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Chojalactones A−C, Cytotoxic Butanolides Isolated from *Streptomyces* sp. Cultivated with Mycolic Acid Containing Bacterium

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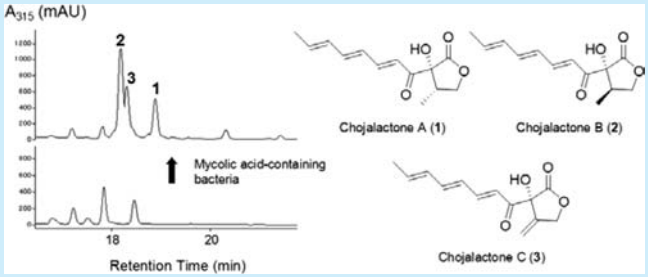
Supporting Information

ABSTRACT: The soil-derived bacterium, *Streptomyces* sp. CJ-5, was cocultured with the mycolic acid-containing bacterium *Tsukamurella pulmonis* TP-B0596. The combined culture method significantly enhanced the production of the secondary metabolites in *Streptomyces* sp. CJ-5, leading to the isolation of three novel butanolide chojalactones A−C (**1**−**3**), with unusual γ-butyrolactone scaffolds. The complete structures, including the absolute configurations of **1**−**3**, were determined based on spectroscopic data and total syntheses. In methylthiazole tetrazolium (MTT) assays, **1** and **2** showed moderate cytotoxicity against P388 cells.



In the history of drug discovery, the genus *Streptomyces* has played an important role as a major source of lead/seed compounds, and numerous useful bioactive compounds have been isolated from this extraordinary genus.[1,2] Furthermore, recent genomic analyses of *Streptomyces* species revealed a greater abundance of biosynthetic gene clusters responsible for secondary metabolites than previously expected based on the number of isolated compounds. Thus, *Streptomyces* strains express only a very small portion of their gene clusters under standard laboratory conditions, and the others remain cryptic.[3] Therefore, the cryptic gene clusters in *Streptomyces* strains should be regarded as an untapped source of bacterial secondary metabolites, and methods for the effective activation of these gene clusters should lead to the isolation of novel bioactive compounds.[4,5]

It was previously reported that several kinds of mycolic acid-containing bacteria are capable of inducing the production of secondary metabolites in a broad range of *Streptomyces* strains by coculture.[6] For example, a mycolic acid-containing bacteria *Tsukamurella pulmonis* induced the new secondary metabolites production in 36.6% of *Streptomyces* strains.[6] Indeed, the use of this methodology has resulted in the isolation of novel bioactive compounds, such as the antimicrobial polycyclic polyketide alchivemycin A from *Streptomyces endus* S-522[6,7] and the cytotoxic indolocarbazole alkaloid arcyriaflavin E from *Streptomyces cinnamoneus* NBRC 13823.[8]

Thus, this "combined-culture" fermentation method is expected to be a simple and powerful tool for the discovery of cryptic natural products. However, its usage is still limited to a small number of *Streptomyces* strains, and further applications are required to demonstrate the feasibility of this attractive method.

In this study, we applied the combined culture method to our collections of soil-derived *Streptomyces* strains. As the mycolic acid-containing bacterium, we employed *Tsukamurella pulmonis* TP-B0596, as in the previous reports.[6,8] Consequently, the production of the secondary metabolites in one of the strains, *Streptomyces* sp. CJ-5, was significantly enhanced, leading to the identification of the novel butanolide chojalactones A−C (**1**−**3**), containing unusual γ-butyrolactone scaffolds. Here, we report the structural elucidations of **1**−**3**, based on spectroscopic analyses and chemical syntheses of all stereoisomers of **1** and **2**.

Streptomyces sp. CJ-5 was cultured with *T. pulmonis* TP-B0596 in A-3 M medium (15 × 100 mL), for 6 days at 30 °C. The cell pellet was extracted with 1:1 methanol/chloroform (800 mL), and the crude extract (0.73 g) was separated by open column silica gel chromatography and semipreparative reversed-phase HPLC. As a result, we obtained chojalactone A (**1**, 0.6 mg) and an inseparable mixture of chojalactones B and C (**2** and **3**, 1.5 mg) (Figure 1). ^{1}H NMR spectra of the mixture (Figure S6) indicated that the ratio of **2** and **3** was 5:2.

The molecular formula of chojalactone A (**1**) was established as $C_{13}H_{16}O_4$ by time-of-flight high-resolution mass spectrometry (TOF-HRMS) (m/z found: 237.1113 $[M + H]^+$ calculated: 237.1127), indicating 6 degrees of unsaturation (Figure S18). ^{1}H NMR, ^{13}C NMR, and HMQC spectra revealed the presence of one hydroxyl group at δ_H 4.32, two methyl groups at δ_H/δ_C 1.03/9.8 and 1.86/18.9 (H-5/C-5 and H-8′/C-8′), one sp^3 methine group at δ_H/δ_C 2.84/39.1 (H-3/C-3), one sp^3 oxymethylene group at δ_H/δ_C 4.08 and 4.54/72.1 (H-4a and 4b/C-4), one sp^3 quaternary carbon attached with

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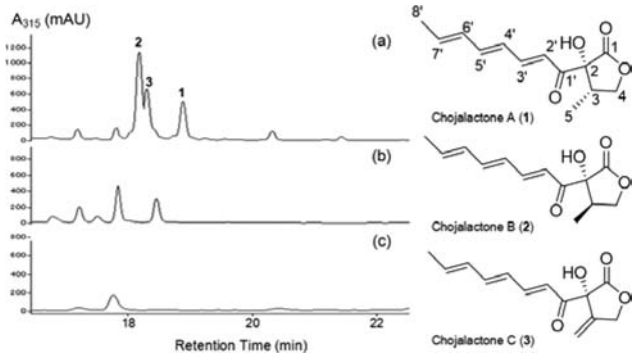

Figure 1. HPLC profiles of 1:1 methanol/chloroform extracts of (a) *Streptomyces* sp. CJ-5 with *T. pulmonis*, (b) *Streptomyces* sp. CJ-5 only, and (c) *T. pulmonis* only, monitored by UV absorption at 315 nm, and structures of chojalactones A−C (**1**−**3**).

oxygen at δ_C 81.5 (C-2), six olefinic methine groups at δ_H/δ_C 6.32/120.0, 7.54/147.9, 6.23/127.7, 6.70/145.7, 6.20/131.4, and 6.06/138.2 (H-2′/C-2′ to H-7′/C-7′), one ester group at δ_C 174.2 (C-1), and one α,β-unsaturated ketone group at δ_C 194.1 (C-1′) (Table S1). The 2,4,6-octa-triene-1-one moiety (C-1′-8′, Figure 1) was established based on the ^{1}H−^{1}H COSY cross peaks through H-2′ to H-8′ (Figure 2), the HMBC

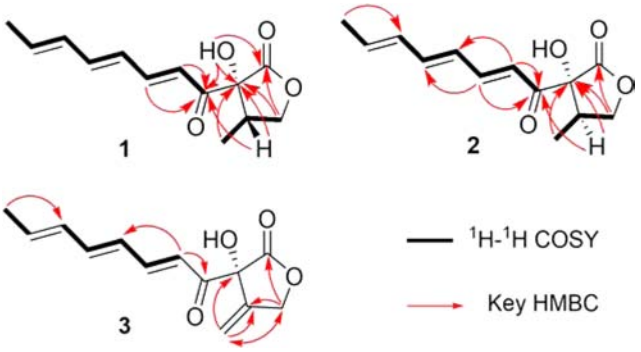

Figure 2. Key ^{1}H−^{1}H COSY and HMBC correlations of **1**−**3**.

correlations of H-3′/C-1′ and H-2′/C-1′ (Figure 2), the downfield proton chemical shift of H-3′ (δ_H 7.54), and the characteristic UV absorption at 337 nm. All *E* configurations of the triene from C-2′ to C-7′ were deduced based on the large $^3J_{HH}$ coupling constants ($^3J_{H2'H3'}$ = 15.0 Hz, $^3J_{H4'H5'}$ = 15.0 Hz, $^3J_{H6'H7'}$ = 15.5 Hz, Table S1). The 2-hydroxy-3-methyl-γ-butyrolactone moiety (C-1−5) was inferred from the ^{1}H−^{1}H COSY cross peaks of H-3/H-4a, H-3/H-4b, and H-3/H-5 (Figure 2) and the HMBC correlations of OH/C-1, OH/C-2, H-3/C-2, H-4a/C-1, H-4a/C-2, H-4b/C-1, H-4b/C-2, and H-5/C-2 (Figure 2). Finally, C-2 and C-1′ were connected on the basis of the HMBC correlations of OH/C-1′ and H-3/C-1′ (Figure 2). Therefore, the planar structure of chojalactone A was determined to be **1**. As noted above, the structural elucidations of chojalactones B (**2**, major component) and C (**3**, minor component) were performed with the mixture. The molecular formulas of **2** and **3** were established as $C_{13}H_{16}O_4$, which is the same molecular formula as **1** (*m*/*z* found: 237.1113 $[M + H]^+$; calculated: 237.1127) and $C_{13}H_{14}O_4$, lacking one H_2 from **1** (or **2**) (*m*/*z* found: 235.0974 $[M + H]^+$; calculated: 235.0970), respectively, based on the TOF-HRMS spectra of the mixture and the intensities of each ion peak (Figure S19).

Both compounds also showed UV absorption at 337 nm, suggesting that a 2*E*,4*E*,6*E*-octa-triene-1-one moiety (C-1′-8′) also exists in both **2** and **3**. In fact, 1D and 2D NMR data indicated the presence of two kinds of 2*E*,4*E*,6*E*-octa-triene-1-one substructures (Figure 2 and Tables S2 and S3). In addition, the ^{1}H NMR, ^{13}C NMR, and HMQC spectra of the mixture indicated the presence of 10 carbon resonances, one methyl group at δ_H/δ_C 1.01/10.7, one sp^3 methine group at δ_H/δ_C 2.80/42.6, two sp^3 oxymethylene groups at δ_H/δ_C 4.03, 4.48/71.0, and 5.06/70.3, two sp^3 quaternary carbons attached with oxygen at δ_C 80.7 and 83.7, one olefinic methylene group at δ_H/δ_C 5.39, 5.43/113.8, one olefinic quaternary carbon at δ_C 140.9, and two ester groups at δ_C 172.9 and 175.8. Interpretation of the ^{1}H−^{1}H COSY and HMBC spectrum suggested the presence of two different γ-butyrolactone moieties, which are 2-hydroxy-3-methyl-γ-butyrolactone in **2** and 2-hydroxy-3-methylene γ-butyrolactone in **3** (Figure 2 and Tables S2 and S3). The HMBC correlation between δ_H 2.80 (H-3 of **2**) and δ_C 195.0 (C-1′ of **2**) supported the connection between one side of the 2*E*,4*E*,6*E*-octa-triene-1-one moiety and the 2-hydroxy-3-methyl-γ-butyrolactone ring (Figure 2), and thus the planar structure of chojalactone B was determined to be **2**. Therefore, the other side of the 2*E*,4*E*,6*E*-octa-triene-1-one moiety resulted in a connection with the remaining 2-hydroxy-3-methylene γ-butyrolactone ring between the C-2 and C-1′ positions to satisfy the molecular formula of **3**, and the planar structure of chojalactone C was determined to be **3**.

In previous research for the stereochemical assignment of ithomiolides, pheromones produced by male ithomiinae butterflies, Schulz reported two synthetic analogues (**4a** and **4b**, Figure 3),[9] differing only in the kinds of acyl chains of **1** and

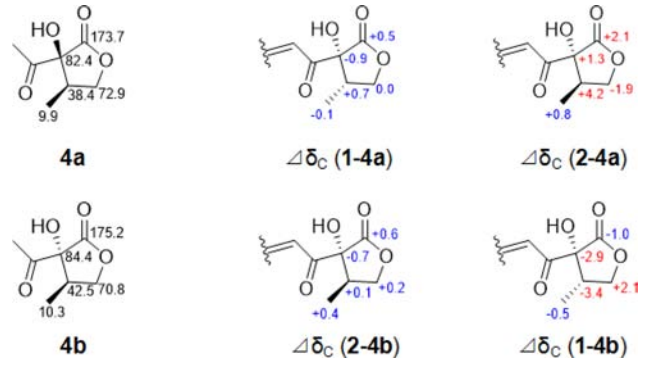

Figure 3. Structures and δ_C (in ppm) of **4a** and **4b**, and the $\Delta\delta_C$ values (in ppm, **1**-**4a**, **2**-**4a**, **1**-**4b**, and **2**-**4b**). The $\Delta\delta_C$ values exceeding ±1.0 ppm are depicted in red, and the others are depicted in blue.

2. Thus, we determined the relative configurations at the C-2 and C-3 positions of **1** and **2**, by comparing the ^{13}C chemical shifts of their γ-butyrolactone moieties (C-1−5) with those of **4a** and **4b**. The values of $\Delta\delta_C$ (**1**-**4a**) and $\Delta\delta_C$ (**1**-**4b**) strongly suggested that **1** has a (2*R*, 3*S*) or (2*S*, 3*R*) configuration; all values of $\Delta\delta_C$ (**1**-**4a**) are in the range of ±1.0 ppm, whereas the values of $\Delta\delta_C$ (**1**-**4b**) exceed the range of ±1.0 in C-2, C-3, and C-4 (Figure 3). In contrast, **2** was assumed to have a (2*R*, 3*R*) or (2*S*, 3*S*) configuration; all values of $\Delta\delta_C$ (**2**-**4b**) are in the range of ±1.0 ppm, and the values of $\Delta\delta_C$ (**2**-**4a**) exceed the range of ±1.0 in C-1, C-2, C-3, and C-4 (Figure 3). Therefore, the relative configurations of chojalactones A and B were determined to be **1** and **2**, as shown in Figure 1.

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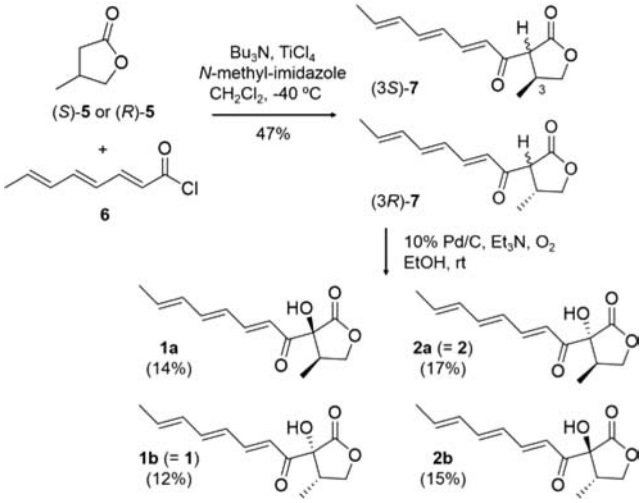
To determine the absolute configurations, we conducted the total syntheses of chojalactones A (**1**) and B (**2**), together with all of their possible stereoisomers. We planned to synthesize all diastereomeric isomers of chojalactones A (**1a** and **1b**) and B (**2a** and **2b**) by the direct α-oxidation of (3*R*)-**7** or (3*S*)-**7**, and both stereoisomers of **7** could be prepared from optically pure γ-butyrolactone **5** and acid chloride **6** by Claisen condensation (Scheme 1). First, Claisen condensation between **5** and **6** was

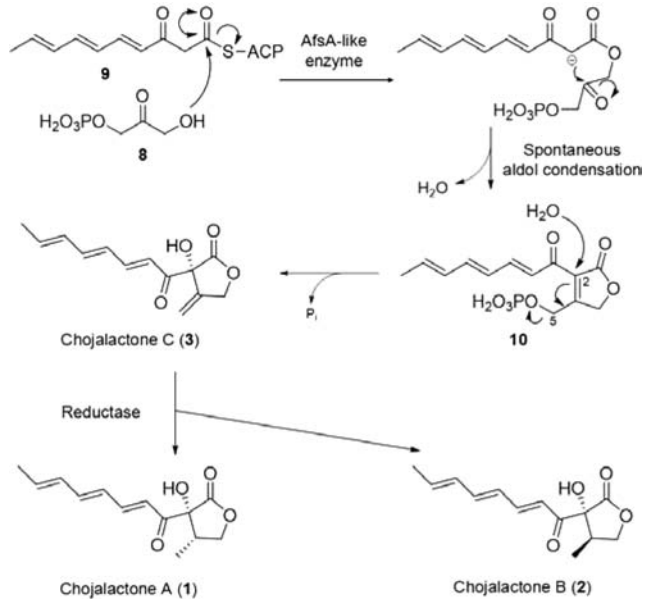
Scheme 1. Synthesis of All Diastereomeric Isomers of Chojalactones A (1a and 1b) and B (2a and 2b)



performed using the Ti-crossed Claisen condensation method developed by Tanabe's group,[10] employing *N*-methyl-imidazole as a key catalyst, and the reaction proceeded under mildly basic conditions (Bu_3N, −40 °C) to yield the desired β-ketolactone **7** (47%). As for the subsequent oxidation step, Sajiki's group previously reported that Pd/C catalyzes direct α-oxidation for a variety of 1,3-dicarbonyl compounds under basic conditions, using molecular oxygen as an oxygen source.[11] Taking advantage of this facile methodology, the direct α-oxidation of **7** was conducted under an O_2 atmosphere, using Et_3N as the base at room temperature. Consequently, we obtained **1a** and **2a** from (3*S*)-**7** (**1a** 14%, **2a** 17%), and **1b** and **2b** were obtained from (3*R*)-**7** (**1b** 12%, **2b** 15%), which suggested that this oxidation process is not stereoselective. Next, chiral-phase HPLC analyses on a Chiralpak AS-RH column for the synthetic standards (**1a**, **1b**, **2a**, and **2b**) and natural chojalactones (**1**−**3**) were performed to elucidate the absolute configurations. All compounds were monitored by UV absorption at 223 nm, and **1a**, **1b**, **2a**, and **2b** were detected at 23.5, 22.1, 18.8, and 20.6 min, respectively (Figure S20), whereas the natural compounds **1**−**3** were detected at 22.1, 18.9, and 17.9 min respectively (Figure S20). These results revealed that chojalactones A (**1**) and B (**2**) are identical to **1b** and **2a**, respectively. Therefore, the absolute configurations of **1** and **2** were determined to be (2*R*, 3*S*) and (2*R*, 3*R*), as shown in Figure 1. The absolute configuration of **3** was predicted to be 2*R*, as discussed below.

Chojalactones A−C (**1**−**3**), containing 2,3-disubstituted γ-butyrolactone skeletons, resemble a well-studied group of signaling molecules, such as A-factor, produced by various *Streptomyces* strains.[12,13] It was assumed that the lactone rings in these signaling molecules are constructed through enzymatic condensation between dihydroxyacetone phosphate (**8**) and the variety of β-ketoacyl-ACPs catalyzed by AfsA or its orthologues,[14,15] and subsequent spontaneous intermolecular aldol condensation (Scheme 2).[14] In accordance with these

Scheme 2. Plausible Biosynthetic Pathway of Chojalactones A−C (1−3)



findings, the biosynthetic pathway for **1**−**3** was proposed, as shown in Scheme 2. Thus, chojalactone C (**3**) would be generated from **8** and β-ketoacyl-ACP **9** by an AfsA orthologue-catalyzed lactone ring formation, followed by water addition coupled with dephosphorylation. Presumably, **3** is then converted into both chojalactones A (**1**) and B (**2**) by reduction of the exomethylene moiety. Considering that the C-2 configurations of **1** and **2** are retained, it is likely that the addition of water to **10** should be an enzymatic process. Therefore, the C-2 configuration of **3** should also be 2*R*.

The bioactivities of all synthetic chojalactone isomers (**1a**, **1b**, **2a**, and **2b**) were examined, and all compounds showed moderate cytotoxicity against P388 murine leukemia cells. The IC_{50} values of **1a**, **1b**, **2a**, and **2b** were determined to be 37, 28, 18, and 17 μM respectively. In contrast, no antimicrobial activities were observed against *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans* for all compounds. Owing to their structural resemblance to A-factor, the roles of the chojalactones as signaling molecules can be envisaged and are currently under investigation.

In this study, we illustrated that the combined-culture method, the coculturing of a *Streptomyces* strain with a mycolic acid containing bacterium (*T. pulmonis*), led to the isolations of the novel cytotoxic butanolide chojalactones A−C (**1**−**3**), with unusual 2-hydroxyl-2-acyl-3-methyl (**1** and **2**) and 2-hydroxyl-2-acyl-3-methylene (**3**) γ-butyrolactone scaffolds. Our results further support the efficacy of the combined-culture method for the discovery of cryptic natural products. In addition, we also succeeded in the total syntheses of **1** and **2**, together with their stereoisomers in the course of the stereochemical assignments of **1**−**3**, using Ti-crossed Claisen condensation[10] and Pd/C-catalyzed direct α-oxidation by molecular oxygen.[11]

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, spectroscopic data of **1**−**3** and **7** and chromatograms of chiral-phase HPLC are described. 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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