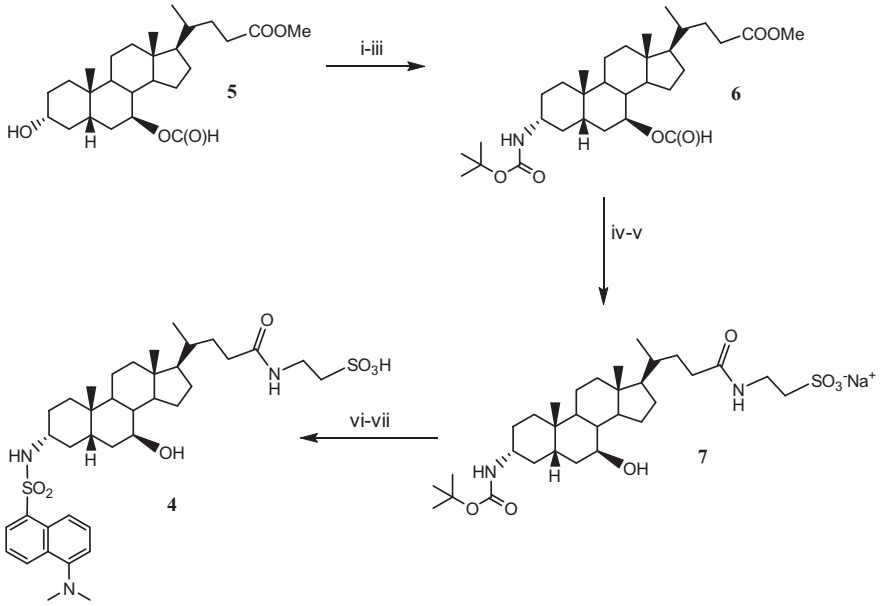


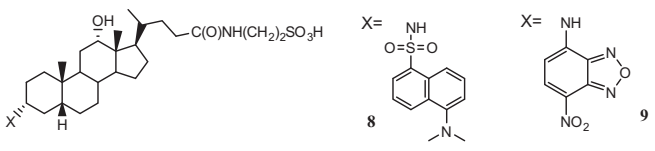
Figure 1. Structural formulae of TUDCA (**1**), UDCA (**2**), and DCA (**3**).



Scheme 1. Reagents and conditions: (i) PPh_3, NBS; (ii) NaN_3, DMPU; (iii) H_2, Pd/C, BOC_2O; (iv) NaOH, H_2O, MeOH; (v) taurine sodium salt, COMU, C18 extraction; (vi) TFA, DCM, (vii) dansyl chloride, DIPEA or solid K_2CO_3.

or purification difficulties due to the high polarity of the ionised sulfonic acid functionality.[18,19] We used 1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), a relatively new reagent for amide coupling.[20] The coupling was combined with solid reverse phase extraction (SPE) for the removal of water-soluble byproducts to give tauro conjugates in excellent yield (>90%).

3α-BOCamino-TUDCA was deprotected using trifluoroacetic acid (TFA) in DCM and then dansyl conjugated with dansyl chloride in the presence of solid K_2CO_3 or in a single phase with *N*,*N*-diisopropylethylamine (DIPEA). In this latter case the product was obtained co-purified with DIPEA. We had earlier tried to produce the target compound by taurine conjugation to 3α-Dns-UDCA but yields were higher when the taurine conjugation was performed earlier in the sequence and the dansyl conjugation performed last, on 3α-amino-TUDCA. Using this kind of approach we were also able to prepare **8** and **9** which are the 3α-Dns- and 3α-NBD-analogues of tauro-DCA.



Compound **4** and the DCA analogues **8** and **9** were characterised by ^1H, ^{13}C NMR and by HPLC and high resolution mass spectroscopy.[21] The dansyl analogues exhibited typical dansyl fluorescence characteristics with absorption maxima at λ_{max} 334 nm. The emission spectra in ethanol (0.02 mg/ml) produced maxima at 508–510 nm. Cell viability effects of 3α-Dns-TUDCA were assessed using the MTT assay on HEK293 and on liver cell line HUH-7. 3α-Dns-TUDCA and TUDCA had similar effects on cell viabilities in the two cell lines showing that the dansyl group incorporation did not significantly increase toxicity relative to TUDCA.

An important property of TUDCA reported in many cell types is its ability to inhibit ER stress responses. The basis for this effect might be promotion of protein folding and inhibition of aggregation, an effect on ER calcium homeostasis or a direct effect on the cellular response to unfolded proteins. The mechanism is not understood and it is worth investigating for several reasons including the prospect of producing a more efficacious or pharmaceutically useful ER stress modulator. One of the reasons for the design of the fluorescent analogue was to investigate TUDCA mechanism but in validating it as a reporter for TUDCA it was necessary to demonstrate it had similar effects on ER stress responses. The effect of TUDCA, UDCA and 3α-Dns-TUDCA (**4**) on ER stress was studied in the HUH-7 cell line in response to tunicamycin (5 μg/ml) or DCA (200 μM). Tunicamycin is an inhibitor of N-linked glycosylation and in eukaryocytes it causes protein misfolding leading to ER stress and activation of the UPR.[22] The UPR has three main arms with transduction through inositol requiring (IRE) 1α, PKR-like ER kinase (PERK) and activating transcription factor (ATF)6α. In the normal state these three transducer proteins are bound to immunoglobulin protein (BiP or glucose-regulated protein 78 (GRP78)). Accumulating unfolded or aggregated protein causes a shift of BiP from IRE, PERK and ATF6, liberating them. IRE1α autoactivation (phosphorylation) triggers its ribonuclease activity causing scission of XBP1, an unstable mRNA strand. This produces the more active XBP1s (spliced XBP). XBP1s increase, or