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Synthesis and Evaluation of Water-Soluble Prodrugs of Ursodeoxycholic Acid (UDCA), an Anti-apoptotic Bile Acid

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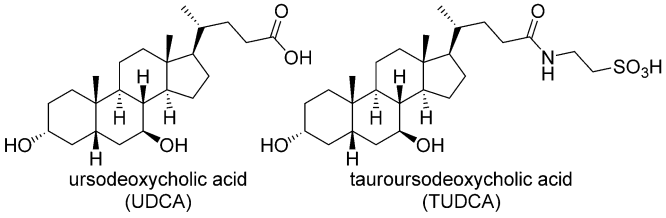
Ursodeoxycholic acid (UDCA) is a bile acid with demonstrated anti-apoptotic activity in both in vitro and in vivo models. However, its utility is hampered by limited aqueous solubility. As such, water-soluble prodrugs of UDCA could have an advantage over the parent bile acid in indications where intravenous administration might be preferable, such as decreasing damage from stroke or acute kidney injury. Five phosphate prodrugs were synthesized, including one incorporating a novel phosphoryloxymethyl carboxylate (POMC) moiety. These prodrugs were highly water-soluble, but showed significant differences in chemical stability, with oxymethylphosphate prodrugs being the most unstable. In a series of NMR experiments, the POMC prodrug was bioactivated to UDCA by alkaline phosphatase (AP) faster than a prodrug containing a phosphate directly attached to the alcohol at the 3-position of UDCA. Both of these prodrugs showed significant anti-apoptotic activity in a series of in vitro assays, although the POMC prodrug required the addition of AP for activity, while the other compound was active without exogenous AP.

Introduction

Ursodeoxycholic acid (UDCA) is a naturally occurring component of bile used clinically to treat primary biliary cirrhosis. In addition to treating various other liver diseases, UDCA and its taurine-conjugated derivative tauroursodeoxycholic acid (TUDCA) are anti-apoptotic agents[1,2] that are known to have protective effects in animal models of multiple disorders, including Huntington's disease,[3] spinal cord injury,[4] cataracts,[5] and acute pancreatitis.[6] The molecular mechanisms underlying



the cytoprotective activities of these molecules are believed to engage a number of different pathways including preventing Bax-induced membrane perturbation in mitochondria, blocking caspase-3 activation, inhibiting calpain and caspase-12 activation caused by endoplasmic reticulum (ER) stress, and by modulating gene regulation.[1,2,7,8]

Previous research from our groups has shown that TUDCA has beneficial effects in animal models of ischemic and hemorrhagic stroke,[9,10] acute kidney injury,[11] and myocardial infarction.[12] However, we were unable to test UDCA in these models because its limited aqueous solubility precluded the administration, preferably intravenously (iv) for these indications, of doses large enough to have an effect. This is unfortunate as UDCA is an FDA-approved drug in the U.S. with an excellent safety record in humans even when used for extended periods of time at high doses,[13–16] making it a particularly attractive potential therapy. With this in mind, we set out to synthesize highly water-soluble prodrugs of UDCA. We chose to focus on synthesizing phosphate ester prodrugs as this modification often increases aqueous solubility by several orders of magnitude. There are several examples of this class of prodrugs already in clinical use, including prednisolone phosphate, fosphenytoin, and fosamprenavir.[17–20] These prodrugs are activated in vivo by ubiquitous endogenous phosphatases. A particular advantage of this class of prodrugs is that they show very little interspecies differences in rates of bioactivation. This stands in contrast to other potential classes of prodrugs of UDCA such as carboxylic acid esters and could make a phosphate prodrug easier to develop.[17]

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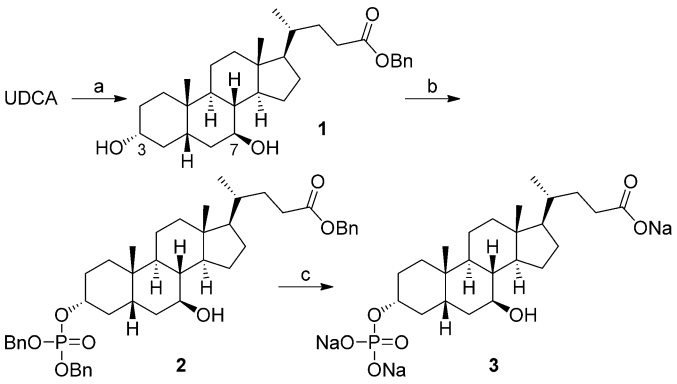
Results and Discussion

Synthesis of prodrugs

Ursodeoxycholic acid has two alcohol moieties to which a phosphate can be directly attached, located at the 3- and 7-positions. We began our synthesis of both of these potential

prodrugs by benzyl protecting the acid of UDCA, which proceeded in high yield using benzyl bromide (BnBr) as the alkylating agent (Scheme 1). Heating the resulting benzyl ester **1** with dibenzyl *N*,*N*-diethylphosphoramidite followed by oxidation with H_2O_2 furnished a phosphate ester which was tentatively assigned the structure **2** based on reports that similar steroidal structures react more readily at the 3-position than at the 7-position.[21,22] This assignment was later confirmed by NMR spectroscopy (see below). Removal of the three benzyl groups of **2** with hydrogen and Pd/C followed by treatment with sodium carbonate yielded the desired 3-substituted phosphate ester prodrug of UDCA (**3**).

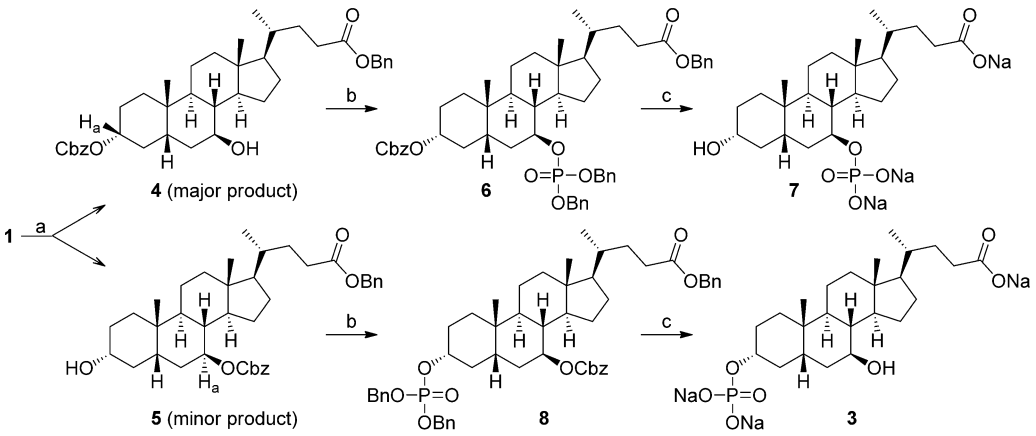


Scheme 1. Synthesis of 3-substituted phosphate prodrug **3**. *Reagents and conditions*: a) BnBr, K_2CO_3, CH_3CN, 80 °C; b) 1. dibenzyl *N*,*N*-diethylphosphoramidite, 1,2,4-triazole, $NaHCO_3$, 1,2-dichloroethane, 65 °C, 2. 30% H_2O_2, 0 °C; c) 1. Pd/C, H_2, MeOH, 2. Na_2CO_3.

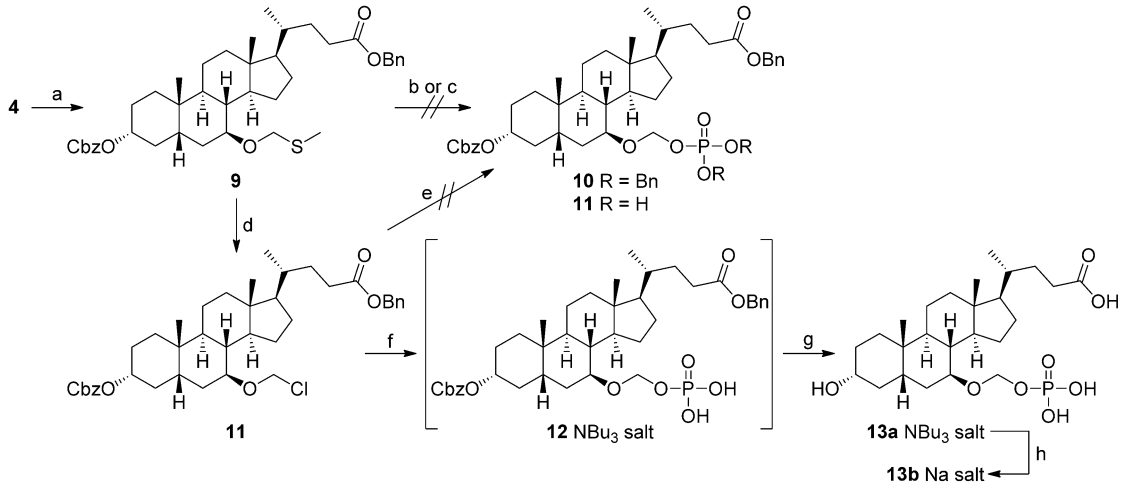
To obtain the 7-substituted phosphate ester prodrug of UDCA we treated benzyl ester **1** with benzyl chloroformate and pyridine in dichloromethane (Scheme 2). This led to a mixture of products, including 3-Cbz-protected alcohol **4** (41%), 7-Cbz-protected alcohol **5** (10%), recovered starting material (41%) and a small amount of 3,7-diCbz-protected material. These products could readily be separated by column chromatography and allowed us to unambiguously confirm the regiochemistry of our prodrugs by ^{1}H NMR analysis, as the signal of the proton next to the Cbz-protected alcohol (H_a in Scheme 2) was a dddd in the major mono-substituted product (consistent with structure **4**) and a ddd in the minor mono-substituted product (consistent with structure **5**). Compound **4** was then converted into 7-substituted phosphate ester prodrug **7** using dibenzyl *N*,*N*-diethylphosphoramidite followed by oxidation with H_2O_2 and then Pd/C catalyzed debenzylation. Similar standard conditions converted **5** into the same 3-substituted phosphate ester prodrug **3** that was obtained using Scheme 1. Both phosphate prodrugs were highly water-soluble, rapidly dissolving at all concentrations tested (up to 20 mg mL^{-1}), and were stable in solution for extended periods of time (>6 months) without any apparent decomposition.

In addition to prodrugs **3** and **7**, where the phosphate is directly linked to one of the alcohols in UDCA, we also set out to synthesize 3- and 7-substituted oxymethylphosphate (OMP) UDCA prodrugs. While often more difficult to synthesize, OMP prodrugs (also referred to a phosphonooxymethyl or POM prodrugs) are typically bioactivated by alkaline phosphatase at a significantly higher rate than their directly linked phosphate ester analogues due to decreased steric hindrance, which would be preferred for rapid treatment of stroke or myocardial infarction.[19] Upon bioactivation, OMP prodrugs release parent drug and formaldehyde in a two-step process.[23]

The synthesis of the 3- and 7-substituted oxymethylphosphate (OMP) prodrugs of UDCA did indeed prove to be considerably more complicated than the synthesis of the directly linked phosphate prodrugs **3** and **7**. Attempts to directly alkylate the UDCA scaffold at either the 3- or 7-positions with either dibenzyl chloromethyl phosphate or chloroiodomethane were unsuccessful. Instead, we turned to a synthetic scheme that had previously be used to synthesize OMP prodrugs, namely methylthiomethyl (MTM) ether formation followed by reaction with N-iodosuccinimide (NIS) and a phosphate.[22,24,25] We successfully synthesized the desired MTM ether intermediate **9** via a Pummerer rearrangement by stirring **4** in DMSO, acetic anhydride (Ac_2O), and acetic acid (AcOH) (Scheme 3).



Scheme 2. Synthesis of 7-substituted phosphate prodrug **7** and alternate route to prodrug **3**. *Reagents and conditions*: a) benzyl chloroformate, pyridine, CH_2Cl_2; b) 1. dibenzyl *N*,*N*-diethylphosphoramidite, 1,2,4-triazole, $NaHCO_3$, Δ, 2. 30% H_2O_2, 0 °C; c) 1. Pd/C, H_2, MeOH, 2. Na_2CO_3.

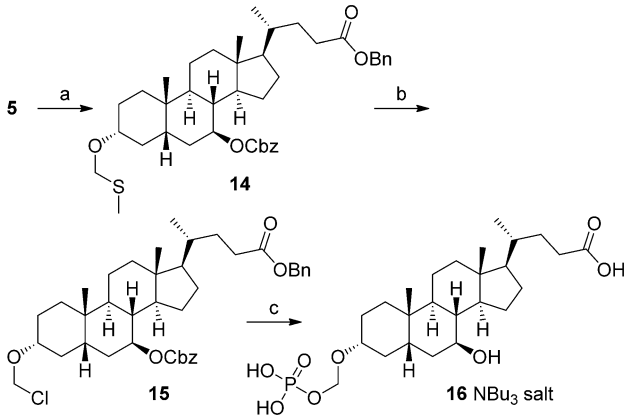


Scheme 3. Synthesis of 7-substituted oxymethylphosphate prodrug **13**. *Reagents and conditions*: a) DMSO, Ac_2O, AcOH; b) dibenzyl phosphate, *N*-iodosuccinimide, molecular sieves; c) H_3PO_4, *N*-iodosuccinimide, molecular sieves d) $SOCl_2$, CH_2Cl_2, 100 °C; e) dibenzyl phosphate, K_2CO_3, $[D_3]CH_3CN$; f) H_3PO_4, NBu_3, CH_3CN; g) Pd/C, H_2, MeOH; h) ion exchange using Na^+-Dowex resin, MeOH/H_2O.

Unfortunately, treating **9** with NIS and either dibenzyl phosphate or H_3PO_4 did not lead to any isolable product. However, we were able to convert the MTM ether **9** into chloroalkyl ether **11** by heating it in CH_2Cl_2 and thionyl chloride. In an NMR experiment, reaction of chloroalkyl ether **11** with dibenzyl phosphate and K_2CO_3 in $[D_3]$acetonitrile led to an impure product (likely **10**) which decomposed before it could be isolated. Similarly, reaction of **11** with either K_3PO_4 or Na_3PO_4 failed to lead to the desired OMP product. We were finally able to successfully substitute **11** by following the example of Komatsu and co-workers, who found that a tri(*n*-butyl)amine salt of phosphate could be successfully reacted with a chloroalkyl ether, presumably because of its improved solubility in organic solvents.[26,27] Thus, stirring **11** with a tri(*n*-butyl)amine salt of phosphate in acetonitrile led to **12**, which was then deprotected using hydrogen and Pd/C in methanol. The crude material was purified by C_{18} column to afford 7-substituted OMP prodrug **13a** as an NBu_3 salt. Similarly, 3-substituted OMP prodrug **16** could be obtained from compound **5** using the same sequence of synthetic steps (Scheme 4).

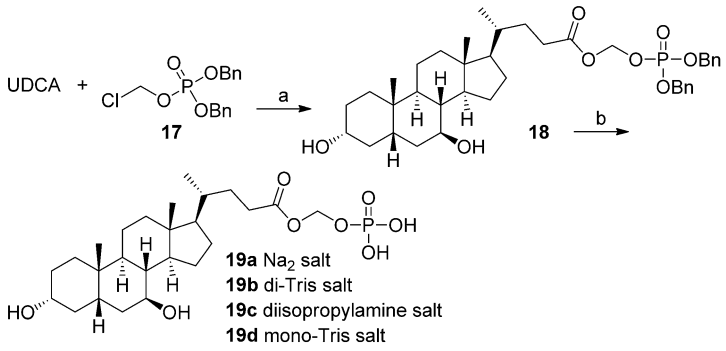
The 3- and 7-substituted oxymethylphosphate prodrugs **16** and **13a** were poorly water-soluble as NBu_3 salts. Therefore, compound **13a** was converted into a sodium salt **13b** by ion-exchange filtration through Dowex resin.[28] The resulting white solid rapidly dissolved in water at all concentrations tested (up to 10 mg mL^{-1}). Unfortunately, a significant portion of the material decomposed when left in D_2O solution overnight.

Due to the combination of chemical instability and the relatively difficult synthesis of the 3- and 7-substituted OMP prodrugs, we instead decided to prepare a prodrug where the OMP group is linked to the carboxylic acid of UDCA instead of one of its alcohols. Such a prodrug could potentially be bioactivated in vivo to parent drug both by alkaline phosphatase and by esterases and has the additional advantage that the phosphate moiety is sterically unhindered (relative to the phosphate group in **3** or **7**), which may increase the rate of enzymatic activation. We are aware of only one example of such a phosphoryloxymethyl carboxylate (POMC) prodrug in the chemical literature, in a recent patent application by Barnes and co-workers.[29] However, no discussion of the properties of the potential prodrug was presented other than to mention that the material was not obtained cleanly. In addition, Stella and co-workers explored related phosphoryloxymethyloxy carbonyl prodrugs of alcohols, aliphatic amines and aromatic amines, but found their potential utility limited by chemical instability.[30]



Scheme 4. Synthesis of 3-substituted oxymethylphosphate prodrug **16**. *Reagents and conditions*: a) DMSO, Ac_2O, AcOH; b) $SOCl_2$, CH_2Cl_2, 100 °C; c) 1. H_3PO_4, NBu_3, CH_3CN, 2. Pd/C, H_2, MeOH.

We began our synthesis of the POMC prodrug by reacting UDCA with K_2CO_3 and dibenzyl chloromethyl phosphate (**17**) to afford ester **18** (Scheme 5). Interestingly, this reaction proceeded in higher yield (81% instead of 22%) and at much lower temperature (room temperature instead of 120 °C) when DMF was used as a solvent instead of acetonitrile. Using DMF instead of acetonitrile also greatly minimized the formation of



Scheme 5. Synthesis of phosphoryloxymethyl carboxylate (POMC) prodrug. *Reagents and conditions*: a) K_2CO_3, DMF; b) 1. Pd/C, H_2, MeOH, 2. Na_2CO_3, isopropylamine or Tris.

benzyl ester **1** as a major side product. Next, benzyl deprotection of **18** using hydrogen and Pd/C followed by treatment with sodium carbonate yielded the desired POMC prodrug **19a** as a disodium salt. Unfortunately, NMR analysis showed this material to contain a significant amount of impurities and several attempts to synthesize this product cleanly failed. However, we were encouraged by a report from Farquhar and coworkers, who isolated a similar compound that they were using as a chemical intermediate as a dicyclohexylammonium salt.[31] When we replaced sodium carbonate with two equivalents of tris(hydroxymethyl)aminomethane (Tris), we able to cleanly isolate the desired product as a diamine salt (**19b**). The di-Tris salt of **19** was highly water-soluble, rapidly dissolving at all concentrations tested (up to 20 mg mL^{-1}), and stable for extended periods of time when stored in a freezer. However, it showed moderate chemical instability in solution at room temperature (only 36% remained after one week in D_2O solution, Table 1). A diisopropylamine salt (**19c**) showed similar chemical stability (34% remained after one week at room temperature in D_2O). However, we noticed that formulations of **19** containing less than two equivalents of amine proved to be significantly more chemically stable in solution. This led us to synthesize the mono-Tris salt of our POMC prodrug, **19d**, which was highly water-soluble (>20 mg mL^{-1}), and decomposed relatively slowly in solution (88% remained after one week at room temperature in D_2O). The increased aqueous stability of the monoanionic prodrug relative to the dianionic prodrug is similar to that seen with Stella's phosphoryloxymethyloxy carbonyl prodrugs and is consistent with his hypothesis that hydrolysis occurs primarily via an intramolecular general base or intramolecular nucleophilic catalysis mechanism.[30] This hypothesis is further supported by data showing that adding an additional equivalent of Tris to **19b** has little effect on its stability in solution (Table 1, entry **19e**). The compound also showed similar stability in pH 7.4 tris buffer, with 81% remaining after one day at RT.

Table 1. Chemical stability of different salt forms of **19**.

Compd	Counter-ion	Equiv	[%] Remaining[a]
19b	Tris	2	36
19c	Isopropylamine	2	34
19d	Tris	1	88
19e	Tris	3	33

[a] Percent of prodrug remaining after seven days at RT in D_2O solution; $n=3$, SD < 2%.

Alkaline phosphatase activation of prodrugs

To determine whether the POMC prodrug was indeed activated under in vitro conditions faster than a prodrug where the phosphate moiety is directly linked to an alcohol, we conducted a series of experiments where we monitored the alkaline phosphatase catalyzed activation of prodrugs **19d** and **3** by inverse-gated decoupled ^{31}P NMR (see Experimental Section for details). As shown in Figure 1, UDCA is more rapidly released from prodrug **19d** under in vitro conditions than prodrug **3**.

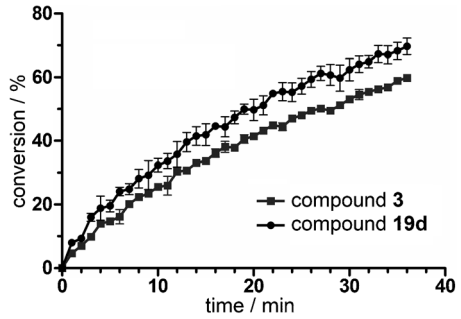


Figure 1. Alkaline phosphatase catalyzed activation of prodrugs **3** and **19d**. Each data point represents the mean ± SEM of three separate experiments.

Evaluation of anti-apoptotic activity of prodrugs

We have previously shown that UDCA significantly inhibits caspase-3 activation and apoptosis induced by transforming growth factor β1 (TGFβ1) in primary rat hepatocytes.[32] To compare the in vitro cytoprotective effects of our new prodrugs with UDCA, we evaluated one of our alcohol-linked phosphate prodrugs (the 3-substituted prodrug **3**) and our POMC prodrug **19d** for their ability to modulate TGFβ1-induced cytotoxicity. Our preliminary results demonstrated that the presence of alkaline phosphatase (AP) in the culture medium does not significantly change TGFβ1, UDCA or compound **3** cytotoxic and cytoprotective properties. However, compound **19d** was cytoprotective only in the presence of AP (data not shown). Therefore, AP was added to the culture medium whenever assessing the cytoprotective potential of **19d**. We first analyzed the ability of **3** and **19d** to inhibit TGFβ1-induced general cell death, as compared with UDCA. The results showed that UDCA, **3** and alkaline phosphatase-activated **19d** inhibited TGFβ1-induced loss of cellular viability by at least 50% ($p < 0.05$) (Figure 2, upper panel). In addition, TGFβ1 also induced a 30% increase in the amount of LDH re-

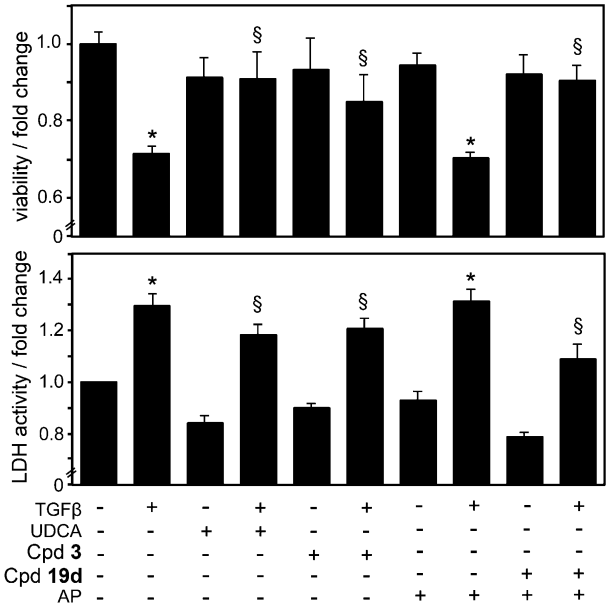


Figure 2. Compounds **3** and **19 d** inhibit TGFβ-induced cytotoxicity in primary rat hepatocytes. Primary rat hepatocytes were incubated with 100 μM UDCA, compound **3**, compound **19 d**, or no addition (control), in the presence or absence of alkaline phosphatase (3 U mL^{-1}) for 12 h. Cells were then exposed to 1 nM TGFβ for 24 h before processing for cell viability assays. Viability was assessed by live-cell protease cleavage of a fluorogenic peptide substrate (top). Culture medium was used for LDH viability assays (bottom). Results are expressed as means ± SEM of at least three different experiments; *$p < 0.01$ from respective control; §$p < 0.05$ from respective TGFβ.

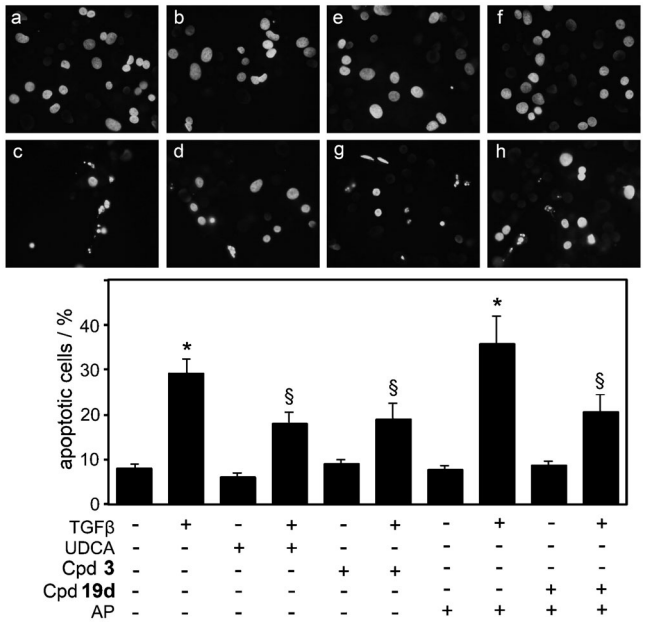


Figure 3. Compounds **3** and **19 d** inhibit TGFβ-induced nuclear fragmentation in primary rat hepatocytes. Primary rat hepatocytes were incubated with 100 μM UDCA, compound **3**, compound **19 d**, or no addition (control), in the presence or absence of alkaline phosphatase (3 U mL^{-1}) for 12 h. Cells were then exposed to 1 nM TGFβ for 24 h before processing for apoptosis assays. Fluorescence microscopy of Hoechst staining in a) control hepatocytes and in cells exposed to either b) compound **3**, c) TGFβ, d) TGFβ + compound **3**, e) AP, f) compound **19 d** + AP, g) TGFβ + AP, or h) compound **19 d** + TGFβ + AP (top). Normal nuclei showed noncondensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Percentage of apoptotic of cells (bottom). Results are expressed as means ± SEM of at least three different experiments; *$p < 0.01$ from respective control; §$p < 0.05$ from respective TGFβ.

leased from cells, an indicator of cellular toxicity ($p < 0.01$) (Figure 2, lower panel). Similarly as before, UDCA, **3** and **19 d**, inhibited TGFβ1-induced cytotoxicity by ~40, 30 and 60%, respectively ($p < 0.05$).

We then specifically evaluated the effects of **3** and **19 d** on modulation of TGFβ1-induced apoptosis by changes in nuclear morphology (Figure 3) and by caspase activity (Figure 4). We confirmed that significant levels of apoptosis occurred in cultured primary rat hepatocytes after incubation with TGFβ1 ($p < 0.01$), with a concomitant increase in caspase-3-like activity ($p < 0.01$). Notably, UDCA, **3** and **19 d** protected against TGFβ1-induced nuclear fragmentation by 50–80% ($p < 0.05$) and caspase-3-like activation by 40–70% ($p < 0.05$). Altogether, these results show that much like UDCA, the newly synthesized UDCA prodrugs **3** and **19 d** display significant cytoprotective properties in vitro.

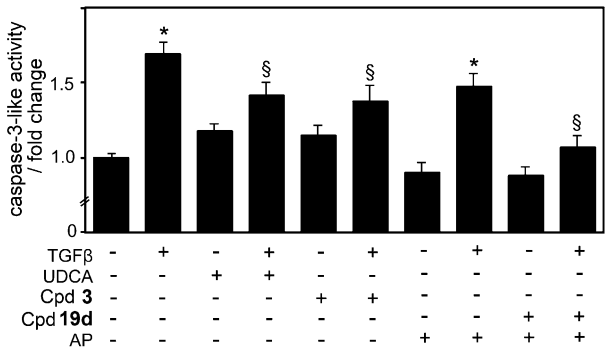


Figure 4. TGFβ-induced caspase-3 activity is inhibited by compound **3** and **19 d**. Primary rat hepatocytes were incubated with 100 μM UDCA, compound **3**, compound **19 d**, or no addition (control), in the presence or absence of alkaline phosphatase (3 U mL^{-1}) for 12 h. Cells were then exposed to 1 nM TGFβ for 24 h before processing for apoptosis assays. Caspase-3-like activity was analyzed by enzymatic cleavage of the proluminescent caspase-3/7 DEVD-aminoluciferin substrate. Results are expressed as means ± SEM of at least three different experiments; *$p < 0.01$ from respective control; §$p < 0.05$ from respective TGFβ.

Conclusions

We have prepared five highly water-soluble prodrugs of the anti-apoptotic bile acid UDCA from three distinct classes: directly linked phosphate esters, oxymethylphosphate (OMP) prodrugs and a novel phosphoryloxymethyl carboxylate (POMC) prodrug. As the OMP prodrugs of UDCA were both difficult to synthesize and chemically unstable, they were not tested in any biological assays. Compound **3**, a directly linked phosphate ester, proved to have similar anti-apoptotic potency to UDCA in our in vitro assays, even without prior bioactivation by alkaline phosphatase. Our POMC prodrug compound **19**, in contrast, was also highly active in these assays, but required activation by exogenous alkaline phosphatase to have an

effect. In our future studies, we intend to evaluate the ability of prodrugs **3** and **19** to decrease apoptosis in an in vivo animal model.

The novel POMC prodrug **19** was bioactivated by alkaline phosphatase to UDCA faster than prodrug **3**, in which the phosphate ester is directly linked to an alcohol. We were unable to isolate **19** cleanly as a sodium salt, but pure mono and diamine salts of **19** could be readily obtained on a large scale (>5 g) in just two steps from the parent carboxylic acid (UDCA). Diamine salts of **19** were somewhat unstable in solution over long periods of time at room temperature, but the mono-Tris salt of **19** decomposed at a much lower rate and was stable for extended periods when stored cold. While the moderate chemical instability of the POMC prodrugs will definitely limit their use, they might still find potential application as prodrugs of carboxylic acids for situations in which cold storage is readily available and high aqueous solubility and rapid release are crucial, such as in acute medical conditions in a hospital environment.

Experimental Section

Chemical stability

In D_2O: Prodrug **19b**, **19c**, or **19d** (4.0 mg) was dissolved in D_2O (1.0 mL) and placed in an NMR tube with a sealed capillary tube containing a solution of phenylphosphonic acid in D_2O as a standard. At $t=0$, a ^{1}H NMR spectrum was obtained, and the proton signal at $\delta=5.51$ ppm was integrated ($I_{t=0}$) relative to the aromatic signals of phenylphosphonic acid. The tube was then stored at RT for 7 days at RT. Another ^{1}H NMR spectrum was taken, and the proton signal at $\delta=5.51$ ppm was again integrated ($I_{t=7}$) as before. The proportion (%) of prodrug remaining was determined as $I_{t=7}/I_{t=0}\times100$. Each experiment was repeated three times. Chemical stability results obtained using ^{31}P NMR were similar to those obtained using ^{1}H NMR. For **19e**, 4.0 mg **19b** was dissolved in D_2O and an additional equivalent of Tris added (the stoichiometry was confirmed by ^{1}H NMR) and the experiment conducted as above.

In pH 7.4 tris-buffered saline: Prodrug **19d** (4.0 mg) was dissolved in H_2O (0.9 mL) and treated with tris-buffered saline (0.1 mL; BM-300 from Boston BioProducts), containing tris (250 mM), KCl (27 mM), and NaCl (1.37 M). A sealed capillary tube containing phenylphosphonic acid dissolved in D_2O was used in NMR experiments as a standard. Chemical stability results were obtained by measuring the disappearance of prodrug relative to the internal standard by ^{31}P NMR. The experiment was repeated three times. The standard deviation (SD) was ±3%.

Biological evaluation

*Activation of prodrugs **3** and **19d***: Alkaline phosphatase from bovine intestinal mucosa (Sigma–Aldrich, P5521-2KU) was dissolved in 2.0 mL of a 0.100 M sodium glycine buffer containing 1.0 mM $ZnCl_2$ and 1.0 mM $MgCl_2$. This stock solution was stored at 4 °C between uses. Compound **19d** (10.0 mg, 0.016 mmol) or compound **3** (8.7 mg, 0.016 mmol) were dissolved in 0.6 mL of a 0.100 M tris glycine buffer solution containing 1.0 mM $ZnCl_2$ and 1.0 mM $MgCl_2$. Neither compound showed decomposition by ^{31}P NMR when left in this buffer solution for 1 h; 50 μL of the previously prepared AP stock solution was further diluted by addition to 0.950 mL of a 0.100 M tris glycine buffer containing 1.0 mM $ZnCl_2$ and 1.0 mM $MgCl_2$; 10.0 μL of this diluted AP solution was added to the prodrug solution by syringe. A series of 42 inverse-gated decoupled ^{31}P NMRs were taken (24 scans each, ~1 min acquisition time). Conversion (%) was determined from the relative integration of the starting material and product peaks, NMR time stamps were used to determine time. Each experiment was repeated three times.

Animal experiments: All experiments involving animals were performed by an Investigator accredited for directing animal experiments (FELASA level C), in conformity with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Experiments received prior approval from the Portuguese National Authority for Animal Health (DGAV).

Cell culture and treatments: Primary rat hepatocytes were isolated from male rats (100–150 g) by collagenase perfusion.[33] Briefly, rats were anesthetized with phenobarbital sodium (100 mg kg^{-1} body weight) injected into the peritoneal cavity. After administration of heparin (200 U kg^{-1} body weight) in the tail vein, the animals' abdomen was opened and the portal vein exposed and cannulated. The liver was then perfused at 37 °C in situ with a calcium-free Hank's Balanced Salt Solution (HBSS) for ~10 min, and then with 0.05% collagenase type IV in calcium-present HBSS for another 10 min. Hepatocyte suspensions were obtained by passing collagenase-digested livers through 125 μm gauze and washing cells in Complete William's E medium (Sigma–Aldrich) supplemented with 26 mM sodium bicarbonate, 23 mM HEPES, 0.01 U mL^{-1} insulin, 2 mM L-glutamine, 10 nM dexamethasone, 100 U mL^{-1} penicillin, and 10% heat-inactivated fetal bovine serum (Invitrogen). Viable primary rat hepatocytes were enriched by low-speed centrifugation at 200 g for 3 min. Cell viability was determined by trypan blue exclusion and was typically 80–85%. After isolation, hepatocytes were resuspended in Complete William's E medium and plated on Primaria tissue culture dishes (BD Biosciences) at 5×10^4 cells cm^{-2}. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 6 h, to allow attachment. Plates were then washed with medium to remove dead cells and incubated in Complete William's E medium supplemented with either 100 μM UDCA, compound **3**, compound **19d** or no addition (control), in the presence or absence of 3 U mL^{-1} of alkaline phosphatase (Invitrogen) for 12 h. Cells were then exposed to 1 nmol L^{-1} recombinant human TGF-β1 (R&D Systems) for 24 h before processing for cell viability and apoptosis assays.

Cell viability assays: LDH, a stable cytosolic enzyme, is released to cell culture media following cell lysis, and can be used as a marker of cytotoxicity. Briefly, to assess LDH release, supernatants taken from a gentle centrifugation of cell culture media at 250 g, were combined in microplates with lactate (substrate), tetrazolium salt (coloring solution), and NAD (co-factor), previously mixed in equal proportions, following the manufacturer's instructions (Sigma–Aldrich). Multi-well plates were protected from light and incubated for 10 min at room temperature. Finally, absorbance was measured at 490 nm, with 690 nm as reference, using a Bio-Rad model 680-microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

To assess cellular viability, the CellTiter-Fluor viability assay was used (Promega, Madison, WI, USA). Briefly, viable cells are measured using a fluorogenic, cell-permeant, peptide substrate (Gly-Phe-AFC), which is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living

cells. Cells were incubated with an equal volume of CellTiter-Fluor Reagent for 30 min at 37 °C and resulting fluorescence (380–400 nm_{Ex}/505 nm_{Em}) measured using a GloMax+ Multi Detection System (Promega).

Apoptosis assays: General caspase-3/7 activity was evaluated using the Caspase-Glo 3/7 Assay (Promega). Briefly, the assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. Cells were incubated with an equal volume of Caspase-Glo 3/7Reagent for 30 min at 37 °C and resulting luminescence measured using a GloMax+ Multi Detection System (Promega).

In addition, Hoechst labeling of cells was used to detect apoptotic nuclei by morphological analysis. Briefly, culture medium was gently removed to prevent detachment of cells. Attached primary rat hepatocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at RT, washed with PBS, incubated with Hoechst dye 33258 (Sigma–Aldrich) at 5 $\mu g\,mL^{-1}$ in PBS for 5 min, washed with PBS, and mounted using Fluoromount-G (SouthernBiotech). Fluorescence was visualized using an Axioskop fluorescence microscope (Carl Zeiss GmbH). Blue-fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin disperse over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as by nuclear fragmentation of condensed chromatin. Five random microscopic fields per sample containing ~150 nuclei were counted, and mean values expressed as the percentage of apoptotic nuclei.

Statistical analysis: Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA) for the analysis of variance and Bonferroni's multiple comparison tests. Values of $p < 0.05$ were considered significant.

Chemistry

General: Microwave experiments were performed on a Biotage Initiator Microwave Synthesizer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. ^{1}H NMR data are reported as follows: chemical shift in parts per million (ppm) downfield of the internal reference tetramethylsilane (TMS), multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, quint = quintet and m = multiplet), coupling constant (*J*) in Hertz (Hz), and integrated value. Coupling constant defined as J_{31P} disappeared when ^{1}H NMR spectra were taken with ^{31}P decoupling. ^{13}C NMR spectra were measured with complete proton decoupling. ^{31}P NMR spectra taken for compound characterization were measured with complete proton decoupling and were referenced to 85% phosphoric acid, which was added to the NMR tube in a sealed capillary tube. LC/MS analysis was carried out using a BEH C_{18} column (2.1 mm × 50 mm, 5 μm) on a Waters Acquity UPLC system with a Waters ZQ mass detector. HRMS analyses were performed on a Waters LCT-TOF High-resolution mass spectrometer. Ursodeoxycholic acid (UDCA) was obtained from Sigma–Aldrich. Dibenzyl chloromethyl phosphate was synthesized by the method of Mäntylä,[34] but is also commercially available from Sigma–Aldrich.

Ursodeoxycholic acid benzyl ester (1): A suspension of UDCA (4.03 g, 10.3 mmol) and K_2CO_3 (4.88 g, 35.3 mmol) in CH_3CN (100 mL) was treated with BnBr (6.00 mL, 50.5 mmol). The reaction mixture was heated to 80 °C for 3 h, filtered, and concentrated in vacuo. Purification by flash chromatography (30→100% EtOAc/hexanes) on silica gel furnished the desired product as a white solid (4.72 g, 95%): ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.39–7.28$ (m, 5H), 5.13 and 5.10 (ABq, $J_{AB} = 12.3$ Hz, 2H), 3.56–3.42 (m, 2H), 2.46–2.36 (m, 1H), 2.36–2.25 (m, 1H), 2.08–1.97 (m, 1H), 1.94–1.76 (m, 5H), 1.67–0.98 (m, 18H), 0.97 (s, 3H), 0.94 (d, $J = 6.4$ Hz, 3H), 0.67 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 12.7$, 18.9, 22.4, 23.9, 27.9, 29.6, 31.1, 32.2, 32.3, 35.2, 36.1, 36.6, 38.0, 38.6, 40.7, 41.5, 44.0, 44.5, 44.8, 56.5, 57.5, 67.2, 71.9, 72.1, 129.2, 129.3, 129.6, 137.7, 175.7 ppm.

3-(Bis(benzyloxy)phosphoryloxy)ursodeoxycholic acid benzyl ester (2): A stirred suspension of **1** (1.497 g, 3.10 mmol), 1,2,4-triazole (450 mg, 6.52 mmol), and $NaHCO_3$ (1.906 g, 22.69 mmol) in 1,2-dichloroethane (30 mL) was treated with dibenzyl *N*,*N*-diethylphosphoramidite (1.00 mL, 3.15 mmol). The reaction mixture was heated overnight to 65 °C. After cooling in an ice bath, THF (12 mL) was added to the mixture, followed by dropwise addition of 30% H_2O_2 (6 mL). After stirring for 5 min., saturated aq $Na_2S_2O_3$ (30 mL) was added slowly (CAUTION: highly exothermic reaction). The mixture was diluted with water (200 mL) and extracted with CH_2Cl_2 (2×200 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification by flash chromatography (30→100% EtOAc/hexanes) on silica gel followed by a second flash chromatography (0→10% MeOH/CH_2Cl_2) on silica gel furnished the desired product as a clear colorless oil (1.1158 g, 48%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.43–7.27$ (m, 15H), 5.12 and 5.09 (ABq, $J_{AB} = 12.4$ Hz, 2H), 5.07–4.96 (m, 4H), 4.29–4.15 (m, 1H), 3.58–3.44 (m, 1H), 2.46–2.33 (m, 1H), 2.33–2.21 (m, 1H), 2.01–1.93 (m, 1H), 1.93–0.93 (m, 23H), 0.91 (s, 3H), 0.91 (d, $J = 6.1$ Hz, 3H), 0.64 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.1$, 18.4, 21.2, 23.2, 26.8, 28.2, 28.6, 31.0, 31.3, 33.9, 34.5, 34.8, 35.2, 36.5, 39.1, 40.1, 42.3, 43.66, 43.73, 54.9, 55.7, 66.1, 69.04, 69.07, 69.10, 69.12, 71.1, 78.6 (d, $J_{31P} = 6.0$ Hz), 127.88, 127.90, 128.2, 128.3, 128.45, 128.54, 135.98, 136.05, 136.10, 174.0 ppm; HRMS: *m/z* $[M+H]^+$ calcd for $C_{45}H_{59}O_7P$: 743.4077, found: 743.4092.

3-(Phosphonatooxy)ursodeoxycholic acid sodium salt (3): A solution of **2** (2.22 g, 2.99 mmol) in MeOH (100 mL) was treated with 10% Pd/C (291 mg). The reaction mixture was stirred at RT under an atmosphere of H_2 maintained using a gas-filled balloon for 2 h and filtered through Celite. Na_2CO_3 (476 mg, 4.49 mmol) dissolved in water (25 mL) was added to the filtrate, and the solution was concentrated in vacuo until most of the MeOH was removed. The remaining solution was lyophilized to afford the desired product as a white solid (1.627 g, quant.): ^{1}H NMR (400 MHz, D_2O): $\delta = 4.04–3.91$ (m, 1H), 3.72–3.62 (m, 1H), 2.29–2.17 (m, 1H), 2.17–2.06 (m, 1H), 2.07–1.97 (m, 1H), 1.95–1.00 (m, 23H), 0.96 (s, 3H), 0.95 (d, $J = 6.0$ Hz, 3H), 0.70 ppm (s, 3H); ^{13}C NMR (100 MHz, D_2O): $\delta = 12.1$, 18.6, 21.5, 23.3, 27.2, 28.8, 29.0, 33.1, 34.1, 35.2, 35.3, 35.7, 35.8, 37.1, 39.6, 40.4, 42.9, 43.5, 44.0, 55.1, 55.7, 71.9, 75.9 (d, $J_{31P} = 5.0$ Hz), 185.5 ppm; ^{31}P NMR (162 MHz, D_2O): 2.51 ppm; HRMS: *m/z* $[M+H]^+$ calcd for $C_{24}H_{41}O_7P$: 473.2668, found: 473.2670.

3-(Benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (4) and 7-(benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (5): A stirred solution of **1** (1.465 g, 3.04 mmol) in dry CH_2Cl_2 (50 mL) was treated with pyridine (0.600 mL, 7.42 mmol) followed by slow addition of benzyl chloroformate (1.00 mL, 7.03 mmol). After stirring for 1 h, additional pyridine (0.300 mL, 3.71 mmol) and benzyl chloroformate (0.600 mL, 4.22 mmol) were added. After 30 min., the reaction mixture was extracted with 1 M aq HCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification by flash chromatography (10→100% EtOAc/hexanes) on silica gel first furnished **4** as a slightly yellow

foam (0.7769 g, 41%), followed by **5** as a slightly yellow foam (181 mg, 10%), which was then followed by recovered **1** as a white solid (603.2 mg, 41%).

3-(Benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (4): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39–7.30 (m, 10H), 5.14 (s, 2H), 5.12 and 5.09 (ABq, J_{AB} = 12.3 Hz, 2H), 4.56 (dddd, J = 5, 5, 11, 11 Hz, 1H), 3.60–3.50 (m, 1H), 2.45–2.34 (m, 1H), 2.33–2.22 (m, 1H), 2.02–1.94 (m, 1H), 1.94–0.98 (m, 23H), 0.95 (s, 3H), 0.91 (d, J = 6.2 Hz, 3H), 0.65 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.1, 18.3, 21.2, 23.3, 26.4, 26.9, 28.6, 31.0, 31.3, 33.0, 34.1, 34.5, 35.2, 36.6, 39.1, 40.1, 42.2, 43.7, 43.8, 54.9, 55.7, 66.1, 69.3, 71.2, 77.9, 128.18, 128.24, 128.3, 128.46, 128.54, 128.6, 135.4, 136.1, 154.5, 174.0 ppm.

7-(Benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (5): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39–7.30 (m, 10H), 5.16 and 5.12 (ABq, J_{AB} = 12.2 Hz, 2H), 5.12 and 5.10 (ABq, J_{AB} = 12.3 Hz, 2H), 4.64 (ddd, J = 5, 11, 11 Hz, 1H), 3.63–3.52 (m, 1H), 2.44–2.34 (m, 1H), 2.32–2.22 (m, 1H), 2.01–1.93 (m, 1H), 1.91–0.96 (m, 23H), 0.94 (s, 3H), 0.90 (d, J = 6.3 Hz, 3H), 0.62 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.0, 18.3, 21.2, 23.2, 25.6, 28.6, 30.2, 31.0, 31.3, 33.0, 33.9, 34.7, 35.2, 37.1, 39.4, 39.9, 40.0, 42.2, 43.6, 55.0, 55.2, 66.1, 69.3, 71.3, 78.5, 128.1, 128.19, 128.24, 128.4, 128.5, 128.6, 135.6, 136.1, 154.6, 174.0 ppm.

3-(Benzyloxycarbonyloxy)-7-(bis(benzyloxy)phosphoryloxy)ursodeoxycholic acid benzyl ester (6): A stirred suspension of **4** (374 mg, 0.61 mmol), 1,2,4-triazole (89.8 mg, 1.30 mmol), and $NaHCO_3$ (263 mg, 3.13 mmol) in CH_2Cl_2 was treated with dibenzyl *N*,*N*-diethylphosphoramidite (0.900 mL, 3.00 mmol). The reaction mixture was heated overnight to 40 °C. After cooling in an ice bath, THF (5 mL) was added to the mixture, followed by dropwise addition of 30% aq H_2O_2 (3 mL). After stirring for 5 min, saturated aq $Na_2S_2O_3$ (20 mL) was added slowly (CAUTION: highly exothermic reaction). The mixture was diluted with CH_2Cl_2 and extracted with water (100 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification by flash chromatography (30% EtOAc/hexanes) on silica gel furnished the desired product as a slightly yellow oil (350.1 mg, 66%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43–7.27 (m, 20H), 5.16 and 5.15 (ABq, J_{AB} = 12.4 Hz, 2H), 5.12 and 5.10 (ABq, J_{AB} = 12.4 Hz, 2H), 5.05–4.91 (m, 4H), 4.51 (dddd, J = 5, 5, 10, 10 Hz, 1H), 4.30–4.17 (m, 1H), 2.45–2.34 (m, 1H), 2.32–2.21 (m, 1H), 1.99–0.99 (m, 24H), 0.93 (s, 3H), 0.90 (d, J = 6.1 Hz, 3H), 0.61 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.1, 18.4, 21.2, 23.2, 26.2, 28.4, 31.0, 31.3, 32.7, 33.8, 34.3, 34.4, 35.2, 39.2, 39.8, 41.8, 41.9, 42.0, 43.7, 54.9, 55.0, 66.1, 68.86, 68.92, 69.0, 69.1, 69.4, 77.5, 79.7, 79.8, 127.86, 127.90, 128.17, 128.22, 128.3, 128.4, 128.47, 128.54, 128.6, 135.4, 136.09, 136.11, 136.15, 136.18, 154.5, 174.0 ppm; LC/MS (ESI+): m/z = 877.7 $[M+H]^+$.

7-(Phosphonatooxy)ursodeoxycholic acid sodium salt (7): A suspension of **6** (1.1984 g, 1.37 mmol) in MeOH (200 mL) was treated with 10% Pd/C (322 mg). The reaction mixture was stirred at RT under an atmosphere of H_2 maintained using a gas-filled balloon for 2 h and then filtered through Celite. Na_2CO_3 (216.2 mg, 2.04 mmol) dissolved in water (25 mL) was added, and the solution was concentrated in vacuo until most of the MeOH was removed. The remaining solution was lyophilized to afford the desired product as a white solid (762.7 mg, quant.): ^{1}H NMR (400 MHz, D_2O): δ = 4.13–3.99 (m, 1H), 3.69–3.55 (m, 1H), 2.30–2.17 (m, 1H), 2.17–2.07 (m, 1H), 2.07–1.92 (m, 3H), 1.92–0.99 (m, 21H), 0.97 (s, 3H), 0.95 (d, J = 6.5 Hz, 3H), 0.69 ppm (s, 3H).^{13}C NMR (100 MHz, D_2O): δ = 12.1, 18.6, 21.5, 23.4, 27.0, 28.8, 29.6, 33.1, 34.1, 35.0, 35.2, 35.4, 35.9, 36.4, 39.5, 40.2, 42.6, 42.7, 44.0, 55.1, 55.3, 72.0, 76.4 (d, J_{31P} = 5.9 Hz), 185.6 ppm; ^{31}P NMR (162 MHz, D_2O): 0.93 ppm; LC/MS (ESI−): m/z = 471.4 $[M-H]^-$.

3-(Benzyloxycarbonyloxy)-7-(methylthiomethoxy)ursodeoxycholic acid benzyl ester (9): A solution of **4** (2.71 g, 4.39 mmol) in DMSO (34 mL) was treated with Ac_2O (21 mL) followed by AcOH (34 mL). After stirring at RT for 24 h, the reaction mixture was diluted with water (500 mL) and neutralized with solid $NaHCO_3$. The mixture was extracted with EtOAc (500 mL). The organic layer was then further washed with water (5×500 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification by flash chromatography (5→30% EtOAc/hexanes) on silica gel furnished the desired product as a slightly yellow oil (1.3966 g, 47%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.40–7.28 (m, 10H), 5.14 (s, 2H), 5.12 and 5.09 (ABq, J_{AB} = 12.4 Hz, 2H), 4.61–4.50 (m, 1H), 4.59 and 4.52 (ABq, J_{AB} = 11.2 Hz, 2H), 3.33 (ddd, J = 5, 11, 11 Hz, 1H), 2.45–2.35 (m, 1H), 2.32–2.22 (m, 1H), 2.17 (s, 3H), 2.00–1.93 (m, 1H), 1.92–0.97 (m, 23H), 0.95 (s, 3H), 0.90 (d, J = 6.2 Hz, 3H), 0.63 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.2, 15.3, 18.4, 21.3, 23.3, 26.3, 26.6, 28.5, 31.0, 31.3, 32.5, 33.0, 34.1, 34.5, 35.2, 39.4, 40.1, 41.5, 42.0, 43.8, 55.0, 55.8, 66.1, 69.4, 73.0, 77.9, 78.1, 128.16, 128.23, 128.3, 128.46, 128.54, 128.6 135.4, 136.2,154.6, 174.1 ppm.

3-(Benzyloxycarbonyloxy)-7-(chloromethoxy)ursodeoxycholic acid benzyl ester (11): A solution of **9** (847 mg, 1.25 mmol) in dry CH_2Cl_2 (20 mL) was treated with 2 M $SOCl_2$ in CH_2Cl_2 (1.9 mL, 3.8 mmol). The reaction mixture was heated in a microwave to 100 °C for 30 min and then concentrated in vacuo. The ^{1}H NMR spectrum of the crude material in $CDCl_3$ showed a new set of doublets δ = 5.56 and 5.47 ppm (J = 5.4 Hz, 1H each)[35] and the disappearance of the AB pattern at δ = 4.59 and 4.52 ppm as well as the SMe peak present in the ^{1}H NMR spectrum of **9** at δ = 2.17 ppm. The crude material was used in the next reaction without further purification.

7-(Phosphonooxymethoxy)ursodeoxycholic acid tributylamine salt (13a): A suspension of H_3PO_4 (586 mg, 5.98 mmol) and 4 Å molecular sieves (2.023 g) in CH_3CN (40 mL) was treated with Bu_3N (5.4 mL, 22.7 mmol). The mixture was stirred overnight and then added to a flask containing crude **11**. After stirring for 24 h, the mixture was filtered through Celite and concentrated in vacuo. The residue was dissolved in MeOH (50 mL) and concentrated in vacuo again. Next, the residue was dissolved in MeOH (50 mL), 10% Pd/C (2.369 g) was added, and the reaction mixture stirred at RT under an atmosphere of H_2 maintained using a gas-filled balloon for 2 h and then filtered through Celite. Additional 10% Pd/C (2.14 g) was added to the filtrate, and the reaction mixture was again stirred under H_2 for an additional 72 h. The reaction mixture was filtered through Celite and concentrated in vacuo. The resulting residue was purified by liquid chromatography (5% CH_3CN/water to 100% CH_3CN, C_{18} column) to yield, after lyophilization, the desired product as a white solid (116.7 mg). There are ~1.4 equiv of NBu_3 present for every equiv of bile acid based on ^{1}H NMR analysis (c.f. integration of CH_3 peak at δ = 0.70 ppm and multiplet at δ = 3.12–3.02 ppm). ^{1}H NMR (400 MHz, CD_3OD): δ = 5.18 (dd, J = 6 Hz, J_{31P} = 6 Hz, 1H), 4.99 (dd, J = 6 Hz, J_{31P} = 8 Hz, 1H), 3.66–3.55 (m, 1H), 3.53–3.42 (m, 1H), 3.13–3.02 (m, 8.2H), 2.35–2.24 (m, 1H), 2.20–2.10 (m, 1H), 2.08–1.98 (m, 1H), 1.94–0.90 (m, 57H), 0.70 ppm (s, 3H); LC/MS (ESI−): m/z = 501.3 $[M-H]^-$.

7-(Phosphonooxymethoxy)ursodeoxycholic acid sodium salt (13b): A 1 cm wide column was filled with 12 cm of DOWEX 50W2 (50–100 mesh, strongly acidic) ion exchange resin.[28] The column was prepared by sequentially washing with MeOH/water (1:1), 1 M aq $NaHCO_3$ (gas evolution), water, and then finally MeOH/water

(1:1). Compound **13a** (115 mg) was dissolved in MeOH/water (1:1) and loaded onto the column, which was eluted with MeOH/water (1:1). The product-containing fractions were lyophilized to furnish the desired product as a white solid (76.4 mg): ^{1}H NMR (400 MHz, D_2O): $\delta = 5.18$ (dd, $J = 5.7$ Hz, $J_{31P} = 6.8$ Hz, 1H), 4.99 (dd, $J = 5.7$ Hz, $J_{31P} = 9.4$ Hz, 1H), 3.74–3.56 (m, 2H), 2.40–2.28 (m, 1H), 2.27–2.14 (m, 1H), 2.08 (m, 24H), 1.00–0.92 (m, 6H), 0.70 ppm (s, 3H).

3-(Methylthiomethoxy)-7-(benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (14): A solution of **5** (1.113 g, 1.80 mmol) in DMSO (17 mL) was treated with Ac_2O (10.5 mL) followed by AcOH (17 mL). After stirring at RT for 24 h, the reaction mixture was diluted with water (500 mL) and neutralized with solid $NaHCO_3$. The mixture was extracted with EtOAc (500 mL). The organic layer was then further extracted with water (5×500 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification by flash chromatography (5→50% EtOAc/hexanes) on silica gel furnished the desired product as a slightly yellow oil (364 mg, 30%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.41$–7.28 (m, 10H), 5.16 and 5.12 (ABq, $J_{AB} = 12.0$ Hz, 2H), 5.12 and 5.10 (ABq, $J_{AB} = 12.4$ Hz, 2H), 4.67–4.58 (m, 1H), 4.65 (s, 2H), 3.57 (dddd, $J = 5$, 5, 10, 10 Hz, 1H), 2.44–2.34 (m, 1H), 2.31–2.22 (m, 1H), 2.15 (s, 3H), 2.00–1.93 (m, 1H), 1.92–0.95 (m, 23H), 0.94 (s, 3H), 0.89 (d, $J = 6.3$ Hz, 3H), 0.62 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.2$, 13.7, 18.3, 21.2, 23.2, 25.6, 26.9, 28.4, 31.0, 31.3, 33.1, 33.4, 34.2, 34.7, 35.2, 39.2, 39.9, 40.0, 42.2, 43.6, 55.0, 55.2, 66.1, 69.3, 72.0, 75.2, 78.5, 128.10, 128.18, 128.24, 128.39, 128.64, 135.6, 136.1, 154.6, 174.0 ppm.

3-(Chloromethoxy)-7-(benzyloxycarbonyloxy)ursodeoxycholic acid benzyl ester (15): A solution of **14** (360 mg, 0.53 mmol) in dry CH_2Cl_2 (20 mL) was treated with 2 M $SOCl_2$ in CH_2Cl_2 (0.8 mL, 1.6 mmol). The reaction mixture was heated in a microwave to 100 °C for 30 min and then concentrated in vacuo. The ^{1}H NMR spectrum of the crude material in $CDCl_3$ showed a new AB pattern at $\delta = 5.55$ and 5.54 ppm ($J = 5.4$ Hz, 2H total) and the disappearance of the singlet at $\delta = 4.65$ ppm as well as the SMe peak present in the ^{1}H NMR spectrum of **14** at $\delta = 2.15$ ppm. The crude material was used without further purification in the next reaction.

3-(Phosphonooxymethoxy)ursodeoxycholic acid tributylamine salt (16): A suspension of H_3PO_4 (248 mg, 2.53 mmol) and 4 Å molecular sieves (0.760 g) in CH_3CN (15 mL) was treated with Bu_3N (2.3 mL, 9.68 mmol). The mixture was stirred overnight and then added to a flask containing crude **15**. After stirring for 72 h, the mixture was filtered and concentrated in vacuo. The residue was dissolved in MeOH (25 mL) and again concentrated in vacuo. Next, the residue was dissolved in MeOH (40 mL), 10% Pd/C (656 mg) was added, and the reaction mixture was stirred under a balloon filled with H_2 for 2 h and then filtered through Celite. A crude NMR of an aliquot showed no reaction. Additional 10% Pd/C (744 mg) was added, and the reaction mixture was stirred under a balloon filled with H_2 for 2 h and filtered through Celite. A crude NMR of an aliquot again showed no reaction. An additional 10% Pd/C (1901 mg) was added, and the reaction mixture was stirred under a balloon filled with H_2 overnight, filtered through Celite, and concentrated under reduced pressure. The resulting residue was purified by liquid chromatography (5% CH_3CN/H_2O to 100% CH_3CN, C_{18} column) to yield 80.7 mg of a white solid after lyophilization. There are ~1.7 equiv of NBu_3 present for every equiv of bile acid based on ^{1}H NMR analysis (c.f. integration of CH_3 peak at $\delta =$ 0.71 ppm and multiplet at $\delta = 3.12$–3.02 ppm). ^{1}H NMR (400 MHz, CD_3OD): $\delta = 5.08$ (d, $J_{31P} = 8.4$ Hz, 2H), 3.75–3.63 (m, 1H), 3.54–3.43 (m, 1H), 3.12–2.98 (m, 10H), 2.34–2.23 (m, 1H), 2.19–2.09 (m, 1H), 2.08–20 (m, 1H), 1.94–0.90 (m, 68H), 0.71 ppm (s, 3H); LC/MS (ESI−): $m/z = 501.3$ $[M-H]^-$.

Ursodeoxycholic acid (bis(benzyloxy)phosphoryloxy)methyl ester (18): A suspension of UDCA (1.46 g, 3.72 mmol) and K_2CO_3 (984 mg, 7.12 mmol) in DMF (10 mL) was treated with dibenzyl chloromethyl phosphate (1.23 g, 3.76 mmol). The mixture was stirred at RT overnight, diluted with water (250 mL), and extracted with EtOAc (3×250 mL) and CH_2Cl_2 (1×250 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification by flash chromatography (40→100% EtOAc/hexanes) on silica gel furnished the desired product as a clear, colorless foamy oil (2.05 g, 81%): ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.42$–7.38 (m, 10H), 5.64 (d, $J_{31P} = 13.8$ Hz, 2H), 5.10 (d, $J_{31P} = 8.3$ Hz, 4H), 3.58–3.44 (m, 2H), 2.41–2.31 (m, 1H), 2.29–2.18 (m, 1H), 2.08–1.98 (m, 1H), 1.96–1.72 (m, 5H), 1.70–1.00 (m, 18H), 0.99 (s, 3H), 0.93 (d, $J = 6.5$ Hz, 3H), 0.71 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta =$ 12.7, 18.9, 22.4, 23.9, 27.9, 29.6, 31.1, 31.6, 31.8, 35.2, 36.1, 36.5, 38.0, 38.6, 40.7, 41.5, 44.0, 44.5, 44.8, 56.4, 57.5, 71.1 (d, $J_{31P} =$ 5.9 Hz), 71.9, 72.1, 83.9 (d, $J_{31P} = 5.7$ Hz), 129.2, 129.7, 129.8, 136.9, 137.0, 173.8 ppm. ^{31}P NMR (162 MHz, CD_3OD): $\delta = -1.59$ ppm; HRMS: m/z $[M+H]^+$ calcd for $C_{39}H_{55}O_8P$: 683.3713, found: 683.3735.

General procedure for the synthesis ursodeoxycholic acid phosphonooxymethoxy ester salts (19b–e): A solution of **18** in MeOH was treated with 10% Pd/C. The reaction mixture was stirred at RT under an atmosphere of H_2 maintained using a gas-filled balloon for 45 min and then filtered through Celite. The appropriate amine (1–2 equiv) was added to the filtrate, and the solution concentrated in vacuo.

Mono-Tris salt **19d**: White solid, 99% yield: ^{1}H NMR (400 MHz, D_2O): $\delta = 5.51$ (d, $J_{31P} = 12.8$ Hz, 2H), 3.74 (s, 6H), 3.60–3.54 (m, 2H), 2.58–2.46 (m, 1H), 2.44–2.32 (m, 1H), 2.09–1.98 (m, 1H), 1.96–1.74 (m, 5H), 1.72–1.02 (m, 18H), 1.01–0.95 (m, 6H), 0.72 ppm (s, 3H); ^{13}C NMR (100 MHz, D_2O): $\delta = 12.6$, 19.0, 22.0, 23.9, 27.2, 29.0, 30.2, 31.1, 31.4, 34.4, 35.4, 35.7, 36.6, 37.3, 39.9, 40.8, 42.8, 43.7, 44.1, 55.3, 56.1, 60.0, 62.1, 71.6, 71.7, 83.6, 176.4 ppm; ^{31}P NMR (162 MHz, D_2O): $\delta = -0.30$ ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{25}H_{43}O_8P$: 501.2617, found: 501.2585.

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