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Facile synthesis of 5β-cholane-*sym*-triazine conjugates starting from metformin and bile acid methyl esters: Liquid and solid state NMR characterization and single crystal structure of lithocholyl triazine

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ABSTRACT

Four bile acid-triazine conjugates: $N^{2'}$,$N^{2'}$-dimethyl-6′-(3α-hydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (lithocholyl triazine, **4a**), $N^{2'}$,$N^{2'}$-dimethyl-6′-(3α,7α-dihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (chenodeoxycholyl triazine, **4b**), $N^{2'}$,$N^{2'}$-dimethyl-6′6′-(3α,12α-dihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (deoxycholyl triazine) (**4c**), and $N^{2'}$,$N^{2'}$-dimethyl-6′-(3α,7α, 12α-trihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (cholyl triazine) (**4d**) have been prepared and characterized by liquid and solid state NMR. An improved synthetic method produced better yields and an easier purification procedure for **4d** than reported in the literature. Single crystal structure of **4a** is reported: empirical formula $C_{28}H_{47}N_5O$, monoclinic $P2_1$ space group with unit cell dimensions, *a* 18.7135(5) Å, *b* 7.4510(2) Å, *c* 19.3073(5) Å, β 95.7290(10)°, volume 2678.65(12) Å^3.

1. Introduction

The most abundant naturally occurring bile acids in higher vertebrates are derivatives of cholanic acid, cyclopentanoperhydrophenanthrene ring-containing steroid consisting of 24 carbon atoms. Bile acids form as end products of cholesterol metabolism in the liver and their formation include several hydroxylations of sterol nucleus followed by a loss of an isopropyl group form the side chain to give primary bile acids which are further modified by the bacteria in the intestines to give secondary bile acids. Bile acids are natural substrates of enterohepatic circulation. Their main role is the digestion of lipids and lipid soluble vitamins [1]. Bile acids and their salts as well their derivatives are found to have numerous applications in biology, medicine and physiology as well as in supramolecular chemistry and nanoscience [2]. Several research groups have recently reviewed the supramolecular and pharmaceutical use of bile acids and their derivatives [3] as well as the utilization of bile acid transportation system for the improved drug delivery [4].

It is known that *N*-substituted 2,4-diamino-6alkyl-*sym*-triazines are formed via condensation of biguanides with acid derivatives such as acid chlorides, esters, anhydrides, and imino esters [5]. Herein we report the synthesis and structural characterization of four bile acid derived triazines (see Scheme 1) utilizing the condensation reaction of bile acid methyl ester with dimethylbiguanide in the presence of sodium methylate, method previously described for the preparation of other triazines [5]. First two molecules (**4a** and **4d**, see Scheme 1) and their characterization were published by us already in 2006 in form of a poster [6]. Marin et al. [7] also published the synthesis of **4d** and two other triazines. In order to conjugate bile acids with dimethylbiguanide they used 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as a coupling agent and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) for neutralization of HCl of metformin, which resulted in a complex mixture of products. They also evaluated the cytostatic activity of these compounds *in vitro* and antitumor effect *in vivo*, respectively. They found ursodeoxycholic acid derived triazine to possess moderate non-specific toxicity similar to that of cisplatin and when administered to tumor-bearing mice, this compound prolonged the survival of these animals, while treatment with cisplatin failed to do so [7]. Inspired by these results we finished the synthesis of other two triazines (**4b** and **4c**, see Scheme 1) with improved yields.

We have also thoroughly studied the structures of the prepared triazines in solution by NMR spectroscopy and mass spectrometry and in solid state by CP/MAS NMR spectroscopy; a technique which nowadays has a significant contribution to the understanding of the structures of polymorphs and solvates of pharmaceutical significance [8]. The study of the polymorphic nature of pharmaceutical solids is particularly important since the different polymorphic forms may have different physical or chemical properties along with the differences in pharmaceutical activity. Studies on the polymorphism of several bile acid derivatives have been reported from our laboratory [9]. Recently we also studied

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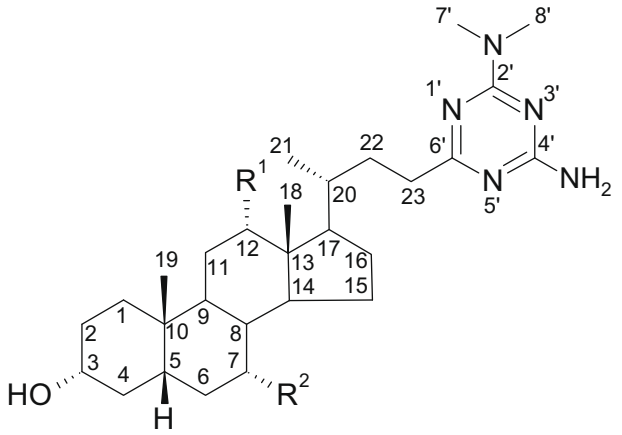


4a: $R^1=R^2=H$
4b: $R^1=H$, $R^2=OH$
4c: $R^1=OH$, $R^2=H$
4d: $R^1=R^2=OH$

Scheme 1. Prepared molecules and the numbering of the carbon and nitrogen atoms.

the polymorphic nature of naturally occurring bile acids [10]. Molecular structure and crystal packing of **4a** was determined utilizing single crystal X-ray crystallography.

2. Experimental

2.1. Spectroscopy

^1H and ^{13}C NMR experiments were run with a Bruker Avance DRX 500 NMR spectrometer equipped with a 5 mm diameter broad band inverse probehead working at 500.13 MHz for ^1H, at 125.76 MHz for ^{13}C, and at 50.68 MHz for ^{15}N, respectively. The $\{^1H\}$-^{13}C NMR spectrum was measured in standard way using composite pulse decoupling (waltz-16). Spectra were measured in $CDCl_3$ and CD_3OD at 303 K and ^1H and ^{13}C chemical shifts referenced to the solvent signals ($\delta = 7.26$ for ^1H and $\delta = 77.0$ ppm for ^{13}C from int. TMS ($CDCl_3$) and $\delta = 3.10$ for ^1H and $\delta = 49.0$ ppm for ^{13}C from int. TMS (CD_3OD)). The nitrogen chemical shifts were measured by PFG ^1H,^{15}N HMBC using 50 ms evolution delay for couplings and ^{15}N NMR spectra were referenced to an external nitromethane in a capillary inserted coaxially inside the NMR-tube ($\delta = 0.0$ ppm). The assignment of the individual ^1H, ^{13}C and ^{15}N signals was carried out utilizing the 2D pulse sequences PFG ^1H,^{13}C HMQC [11] and PFG ^1H,^{13}C HMBC [12] and PFG ^1H,^{15}N HMBC [13] and by referring them with those reported in the literature for similar type of compounds [14].

For CP/MAS NMR spectroscopy the compounds were crystallized from various solvents: **4a** from methanol and *p*-xylene, **4b** from *p*-xylene and DMF–CH_3CN, **4c** from *p*-xylene and EtOAc–CH_3CN and CH_3CN and **4d** from CH_3OH, respectively. The $^{13}C\{^1H\}$CP/MAS and $^{15}N\{^1H\}$CP/MAS NMR spectra were recorded on a Bruker AV 400 spectrometer equipped with a 4 mm standard bore CP/MAS probehead whose X channel was tuned to 100.62 MHz for ^{13}C and 40.55 MHz for ^{15}N, respectively. The other channel was tuned to 400.13 MHz for broad band ^1H decoupling. Approximately 100 mg of dried and finely powdered samples were packed in the ZrO_2 rotor closed with Kel-F cap and spun at 10 kHz rate. The $^{13}C\{^1H\}$CP/MAS NMR was carried out for all samples under Hartmann–Hahn conditions with TPPM decoupling. The $\pi/2$ pulse for proton and carbons were found to be 4.0 and 5 μs at power levels of −5.0 and −4.0 dB, respectively. The experiments were conducted at contact time of 2 ms. A total of 10,000 scans were recorded with 4 s recycle delay for each sample. All FIDs were processed by exponential apodization function with line broadening of 20–40 Hz prior to FT. The $^{15}N\{^1H\}$CP/MAS NMR experiments were carried out for all samples at a 10 kHz spinning rate under Hartmann–Hahn condition. The $\pi/2$pulses for proton and nitrogen were found to be 4.2 and 5 μs at power levels of –4.6 and −0.8 dB, respectively. The optimized contact time of 2 ms was used for efficient polarization transfer with a 4 s recycle delay to acquire the CP/MAS spectra. A total of 70,000 scans were acquired obtain the CP/MAS spectra. The ^{13}C and ^{15}N CPMAS chemical shifts were referenced with those of glycine standard measured before the each sample of **4a**–**4d**.

Molecular masses of the synthesis products were confirmed by using Micromass LCT ESI-TOF mass spectrometer in positive ion mode. Samples were prepared in HPLC grade solvents and diluted to concentrations ~0.1–1.0 μg/L. Under these conditions all the compounds yielded abundant molecular ion peaks by coordinating hydrogen, sodium, and/or potassium ions ($[M+H]^+$, $[M+Na]^+$ and/or $[M+K]^+$, respectively).

2.2. X-ray crystallography

Crystals of **4a** suitable for single crystal X-ray crystallography were grown in ethyl acetate/hexane (1:1) solution. Data was collected at 123(2) K on a Bruker-Nonius KappaCCD diffractometer with graphite monochromated Cu-K_α radiation ($\lambda = 1.54184$). COLLECT [15] data collection software was utilized and data was processed with DENZO-SMN [16]. The reflections were corrected for Lorenz polarization effects but absorption correction was not used. Structure was solved by direct methods (SHELXS-96 [17]) and refined anisotropically by full matrix least squares on F values (SHELXL-97 [17]). The hydrogen atoms, except N–H and O–H, were calculated to their idealized positions and those attached to N or O were located from electron density map. Hydrogen atoms were refined only isotropically with isotropic temperature factors (1.2 or 1.5 times the parent atom factor). PLATON [18]-program was used in validation of the structure. Figures were drawn using Ortep-3 for Windows [19] and Mercury [20]. Crystallographic data (excluding structure factors) have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC-738340 for **4a**. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ UK.

2.3. Synthesis and purification

All bile acids were purchased from commercial sources and were used as such in synthetic procedures. All the used solvents were of an analytical grade and water was deionized before use. The methyl esters of bile acids were prepared according to known sulphuric acid catalyzed method [21]. Silica 0.043–0.060 Å was used in column chromatographic purifications.

2.3.1. *$N^{2'}$,$N^{2'}$-dimethyl-6′-(3α-hydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (lithocholyl triazine, **4a**)*

Sodium (0.14 g; 6.0 mmol) was placed in a round bottom 100 mL flask, fitted with a reflux condenser and a dropping funnel, and portion (20 mL) of methanol added at once under N_2 atmosphere. When the reaction had ceased, another portion (20 mL) of methanol was added after which dimethylbiguanide hydrochoride (0.83 g; 5.0 mmol) in methanol was added dropwise. The resulting reaction mixture was stirred for 30 min at ambient temperature. A solution of methyl lithocholate (1.95 g; 5.0 mmol)

in methanol was added to the reaction mixture through the dropping funnel. Reaction mixture was then heated to reflux and refluxing continued for 48 h under N_2. Mixture was then cooled to −5 °C and precipitated product was filtered, washed with methanol and dried *in vacuo* to give 0.71 g of **4a** as white powder (yield 29%).

^{1}H NMR ($CDCl_3$ 500 MHz) δ ppm: ^{1}H NMR ($CDCl_3$ 500 MHz) δ ppm: 5.04 (br. s, 2H, 4NH_2), 3.66 (m, 1H, 3C*H*), (br. s, 6H, 7′/8′CH_3), 2.58–2.34 (2H, ddq$_{AB}$, δH_A = 2.55 ppm and δH_B = 2.37 ppm, J_{ab} = −14.0 Hz), 1.87–0.98 (steroidal –C*H* and –CH_2, –O*H*), 0.98 (d, 3H, J = 6.4 Hz, 21CH_3), 0.93 (s, 3H, 19CH_3), 0.66 (s, 3H, 18CH_3). ^{13}C NMR ($CDCl_3$ 126 MHz) δ ppm: 179.2 (C6′), 166.9 (C2′), 165.8 (C4′), 72.0 (C3), 56.7 (C14), 56.4 (C17), 42.9 (C13), 42.3 (C5), 40.6 (C9), 40.4 (C12), 36.7 (C4), 36.2 (C7′/8′), 36.0 (C20), 35.9 (C23), 35.8 (C8), 35.5 (C1), 34.7 (C10), 33.8 (C22), 30.8 (C2), 28.4 (C16), 27.4 (C6), 26.6 (C7), 24.4 (C15), 23.5 (C19), 21.0 (C11), 18.7 (C21), 12.2 (C18). ESI-TOF MS (MeCN) *m/z*: $C_{28}H_{47}N_5O$. Found: $[M+H]^+$ 470.35 ([**4a**+H]$^+$ requires 470.73) $[M+Na]^+$ 492.33 ([**4a**+Na]$^+$ requires 492.71). Anal. calcd. for $C_{28}H_{47}N_5O_1{\cdot}½CH_3OH$: C 70.47; H 10.17; N 14.42. Found: C 70.83; H 10.00; N 14.24.

2.3.2. General method for preparation of other triazines

2.3.2.1. N$^{2'}$,N$^{2'}$-dimethyl-6′-(3α,7α-dihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (chenodeoxycholyl triazine, 4b). Sodium (0.184 g; 8.0 mmol) was placed in a round bottom 250 mL flask, fitted with a reflux condenser and a dropping funnel, and portion (15 mL) of methanol added at once under N_2 atmosphere. When the reaction had ceased, another portion (15 mL) of methanol was added after which dimethylbiguanide (1.325 g; 8.0 mmol) in 30 mL of methanol was added dropwise. Resulting reaction mixture was stirred for 30 min. Solution of methyl chenodeoxycholate (1.626 g; 4.0 mmol) in 10 mL of methanol was added to the reaction mixture through the dropping funnel. Reaction mixture was then heated to reflux and refluxing continued for 19 h under N_2. Mixture was then concentrated by evaporation and residue dissolved in 120 mL of $CHCl_3$, solution washed with 5 × 40 mL of H_2O and 2 × 40 mL of brine, dried over $MgSO_4$ and evaporated to dryness. Crude product was purified by column chromatography using EtOAc:MeOH (9:1) as an eluent. Fractions containing the product were combined, evaporated and dried *in vacuo* to give 0.727 g of **4b** as a white powder (yield 37%).

^{1}H NMR ($CDCl_3$ 500 MHz) δ ppm: 5.10 (br. s, 2H, 4NH_2), 3.83 (m, 1H, 7C*H*), 3.446 (m, 1H, 3C*H*) 3.12 (br. s, 6H, 7′/8′CH_3), 2.58–2.34 (2H, ddq$_{AB}$, δH_A = 2.56 ppm and δH_B = 2.36 ppm, J_{ab} = 13.6 Hz), 2.24–0.94 (steroidal –C*H* and –CH_2, –O*H*), 0.98 (d, 3H, J = 6.5 Hz, 21CH_3), 0.90 (s, 3H, 19CH_3), 0.65 (s, 3H, 18CH_3). ^{13}C NMR ($CDCl_3$ 126 MHz) δ ppm: 178.9 (C6′), 166.6 (C2′), 165.6 (C4′), 72.0 (C3), 68.5 (C7), 56.1 (C17), 50.5 (C14), 42.7 (C13), 41.6 (C5), 39.9 (C4), 39.7 (C12), 39.5 (C8), 39.5 (C22), 36.1 (C7′/8′), 35.7 (C23), 35.6 (C20), 35.4 (C1), 35.1 (C10), 34.6 (C6), 32.9 (C9), 30.7 (C2), 28.2 (C16), 23.7 (C15), 22.8 (C19), 20.6 (C11), 18.5 (C21), 11.8 (C18). ESI-TOF MS (MeOH/HCOOH 0.1%), *m/z*: $C_{28}H_{47}N_5O_2$. Found: $[M+H]^+$ 486.41 ([**4b**+H]$^+$ requires 486.38) $[M+Na]^+$ 508.39 ([**4b**+Na]$^+$ requires 508.36). Anal. calcd. for $C_{28}H_{47}N_5O_2$: C 69.24; H 9.75; N 14.42. Found: C 69.12; H 9.67; N 14.30.

2.3.2.2. N$^{2'}$,N$^{2'}$-dimethyl-6′-(3α,12α-dihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (deoxycholyl triazine) (4c). Yield 0.653 g of white powder (34%). ^{1}H NMR ($CDCl_3$ 500 MHz) δ ppm: 5.09 (br. s, 2H, 4NH_2), 3.99 (br. t, 1H, 12C*H*), 3.59 (m, 1H, 3C*H*), 3.11 (br. s, 6H, 7′/8′CH_3), 2.60–2.36 (ddq$_{AB}$, δH_A = 2.57 ppm and δH_B = 2.38 ppm, J_{ab} = −13.7 Hz), 1.91–0.93 (steroidal –C*H* and –CH_2, –O*H*), 1.02 (d, 3H, J = 6.0 Hz, 21CH_3), 0.90 (s, 3H, 19CH_3), 0.67(s, 3H, 18CH_3). ^{13}C NMR ($CDCl_3$ 126 MHz) δ ppm: 178.9 (C6′), 166.6 (C2′), 165.6 (C4′), 76.7 (C12), 71.8 (C3), 48.3 (C14), 47.5 (C17), 46.6 (C13), 42.1 (C5), 36.5 (C4), 36.1 (C8), 36.1 (C7′/8′), 35.8 (C23), 35.4 (C20), 35.3(C1), 34.1 (C10), 33.7 (C9), 33.6 (C22), 30.6 (C2), 28.6 (C11), 27.5 (C16), 27.2 (C6), 26.2 (C7), 23.7 (C15), 23.1 (C19), 17.6 (C21), 12.8 (C18). ESI-TOF MS (MeOH/HCOOH 0.1%) *m/z*: $C_{28}H_{47}N_5O_2$. Found: $[M+H]^+$ 486.44 ([**4c**+H]$^+$ requires 486.38). Anal. calcd. for $C_{28}H_{47}N_5O_2$: C 69.24; H 9.75; N 14.42. Found: C 69.23; H 9.69; N 14.26.

2.3.2.3. N$^{2'}$,N$^{2'}$-dimethyl-6′-(3α,7α,12α-trihydroxy-5β-24-norcholyl)-1′,3′,5′-triazine-2′,4′-diamine (cholyl triazine) (4d). Yield 0.785 g of **4d** as white powder (yield 39%). ^{1}H NMR (CD_3OD 500 MHz) δ ppm: 3.95 (m, 1H, 12C*H*), 3.78 (m, 1H, 7C*H*) 3.78 (m, 1H, 3C*H*), 3.11 (br. s, 6H, 7′/8′CH_3), 2.56–2.32 (ddq$_{AB}$, δH_A = 2.35 ppm and δH_B = 2.53 ppm, J_{ab} = −13.3 Hz), 2.30–0.93 (steroidal –C*H* and –CH_2), 1.06 (d, 3H, J = 6.0 Hz, 21CH_3) 0.91 (s, 3H, 19CH_3) 0.70 (s, 3H, 18CH_3). ^{13}C NMR ($CDCl_3$ 126 MHz) δ ppm: 179.2 (C6′), 166.8 (C2′), 165.7 (C4′), 73.1 (C12), 72.1 (C3), 68.6 (C7), 47.3 (C17), 46.7 (C13), 42.0 (C14), 41.7 (C5), 39.9 (C4), 39.8 (C8), 36.3 (C7′/8′), 35.8 (C23), 35.7 (C20), 34.8 (C6), 34.9 (C10), 35.5 (C1), 33.8 (C22), 30.7 (C2), 28.4 (C11), 27.7 (C16), 26.8 (C9), 23.4 (C15), 22.7 (C19), 17.8 (C21), 12.8 (C18). ESI-TOF MS (MeCN) *m/z*: $C_{28}H_{47}N_5O_3$. Found: $[M+H]^+$ 502.40 ([**4d**+H]$^+$ requires 502.73), 524.39 (([**4d**+Na]$^+$ requires 524.71), 540.37 ([**4d**+K]$^+$ requires 540.82). Anal. calcd. for $C_{28}H_{47}N_5O_3{\cdot}½H_2O$: C 65.85; H 9.47; N 13.71. Found: C 66.20; H 9.40; N 13.72.

3. Results and discussion

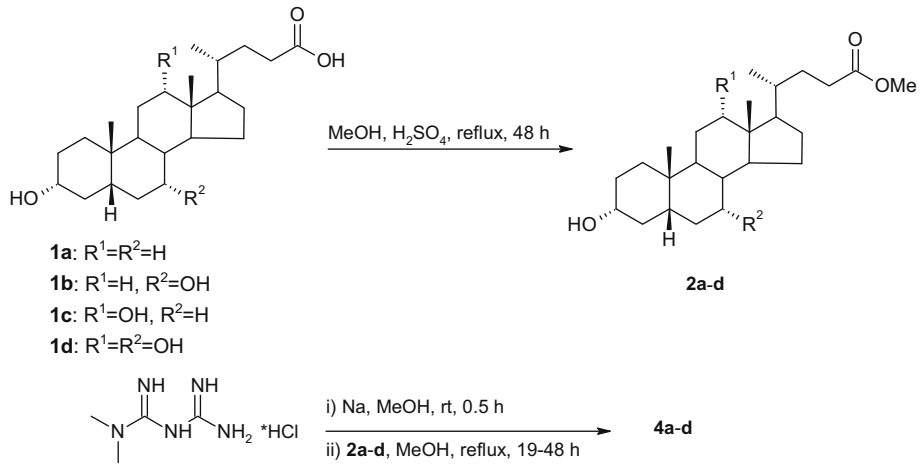
3.1. Synthesis

The synthetic route to bile acid triazines is outlined in the scheme 2. The preparation of bile acid triazines started by conversion of each bile acid **1a–d** to their methyl esters **2a–d** utilizing Fischer esterification reaction which is widely used in preparation of simple bile acid alkyl esters [21]. The formation of the bile acid substituted triazine ring was condensation reaction of dimethylbiguanide with bile acid ester in the presence of sodium methylate. Two equivalents of both dimethyl biguanide hydrochloride and base against the ester were used and the reaction mixtures were refluxed from 19 to 48 h. While, according to Marin et al. [7] the EEDQ/DCU-coupling method produced a complex mixture of products thus requiring two successive column chromatographic purifications, here the main impurity in the crude product was bile acid ester and only one column chromatographic purification was sufficient enough to give pure products **4b–d**. In the case of **4a**, the product was precipitated out from the reaction mixture at −5 °C and it was purified simply by washing the precipitate with methanol. Yields of **4a–d** varied from 29% to 39%. Better yields were thus obtained using this method compared to the previously reported method where for example the yield of **4d** was 21%.

3.2. NMR spectroscopy

^{13}C and ^{15}N NMR chemical shifts and selected ^{1}H NMR chemical shifts for **4a–d** in $CDCl_3$ and/or CD_3OD are presented in the Tables 1–3. Our assignment of the ^{13}C NMR chemical shifts for **4d** in CD_3OD is in agreement with that made by Marin et al. [7].

Very different types of nitrogen atoms are present in these compounds, the endocyclic *s*-triazine N-atoms (N(1′), N(3′) and N(5′)), the aniline-like N-atom (N(4′)) and tertiary non-aromatic N-atom (N(2′)). When the samples were measured in $CDCl_3$, the carbon attached to –NH_2 (C4′) is more shielded compared to the carbon attached to N(CH_3)$_2$ (C2′), but when the sample **4d** was measured in CD_3OD the order of their chemical shifts was opposite, illustrating the importance of the solvent effect. The signal for protons of C(7′/8′) is very broad in solution at 303 K which probably results from dynamics of the methyl groups bound to nitrogen. This disables the observation of the ^{15}N chemical shift for N(2′). When the samples of **4b** and **4c** in $CDCl_3$ and **4d** in CD_3OD were heated to 333 or



Scheme 2. Preparation of the bile acid triazines.

Table 1
^{13}C NMR chemical shifts of **4a**–**c** in $CDCl_3$ and **4d** in $CDCl_3$ and CD_3OD at 303 K.

C	4a	4b	4c	4d ($CDCl_3$)	4d (CD_3OD)
(C1)	35.5	35.4	35.3	35.5	36.6
(C2)	30.8	30.7	30.6	30.7	31.4
(C3)	72.0	72.0	71.8	72.1	73.1
(C4)	36.7	39.9	36.5	39.9	40.7
(C5)	42.3	41.6	42.1	41.7	43.4
(C6)	27.4	34.6	27.2	34.8	35.3*
(C7)	26.6	68.5	26.2	68.6	69.2
(C8)	35.8	39.5	36.1*	39.8	41.2
(C9)	40.6	32.9	33.7	26.8	28.1
(C10)	34.7	35.1	34.1	34.9	35.3*
(C11)	21.0	20.6	28.6	28.4	29.8
(C12)	40.4	39.7	76.7	73.1	74.2
(C13)	42.9	42.7	46.6	46.7	47.7
(C14)	56.7	50.5	48.3	42.0	43.2
(C15)	24.4	23.7	23.7	23.4	24.4
(C16)	28.4	28.2	27.5	27.7	28.9
(C17)	56.4	56.1	47.5	47.3	48.5
(C18)	12.2	11.8	12.8	12.8	13.2
(C19)	23.5	22.8	23.1	22.7	23.3
(C20)	36.0	35.6	35.4	35.7	37.2
(C21)	18.7	18.5	17.6	17.8	18.0
(C22)	33.8	39.5	33.6	33.8	35.3
(C23)	35.9	35.7	35.8	35.8	36.7
(C6′)	179.2	178.9	178.9	179.2	180.0
(C2′)	166.9	166.6	166.6	166.8	166.8
(C4′)	165.8	165.6	165.6	165.7	168.0
(C7′/8′)	36.2	36.1	36.1*	36.3	36.6

* Peaks overlapped.

337 K, respectively, the signal for C(7′/8′)–H sharpened significantly and the coupling from the methyl protons to N(2′) could be detected. Only in the case of **4a** in $CDCl_3$ at 333 K still no coupling was detected. However, in the CP/MAS NMR ^{15}N nucleus can be detected directly without the magnetization transfer from the proton, and thus the chemical shifts also for N(3′) and N(2′) are observed in solid state for **4a**. Table 3 lists the ^{15}N NMR chemical shifts for **4a**–**c** in in $CDCl_3$, for **4d** in CD_3OD and for **4a** also in solid state.

For CP/MAS experiments, the compounds were crystallized from various solvents and solvent-mixtures including methanol, *p*-xylene, acetonitrile, ethyl acetate, and dimethylformamide (DMF)-acetonitrile. ^{13}C CP/MAS spectra of **4a** crystallized both from methanol and from *p*-xylene were of good quality with sharp peaks. The steroidal methylene and methine regions are generally crowded and not well resolved compared to the other regions. Because of the varying number of hydroxyl groups and the triazine ring, we divide the ^{13}C CP/MAS spectra resonances to heteroaromatic region (160–180 ppm), hydroxyl region (60–80 ppm), and aliphatic region containing tertiary, methylene and methine carbons (20–50 ppm) and methyl carbons (10–20 ppm). Table 4 collects some selected ^{13}C NMR chemical shifts of **4a**–**4d** in solid state.

Fig. 1 presents selected spectra from the ^{13}C CP/MAS experiments. Crystallization of **4a** from both methanol and *p*-xylene resulted in highly crystalline material from which the ^{13}C CP/MAS spectra with sharp peaks could be obtained. Also the ^{15}N CP/MAS spectrum was of high quality enabling the assignment of the ^{15}N chemical shifts in solid state. However, ^{13}C CP/MAS spectra of **4a** from different solvents were similar.

Crystallization of **4b** from *p*-xylene afforded as well crystalline material which seems to be a mixture of at least two polymorphic forms. In the contrary, crystals obtained from DFM-CH_3CN show only one form which ^{13}C CP/MAS spectrum show sharp signals. The ^{13}C CP/MAS spectrum displayed *doublet* resonance for most of the carbons which probably arises from the existence of two non-equivalent molecules in the asymmetric unit in its crystal lat-

Table 2
Selected ^{1}H chemical shifts of **4a**–**c** in $CDCl_3$ and **4d** in CD_3OD at 303 K.

H	4a	4b	4c	4d
C(3)–H	3.62 (m, 1H)	3.45 (m, 1H)	3.59 (m, 1H)	3.36 (m, 1H)
C(7)–H	–	3.83 (t, 1H)	–	3.78 (m, 1H)
C(12)–H	–	–	3.99 (br. t, 1H)	3.95 (m, 1H)
C(18)–H	0.66 (s, 3H)	0.65 (s, 3H)	0.67 (s, 3H)	0.70 (s, 3H)
C(19)–H	0.93 (s, 3H)	0.90 (s, 3H)	0.90 (s, 3H)	0.91 (s, 3H)
C(21)–H	0.98 (d, 3H, J = 6.4 Hz)	0.98 (d, 3H, J = 6.5 Hz)	1.02 (d, 3H, J = 6.0 Hz)	1.06 (d, 3H, J = 6.0 Hz)
C(23)–H	2.34–2.58 (ddq$_{AB}$, δH_A = 2.55 ppm and δH_B = 2.37 ppm, J_{ab} = −14.0 Hz)	2.34–2.58 (ddq$_{AB}$, δH_A = 2.56 ppm and δH_B = 2.36 ppm, J_{ab} = −13.6 Hz)	2.36–2.60 (ddq$_{AB}$, δH_A = 2.57 ppm and δH_B = 2.38 ppm, J_{ab} = −13.7 Hz)	2.32–2.56 (ddq$_{AB}$, δH_A = 2.35 ppm and δH_B = 2.53 ppm, J_{ab} = −13.3 Hz)
C(7′/8′)–H	3.12 (br. s, 2H)	3.12 (br. s, 2H)	3.11 (br. s, 2H)	3.11 (br. s, 2H)
N(4′)–H	5.04 (br. s, 2H)	5.10 (br. s, 2H)	5.09 (br. s, 2H)	Not obs.

Table 3
^{15}N chemical shifts (from external CH_3NO_2) of **4a–c** in $CDCl_3$ at 303 K/333 K, **4d** in CD_3OD at 337 K and **4a** in solid state at 296 K.

N	4a	4a	4b	4c	4d
N(1′)	−168.8[a]	−173.7	−169.3[a]/−168.1[b]	−169.7[a]/−168.3[b]	−171.8[c]
N(2′)	Not obs.[a]	−309.5[*]	Not obs.[a]/−305.7[b]	Not obs.[a]/−305.7[b]	−305.4[c]
N(3′)	Not obs.[a]	−206.8	−198.4[a]/Not obs.[b]	−198.7[a]	Not obs.[c]
N(4′)	−304.0[a]	−299.0[*]	−303.8[a]/−303.6[b]	−303.6[a]/−304.0[b]	Not obs.[c]
N(5′)	−178.9[a]	−187.2	−177.0[a]/−174.8[b]	−177.3[a]/−175.1[b]	−181.3[c]

[a] 303 K.
[b] 333 K.
[c] 337 K.
[*] Shifts may be interchanged.

Table 4
Selected ^{13}C NMR chemical shifts of **4a–d** from CP/MAS NMR experiments at 303 K.

C	**4a** (MeOH)	**4a** (*p*-xylene)	**4b** (DMF–CH_3CN)	**4c** (CH_3CN)	**4d** (MeOH)
(C3)	70.1	70.5	73.1/70.1	71.4	–[b]
(C7)	–	–	66.7/65.0	–	–[b]
(C12)	–	–	–	73.2	–[b]
(C18)	15.0	15.5	12.4/11.8	12.9	11.6
(C19)	23.9	24.4	22.8	20.5	23.5
(C21)	18.9	19.3	18.1/17.8	15.8	17.4
(C6′)	176.4	177.0	178.5	179.1	177.1
(C2′)	165.2[a]	165.6[a]	167.3[c]	167.1[c]	165.9[c]
(C4′)	165.2[a]	165.6[a]	164.5[c]	164.1[c]	163.8[c]

[a] Peaks overlap.
[b] Could not be assigned.
[c] Could be interchanged.

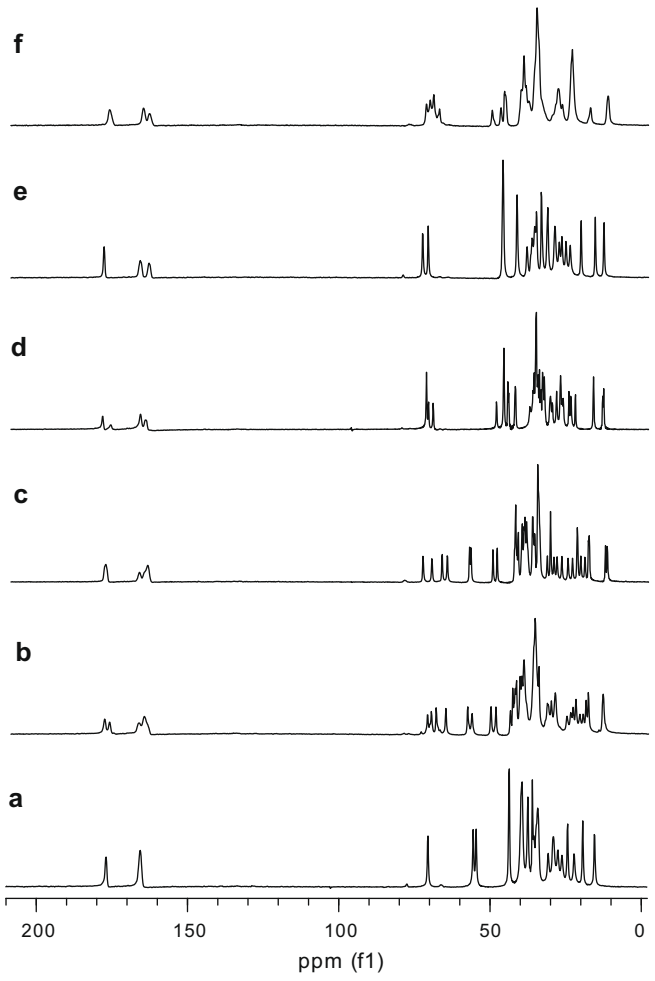


Fig. 1. ^{13}C CP/MAS spectra of (a) **4a** from *p*-xylene, (b) **4b** from *p*-xylene, (c) **4b** from DMF-acetonitrile, (d) **4c** from ethyl acetate-acetonitrile, (e) **4c** from acetonitrile, and (f) **4d** from methanol measured at 100 MHz at 303 K.

tice. We have found similar behaviour with the naturally occurring bile acids: the crystals containing two molecules per asymmetric unit always have the *doublet* resonance patterns in their ^{13}C CP/MAS spectra. This has been explained to be due to the differences in the side chain as well as in the hydrogen bonding modes of these non-equivalent molecules [10]. In the case of crystals of **4b** from DMF–CH_3CN largest differences in the chemical shift values between the two peaks in the *doublet* resonances are found in the hydroxyl region which supports the existence of two non-equivalent molecules with different hydrogen bonding systems. However, more experiments are needed in order to prove this hypothesis. Unfortunately the quality of the crystals obtained from **4b** was not suitable for structural analysis by single crystal X-ray diffraction.

Crystallization of **4c** from EtOAc–CH_3CN afforded again highly crystalline material, but despite of the careful drying of the substance there are extra peaks in CP/MAS spectrum resulting from the residual EtOAc present in the sample. When the same sample was crystallized from pure CH_3CN it resulted in crystalline material, though the quality of the spectrum was not as high but most of the peaks were still well resolved. This spectrum differs slightly from the previous one especially in the hydroxyl region. This could be explained by different hydrogen bonding mode in these crystals.

In the case of **4d** crystallization of the sample turned out to be demanding. Finally, crystalline material was obtained by crystallization of **4d** from methanol, however, its crystallinity was low compared to the other samples and as a result the resolution of the peaks in the ^{13}C CP/MAS spectrum was poor. Most of the characteristic peaks could still be assigned from this spectrum. Instead of the expected three peaks, there are four peaks at the hydroxyl region. This cannot be explained by residual solvent, since the resonance for methanol should be more shielded. Thus the probable explanation is that also in the case of **4d** there exists more than one polymorphic form in this solid.

These preliminary studies show that **4a–4c** form highly crystalline material upon recrystallization from various solvents and at least in the case of **4b** and **4c** there can be found more than one

Table 5
Crystallographic data and parameters for **4a**.

	4a
Empirical formula	$C_{28}H_{47}N_5O$
Molecular weight	469.71
Crystal system	Monoclinic
Space group	$P2_1$
a (Å)	18.7135(5)
b (Å)	7.4510(2)
c (Å)	19.3073(5)
α (°)	90
β (°)	95.7290(10)
γ (°)	90
Volume (Å^3)	2678.65(12)
Z	4
Density$_{calc}$ (g cm^{-3})	1.165
T (K)	123(2)
Abs. coefficient (mm^{-1})	0.555
F(0 0 0)	1032
θ range (°)	3.14–67.08
Index ranges	$-22 \le h \le 20$ $-8 \le k \le 8$ $-22 \le l \le 22$
Reflections collected	10158
Internal consistency	0.0861
Data/restraints/parameters	4913/7/632
Goodness-of-fit on P^2	0.987
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.0676$ $wR_2 = 0.1748$
R indices (all data)	$R_1 = 0.0744$ $wR_2 = 0.1856$
Extinction coefficient	0.0050(9)
Largest diffraction peak and hole (e Å^{-3})	0.290 and −0.354

form i.e. polymorph or solvate. In order to explore the potential polymorphic nature of these solids, it is, however, necessary to perform a more detailed study. Thus extensive crystallization from several solvents followed by the study of the solids by CP/MAS NMR spectroscopy, powder X-ray diffraction and when possible also structure determination by single crystal X-ray crystallography are needed.

3.3. X-ray crystallography

Table 5 summarizes the crystal data and parameters for **4a**. Selected geometrical parameters are presented in the Table 6. Compound **4a** crystallizes in monoclinic space group $P2_1$ with two crystallographically independent molecules in an asymmetric unit. In the Fig. 2 the molecular structure of molecule A is shown.

Comparison of the molecule A and molecule B shows that they are similar on their steroidal skeleton and the differences are found in the side chain. Conformation of the bile acid side chain can be described using the value of the C17–C20–C22–C23 dihedral angle or more comprehensively by examining all of the four dihedral angles in the side chain using letters *t* (trans), *g* (gauche) or *i* (intermediate) [22]. Here we use only three dihedral angles because of the shortening of the side chain with one carbon, when the ring is formed. In the case of **4a** both molecules A and B have the side chain conformation *ttt*, but the dihedral angle C17A–C20A–C22A–C23A is 5.7° larger than the dihedral angle C17B–C20B–C22B–C23B. Also other dihedral angles in the side chain differ to some extent. This causes a slightly different twist of the side chain and triazine part in two independent molecules. Although the differences are quite small and the structure resembles orthorhombic $P2_12_12_1$ space group, the structure could not be solved in this higher symmetry. Addsym in PLATON [18], which uses the Le Page algorithm for missing symmetry, was used to check the structure for missing symmetry, but no higher crystallographic symmetry was found. Instead the program suggested the existence of potential pseudosymmetry, i.e. slightly distorted higher symmetry, in these crystals. Crystal packing and the hydrogen bonding network of **4a** are illustrated in the Fig. 3. Hydrogen bonding geometries are listed in the Table 6. Both molecules A and B form similar hydrogen bonds. Three different types of classical hydrogen bonds are found in these crystals.

4. Conclusions

Condensation reaction between bile acid esters and dimethylbiguanide in the presence of sodium methoxide is shown to be a facile method in preparation of cholane–triazine conjugates. The

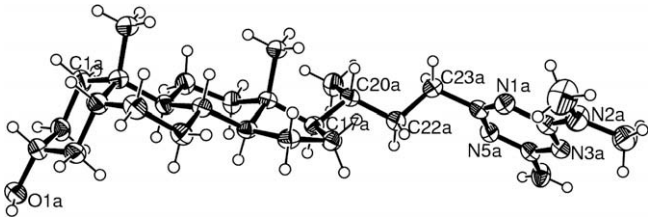


Fig. 2. Ortep-3 plot of the molecular structure of **4a** (molecule A) showing thermal ellipsoids with 50% probability.

Table 6
Selected geometrical parameters of **4a**.

X–Y	D(X–Y) (Å)	W–X–Y–Z	(WXYZ)/°	D–H···A	d(D···A) (Å)	(DHA) (°)
C(6′A)–N(1′A),	1.317(5)/	C13A–C17A–C20A–C22A/	170.6(3)/	N(4A)–HNA2···N(3A)#1	2.977(5)	136(5)
C(6′B)–N(1′B)	1.313(5)	C13B–C17B–C20B–C22B	172.6(3)			
N(1′A)–C(2′A),	1.367(5)/	C17A–C20A–C22A–C23A/	−163.8(3)/	N(4A)–HNA1···O(1A)#2	2.884(4)	164(6)
N(1′B)–C(2′B)	1.366(5)	C17B–C20B–C22B–C23B	−158.1(3)			
C(2′A)–N(3′A),	1.340(5)/	C20A–C22A–C23A–C6′A/	173.6(3)/	O(1A)–H(1OA)···N(5A)#3	2.794(5)	169(5)
C(2′A)–N(3′A)	1.339(5)	C20B–C22B–C23B–C6′B	175.2(3)			
N(3′A)–C(4′A),	1.338(5)/	C22A–C23A–C6′A–N1′A/	−92.8(4)/	N(4B)–HNB1···O(1B)#4	2.875(4)	162(5)
N(3′B)–C(4′B)	1.342(5)	C22B–C23B–C6′B–N1′B	−94.0(4)			
N(5′A)–C(6′A),	1.339(5)/	C22A–C23A–C6′A–N5′A	86.6(4)/	N(4B)–HNB2···N(3B)#5	2.993(5)	133(4)
N(5′B)–C(6′B)	1.351(5)		86.2(4)			
C(2′A)–N(2′A),	1.340(5)/	N1′A–C2′A–N2′A–C7′A/	−178.4(4)/	O(1B)–H(1OB)···N(5B)#6	2.803(5)	163(4)
C(2′B)–N(2′B)	1.344(5)	N1′B–C2′B–N2′B–C7′B	−177.9(4)			
C(4′A)–N(4′A),	1.336(5)/	N1′A–C2′A–N2′A–C8′A/	11.1(6)/			
N(4′B)–N(4′B)	1.336(5)	N1′B–C2′B–N2′B–C8′B	12.0(6)			

Symmetry transformations used to generate equivalent atoms: #1 $-x, y + 1/2, -z + 2$; #2 $x - 1, y, z$; #3 $-x + 1, y - 1/2, -z + 2$; #4 $x + 1, y, z$; #5 $-x + 2, y + 1/2, -z + 1$; #6 $-x + 1, y - 1/2, -z + 1$.

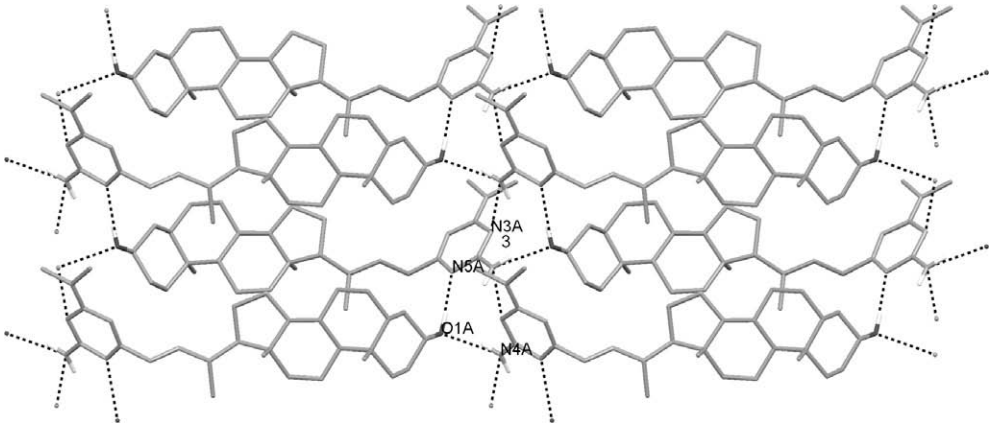


Fig. 3. Crystal packing view along *c*-axis illustrating the hydrogen bonding network in crystals of **4a**. Non-bonding hydrogens are omitted for clarity.

method reported in this study may be useful also in preparation of some other nitrogen containing bile acid derivatives with potential pharmaceutical use. Owing to the pharmaceutical potential of the prepared cholane–triazine conjugates, also their polymorph and solvate forming properties have been preliminary screened by measuring their ^{13}C and ^{15}N CP/MAS NMR spectra, which are compared with liquid state NMR chemical shifts. These studies arise questions about the polymorphic or solvate forming nature of these compounds and we hope we will be able to present more detailed study of the polymorphic nature of these and similar compounds which are currently being prepared. The information gained from the solution NMR and solid state NMR here may serve as reference material when dealing with some similar type of compounds.

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