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Antimicrobial cholic acid derivatives from the Pitch Lake bacterium *Bacillus amyloliquefaciens* UWI-W23

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ABSTRACT

Six cholic acid derivatives (**1–6**) were isolated from broth cultures of *Bacillus amyloliquefaciens* UWI-W23, an isolate from the Trinidad Pitch Lake. The compounds were extracted via solvent extraction and/or XAD resin adsorption and purified using silica gel column chromatography. Their structures were elucidated using 1D, 2D NMR and ESI-MS spectrometry and FT-IR spectrophotometry. One of the compounds, taurodeoxycholate (**2**) is for the first time being reported from a bacterial source while deoxycholate (**4**) is for the first time being reported from a Gram-positive bacterium. The other compounds have not been previously isolated from *Bacillus* spp. viz. cholate (**1**), taurocholic acid (**3**); glycodeoxycholic acid (**5**) and glycocholic acid (**6**). All six compounds exhibited antimicrobial activity against *P. aeruginosa* and *B. cereus* with MICs ranging from 7 to 250 μg/mL. Cholate (**1**) also showed activity against MRSA (MICs = 125 μg/mL) and glycocholic acid (**6**) against *S. cerevisiae* (MICs = 15.6 μg/mL).

1. Introduction

With the continued misuse of antibiotics, there is an overwhelming increase in antibiotic resistant infectious microorganisms [1]. As such, there is need for the continued search for and development of novel drugs to counteract this problem. One approach to this search for new antimicrobials has been, to explore microorganisms from unique environments. In our continued work in this area, we have isolated several bioactive cholic acid derivatives (**1–6**) from *Bacillus amyloliquefaciens* UWI-W23, a bacterial strain originating from the sulphurous aqueous ponds of the Trinidad Pitch Lake.

Cholic acid and chenodeoxycholic acid are primary bile acids, which are formed from the degradation of cholesterol in the liver and stored in the gall bladder of vertebrates [2]. The primary bile acids are secreted into the intestine upon consuming a meal. They function in the digestion of fats, and in the prevention of bacterial proliferation [3]. Intestinal bacteria, including members of *Clostridium* spp., are known to remove the 7α-hydroxyl group of these primary bile acids to form the secondary bile acids, deoxycholic acid and lithocholic acid, respectively [4].

Bile acids have been reported in recent times to act as hormones which regulate glucose and lipid metabolism in addition to inflammatory responses [4]. They are usually bacteriostatic against Gram-positive bacteria and as such taurocholic acid is the major component of the well marketed MacConkey agar, a selective culture medium for Gram-negative bacteria. They are also used in asymmetric synthesis and in the production of cholaphanes as well as in pharmacological applications, for example, as drug delivery agents [5].

Bile acids are known to conjugate with amino acids, taurine and glycine in the liver, and this results in their increased water solubility. The conjugates are amphipathic compounds, and this accounts for their ability to emulsify fats and destabilize bacterial membranes [6]. Our isolated cholic acid derivatives thus, have potential to be developed into potent antimicrobial compounds.

There has been great debate over the years on whether bacteria can produce steroid compounds. However, in 1995 and 2005 the first reports were made, confirming the production of steroidal compounds, particularly, bile acids by prokaryotes [6,7]. However, such reports were limited to marine isolates belonging to the genera *Myroides, Aeromicrobium*, *Donghaeana*, *Dokdonia*, *Polaribacter*, *Maribacter*, *Hahella* and *Rhodococcus* as well as the soil bacterium *Streptococcus faecium* [6–8]. The cholic acid derivatives viz; cholate (**1**), taurodeoxycholate (**2**), taurocholic acid (**3**), deoxycholate (**4**), glycodeoxycholic acid (**5**) and glycocholic acid (**6**), were proven to be absent from the culture medium used, and thus it can be concluded that they are secondary metabolites of *Bacillus amyloliquefaciens* UWI-W23. None of these compounds have been previously reported from *Bacillus* spp. Additionally, compound **2** has never been isolated from any bacterial

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strain and compound **4** has not been isolated previously from a Gram-positive bacterium.

2. Experimental

2.1. General methods

UWI-W23 was cultured under standard sterile conditions, using a Gyromax 777R Amerex Orbital incubator rotary shaker. Cultures were grown using nutrient agar (Oxoid CM 0003), containing Lab-Lemco powder 1 g/L, yeast extract 2 g/L, peptone 5 g/L, sodium chloride 5 g/L, agar 15 g/L) and nutrient broth (Oxoid CM1), containing the same components of CM 0003 except for agar. NMR data were obtained using Bruker Ultrashield Avance III-600 and 300 spectrometers and a Bruker Avance DRX-400 NMR spectrometer. Samples were prepared in CD_3OD with TMS as the internal standard. HRESI-MS were measured on a Bruker MicrOTOF-Q mass spectrometer and samples were dissolved in MeOH/H_2O. IR spectral data were obtained using a PerkinElmer FT-IR RX-I spectrophotometer, with samples prepared in NaCl. Column chromatography was performed using Baker normal phase silica gel (60–200 mesh). TLC was performed on 0.25 mm thick layers of aluminum-backed silica gel plates using solvent systems of 70:30 and 60:40 ($CHCl_3$:MeOH). Chromatograms were observed using ammonium molybdate (5 g ammonium molybdate in 10% H_2SO_4) as a visualization agent.

2.2. Bacterial isolate

Bacillus amyloliquefaciens UWI-W23 was obtained from the culture collection of the Department of Life Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago. The organism was an isolate originating from a sample of pond water collected at the La Brea Pitch Lake, Trinidad and was identified by partial 16S rRNA gene sequence analysis.

2.3. Extraction and isolation

Bacillus amyloliquefaciens UWI-W23 was grown on nutrient agar plates (90 mm) for 24 h at 30 °C and the plates were then cut into small pieces (~1 cm^2) before transferring into sterile nutrient broth at a rate of 1 plate per 200 mL broth. The inoculated broth was incubated in a shaker at 30 °C at 150 rpm for 24 h. This seed culture was then used to inoculate larger batches (~2–3 L) of sterile nutrient broth (70 mL seed culture per 1 L) which were then incubated as above, but for 18 days.

For the isolation of **1**; 40 L of cultured broth was centrifuged and filtered through muslin, followed by extraction with EtOAc (2 × 300 mL EtOAc per 1 L). The EtOAc-soluble material (1 g) was subjected to fractionation by silica gel column chromatography (EtOAc/MeOH). Cholate (**1**) (3.7 mg) was eluted with EtOAc/MeOH (75:25–70:30 v/v).

For the isolation of **2** and **3**; 10 L of cultured broth was filtered through muslin, followed by extraction with Pet. ether (2 × 300 mL Pet. ether per 1 L). The Pet. ether-soluble material (0.67 g) was subjected to fractionation by silica gel column chromatography ($CHCl_3$:MeOH). Taurodeoxycholate (**2**) (3.1 mg) and Taurocholic acid (**3**) (2.2 mg) were eluted with $CHCl_3$/MeOH (65:35 and 65:35–60:40 v/v, respectively).

For the isolation of **4–6**; 9 L of cultured broth was filtered through muslin and then passed through a column packed with XAD-7 resin (600 g). The fraction eluted with 400 mL of MeOH:H_2O (60:40 v/v) (0.8 g) was then subjected to fractionation by silica gel column chromatography. Deoxycholate (**4**) (1 mg), glycodeoxycholate (**5**) (5 mg) and glycocholic acid (**6**) (12 mg) were eluted with $CHCl_3$/MeOH (85:15, 60:40 and 55:45 v/v, respectively).

Table 1
^{1}H NMR Data of Cholic Acid Derivatives **1–6** (δ in ppm, J in Hz, CD_3OD).

Position	**1**[a]	**2**[b]	**3**[b]	**4**[c]	**5**[a]	**6**[b]
1	0.99 m	0.98 m	0.99 m		0.99 m	0.98 m
	1.81 m	1.79 m	1.81 m		1.78 m	1.80 m
2	1.43 m	1.41 m	1.43 m		1.42 m	1.45 m
	1.59 m	1.60 m	1.61 m		1.60 m	1.60 m
3	3.38 m	3.51 m	3.35 m	3.53 m	3.54 m	3.37 m
4	1.65 m	1.43 m	1.68 m		1.47 m	1.65 m
	2.29 m	1.79 m	2.27 m		1.81 m	2.30 m
5	1.38 m	1.38 m	1.37 m		1.40 m	1.38 m
6	1.53 m	1.28 m	1.50 m		1.29 m	1.53 m
	1.96 m	1.88 m	1.92 m		1.89 m	1.97 m
7	3.81 m	1.16 m	3.80 m		1.18 m	3.79 m
		1.43 m			1.44 m	
8	1.55 m	1.43 m	1.57 m		1.43 m	1.55 m
9	2.24 m	1.88 m	2.26 m		1.90 m	2.55 m
11	1.58 m	1.51 m	1.59 m		1.53 m	1.58 m
12	3.98 m	3.95 m	3.96 m	3.96 m	3.96 m	3.95 m
14	1.99 m	1.60 m	1.98 m		1.63 m	2.00 m
15	1.13 m	1.08 m	1.11 m		1.10 m	1.11 m
	1.75 m	1.59 m	1.77 m		1.62 m	1.75 m
16	1.34 m	1.28 m	1.29 m		1.29 m	1.31 m
	1.93 m	1.88 m	1.90 m		1.89 m	1.90 m
17	1.86 m	1.83 m	1.89 m		1.85 m	1.87 m
18	0.71 s	0.70 s	0.71 s	0.71 s	0.72 s	0.71 s
19	0.92 s	0.92 s	0.92 s	0.93 s	0.94 s	0.92 s
20	1.40 m	1.43 m	1.40 m		1.47 m	1.44 m
21	1.02 d	1.01 d	1.02 d	1.01 d	1.03 d	1.03 d
	(6.6)	(6.4)	(6.4)	(5.8)	(6.5)	(6.4)
22	1.33 m	1.31 m	1.38 m		1.36 m	1.37 m
	1.83 m	1.76 m	1.79 m		1.80 m	1.80 m
23	2.08 m	2.10 m	2.13 m		2.17 m	2.19 m
	2.26 m	2.25 m	2.26 m		2.33 m	2.31 m
25		3.59 t	3.58 t		3.79 s	3.83 s
		(7.2)	(6.8)			
26		2.95 t	2.96 t			
		(7.2)	(6.8)			

[a] Measured at 600 MHz.
[b] Measured at 400 MHz.
[c] Measured at 300 MHz. s, singlet; d, doublet; t, triplet, m, multiplet.

Table 2
^{13}C NMR Data of Cholic Acid Derivatives **1–6** (δ in ppm, CD_3OD).

Position	**1**[a]	**2**[b]	**3**[b]	**5**[a]	**6**[b]
1	36.7	36.6	36.5	36.5	36.5
2	31.4	31.1	31.2	31.1	31.2
3	73.1	72.6	72.9	72.6	72.9
4	40.7	37.2	40.5	37.2	40.5
5	43.4	43.7	43.2	43.7	43.2
6	36.0	28.4	35.9	28.4	35.9
7	69.3	27.5	69.1	27.5	69.1
8	41.2	37.5	41.0	37.5	41.1
9	28.0	34.8	27.9	34.9	27.9
10	36.0	35.3	35.9	35.3	35.9
11	29.7	29.9	29.6	29.9	29.6
12	74.3	74.1	74.1	74.1	74.1
13	47.7	47.6	47.5	47.6	47.5
14	43.1	49.3	43.0	49.4	43.0
15	24.4	24.9	24.2	24.9	24.2
16	28.9	28.6	28.7	28.4	28.7
17	48.6	48.2	48.2	48.1	48.1
18	13.2	13.2	13.0	13.2	13.0
19	23.3	23.7	23.2	23.7	23.2
20	37.5	36.9	36.9	37.2	36.9
21	18.0	17.7	17.8	17.8	17.8
22	34.2	33.1	33.2	33.1	33.1
23	36.1	34.2	34.3	34.0	34.0
24	unseen	176.1	176.7	177.0	173.6
25		36.5	36.6	43.7	43.0
26		51.5	51.5	177.0	175.7

[a] Measured at 150 MHz.
[b] Measured at 75 MHz.