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16α-Hydroxycholic Acid: *In Vivo* Microbial Transformation Product of Cholic Acid

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Authors' contributions

This work was carried out in collaboration between all authors. This investigation is a result of collaborative work between all authors. Author SAA was the principal author for the grant and designed the work. Author SO carried out the lab. work after consultation with SAA and MA. Author MA interpreted the results, spectral data, manuscript writing and its submission. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: This study describes the transformation of cholic acid to hydroxylated cholic acid metabolites that could not be easily synthesized.
Study Design: The transformation was catalyzed by thermophilic *Geobacillus stearothermophilus* comb. nov., isolated from oil contaminated soil in Kuwait. Cholic acid, as the sole source of carbon, was added to *G. stearothermophilus* cells in phosphate buffer pH 7 and shaken at 65ºC for 5 days.
Methodology: The cholic acid transformation products were extracted with ethyl acetate, purified on preparative TLC plates and their molecular structures were established from their spectral data.
Results: The bacterium could selectively oxidize hydroxyl-groups at C3 and C7 while leaving the C12-hydroxyl-group unoxidized, in cholic acid. Five commonly found metabolites of cholic acid and a novel transformation product, 16α-hydroxycholic acid, were identified.
Conclusion: Our results indicate that *G. stearothermophilus* can hydroxylate/oxidize a steroid nucleus at various ring positions, and has a unique ability for hydroxylation at C16α in cholic acid.

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Keywords: Biotransformation; cholic acid; G. stearothermophilus; hydroxylation; stereospecific.

1. INTRODUCTION

Bile acids and their derivatives are important molecules for the development of pro-drugs, drug absorption and lipid metabolism [1-3]. Stereochemistry of these molecules is critical for differential binding affinities with protein targets. These bindings require a delicate balance between cholesterol and bile acids initiating a range of biological responses [4]. Modified steroids (oxysterols) work as signaling molecules due to their ability to interact and trigger nuclear receptors [5]. However, a steroid molecule may have many isomeric structures and only one of the isomer can bind to its receptor and demonstrate physiological activity. Conversely, chemical synthesis of the isomeric steroids can be hard to accomplish. It is, therefore, important to consider alternate methods that can generate stereoisomeric structures with a capacity to bind specific receptors for potential therapeutic applications. This structural specificity can be easily sustained by enzyme-catalyzed transformation of steroids and the target has been accomplished by using both bacterial and fungal enzymes. While fungal and mesophilic bacterial enzymes catalyze transformations at ambient temperature, use of thermophilic bacterial enzymes makes it simpler to handle at elevated temperatures for their industrial use. Many reviews have outlined an extensive list of advantages in the use of thermophilic organisms in biotechnology because the enzymes of thermophiles are thermostable with many advantages for their use in biotechnology [6-9].

Stereospecific modification of steroid hormones and phytosterols has been achieved using numerous mesophilic and thermophilic bacterial strains [10-14]. Bacterial flora, naturally present within the biosystems, and also fungal strains are known to modify steroid molecules [15-25]. *In vivo* transformation of steroids into valuable drugs is of great industrial interest and in this endeavor; genes encoding these enzymes have been identified [26-29]. The steroid transformations through oxidative chain and ring fission are catalyzed by dehydrogenases and isomerase, reactions that are challenging in the synthesis of steroids [30-34]. Modified bile acids including lithocholic acid (LCA) and deoxycholic acid (DCA) are important biomarkers in the stimulation of colorectal cancer [35] while ursodeoxycholic acid (UDCA), with a β-hydroxl group at C-7, has been used to dissolve cholesterol gall-stones and in liver dysfunction such as hepatitis C and biliary cirrhosis [36,37].

Bile and its component bile acids are abundantly available from slaughter houses and association of bile acids in certain pathologies and microbial transformations has been demonstrated [38-40]. Many of the bile acid derivatives are important intermediates in the synthesis of steroid drugs. Thus, 12-hydroxy-3-oxo-1,4-pregnadiene-20-carboxylic acid, is a useful intermediate for the formation of steroid hormones and other therapeutically useful steroids [34].

We have studied *Geobacillus stearothermophilus*, a thermophile, isolated from oil contaminated soil in Kuwait for the past several years and have shown its inordinate potential for the transformation of steroid hormones, bile acids (chenodeoxycholic acid) and aromatic amino acids [41-46]. Here, we extend our studies to report transformation of cholic acid catalyzed by *G. stearothermophilus*. In our previous studies, we observed that *G. stearothermophilus* could hydroxylate steroid molecules at C3, 7 or 12 and also could produce B-seco compounds by a ring fission at C9, 10. It is known that Cholic acid is derived from cholesterol through hydroxylation by cytochrome P450 gene CYP7A1. Important steroid modifications such as, hydroxylation of 11-dehydrocortisol to give cortisol by 11β-

hydroxylase, gene CYP11B1 and the biosynthesis of aldosterone catalyzed by steroid 21-hydroxylase, gene CYP21A2, are well established examples of steroid hydroxylations to give therapeutically important molecules. In the present study we have studied the potential of *G. stearothermophilus*, for its use in the transformation of cholic acid. The rationale for using cholic acid was to investigate hydroxylation in a steroid molecule like cholic acid where C3,7,12 positions were already blocked by hydroxyl groups making this molecule different from other steroids.

2. MATERIALS AND METHODS

All organic solvents used for extraction were of analytical grade. Solvents and TLC plates were purchased from E. Merck (Darmstadt, Germany). Cholic acid and metabolite standards were of 99% purity and were purchased from Sigma-Aldrich (USA). Media components and salts were of high purity and were purchased from Fluka (Switzerland). FT-IR spectra were obtained on a Jasco-6300 spectrophotometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained using Bruker 400 and 600 MHz (Bruker AC 400 and Bruker Advance II 600) spectrometers respectively, as D_4-methanol solutions. Mass spectra were obtained in EI mode at 70 eV ionization potential using a GC/MS DFS-Thermo Finnigan and V.G. Analytical Ltd. (Manchester, England) model #305 mass spectrometer-2025 interfaced with NIST library database. The study followed principles in the declaration of Helsinki.

2.1 Organism

The organism used for these studies was parental strain of *G. stearothermophilus* and its mutant strain obtained by treatment of the parental strain with 1-methylnitrosoguanidine followed by 3 min. UV exposure at λ 254 nm. *G. stearothermophilus* has the capacity to use different types of organic compounds as carbon source for its growth. The bacterium identity and its charateristics have been decribed previously [44].

The parental strain was maintained at 4ºC on tryptone and yeast extract agar plates as described previously [47]. Seed cultures, (50mL/L) of *G. stearothermophilus* parental and mutant strains were grown separately on tryptone yeast extract (TYE) medium at 65ºC for 14 h in a shaker incubator under aerobic conditions. The cultures were immediately transferred to fresh 500mL TYE medium in 2 L baffled flasks and were shaken at 200 rpm and incubated at 65ºC until the mid-log phase of growth (3.5-4h). The cells were collected by centrifugation at 8000 rpm for 20 min, followed by their suspension in 5-10mL of 50mM phosphate buffer (pH 7). The suspension was transferred to a flask containing 200mL of the 50 mM phosphate buffer, pH 7, containing 10% of Castenholz mineral salt solution as described previously [44].

2.3 Transformation Experiments

Transformation studies were carried out in three cotton plugged Erlenmeyer flasks (250mL) containing sterile 100mL of 50 mM phosphate buffer pH 7. Cholic acid was added (20mg/100mL) into two flasks and sterilized by autoclaving at 121ºC for 30 minutes. Bacterial cells, suspended in phosphate buffer (10mL), were added to one of these flasks, and the other flask with cholic acid but without bacterial cells, was taken as control. The third sterilized flask contained only bacterial cells suspended in buffer and was taken as a negative control. The flasks were incubated at 65ºC for 5 days. Large-scale transformation

of cholic acid was carried out in 3.5L buffer under similar conditions. The effect of variable incubation time, temperature and pH on the transformation was studied.

2.4 Purification and Identification of Cholic Acid Metabolites

The 5-day old culture was harvested and the broth, after acidification, was exhaustively extracted with ethyl acetate. The pooled organic extracts were dried over anhydrous sodium sulfate, filtered and the solvent was evaporated under reduced pressure on a rotary evaporator. The semisolid residue, thus obtained, was re-dissolved in methanol and chromatographed on 20x20cm Kieselgel-60 TLC plates. The crude mixture was resolved on TLC plates, Fig.1, using ethylacetate:iso-octane:acetic acid (20:5:1 v/v/v). Cholic acid metabolites which did not resolve in the first solvent mixture, were resolved in benzene:dioxane:methanol (30:20:1 v/v/v). The transformation products on TLC plates were visualized by staining with 10% ethanolic solution of phosphomolybdic acid, followed by heating at 120ºC for 10 minutes.

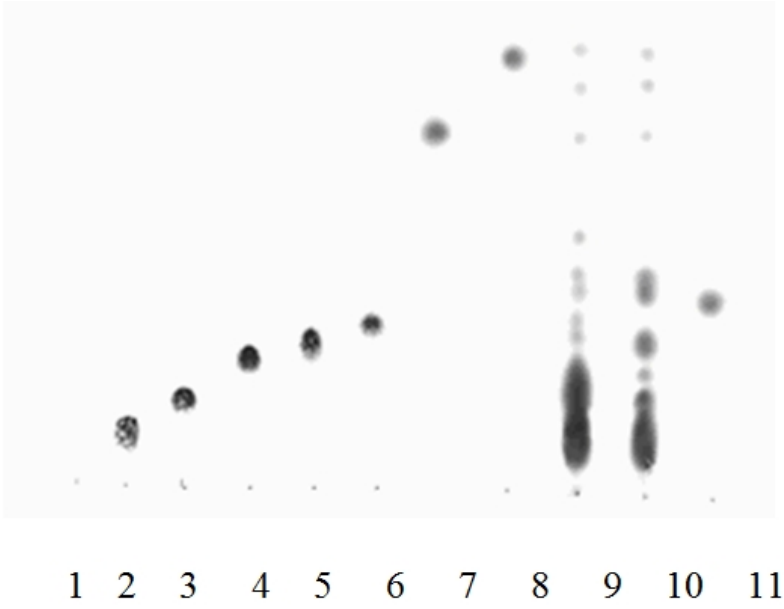

Fig. 1. TLC analysis of 5 day transformation products of cholic acid by *G. stearothermophilus* and mutant strain

From left to right: lane 1: Control (cells without cholic acid); lane 2: Control (cholic acid without cells); lane 3: Compound 1; lane 4: Compound 2 ; lane 5: Compound 3; lane 6: compound 4; lane 7: Compound 5; lane 8: compound 6; lane 9: Cholic acid transformation by parent strain; lane 10: Cholic acid transformation by mutant strain; lane 11: compound 7.

Separation of the large-scale experimental products was achieved on preparative TLC plates and the chromatograms were stained at one edge of the plate, while the remaining plate was masked with a glass sheet. Well-resolved bands, on the corresponding unstained plate, were marked and cut from the plates. Cholic acid metabolites were eluted from the adsorbent by extraction with a mixture of chloroform:methanol (4:1 v/v). Fractions containing identical products were pooled and the solvent was carefully evaporated to dryness at 25ºC

under a gentle stream of nitrogen gas. TLC analyses confirmed the purity of individual metabolites and samples were submitted for spectral data.

Scale-up transformations were also carried out with the mutant strain. It showed intense spots of the metabolites on TLC plates (Fig. 1). Extraction, isolation and purification of the products of mutant strain were carried out as described for the parent strain. In addition, the separation and studies of cholic acid metabolites were also achieved using GC/MS. An ethyl acetate solution of the crude mixture was injected onto an OV1 GC fused silica capillary column (30 m × 0.25 mm i.d.×0.11 µm film thickness) for GC-MS analysis using an Agilent model #6890 gas chromatograph with auto-sampler, mass selective detector model #5973 and Chemstation data system model #6890. Helium gas pressure was maintained at 19.0 psi with a flow rate of 2 mL min^{-1}. The initial oven temperature was maintained at 80ºC for 4 min, followed by a gradual increase of 3ºC min^{-1} to 250ºC, and then the temperature was maintained at 250ºC for 5 min. The total run time was 45.67 min. A typical GC chromatogram is shown in Fig. 2. The MSD Chemstation data analysis with the Agilent library was used for identification of each metabolite.

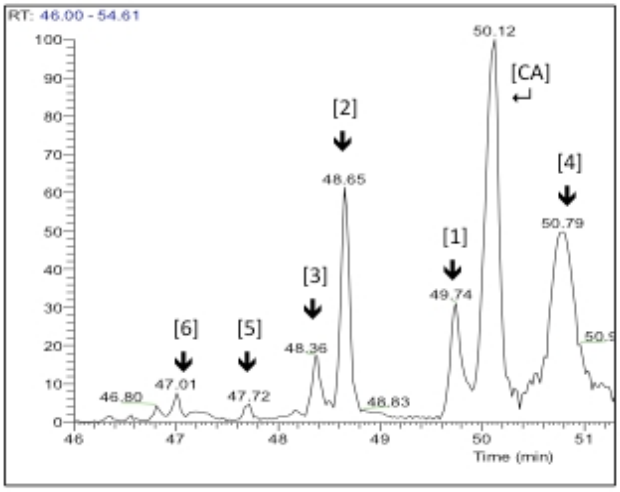

Fig. 2. GC

(1): Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate; (2): 3α-12α-dihydroxy-7-oxo-5β-cholan-24-oic acid; (3): 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid; (4): 3α,7α,12α-16α-tetrahydroxy-5β-cholan-24-oic acid; (5): 3α,7α,12α-trihydroxy-5β-pregnane-20-oic acid; (6): Methyl 3α-methoxy-7α-hydroxy-12-cholen-24-oate.

Purified metabolites were identified from their spectral data including ^{1}H-NMR, ^{13}C-NMR and other spectral data and confirmed by comparing the spectral data with the standards run under identical conditions.

3. RESULTS AND DISCUSSION

Overall yield of the purified products ranged 2-6%. Although yields were not encouraging but we achieved our objective to prove that if C3,7,12 positions were blocked, the hydroxyl group would enter at C16 in a steroid molecule. Optimization of the metabolites was studied by variation in inoculation time, temperature and pH. Six transformation products of cholic acid were identified from their spectral data. These products were: Methyl 3α,7α,12α-

trihydroxy-5β-cholan-24-oate (1); 3α-12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (2); 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid (3); 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid (4); 3α,7α,12α-trihydroxy-5β-pregnane-20-oic acid (5); Methyl 3α-methoxy-7α-hydroxy-12-cholen-24-oate (6). Their TLC and GC resolutions are shown in Fig.1 and 2 respectively, and their molecular structures are given in Fig. 3.

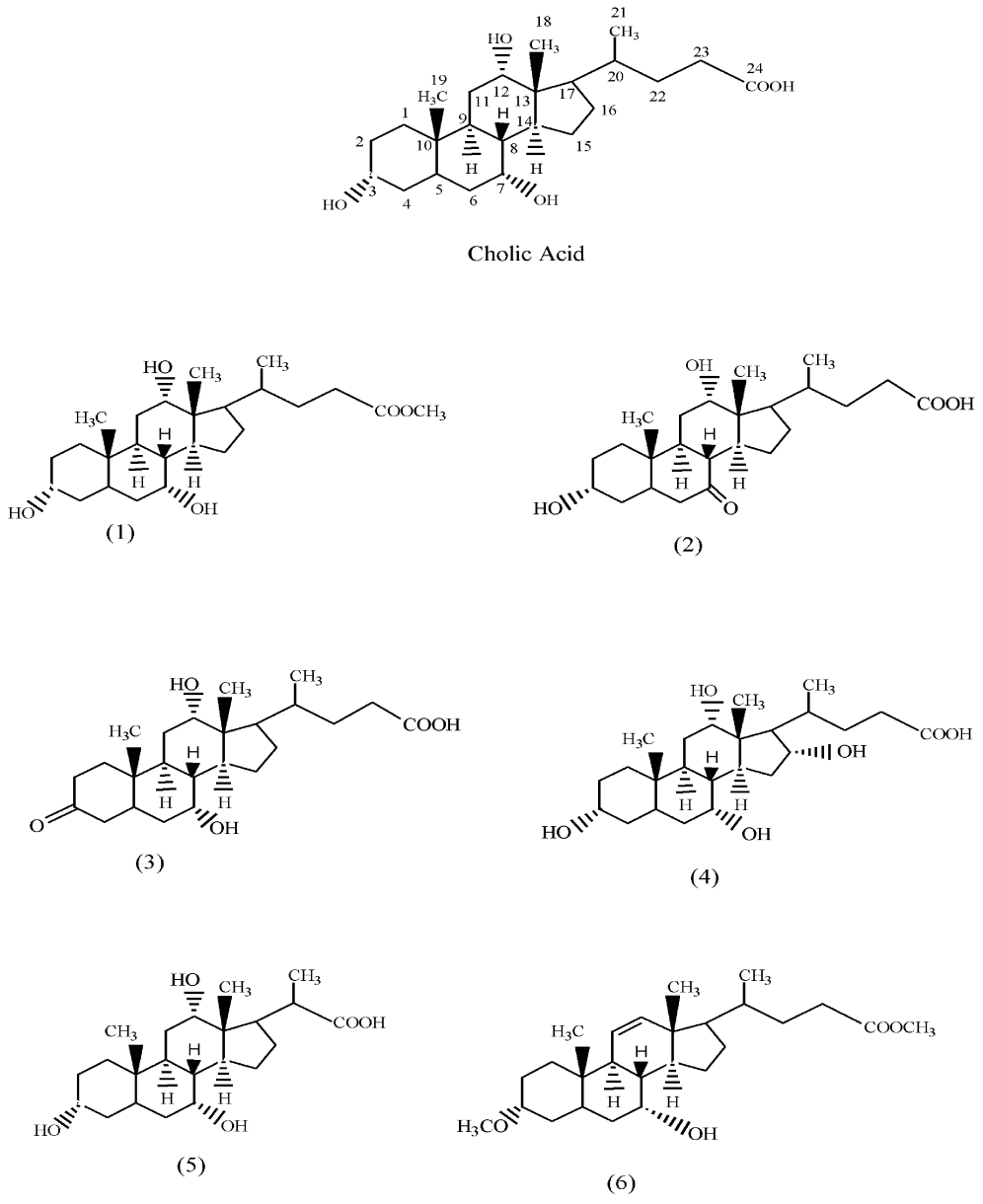

Fig. 3. Molecular structures of the metabolites

(1): Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate; (2): 3α-12α-dihydroxy-7-oxo-5β-cholan-24-oic acid; (3): 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid; (4): 3α,7α,12α-16α-tetrahydroxy-5β-cholan-24-oic acid; (5): 3α,7α,12α-trihydroxy-5β-pregnane-20-oic acid; (6): Methyl 3α-methoxy-7α-hydroxy-12-cholen-24-oate.

3.1 Methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (1)

This compound after its purification on TLC plates, was identified from its typical spectral data. An absorption at 1739 cm^{-1} in its infrared spectrum was attributed to methyl ester group in the molecule. A signal (>C=O) at 50.64 ppm in the proton decoupled ^{13}C-NMR, a singlet for 3H at 3.66 ppm and an absence of carboxyl hydrogen in the ^{1}H-NMR all supported esterification of the carboxyl group at C24 of cholic acid. The most prominent signals (ppm; D_4-methanol) in the ^{1}H-NMR appeared at 0.72, C18-CH_3; 0.931, C19-CH_3; 1.024, C21-CH_3; 3.96, 12-C<u>H</u><; 3.84, 7-C<u>H</u><; 3.36, 3-C<u>H</u><.

Its MS showed a molecular ion at *m/z* 422.3023 consistent with $C_{25}H_{42}O_5$, methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate. The mass spectrum showed a consistent loss of three water molecules conforming three hydroxyl groups in the molecule. Its ^{13}C-NMR data is given in Table 1 and its MS fragmentation appeared at *m/z* 422(1%); 404(22%); 386(73%); 368(38%); 271(100%) 253(75%). Methyl cholate has been earlier reported as a bacterial transformation product of cholic acid [48,49]. The possibility of formation of this compound (2.3% yield) during the extraction process using chloroform:methanol mixtures was checked. The standard cholic acid acid under these extraction conditions did not yield methylcholate. Therefore we assume that this compound was a transformation product of cholic acid. The ester-methyl group may have been contributed by C1 donor like methionine.

3.2 3α,12α,-dihydroxy-7-oxo-5β-cholan-24-oic acid (2)

This compound (yiled 4.6%) showed a typical carbonyl frequency at 1734 cm^{-1} characteristic of a saturated ring carbonyl function. The position of 7 oxo-group was confirmed from the appearance of a new signal at 213.5 ppm in its ^{13}C-NMR, while position of the hydroxyl bearing carbons C3 and C12 remained unchanged and were comparable with the standard cholic acid. Further support for the C7 oxo-group came from the ^{1}H-NMR spectrum where the C<u>H</u>OH signal for cholic acid at C7 was missing. The proton signals (ppm; D_4-methanol) appeared at 8.50, -COO<u>H</u> (exchangeable); 0.784, C18-C<u>H</u>$_3$; 1.23, C19-C<u>H</u>$_3$; 1.05, C21-C<u>H</u>$_3$; 4.06, 12-C<u>H</u><; 3.59, 3-C<u>H</u><.

The mass spectral evidence (*m/z* 406.2719, $C_{24}H_{38}O_5$) was also consistent with the proposed molecular structure. The ^{13}C-NMR data of the compound is given in Table 1 and its MS fragmentation appeared at *m/z* 406(1%); 388(4%); 378(5%); 370(7%); 342(6%); 269(5%); 221(12%); 97(55%); 83(63); 69(90); 60(100%). The transformation of cholic acid to metabolite (2) has been reported in many microbial transformation studies [50,51].

3.3 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid (3)

This compound (1.74% yield), being an epimer of 3α-12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (2) described above, was easily identifiable from its ^{13}C-NMR data given in Table 1. The proton NMR signals (ppm; D_4-methanol) appeared at 8.50 –COO<u>H</u>; 0.775, C18-C<u>H</u>$_3$; 0.939, C19-C<u>H</u>$_3$; 1.01, C21-C<u>H</u>$_3$; 3.89, 7-C<u>H</u><; 4.06, 12-C<u>H</u><. Its molecular ion appeared at *m/z* 406.2718 consistent with $C_{24}H_{38}O_5$ and the fragmentation pattern was consistent with the proposed molecular structure. All spectral features of this compound were comparable with its epimer 3α-12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (2). Its MS fragmentation appeared at *m/z* 406(1%); 388(3%); 370(4%); 355(8%); 337(5%); 300(8%); 287(10%); 269(19%); 135(42%); 97(73%) 83(95%); 69(70%); 57(100%). Many microbial transformation studies

have shown that these two epimeric products (2 & 3) are co-metabolites of cholic acid transformations [50,52].

Table 1. ^{13}CNMR of cholic acid metabolites run as D_4-methanol solution. Values are given in ppm

Carbon #	CA	Cholic acid Metabolites					
		1	2	3	4	5	6
1	35.15	35.64	34.38	36.38	35.05	34.53	26.84
2	29.79	29.9	29.22	36.16	30.31	25.94	25.47
3	67.69	68.2	70.22	215.2	68.27	67.09	81.39
4	39.05	39.61	40.36	45.14	34.98	33.70	26.16
5	41.8	42.35	40.46	43.49	39.06	34.01	35.39
6	34.53	35.05	44.85	33.52	35.88	32.16	31.36
7	71.50	72.0	213.5	67.35	72.03	72.14	70.81
8	39.63	40.16	49.20	39.56	42.15	39.14	34.29
9	26.47	27.03	36.78	27.26	46.64	27.58	46.34
10	34.48	35.0	36.02	34.60	35.64	34.16	33.57
11	28.2	28.73	32.62	28.29	28.7	26.83	138.2
12	72.64	73.15	71.52	72.39	73.20	74.38	126.6
13	46.12	46.63	46.10	46.89	42.31	45.65	42.25
14	41.58	42.15	46.12	41.44	46.64	41.05	46.46
15	22.89	23.37	22.69	26.67	27.01	21.1	22.80
16	27.30	27.81	27.38	27.36	27.84	25.67	23.10
17	46.65	47.13	48.17	48.05	47.20	47.65	48.16
18	11.71	12.11	11.79	11.54	64.49	11.13	18.56
19	21.88	22.3	21.73	20.61	16.75	19.41	19.96
20	35.38	35.91	35.64	35.89	31.01	35.45	30.62
21	16.33	16.73	16.35	16.31	12.15	15.92	16.85
22	30.64	31.0	30.95	29.24	31.40		3026
23	30.94	31.39	30.93	29.27	31.49		30.33
24	176.81	175.7	176.3	176.5	175.5	178.5	175.1
$COOCH_3$							50.64
$-OCH_3$							54.51

3.4 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid (4)

This compound was the major transformation product (6.2% yield) and being a rare metabolite of cholic acid, it was extensively studied. Its molecular ion appeared at *m/z* 424.2827, indicative of its molecular formula $C_{24}H_{40}O_6$. This indicated an additional oxygen atom in cholic acid molecule. The structure of this metabolite was established from detailed analyses of ^{1}H-NMR and ^{13}C-NMR spectra. In the ^{1}H-NMR spectra, all three methyl groups at C18, C19, and C21 appeared as singlets at 0.73, 0.94, 1.04 ppm respectively, each for 3H, with a close resemblance to cholic acid signals. This indicated that the additional oxygen did not enter at any one of these angular carbons. However, when the ^{1}H-NMR of the new compound was compared with cholic acid (Fig 4a), an additional quartet was observed at 3.988 ppm that integrated for one proton (Fig 4b). The other proton signals (D_4-methanol, ppm) appeared at 8.5, COO<u>H</u>; 3.529, multiplet, -C<u>H</u>(OH)- at C3; 3.791, quartet, -C<u>H</u>(OH)- at C7; 3.941, quartet, -C<u>H</u>(OH)- at C12; 4.03,-C<u>H</u>(OH)- at C16α. The stereospecific introduction of the hydroxyl group at C16α was established from the coupling constant of the

triplet for >C<u>H</u>16 split by the >CH_2 at C15. This established the α-stereochemistry of the C16 (OH) group.

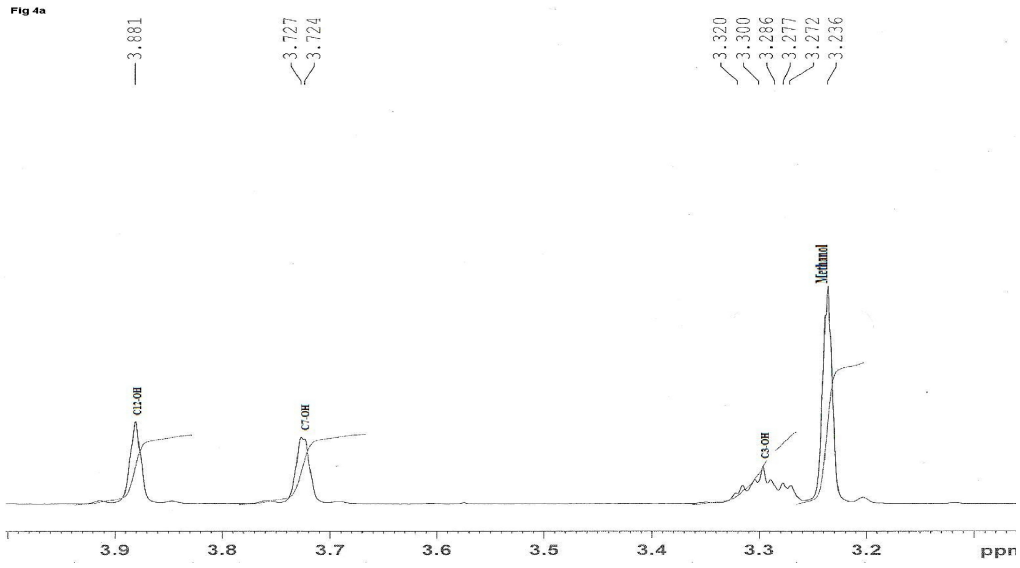

Fig. 4a. ^{1}HNMR of 3,7,12-trihydroxycholan-24-oic acid. The signals show CHO<u>H</u> protons at C3, C7 and C12

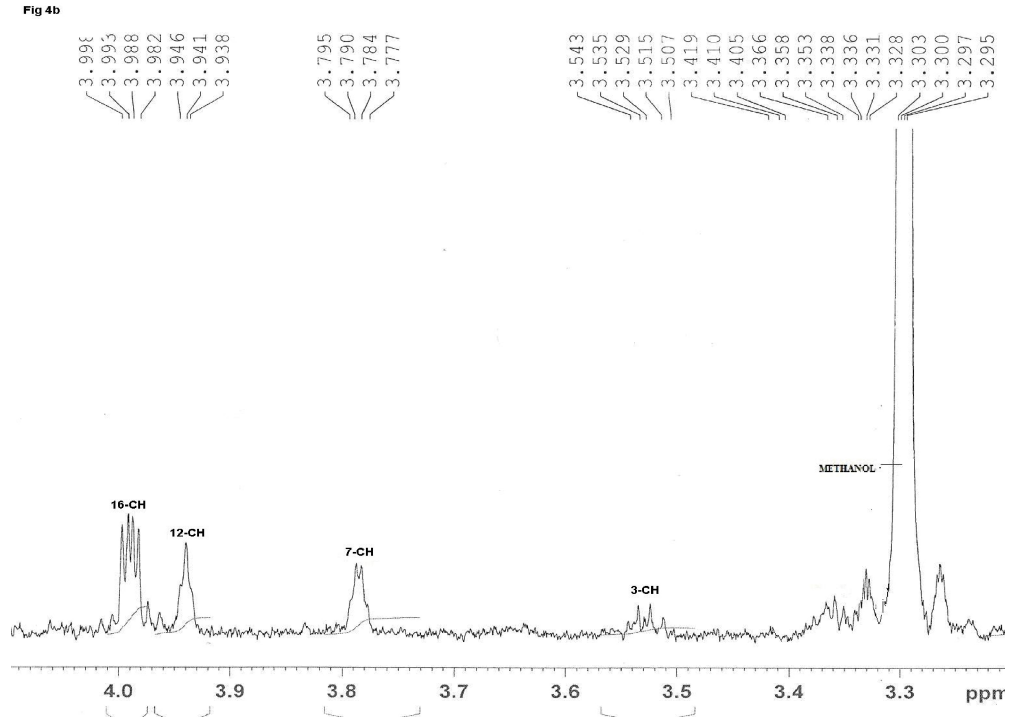

Fig. 4b. ^{1}HNMR of 3,7,12,16-tetrahydroxycholan-24-oic acid. The signals show mthylene protons

Confirmation to this came from the ^{13}C-NMR data of the 16α-hydroxy cholic acid where four distinct carbon signals corresponding to –CHOH- appeared at 64.4911; 68.2777; 72.0347 and 73.2057 ppm for C3, C7, C12 and C16 respectively (Fig 5a). This again confirmed the position of the additional –OH group at C16. The ^{13}CNMR of cholic acid showed only three signals at 67.69; 71.50 and 72.64 ppm for -CHOH- at C3, C7, C12 respectively (Fig 5b). This spectral data were in sharp contrast to other cholic acid metabolites where C-16 (>CH_2) appeared at around 23-27 ppm, Table 1. All other ^{13}C-NMR spectral data were comparable with the standard cholic acid given in Table 1 confirming the additional –OH group at C16. The MS fragmentation of this metabolite appeared at *m/z* 424(0.3%); 408(1%); 390(3%); 372(87%); 271(100%); 253(86%); 226(31%), Fig. 6.

Modifications through hydroxylation processes can take place at many positions around the steroid molecule [53]. The enzyme-catalyzed hydroxylation can take place stereospecifically either/or α/β position to produce one of the isomer[13]. Various types of cytochrome P-450 mono-oxygenases catalyze the NADPH/NADH dependent site-specific hydroxylations. Natural occurrence in python bile, synthesis and characteristics of 16α-hydroxycholic acid (pythocholic acid) have been reported [54-57]. A cytochrome P-450 from *S. roseochrompgenes* strain 10984 has also been purified that can hydroxylate progesterone at C-16α-position. Fungal transformation of progesterone into 16α-hydroxyprogesterone has also been demonstrated using *Sepedoniumam ampullosporum* CMI strain # 203 033 [21]. However this is the first time, hydroxylation of cholic acid at C-16α catalyzed by a thermophilic bacterium has been demonstrated.

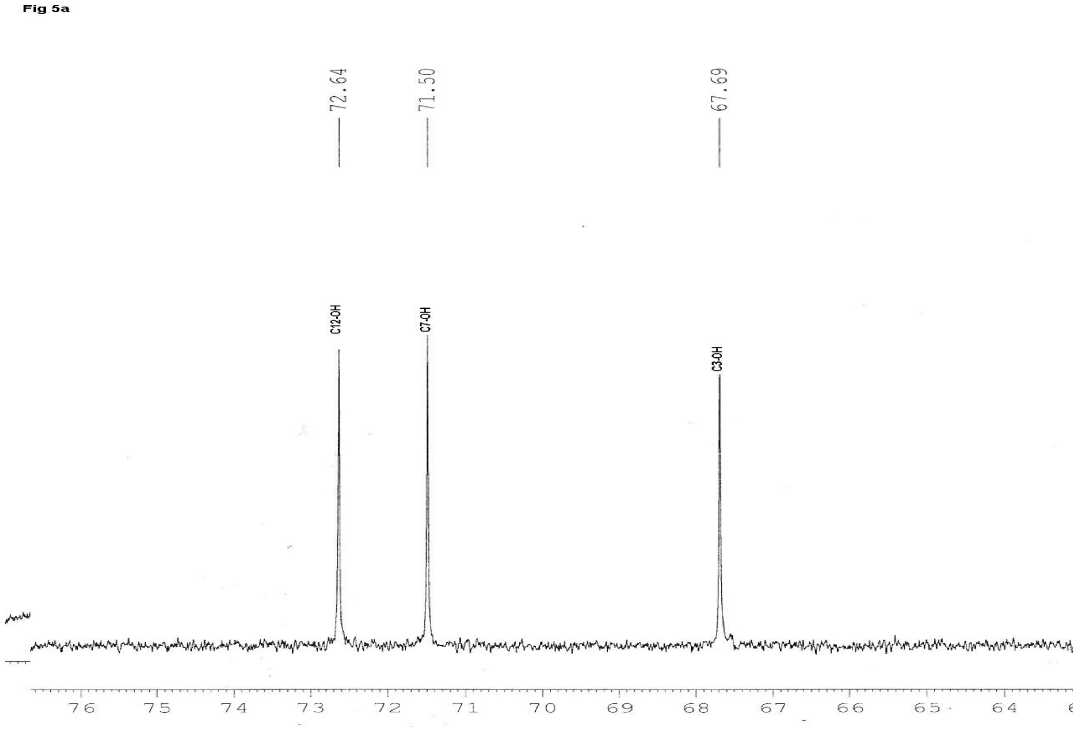

Fig. 5a. ^{13}CNMR of 3,7,12- trihydroxycholan-24-oic acid. The signals show the methylene carbons attached to hydroxyl groups

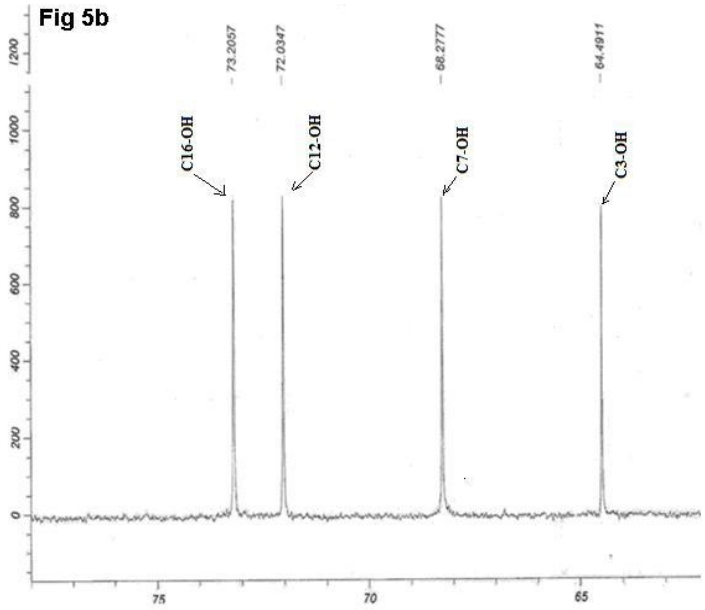

Fig. 5b. ^{13}CNMR of 3,7,12,16-tetrahydroxycholan-24-oic acid. The signals show the methylene carbons attached to the hydroxyl groups

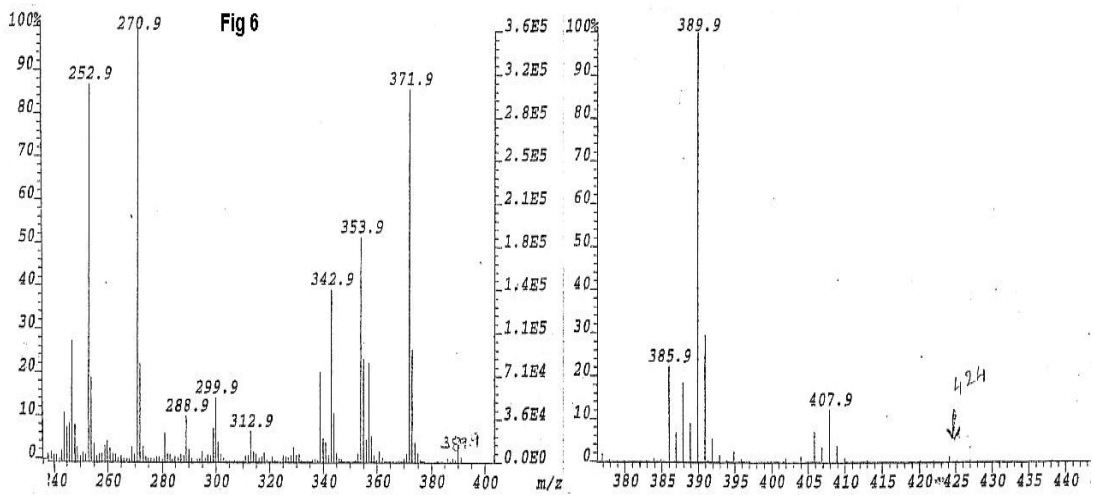

Fig. 6. Mass spectral fragmentation pattern of 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid

3.5 3α,7α,12α-trihydroxy-5β-pregnane-20-oic acid (5)

This compound was recovered in 2.7% yield. The molecular ion of this compound appeared at *m/z* 380.2549, consistent with the formula $C_{22}H_{36}O_5$. From its molecular formula it was evident that cholic acid had lost C_2H_4 neutral fragment from the side chain degradation. An infrared absorption band at 1705.58 cm^{-1} indicated C-20 carboxyl group in the side chain.

This was supported by a strong exchangeable singlet at 8.05 ppm and a signal at 178.5 ppm in the ^{1}H- and ^{13}C-NMR spectra respectively.

The proton NMR signals (ppm; D_4-methanol) appeared at 0.762, C18-C$\underline{H}_3$; 0.982, C19-C$\underline{H}_3$; 1.07, C21-C$\underline{H}_3$; 3.391, 3-C$\underline{H}$<; 3.814, 7-C$\underline{H}$<; 3.920, 12-C$\underline{H}$<. The propionic acid residue at C-17 of the pregnane structure (2.007 and 1.996 for C20$\underline{H}$; 1.843 (C17$\underline{H}$) was supported by the quartet for –C$\underline{H}$-CH_3 group at 3.814 ppm and a doublet at 1.07 ppm for the methyl group at C-20. MS fragmentation of this compound appeared at *m/z* 380.2549 (2) 69(100) 131 (60); 181(52); 281(8). Degradation of the side chain is a common phenomenon in microbial metabolism of bile acids [50].

3.6 Methyl 3α-methoxy-7α-hydroxy-11-cholen-24-oate (6)

This compound (2.3% yield) showed its molecular ion at *m/z* 418.3082 suggesting its molecular formula $C_{26}H_{42}O_4$. Its ^{1}H-NMR spectrum showed two singlets for 3H each at 3.66 and 3.34 ppm characteristic of cholic acid methyl ester -$COOCH_3$ and -OCH_3 groups at C24 and C3, respectively. Both of these groups showed their carbon signals at 50.64 and 54.51 ppm in their ^{13}C-NMR spectrum. The other proton NMR signals (ppm; D_4-methanol) appeared at 0.861, C18-C$\underline{H}_3$; 0.905, C19-C$\underline{H}_3$; 0.974, C21-C$\underline{H}_3$; 3.55, 7-C$\underline{H}$<; 3.59, 3-C$\underline{H}$<; 5.31, C11-C$\underline{H}$=; 5.53, C12 –C$\underline{H}$=. The double bond between C-11-C12 appeared as a complex multiplet for 2H. The ^{13}C-NMR data are presented in Table 1. Its MS fragmentation appeared at *m/z* 418.3082 (1%); 400(4%-H_2O); 388 (38%); 371(41%); 271(100%) 226(61%). This compound has been reported as cholic acid transformation product catalyzed by human intestinal microflora [58].

The formation of 3α,7α,12α-trihydroxy-5β-pregnane-20-oic acid (5) indicates that the organism has the capacity to degrade side chain in cholic acid. However, among the transformation products, no indication of ring fission to produce seco-steroids, was noticed. Preferential oxidation of the hydroxyl groups at C3 and C7, while leaving intact the hydroxyl function at C12/C16, seems to be the principal characteristic of this organism. However, the unique feature of the organism is to introduce a hydroxyl function at C16 producing 16α-hydroxy-cholic acid which could not be obtained through conventional chemical synthesis. The 16-hydroxysteroid epimerase is known to convert 16α-hydroxyl group into 16β-hydroxyl group in a steroid molecule [59].

Streptomyces roseochromogenes has the capacity to introduce 2β and 16α-hydroxyl-group in a steroid molecule [60]. Both 16α and 16β-hydroxysteroids are important intermediates in the synthesis 16-oxygenated estrogens used as markers of fetus well being during pregnancy. The physiology and pathology of 16-hydroxyestrogens has been eloquently reviewed [61]. The 16α-hydroxysteroids may also be useful intermediates in the synthesis of 16α-hydroxyprednisolone, fluorinated 16α-hydroxyprednisolones and other 16α-hydroxycorticoids. Our results show that thermophiles such as *G. stearothermophilus* can be used for 16α-hydroxylation of steroids, for drug development.

4. CONCLUSION

G. stearothermophilus has a good potential for transformation of bile acids into an uncommon product such as 16α-hydroxycholic acid which is a novel metabolite of cholic acid not easily accessiable by chemical synthesis.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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DECLARATION OF INTEREST

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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