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Chrolactomycins from the Actinomycete *Actinospica*

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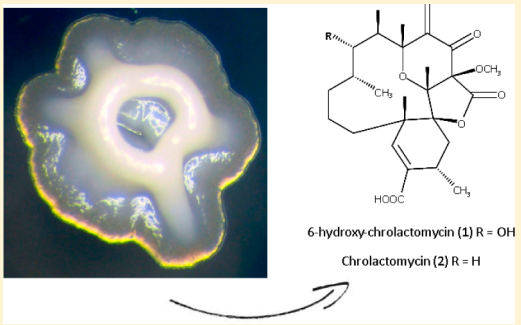
Supporting Information

ABSTRACT: Examination of the metabolites produced by an *Actinospica* strain led to the identification of 6-hydroxychrolactomycin (compound **1**), which is produced along with minor amounts of chrolactomycin (compound **2**). The structure of **1** was established on the basis of extensive spectroscopic analysis, including one- and two-dimensional NMR. Compound **1** showed antimicrobial activity against Gram-positive bacteria, although it was generally less active than **2**.



The genus *Actinospica* was recently described[1] as part of the new order *Catenulisporales*[2] within the Actinobacteria. As part of a screening campaign to identify new bioactive molecules from newly described actinomycete taxa,[3−5] we analyzed the metabolites produced by *Actinospica* strain gamma-22, a strain closely related to *Actinospica robiniae* GE134769^T,[1−3] and we now report the novel metabolite 6-hydroxychrolactomycin (**1**), produced together with small amounts of chrolactomycin (**2**) (Figure 1). These and the recently reported catenulipeptin[6] represent the first metabolites described from members of the *Catenulisporales*.

Actinospica strain gamma22 was grown in half-strength AF medium for 6 days at 28 °C, and an ethyl acetate extract of the cleared broth showed activity against *Staphylococcus aureus*. Upon fractionation by reversed-phase MPLC, anti-staphylococcal activity was associated with two major peaks eluting at 4.5 and 6.3 min, with relative peak areas of 6:4 (Figure 2). The 6.3 min peak, which exhibited a molecular weight of 432 Da and a UV maximum at 210 nm, was tentatively identified as **2** by database searches. Its NMR spectrum (not shown) was fully consistent with that reported for chrolactomycin **2**, a metabolite produced by *Streptomyces* sp. 569N-3.[7]

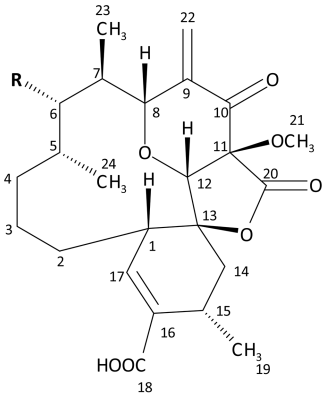


Figure 1. Chemical structure of 6-hydroxychrolactomycin (**1**) and chrolactomycin (**2**).

The 4.5 min peak was identified as 6-hydroxychrolactomycin (**1**). It exhibited a similar characteristic UV maximum at 210 nm and a molecular formula of $C_{24}H_{32}O_8$, which was deduced from HRESIMS. This indicated the presence of an additional oxygen atom in **1** with respect to **2**. The ^{1}H and ^{13}C NMR data for **1** are shown in Table 1 and Figures S1 and S2. Attached proton test (APT) experiments (Figure S2) displayed 24 carbon resonances, including seven quaternary carbons, two of them matching acid/ester/lactone-type carboxyls (δ_C 169.1 and 168.2) and one matching a ketone signal (δ_C 190.1). Eight methine groups, five methylenes, one of them corresponding to a terminal double bond (δ_C 121.0), and four methyl groups, one of them corresponding to a methoxy moiety (δ_C 53.5), were also detected. The chrolactomycin skeleton was confirmed through the study of HSQC (Figure S3) and HMBC (Figure S4); in particular, correlations of H-8 (δ_H 4.46) to C-22 and C-23 (δ_C 121.0 and δ_C 13.3); of H-12 (δ_H 4.61) to C-1 (δ_C 42.1), C-10 (δ_C 190.1), C-11 (δ_C 78.9), C-13 (δ_C 83.0), and C-14 (δ_C 34.0); and of H-19 (δ_H 1.08) to C-14 (δ_C 34.0), C-15 (δ_C 27.2), and C-16 (δ_C 133.6). The only difference between the spectra of **1** and **2** is the presence of a hydroxy methine, which

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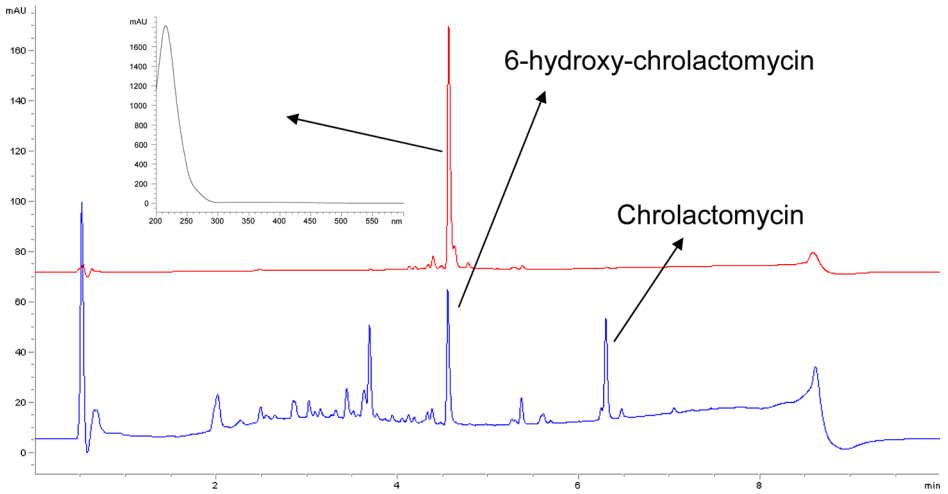

Figure 2. HPLC profiles of the extracted fermentation broth (blue) and of purified 6-hydroxychrolactomycin (**1**, red). UV−vis spectrum of **1** is shown.

Table 1. ^{1}H and ^{13}C NMR Spectral Data of 6-Hydroxychrolactomycin Measured at 400 MHz in CD_3OD

position	δ_C, type	δ_H (J in Hz)
1	42.1, CH	2.40, m
2	31.8, CH_2	1.27, m
		1.65, m
3	23.4, CH_2	1.66, m
4	34.4, CH_2	0.98, m
		1.62, m
5	35.3, CH	2.14, m
6	73.9, CH	3.55, d (8.7 Hz)
7	38.5, CH	2.10, m
8	80.2, CH	4.46, d (9.8 Hz)
9	143.7, C	
10	190.1, C	
11	78.9, C	
12	83.6, CH	4.61, s
13	83.0, C	
14	34.0, CH_2	1.34, m
		1.78, dd (10.5, 14.9 Hz)
15	27.2, CH	2.59, m
16	133.6, C	
17	138.5, CH	6.72, dd (1.8, 6.0 Hz)
18	169.1, C	
19	19.3, CH_3	1.09, d (6.8 Hz)
20	168.2, C	
21	53.5, CH_3	3.60, s
22	121.0, CH_2	5.83, d (1.5 Hz)
		6.44, d (1.0 Hz)
23	13.3, CH_3	1.12, d (6.6 Hz)
24	13.7, CH_3	0.85, d (7.1 Hz)

was assigned to position 6 from the correlation of H-23 (δ_H 1.12) and H-24 (δ_H 0.85) to C-6 (δ_C 73.9). COSY and TOCSY correlations (Figure S5) supported this structure, while NOESY correlations (Figure S6) showed that the relative configurations of all the stereogenic centers (C-1, C-5, C-7, C-8, C-11, C-12, C-13, and C-15) were identical to those of **2**. Moreover, the NOESY correlation between H-6 and H-8 revealed that they were on the same plane, elucidating the relative stereochemistry of C-6 as shown in Figure 3.

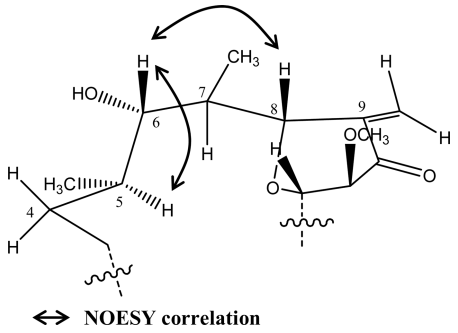

Figure 3. NOESY correlations occurring near C-6 in **1**.

1 showed activity comparable to **2** against *Micrococcus luteus* and *Streptococcus pyogenes* but was less active than **2** against other Gram-positive bacteria (Table 2). No activity was observed against Gram-negative bacteria or *Candida albicans*.

Table 2. Antimicrobial Activities of 1, 2, and Vancomycin (Van) against Gram-Positive Bacteria Expressed As MIC Values (μg/mL)

strain	code	**1**	**2**	Van
Staphylococcus aureus	ATCC6538P	32	2	0.5
Staphylococcus aureus	ATCC29213	64	2	0.5
Micrococcus luteus	108	8	8	0.125
Bacillus subtilis	168	128	8	0.25
Streptococcus pyogenes	49	8	16	0.5

Compounds containing condensed macrolactones are relatively rare. In addition to **1** and **2**, other examples include okilactomycin[8] and its recently reported new congeners.[9] However, none of these compounds contains a 6-hydroxy functional group as found in **1**. It should be noted that the C-6 carbon in okilactomycin derives from C-1 of a propionate unit, presumably through a polyketide pathway.[10] The *Actinospica* strain gamma22 is unusual in being able to insert a hydroxyl at position C-6, although the deoxy analogue **2** is also produced, and it remains to be established whether hydroxylation occurs during or after polyketide synthesis.

Chrolactomycin and okilactomycin have been reported to possess antibacterial and cytotoxic activities, although their

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mode of action has not been fully established. There are reports suggesting inhibition of telomerase activity for chrolactomycin,[11] while okilactomycin appears to preferentially inhibit RNA synthesis in bacteria.[9] In addition to validating our investigation into this rare and unusual actinomycete, the new chrolactomycin congener described here may contribute to understanding structural−activity relationships in this poorly explored class, possibly providing starting material for semisynthesis.

EXPERIMENTAL SECTION

General Experimental Procedures. Analytical HPLC-MS was performed using an Agilent 1100 Series liquid chromatograph equipped with an Ascentis Express Supelco RP_{18} column, 2.7 μm (50 × 4.6 mm), eluted at 1 mL/min and at 40 °C. Samples were run with a linear gradient from 5% to 100% phase B in 6 min, using 0.05% trifluoroacetic acid in water (v/v) and acetonitrile (v/v) as phases A and B, respectively. The effluent from the column was split between a photodiode array detector and an ESI interface of a Bruker Esquire3000 Plus ion trap mass spectrometer operating in the positive-ion mode. ^{1}H and ^{13}C 1D- and 2D-NMR spectra (COSY, TOCSY, NOESY, HSQC, HMBC) were measured in CD_3OD at 25 °C using a Bruker AMX 400 MHz spectrometer.

LC-HRMS analyses were performed on a Surveyor Accela HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) connected to an Exactive benchtop mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with an NSI-ESI ion source. Samples were injected on a C_8 reversed-phase column (BioBasic C_8, 100 × 0.18 mm, 5 μm, 300A, Thermo Fisher Scientific) and were eluted through an acetonitrile gradient (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile): the gradient profile was 5% eluent B for 3 min, 5% to 65% B in 17 min, 65% to 95% B in 5 min; the flow rate was 100 μL/min split in order to achieve a final flux of 2 μL/min. The observed peak was at m/z 449.2162 $[M + H]^+$ (calcd for $C_{24}H_{33}O_8$, 449.2170).

Strain Cultivation and Metabolite Identification. *Actinospica* sp. gamma22 belongs to the NAICONS strain collection. The sequence of 16S rRNA has been deposited in GenBank with accession number AM157355. It was maintained on oatmeal agar plates.[12] For liquid cultures, a loopful of mycelium was scraped from an agar plate and inoculated into 15 mL of half-strength AF medium[12] in a 100 mL baffled flask. A 10% inoculum from a 6-day culture was introduced into 100 mL of fresh medium. All liquid cultures were incubated for 6−7 days at 30 °C on a rotary shaker at 200 rpm.

A total of 6 × 100 mL cultures were harvested, pooled, and centrifuged at 3000 rpm for 10 min to separate the mycelium from the clear broth. The latter (580 mL) was extracted three times with 200 mL of ethyl acetate, and the combined organic phases were dried under reduced pressure and dissolved in 4 mL of 50% dimethylformamide in H_2O (v/v). The sample was resolved on a 20 g reversed-phase Puriflash PF-C_{18} (50 μm particle size) by using a CombiFlash RF Teledyne ISCO medium-pressure chromatography system. The column was previously conditioned with a mixture of phase A/phase B (95:5) (v/v) and then eluted at 30 mL/min with a 15 min linear gradient from 5% to 95% phase B (phase A was water with 0.1% TFA, and phase B was acetonitrile). Antimicrobial activity was monitored by agar diffusion as described below. The active fractions were pooled, concentrated under vacuum, and lyophilized, yielding 20 and 8 mg of purified **1** and **2**, respectively.

Antimicrobial Assays. For determining antimicrobial activity, 100 μL of ethyl acetate extracts were evaporated under vacuum, resuspended in 100 μL of 10% dimethylsulfoxide, and deposited (10 μL) onto Muller Hinton agar (Difco Laboratories, Detroit, MI, USA) plates, previously inoculated with 10^5 cfu/mL of *S. aureus* L100. Plates were incubated overnight at 37 °C. MICs were determined by broth microdilution in sterile 96-well microtiter plates according to CLSI guidelines,[13] using as growth medium Mueller Hinton broth containing 20 mg/L $CaCl_2$ and 10 mg/L $MgCl_2$ for all strains except for *Streptococcus pyogenes*, which was grown in Todd Hewitt broth (Difco Laboratories). Compounds **1** and **2** were dissolved in dimethylsulfoxide and vancomycin (Sigma Aldrich) in water, as 10 mg/mL stock solutions, and diluted in the appropriate culture medium immediately before testing. Bacterial strains were inoculated at 5 × 10^5 cfu/mL from frozen stocks. All strains were from the NAICONS collection unless indicated otherwise in Table 2. Plates were incubated at 37 °C and read after 20−24 h. The MIC was defined as the lowest compound concentration causing complete suppression of visible growth.

ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, APT, HSQC, HMBC, TOCSY, and NOESY spectra of 6-hydroxychrolactomycin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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