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**PAPER**

# Continuous flow synthesis and scale-up of glycine- and taurine-conjugated bile salts†

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A multi-gram scale protocol for the *N*-acyl amidation of bile acids with glycine and taurine has been successfully developed under continuous flow processing conditions. Selecting ursodeoxycholic acid (UDCA) as the model compound and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) as the condensing agent, a modular mesoreactor assisted flow set-up was employed to significantly speed up the optimization of the reaction conditions and the flow scale-up synthesis. The results in terms of yield, in line purification, analysis, and implemented flow set-up for the reaction optimization and large scale production are reported and discussed.

## Introduction

In recent years, the research in the bile acid (BA) field has undergone a profound evolution.[1] From simple natural detergents involved in the absorption of dietary lipids and fat-soluble nutrients, they are now recognized as crucial signalling molecules that participate in multiple paracrine and endocrine functions related to the homeostasis of lipid and cholesterol levels, control of energetic expenditure, and regulation of the immune system. The redefinition of the patho-physiological role of BAs has led to a renewed interest in their use for medicinal chemistry programmes and for biochemical and therapeutic investigations.[2,3] This makes of high priority the search for novel efficient synthesis and valuable analytical protocols, useful for the preparation, identification and characterization of this important class of compounds.

In this framework, with the aim to combine novel advanced synthetic technologies with our knowledge in the BA chemistry and analysis, herein, we propose an improved synthesis and scale-up of naturally-occurring conjugated bile salts using a modular mesoreactor assisted flow set-up, along with a validated HPLC method.

## Background

BAs represent the principal catabolic product of hepatic cholesterol. Before the secretion into the gall bladder, they are conjugated at the carboxyl group with glycine (**3**) (75%) and taurine (**4**) (25%) and stored until the next meal.[4] The conjugation which is carried out by the enzyme BA CoA amino acid *N*-acyl transferase (BAAT), alters the physicochemical properties of BAs making them more hydrophilic and fully ionized at physiological pH. This process contributes to modify their critical micellar concentration (CMC) into the bile, preventing their diffusion through the epithelium of the biliary tree and the passive reabsorption from the small intestine.[5] Once requested for digestion, both glyco- and tauro-conjugates are released into the duodenum and recycled through the enterohepatic circulation where they go through further metabolic enzymatic transformations.

In humans, defects and alterations in BA *N*-acyl amidation are often the cause of severe cholestatic liver diseases[1,6] and may contribute to the overproduction of potentially toxic secondary BAs.[7] In this regard, it has become critically important to have ready access to BA conjugates as diagnostic tools for the detection of errors in BA synthesis and their metabolism, as well as for the spectrometric determination of BA plasma levels and to study bacterial overgrowth in the gastrointestinal tract. It is worth emphasizing that the reference standards used in such analytical and pharmacokinetical assays need to be of high purity, meaning that the synthetic approach is of paramount importance.

Currently, a number of synthetic methods for the preparation of *N*-acyl amidated BAs are available,[8] although most of them suffer from several problems including the presence of protection–deprotection extra steps, low yields, laborious protocols, the use of drastic reaction conditions, and complicated work-ups and purifications. In particular, the purification process is often difficult, time consuming and expensive due to the high polarity and relative insolubility of BA conjugates and to the cost of the reversed-phase liquid chromatography required to obtain pure products (>95%). Whilst the use of enzymatic methodologies still remains elusive, improved syntheses of taurine- and glycine-conjugated BAs have recently been described using diethyl phosphonocyanidate as an alternative coupling agent[9] and microwave heating.[10]

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In continuation of our interest in the development of novel synthetic methodologies for the preparation of biologically active steroids,[11] we report the continuous flow synthesis of naturally-occurring conjugated bile salts (BSs). Particularly, the major goals of this study will focus on the synthetic efficiency, the product quality, and the cost of the process (Table 1). To accomplish this, we have studied four consequential and integrated phases. These include: (a) design of a convenient flow set-up, (b) validation of the HPLC method for the quantitative determination of the reaction yield, (c) optimization of the reaction conditions, (d) scale-up synthesis.

Commencing with our experience of BA *N*-acyl amidation and with classical laboratory batch-screen,[12] we identified 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, **2**) as the most promising activating agent to achieve the desired outcome. In addition, given the importance of ursodeoxycholic acid (UDCA, **1a**) and its conjugates as commercially available therapeutic agents,[13] we selected the reaction of **1a** with glycine (**3**) in the presence of EEDQ (**2**) as the model reaction for the implementation of the flow set-up and for the fine tuning of the reaction conditions (Scheme 1). Once the optimal flow set-up and experimental conditions were established, the reaction was then applied to primary and secondary human BAs **1b–d**.

## Results and discussion

### Flow set-up

The flow experiments were performed in a commercially available flow mesoreactor equipped with a two-loop injection system (2 ml each), two pumps dedicated to an aqueous and organic reservoir of solvent, respectively, a 10 ml PTFE reactor heater, a back pressure regulator (BPR, 100 psi), a UV detector and fraction collector (Fig. 1). During the optimization of the experimental conditions, the reactions were performed by loop injection of two stock solutions: the first one contained UDCA (**1a**) and EEDQ (**2**) dissolved in the organic solvent of choice, and the second a solution of glycine (**3**) in water. In this phase, the base was added either in the organic or alternatively in the aqueous stock solution according to its relative solubility in the reaction medium (Table 2). After the injection and the switching of the valves of R2+ system through the loops, the two solutions were mixed in a T-junction, pumped through the coil reactor, warmed at the selected temperature, and the output collected using a fraction collector (Fig. 1).

**Table 1** Study goals

| Effect on synthesis | Target/constraint |
|---|---|
| Efficiency | Maximize the yield of reaction |
| Product quality | Reduce the levels of impurities and by-products |
| Costs | (a) Avoid work-up and chromatographic purification especially for large scale synthesis<br>(b) Avoid protection/deprotection extra-steps<br>(c) Increase the productivity minimizing the reaction time |

The conversion of the substrate and the relative reaction yield was determined by quantitative HPLC analysis. This approach was suitable to screen different reaction conditions using the minimum amount of starting material. Indeed, the presence of the injection loop system and the connection with a fraction collector and a UV detector, allowed us to process several small samples in succession using the same solvent system and varying the base or the starting material. The in line UV detector was instrumental to detect the presence or the absence of the quinoline released from the reaction of EEDQ (**2**). Thus, the absence of quinoline from the collected output indicated the end of the reaction and the start of the next experiment.

### HPLC analysis

The employment of flow chemistry in the process optimization of the BA *N*-acyl amidation required the validation of a HPLC

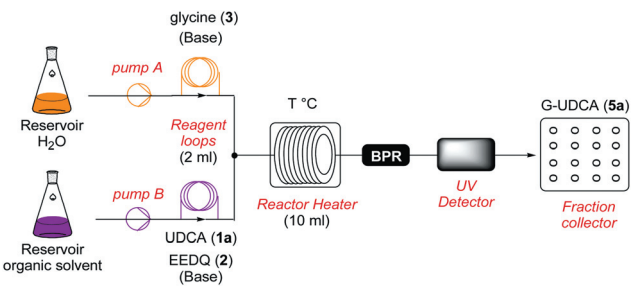
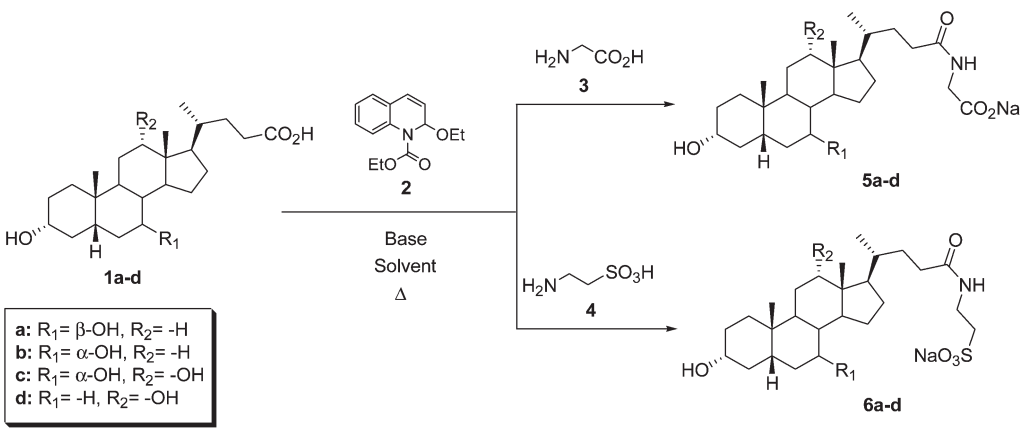



**Fig. 1** Flow set-up used during the optimization of the reaction conditions.




**Scheme 1** General scheme for the synthesis of glycine- and taurine-conjugated bile salts.

**Table 2** Solvent system and base effect[a]

| Entry | Base (solvent)[b] | Solvent system (v/v/v) | G-UDCA (**5a**)[c] (% yield) |
|---|---|---|---|
| 1 | $Et_3N$ (o) | $H_2O$–THF (1 : 1) | 78[d] |
| 2 | Pyr (o) | $H_2O$–THF (1 : 1) | 49 |
| 3 | NaOH (w) | $H_2O$–THF (1 : 1) | 84 |
| 4 | *t*BuOK (w) | $H_2O$–THF (1 : 1) | 78 |
| 5 | $Et_3N$ (o) | $H_2O$–$CHCl_3$ (1 : 1)[f] | EtUDCA |
| 6 | $Et_3N$ (o) | $H_2O$–dioxane (1 : 1) | 72[d] |
| 7 | Pyr (o) | $H_2O$–dioxane (1 : 1) | 46 |
| 8 | NaOH (w) | $H_2O$–dioxane (1 : 1) | 62 |
| 9 | NaOH (w) | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | 92 |
| 10 | $Et_3N$ (o) | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | 79[d] |
| 11 | *t*BuOK (w) | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | 87 |
| 12 | Pyr (o) | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | 78[d] |
| 13 | NaOH (w)[e] | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | Traces |

[a] UDCA : EEDQ : glycine : base: 1 : 2 : 2.5 : 2; combined flow rate: 0.3 ml $min^{-1}$; *T*: 120 °C. [b] The base was dissolved in the organic solvent of choice (o) or in water (w). [c] Determined by HPLC analysis of the crude reaction mixture. [d] Presence of impurities. [e] UDCA was salified with NaOH in the water phase and processed under flow set-up depicted in Fig. 1. [f] BPR: 250 psi.

**Table 3** Temperature and flow rate effect[a]

| Entry | *T* (°C) | Flow rate (ml $min^{-1}$) | G-UDCA (**5a**)[b] (% yield) |
|---|---|---|---|
| 1 | 30 | 0.3 | 89 |
| 2 | 55 | 0.3 | 90 |
| 3 | 80 | 0.3 | 95 |
| 4 | 100 | 0.3 | 90 |
| 5 | 130 | 0.3 | 93[c] |
| 6 | 140 | 0.3 | 94[c] |
| 7 | 150 | 0.3 | 85[c] |
| 8 | 80 | 0.2 | 87 |
| 9 | 80 | 0.5 | 85 |
| 10 | 80 | 0.7 | 89 |
| 11 | 80 | 1.0 | >95 |
| 12 | 80 | 1.5 | 91 |
| 13 | 80 | 2.0 | 89 |
| 14 | 80 | 3.0 | 90 |
| 15 | 80 | 4.0 | 85 |

[a] UDCA : EEDQ : glycine : NaOH: 1 : 2 : 2.5 : 2; $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1, v/v/v). [b] Determined by HPLC analysis of the crude reaction mixture. [c] Presence of impurities (<10%).

method for the determination of the reaction yields as well as of the purity grade of the synthesized conjugates. In this context, a unique HPLC method was successfully established and validated for UDCA (**1a**), CDCA (**1b**), CA (**1c**), DCA (**1d**), as well as for the corresponding glyco- (**5a–d**) and tauro-conjugated (**6a–d**) forms. Because of the shared absence of relevant chromophoric moieties in the sample structure, an evaporative light scattering detector (ELSD)[14] was profitably utilized for the analysis of such steroidal species. For each of the investigated compounds, all the runs were contemporarily carried out on the free and the two relative conjugated species. The different ELSD response of the free and the corresponding conjugated BAs imposed to build-up separate calibration curves. In all the cases, very good precision and accuracy (evaluated both in the short and long period) along with remarkably low LOD and LOQ values were obtained.

### Reaction optimization

With the aim to find the experimental conditions that would ensure the maximum conversion, the minimum formation of side-products and the largest productivity (Table 1), the optimization of the reaction conditions was performed through a traditional experimental approach varying the solvent system, the base, the temperature and the residence time (flow rate) (Tables 2 and 3). The reactions were conducted using a 0.2 M organic solution of UDCA (**1a**) (1 equiv.) and EEDQ (**2**) (2 equiv.) (pump A) along with an aqueous solution of glycine (**3**) (2.5 equiv.) (pump B) (Fig. 1). As anticipated, before the reactions were carried out, the base (2 equiv.) was added to the organic or aqueous stock solution according to its solubility in the medium of choice. In addition, to ensure the complete solubilization of the reagents the resulting mixtures were mixed at 50 °C for 10 min and then cooled to room temperature before the loading into the loop.

As a starting point, we focused our attention on choosing the appropriate organic solvent and base (Table 2). The two reservoirs containing the aqueous and organic solution were degassed for 5 min and connected to the respective dedicated pump with the flow rate fixed at 0.3 ml $min^{-1}$ (pump A 0.15 ml $min^{-1}$ + pump B 0.15 ml $min^{-1}$). The temperature of the coil reactor in the R4 system was fixed at 120 °C and the solutions containing the reagents were injected into the sample loops and processed. As anticipated, the in line UV detector was instrumental in showing the quinoline released by the reaction of EEDQ (**2**) and in initializing the fraction collector, where the output of the reactions was collected in 9 ml tubes. The pH of fractions containing the product was adjusted to 2 by addition of 3 N HCl, and the crude reaction mixture was then analyzed by HPLC.

As shown in Table 2, the reaction can be performed in a fair number of solvents and bases, and provided **5a** in good to high yield. As a general trend, the reactions carried out in THF (Table 2, entries 1–4) afforded G-UDCA (**5a**) in higher yield compared to those performed in dioxane (Table 2, entries 6–8), while the employment of chloroform as the organic solvent led exclusively to the formation of the ethyl ursodeoxycholate (EtUDCA, Table 2, entry 5). In addition, the use of pyridine as the base proved to be detrimental for the reaction outcome (Table 2, entries 2, 7 and 12). The best results were obtained with a $CH_3CN$–*t*-BuOH (3 : 1, v/v) solution, where the formation of the activated mixed anhydride of UDCA was faster and proceeded in high conversion (Table 2, entries 9–12). Furthermore, we have observed that moving the UDCA from the organic solvent to the aqueous phase *via* salification with NaOH, gave only traces of the desired product (Table 2, entry 13).

At this stage, the experimental conditions reported in Table 2, entry 9 (base: NaOH, solvent system: $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1, v/v/v) were selected for the second step of the optimization study to evaluate the effect of the temperature and the flow rate on the reaction outcome. The choice was guided by the following considerations:

(a) Reaction conversion >90% and no side-product formation. This will avoid difficult and high cost reversed-phase chromatographic purifications required to achieve the product with high purity (>95%).

(b) Green solvents. The solvent systems were initially selected considering the relative solubility of the reagents and products formed: they must avoid the precipitation of reagents and products in the tube reactor. Once the diverse efficiency of each solvent system was defined in terms of reaction yield and by-products, we then considered the relative 'green order':[15] $H_2O$ > *t*-BuOH > MeCN = THF > dioxane > $CH_3Cl$.

(c) Precipitation of the product in the acidic media. Before running the HPLC analysis, the output collected in the fraction collector was acidified at pH 2 with 3 N HCl. In the case of reaction mixtures performed in $H_2O$–$CH_3CN$–*t*-BuOH solution, the precipitation of a white solid was observed, suggesting the possibility to purify the conjugates by precipitation and without the use of tedious and expensive reversed-phase chromatography. This observation is of great importance especially in a view to apply the method to a large scale synthesis.

Based on these remarks and using the flow set-up illustrated in Fig. 1, the $CH_3CN$–*t*-BuOH solution of UDCA (**1a**) and **2** was reacted with an equal volume of an aqueous solution of glycine (**3**) and NaOH to evaluate the effect of the reactor coil temperature and of the flow rate. Initially, the reactions were performed at temperatures ranging from 30 to 150 °C with the flow rate fixed at 0.3 ml $min^{-1}$. It emerges from the results reported in Table 3 (entries 1–7) that although the influence of temperature on the reaction outcome is not significant, the corresponding formation of side products was observed with higher temperatures.

Subsequently, with the aim to reduce the reaction time while maintaining a high conversion yield, we evaluated the influence of the residence time. Thus, the stock solutions were reacted at the temperature that gave the best reaction conversion without the formation of by-products (80 °C), employing diverse flow rates (Table 3, entries 8–15). As a result, a high efficiency was maintained with a flow rate up to 4 ml $min^{-1}$ and a residence time of 2.5 min.

In summary, our optimal flow conditions were 0.2 M concentration, 2 equiv. of NaOH as the base, $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1, v/v/v) as the solvent system, a combined reagent flow rate of 1 ml $min^{-1}$ and a residence time in the heated zone (80 °C) of approximately 10 min.

### Scale-up

Having established the optimal flow conditions, the reaction was scaled-up with a factor of 100 using the same modular flow reactor. In this regard, we thought to equip the flow set-up with an in line purification system consisting of a Michel-Miller chromatography column (*L* × I.D. 300 mm × 21 mm, for 20 g of resin) packed with a sulphonic acid resin (PS-SA, Amberlyst A-15) (Fig. 2). The presence of this solid supported scavenger was instrumental to catching the excess of **3** and part of the quinoline (derived from **2**). The real power of this in line purification method consists of the precipitation of the pure G-UDCA (**5a**) by addition of a solution of 3 N HCl, making the process independent from a further off line purification.

Thus, 15.7 g (40 mmol) of UDCA (**1a**) were premixed with EEDQ (**2**) in 200 ml of $CH_3CN$–*t*-BuOH and processed in the usual fashion. The collected output was acidified with 3 N HCl to pH 2 and partially concentrated. The precipitate (16.3 g) was filtered and washed with acetone. In this way, up to 91% of pure

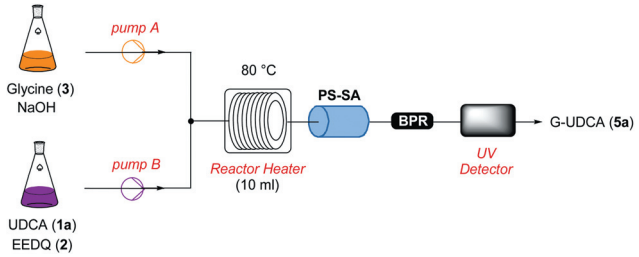



**Fig. 2** Flow set-up for the scale-up synthesis of G-UDCA (**5d**).

**5a** (HPLC purity >98%) was obtained. This result clearly demonstrates the potentiality of flow-based techniques for the preparation of these valuable materials in a bulk quantity.

### *N*-Acyl amidation of human bile acids

Finally, we explored the versatility of our methodology in the synthesis of other human glyco- and tauro-conjugated BSs **5b–d** and **6a–d** (Scheme 1, Table 4) using the flow set-up illustrated in Fig. 1, equipped with the in line purification system (PS-SA, Amberlyst A-15). Thus, the glyco-conjugation of CDCA (**1b**) and DCA (**1d**) afforded the desired products **5b,d** in good yield (78% and 75%, respectively), while a quantitative conversion was obtained with CA (**1c**) (Table 3). In this regard, it is worth noting that in the case of DCA (**1d**) and CA (**1c**), a 3 : 1 : 0.5 (v/v/v) solution of $CH_3CN$–*t*-BuOH–DMSO was needed in order to ensure the complete solubilization of the reagents. Importantly, our flow synthetic protocol was very efficient also for the preparation of the corresponding tauro-conjugates **6a–d**, affording the desired products in nearly quantitative yield (Table 4).

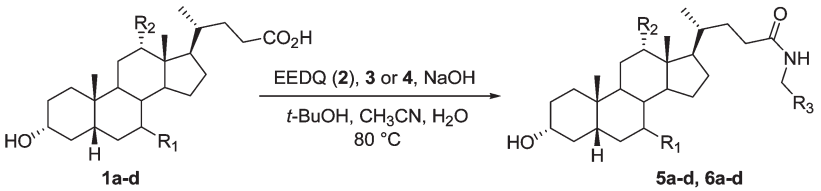
## Conclusions and perspectives

The development of flow synthetic approaches and the application of novel technologies for the preparation of biological active compounds has attracted considerable interest over recent years,[16] especially for large scale product production.[17] With the aim to reduce the costs, maximize the synthetic efficiency, and avoid hazardous reagents and laborious work-up and purifications, classical batch synthetic processes are usually scaled-up by using larger size reactors and by the optimization of the experimental protocol. This process, which is usually time-consuming and expensive, in the case of microreactor flow technology becomes faster, reliable, reproducible, economic and safe.

In this context, we have brought together for the first time BAs with flow chemical technology. In particular, a one pot process for the production of highly pure glyco- and tauro-conjugated BSs is described using a continuous flow approach. Reactions were performed using an organic solution of BAs and EEDQ (**2**) along with an aqueous solution of the amino acid and NaOH, a reagent flow rate of 1 ml $min^{-1}$ and a residence time in the heated zone (80 °C) of approximately 10 min. Advantages of our method compared to previous batch mode approaches include the low cost and efficiency gained through the avoidance of laborious work-up and purifications, the rapid optimization and precise control of the reaction conditions, the possibility to scale-up and continually process material on-demand with high

**Table 4** *N*-Acyl amidation of BAs in flow condition mode[a]




| Educt | $R_1$ | $R_2$ | $R_3$ | Solvent system[a] (v/v/v) | Products[b] (% yield) | Isolated yield[c] (%) |
|---|---|---|---|---|---|---|
| **1a** | β-OH | –H | $-CO_2H$ | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | **5a** (>95) | 91 |
| **1a** | β-OH | –H | $-CH_2SO_3Na$ | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | **6a** (>95) | 95 |
| **1b** | α-OH | –H | $-CO_2Na$ | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | **5b** (82) | 78 |
| **1b** | α-OH | –H | $-CH_2SO_3Na$ | $H_2O$–MeCN–*t*-BuOH (4 : 3 : 1) | **6b** (>95) | 93 |
| **1c** | α-OH | –OH | $-CO_2Na$ | $H_2O$–$CH_3CN$–*t*-BuOH–DMSO (4 : 3 : 1 : 0.5) | **5c** (>95) | 95 |
| **1c** | α-OH | –OH | $-CH_2SO_3Na$ | $H_2O$–$CH_3CN$–*t*-BuOH/DMSO (4 : 3 : 1 : 0.5) | **6c** (91) | 88 |
| **1d** | –H | –OH | $-CO_2Na$ | $H_2O$–$CH_3CN$–*t*-BuOH–DMSO (4 : 3 : 1 : 0.5) | **5d** (80) | 75 |
| **1d** | –H | –OH | $-CH_2SO_3Na$ | $H_2O$–$CH_3CN$–*t*-BuOH–DMSO (4 : 3 : 1 : 0.5) | **6d** (>95) | 95 |

[a] All reactions were processed under flow set-up depicted in Fig. 1. [b] Determined by HPLC analysis of the crude reaction mixture. [c] Determined after purification by reverse phase (RP-18) chromatography.

yield and purity. The rapid and easy access to BA conjugates not only will help the candidate selection of BA analogs as therapeutic agents in the treatment of metabolic and liver diseases, but it will also fill the constant request of these compounds for biological studies and diagnostic purposes.

## Experimental procedures

### General methods

$^1$H NMR spectra were recorded at 400 MHz, $^{13}$C NMR spectra were recorded at 100.6 MHz using the solvents indicated below. Chemical shifts are reported in ppm. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet, dddd, double double double doublet, t, triplet, dt, double triplet, qt, quartet triplet, bs, broad signal. All experiments were performed using a commercially available Vapourtec R2+/R4 module. BAs reference standard were purchased from CalBiochem (purity >96%). TLC was performed on aluminium backed silica plates (silica gel 60 F254). All the reagents were of analytical grade. HPLC-grade water was obtained from a tandem Milli-Ro/Milli-Q apparatus. The analytical HPLC measurements were made on a Shimadzu LC-20A Prominence equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector and a Rheodyne 7725i injector with a 20 µL stainless steel loop. A Varian 385-LC evaporative light scattering detector (ELSD) was utilized for the analyses. The analog-to-digital conversion of the output signal from the ELSD was allowed by a common interface device. The adopted ELSD conditions for the analysis of all BAs were: 30 °C nebulization temperature, 50 °C evaporation temperature, 1.5 L min$^{-1}$ gas flow rate (air) and 2.0 as the gain factor. A GraceSmart RP18 column 250 × 4.6 mm I.D., 5 µm, 100 Å was used as the analytical column. The column temperature was controlled through a Grace heather/chiller thermostat.

**General method for the preparation of glyco- and tauro-conjugated BSs 5a–d and 6a–d.** BA (0.5 mmol) and EEDQ (1 mmol) were dissolved in a mixture of $CH_3CN$–*t*-BuOH (3 : 1, v/v), while the amino acid (1.25 mmol) and NaOH (1 mmol) were solubilized in water. Both solutions made up to 2.5 ml, were mixed at 50 °C for 10 min, cooled at room temperature and then loaded into the corresponding loop. A degassed solution of $CH_3CN$–*t*-BuOH (3 : 1, v/v) and a reservoir of water were connected with pump A and B (see Fig. 1) and the flow rate was fixed at 1 ml min$^{-1}$ (0.5 ml min$^{-1}$ + 0.5 ml min$^{-1}$). After switching the sample loops, the mixtures exited were joined in a T-piece, entered in a 10 ml PTFE coil reactor warmed at 80 °C fitted with the back pressure regulator (100 psi), directed in a Michel-Miller chromatography column (L × I.D. 85 mm × 8 mm) packed with sulphonic acid resin (3 g Amberlyst A-15), and the output was recovered in a fraction collector. A small sample of the reaction was acidified at pH 2 with HCl 3 N and analyzed by HPLC, while the crude reaction mixture was concentrated, salified by using a 10% aqueous solution of NaOH (10%) and passed through a SNAP RP-18 (25 g) in Biotage SP-1 system.

Glyco-chenodeoxycholic acid, sodium salt (**5b**)[18] was obtained in 75% isolated yield as white solid, mp: 161–165 °C. RP-HPLC Rt = 11.37 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 3.2 (1 H, m, C(3)H), 3.4 (2 H, d, $J$ = 7 Hz, C(25)$H_2$), 3.6 (1 H, s, C(7)H), 4.1 (1 H, bs s, OH), 4.4 (1 H, bs s, OH), 7.2 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 11.7, 18.4, 20.3, 22.7, 23.2, 27.8, 30.6, 31.6, 32.3, 32.4, 34.7, 34.8, 35.2, 35.3, 39.2, 39.6, 40.1, 41.5, 41.9, 44.0, 50.0, 55.7, 66.2, 70.3, 171.7, 172.3.

Glyco-cholic acid, sodium salt (**5c**)[18] was obtained in 95% isolated yield as white solid, mp: >250 °C. RP-HPLC Rt = 5.79 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 3.2 (1 H, m, C(3)H), 3.2 (2 H, d, $J$ = 7 Hz, C(25)$H_2$), 3.6 (1 H, s, C(7)H), 3.8 (1 H, s, C(12)H), 4.0 (1 H, bs s, OH), 4.2 (1 H, bs s, OH), 4.3 (1 H, bs s, OH), 7.1 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.4, 17.2, 22.6, 22.8, 26.2, 27.3, 28.5, 30.4, 31.7, 32.7, 34.4, 34.9, 35.3, 35.3, 40.2, 41.4, 41.6, 44.0, 45.8, 46.2, 66.3, 70.5, 71.1, 171.7, 171.8.

Glyco-deoxycholic acid, sodium salt (**5d**)[18] was obtained in 73% isolated yield as white solid, mp: 203–209 °C. RP-HPLC Rt = 13.07 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 2.6 (2 H, dd, C(3)H), 3.3 (1 H, m, C(3)H), 3.7 (2 H, d, $J$ = 7 Hz, C(25)$H_2$), 3.8 (1 H, s, C(12)H), 4.2 (1 H, bs s, OH), 4.4 (1 H, bs s, OH), 8.1 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.5, 17.1, 23.1, 23.5, 26.1, 27.0, 27.2, 28.6, 30.2, 32.2, 33.0, 33.8, 35.0, 35.2, 35.7, 36.3, 40.5, 41.6, 46.0, 46.3, 47.5, 70.0, 71.1, 171.5, 173.0.

Tauro-ursodeoxycholic acid, sodium salt (**6a**)[18] was obtained in 95% isolated yield as white solid, mp: 294–201 °C. RP-HPLC Rt = 4.04 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.9 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 2.6 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 7.5 Hz, C(26)$H_2$), 3.3–3.4 (4 H, m, C(3)H, C(25)$H_2$, C(7)H). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.1, 18.5, 20.9, 23.3, 36.7, 28.2, 30.3, 31.6, 32.6, 33.8, 34.9, 35.0, 35.5, 37.3, 37.7, 38.7, 39.9, 42.2, 43.0, 43.1, 50.6, 54.7, 55.9, 69.5, 69.8, 172.2.

Tauro-chenodeoxycholic acid, sodium salt (**6b**)[18] was obtained in 93% isolated yield as white solid, mp: 163–165 °C. RP-HPLC Rt = 6.48 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 2.6 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 7.5 Hz, C(26)$H_2$), 3.2 (1 H, m, C(3)H), 3.3 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 8.2 Hz, C(25)$H_2$), 3.6 (1 H, s, C(7)H), 4.1 (1 H, bs s, OH), 4.4 (1 H, bs s, OH), 7.7 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 11.6, 18.4, 20.3, 22.7, 23.2, 27.8, 30.7, 31.5, 32.3, 32.6, 34.7, 34.8, 35.0, 35.3, 35.4, 39.1, 39.6, 41.5, 41.9, 50.0, 50.6, 55.6, 66.2, 70.4, 172.2.

Tauro-cholic acid, sodium salt (**6c**)[18] was obtained in 88% isolated yield as white solid, mp: 228–230 °C. RP-HPLC Rt = 4.21 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 2.6 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 7.5 Hz, C(26)$H_2$), 3.2 (1 H, m, C(3)H), 3.3 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 8.2 Hz, C(25)$H_2$), 3.6 (1 H, m, C(7)H), 3.8 (1 H, m, C(12)H), 3.9 (1 H, bs d, OH), 4.0 (1 H, bs d, OH), 4.3 (1 H, bs d, OH), 7.7 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.4, 17.1, 22.6, 22.8, 26.2, 27.3, 28.6, 30.4, 31.6, 32.7, 34.4, 34.9, 35.2, 35.3, 35.5, 41.4, 41.6, 45.8, 46.1, 50.6, 66.3, 70.5, 71.0, 172.3.

Tauro-deoxycholic acid, sodium salt (**6d**)[18] was obtained in 95% isolated yield as white solid, mp: 170–175 °C. RP-HPLC Rt = 7.40 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.8 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 2.6 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 7.5 Hz, C(26)$H_2$), 3.3 (1 H, m, C(3)H), 3.3 (2 H, dd, $J_1$ = 6 Hz, $J_1$ = 8.2 Hz, C(25)$H_2$), 3.8 (1 H, s, C(12)H), 4.2 (1 H, bs d, OH), 4.4 (1 H, bs d, OH) 7.7 (1 H, bs pst, NH). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.5, 17.1, 23.1, 23.5, 26.1, 27.0, 27.2, 28.6, 30.3, 31.6, 32.7, 33.0, 33.8, 35.1, 35.2, 35.5, 35.7, 36.3, 41.7, 46.0, 46.2, 47.5, 50.6, 70.0, 71.0, 172.2.

**General procedures for the scaled-up synthesis of glyco-UDCA (5a).**[19] UDCA (15.7 g, 40 mmol) and EEDQ (80 mmol) were dissolved in a solution of $CH_3CN$–*t*-BuOH (3 : 1, v/v) and the volume was made up to 200 ml. The resulting mixture was premixed at 50 °C for 10 min, and pumped at 0.500 ml $min^{-1}$. An aqueous solution (200 ml) of glycine (100 mmol) and NaOH (80 mmol) was premixed at 50 °C for 10 min and pumped at 0.5 ml $min^{-1}$. The two solutions were joined in a T-piece and entered in a 10 ml PTFE coil reactor warmed at 80 °C, fitted with back pressure regulator (100 psi), and then directed in a Michel-Miller chromatography column (L × I.D. 300 mm × 21 mm) packed with sulphonic acid resin (20 g Amberlyst A-15). The output was collected, partially concentrated in vacuum and acidified at pH 2 with HCl 3 N. The white solid was collected, washed with acetone to obtain 16.28 g (36 mmol, 91%) of pure glyco-UDCA (**5a**), mp: 225–229 °C. RP-HPLC Rt = 6.31 min. $\delta_H$ (400 MHz; $d_6$-DMSO) 0.6 (3 H, s, C(18)$H_3$), 0.9 (3 H, s, C(19)$H_3$), 0.9 (3 H, d, C(21)$H_3$), 3.3 (1 H, m, C(3)H), 3.3 (1 H, s, C(7)H), 3.7 (2 H, d, $J$ = 7 Hz, C(25)$H_2$), 8.1 (1 H, bs pst, NH), 12.2 (1 H, bs s, COOH). $\delta_C$ (100 MHz; $d_6$-DMSO) 12.1, 18.5, 20.9, 23.3, 26.7, 28.2, 30.2, 31.6, 32.1, 33.8, 34.9, 34.9, 37.3, 37.7, 38.8, 40.5, 42.2, 43.0, 43.1, 54.8, 55.9, 69.5, 69.8, 171.4, 173.0.

### RP-HPLC isocratic analysis

The chromatographic analyses were directly executed on each of the reaction mixtures. The mobile phase was prepared by dissolving 100 mM $HCO_2NH_4$ in a $H_2O$ : MeCN – 60 : 40 (v/v) mobile phase; then, the apparent pH [$^s_w$pH, that is the one measured in the employed hydro-organic mobile phase (s), while the calibration of the pH system was done in water (w)] was adjusted to 3.5 with $HCO_2H$. The analyses were carried out at a 1.0 ml $min^{-1}$ flow rate after previous conditioning by passing through the column the selected mobile phase for at least 30 min at the same eluent velocity. Before being used, all the mobile phases were always filtered through a 0.22 μm Millipore filter (Bedford, MA, USA) and then degassed with 20 min sonication. All the analyses were conducted at a 25 °C column temperature.

## Notes and references


1 For review see: (*a*) P. Lefebvre, B. Cariou, F. Lien, F. Kuipers and B. Staels, *Physiol. Rev.*, 2009, **89**, 147; (*b*) C. Thomas, R. Pellicciari, M. Pruzanski, J. Auwerx and K. Schoonjans, *Nat. Rev. Drug Discovery*, 2008, **7**, 1; (*c*) A. F. Hofmann and L. R. Hagey, *Cell. Mol. Life Sci.*, 2008, **65**, 2461.

2 (*a*) R. Pellicciari, A. Gioiello, P. Sabbatini, F. Venturoni, R. Nuti, C. Colliva, G. Rizzo, L. Adorini, M. Pruzanski, A. Roda and A. Macchiarulo, *ACS Med. Chem. Lett.*, 2012, DOI: 10.1021/ml200256d; (*b*) R. Sharma, A. Long and J. F. Gilmer, *Curr. Med. Chem.*, 2011, **18**, 4029; (*c*) A. Gioiello, A. Macchiarulo, A. Carotti, P. Filipponi, G. Costantino, G. Rizzo, L. Adorini and R. Pellicciari, *Bioorg. Med. Chem.*, 2011, **19**, 2650; (*d*) Y. Iguchi, M. Yamaguchi, H. Sato, K. Kihira, T. Nishimaki-Mogami and M. Une, *J. Lipid Res.*, 2010, **51**, 1432; (*e*) R. Pellicciari, A. Gioiello, A. Macchiarulo, C. Thomas, E. Rosatelli, B. Natalini, R. Sardella, M. Pruzanski, A. Roda, E. Pastorini, K. Schoonjans and J. Auwerx, *J. Med. Chem.*, 2009, **52**, 7958; (*f*) K. Kuhajda, S. Kevresan, J. Kandrac, J. P. Fawcett and M. Mikov, *Eur. J. Drug Metab. Pharmacokinet.*, 2006, **31**, 179; (*g*) R. Pellicciari, G. Costantino, E. Camaioni, B. M. Sadeghpour, A. Entrena, T. M. Willson, S. Fiorucci, C. Clerici and A. Gioiello, *J. Med. Chem.*, 2004, **47**, 4559; (*h*) R. Pellicciari, S. Fiorucci, E. Camaioni, C. Clerici, G. Costantino, P. R. Maloney, A. Morelli, D. J. Parks and T. M. Willson, *J. Med. Chem.*, 2002, **45**, 3569.

3 (*a*) T. W. H. Pols, M. Nomura, T. Harach, G. Lo Sasso, M. H. Oosterveer, C. Thomas, G. Rizzo, A. Gioiello, L. Adorini, R. Pellicciari, J. Auwerx and K. Schoonjans, *Cell Metab.*, 2011, **14**, 747; (*b*) T. Mizuochi, A. Kimura, I. Ueki, T. Takahashi, T. Hashimoto, A. Takao, Y. Seki, H. Takei, H. Nittono, T. Kurosawa and T. Matsuishi, *Pediatr. Res.*, 2010, **68**, 258; (*c*) C. Thomas, A. Gioiello, L. Noriega, A. Strehle, J. Oury, G. Rizzo, A. Macchiarulo, H. Yamamoto, C. Mataki, M. Pruzanski, R. Pellicciari, J. Auwerx and K. Schoonjans, *Cell Metab.*, 2009, **10**, 167;

(*d*) E. Gonzales, M. F. Gerhardt, M. Fabre, K. D. Setchell, A. Davit-Spraul, I. Vincent, J. E. Heubi, O. Bernard and E. Jacquemin, *Gastroenterology*, 2009, **137**, 1310; (*e*) H. Ichimiya, H. Nazer, T. Gunasekaran, P. Clayton and J. Sjovall, *Arch. Dis. Child*, 1990, **65**, 1121; (*f*) K. N. Lazaridis, G. J. Gores and K. D. Lindor, *J. Hepatol.*, 2001, **35**, 134; (*g*) G. Paumgartner and U. Beuers, *Clin. Liver Dis.*, 2004, **8**, 67.

4 C. N. Falany, M. R. Johnson, S. Barnes and R. B. Diasio, *J. Biol. Chem.*, 1994, **269**, 19375.

5 A. Roda, A. F. Hofmann and K. J. Mysels, *J. Biol. Chem.*, 1983, **258**, 6362.

6 (*a*) A. F. Hofmann and B. Strandvik, *Lancet*, 1988, **2**, 311.

7 (*a*) N. M. Delzenne, P. B. Calderon, H. S. Taper and M. B. Roberfroid, *Toxicol. Lett.*, 1992, **61**, 291; (*b*) R. H. Palmer, Toxic effects of lithocholate on the liver and biliary tree, in *The Hepatobiliary System. Fundamental and Pathological Mechanisms*, ed. W. Taylor, Plenum Press, New York, 1976, p. 227.

8 (*a*) K. Y. Tserng, D. L. Hackey and P. D. Klein, *J. Lipid Res.*, 1977, **18**, 404; (*b*) J. Goto, K. Suzaki and T. Nambara, *Chem. Pharm. Bull.*, 1980, **28**, 1258; (*c*) T. Niwa, T. Koshiyama, J. Goto and T. Nambara, *Steroids*, 1992, **57**, 522; (*d*) R. Shaw and W. H. Elliott, *Lipids*, 1980, **15**, 805; (*e*) P. J. Henly and R. W. Owen, *J. Steroid Biochem.*, 1988, **31**, 443.

9 T. Momose, T. Tsubaki, T. Iida and T. Nambara, *Lipids*, 1997, **32**, 775.

10 B. Dayal, K. R. Rapole, S. R. Wilson, S. Shefer, G. S. Tint and G. Salen, *Synlett*, 1995, 861.

11 (*a*) A. Gioiello, R. Sardella, E. Rosatelli, B. M. Sadeghpour, B. Natalini and R. Pellicciari, *Steroids*, 2012, **77**, 250; (*b*) A. Gioiello, P. Sabbatini, E. Rosatelli, A. Macchiarulo and R. Pellicciari, *Tetrahedron*, 2011, **67**, 1924.

12 (*a*) A. Roda, B. Grigolo, R. Aldini, P. Simoni, R. Pellicciari, B. Natalini and R. Balducci, *J. Lipid Res.*, 1987, **28**, 1384; (*b*) R. Pellicciari, S. Cecchetti, B. Natalini, A. Roda, B. Grigolo and A. Fini, *J. Med. Chem.*, 1985, **28**, 239.

13 (*a*) P. Angulo, *Curr. Gastroenterol. Reports*, 2002, **4**, 37–44; (*b*) A. Crosignani, P. M. Battezzati, K. D. R. Setchell, P. Invernizzi, G. Covini, M. Zuin and M. Podda, *Dig. Dis. Sci.*, 1996, **41**, 809.

14 (*a*) B. Natalini, R. Sardella, A. Gioiello, G. Carbone, M. Dawgul and R. Pellicciari, *J. Sep. Sci.*, 2009, **32**, 2022; (*b*) B. Natalini, R. Sardella, A. Gioiello, E. Rosatelli, F. Ianni, E. Camaioni and R. Pellicciari, *Anal. Bioanal. Chem.*, 2011, **401**, 267.

15 K. Alfonsi, J. Colberg, P. J. Dunn, T. Fevig, S. Jennings, T. A. Johnson, H. P. Kleine, C. Knight, C. A. Nagy, D. A. Perry and M. Stefaniak, *Green Chem.*, 2008, **10**, 31.

16 For selected reviews see: (*a*) L. M. Sanz and F. Susanne, *J. Med. Chem.*, 2012, DOI: 10.1021/jm2006029; (*b*) M. Deal, Continuous Flow Chemistry in Medicinal Chemist, in *New Synthetic Technologies in Medicinal Chemistry*, ed. E. Farrant, RSC, Cambridge (UK), 2011, ch. 5, p. 90; (*c*) M. Baumann, I. R. Baxendale and S. V. Ley, *Mol. Diversity.*, 2011, **15**, 613; (*d*) C. Wiles and P. Watts, *Future Med. Chem.*, 2009, **1**, 1593; (*e*) C. Wiles and P. Watts, *Expert Opin. Drug Discovery*, 2007, **2**, 1487.

17 For selected review see: (*a*) M. Warman, Continuous Processing in Secondary Production, in *Chemical Engineering in the Pharmaceutical Industry: R&D to Manufacturing*, ed. D. J. am Ende, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2011, ch. 43, p. 837; (*b*) L. Proctor, P. J. Dunn, J. M. Hawkins, A. S. Wells and M. T. Williams, Continuous Processing in Pharmaceutical Industry, in *Green Chemistry in the Pharmaceutical Industry*, ed. P. J. Dunn, A. S. Wells and M. T. Williams, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2010, ch. 11, p. 221; (*c*) V. Hessel, *Chem. Eng. Technol.*, 2009, **32**, 1655; (*d*) D. M. Roberge, B. Zimmermann, F. Rainone, M. Gottsponer, M. Eyholzer and N. Kockmann, *Org. Process Res. Dev.*, 2008, **12**, 905.

18 O. B. Ijare, B. S. Somashekar, Y. Jadegoud and G. A. N. Gowda, *Lipids*, 2005, **40**, 1031.

19 T. Kanazawa, *Proc. Jpn. Acad.*, 1954, **30**, 391.