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Rhodopyran, a carboxylated hexahydrocyclopenta[b]pyran from *Rhodococcus*

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Abstract

A new bicyclic metabolite, rhodopyran (**1**), was isolated from the culture broth of *Rhodococcus* sp. strain RD066637. The planar structure was elucidated by 1D/2D NMR analyses and HRMS spectrometry, and the relative configuration was assigned from vicinal coupling constants and NOESY correlations, while the absolute configuration remains undetermined. Rhodopyran represents a structurally unusual carboxylated hexahydrocyclopenta[b]pyran and expands the known metabolite diversity of *Rhodococcus*, highlighting this underexplored genus as a source of uncommon natural-product scaffolds.

Keywords

bicyclic compound; cyclopenta[b]pyran; natural products; *Rhodococcus*; secondary metabolites

Introduction

Actinomycetes have long been recognized as prolific producers of structurally diverse secondary metabolites, with *Streptomyces* historically serving as the major source of chemically characterized compounds. In contrast, non-streptomycete actinomycetes remain comparatively underexplored, and the genus *Rhodococcus* is one such promising but less extensively investigated source. Genome analyses of *Rhodococcus* have revealed diverse biosynthetic gene clusters, including nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and terpene-related pathways [1,2]. Comparative genomic and taxogenomic studies have also highlighted the genomic variability of this genus and its mosaic genomic architecture [3,4]. In addition, antibiotic biosynthesis following horizontal gene transfer from *Streptomyces* to *Rhodococcus* has been demonstrated for rhodostreptomycins [5].

Chemically characterized *Rhodococcus* metabolites include rhodostreptomycins [5], a quinoline antibiotic [6], rhodopeptins [7], lariatins [8], rhodochelin [9], and the pluramycin-class metabolite rausuquinone [10]. These examples illustrate the chemical diversity of the genus, but the metabolite space of *Rhodococcus* remains far less explored than that of *Streptomyces*. In our continuing cultivation-based survey of *Rhodococcus* metabolites, strain RD066637 was found to produce a new cyclopenta[b]pyran metabolite, rhodopyran (**1**) (Figure 1). Herein, we describe the isolation, structure elucidation, relative configuration, and structural uniqueness of **1**.

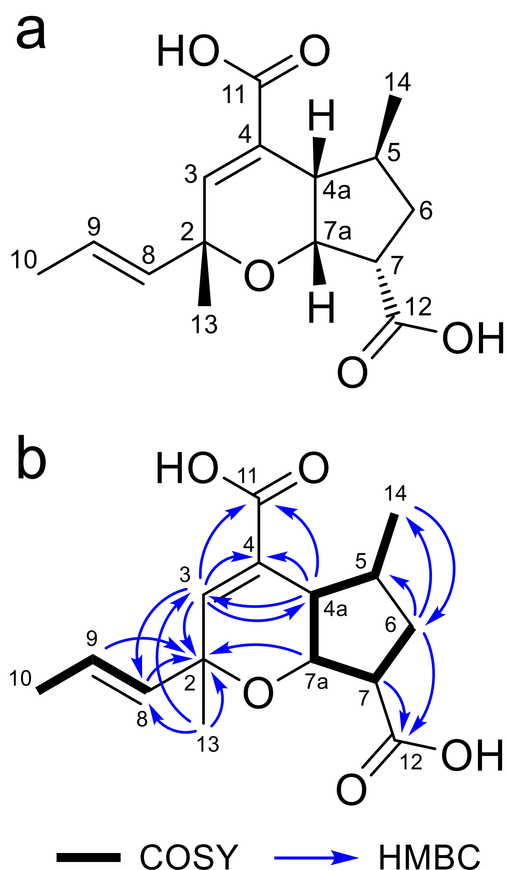

Figure 1: Structure (relative configuration) and 2D NMR analysis for rhodopyran (**1**). a Planar structure. b COSY and key HMBC correlations.

Results and Discussion

Strain RD066637 was precultured in ISP 2 medium at 30 °C and then transferred to A3M production medium (30 °C, 5 days). The whole culture was extracted with 1-butanol. The concentrate was fractionated by silica gel column chromatography, and preparative HPLC afforded rhodopyran (**1**, 22.7 mg from 2 L of culture).

Compound **1** was obtained as an optically active colorless powder ($[\alpha]_D^{25}$ −69, (*c* 0.01, MeOH)). HRESIQTOFMS showed *m/z* 279.1237 $[M–H]^-$, consistent with the molecular formula $C_{15}H_{20}O_5$. The IR spectrum showed a strong band at 1688 cm^{-1}, consistent with carboxyl carbonyl groups. The ^1H NMR spectrum displayed a deshielded olefinic singlet at δ_H 6.72; a pair of coupled olefinic protons at δ_H 5.73 and 5.48; an oxygenated methine at δ_H 4.36; a deshielded methine at δ_H 3.16; methine signals at δ_H 2.36 and 1.97; methylene resonances at δ_H 2.53 and 1.44; and three methyl groups at δ_H 1.67, 1.27, and 1.11. The ^{13}C NMR spectrum exhibited 14 signals, including two carbonyl carbons (δ_C 176.1, 170.6), four olefinic carbons (δ_C 142.9, 135.5, 130.2, 125.0), an oxygenated quaternary carbon (δ_C 75.6), an oxygenated methine (δ_C 77.2), and three methyl carbons (δ_C 24.3, 21.1, 17.9) (Table 1). The remaining carbon resonance (C-7, δ_C 49.4) was obscured by overlapping with the solvent signal in the ^{13}C NMR spectrum (Figure S2); however, its presence was confirmed by HSQC and HMBC correlations, as described below (Figures S2, S4, and S5).

Table 1: NMR spectroscopic data for rhodopyran (**1**) in CD_3OD.

position	δ_C[a], type	δ_H, mult (*J* in Hz)[b]	HMBC[b,c]
2	75.6, C		
3	142.9, CH	6.72, s	2, 4, 4a, 8, 11
4	130.2, C		
4a	47.0, CH	2.36, dd (8.6, 3.2)	3, 4, 11, 14
5	40.4, CH	1.97, m	
6	35.3, CH_2	2.53, ddd (13.0, 9.1, 9.1): α	4a, 5, 7, 7a, 12, 14
		1.44, ddd (13.0, 9.6, 6.2): β	4a, 5, 7, 7a, 12, 14
7	49.4, CH	3.16, ddd (9.6, 9.1, 4.6)	6, 7a, 12
7a	77.2, CH	4.36, dd (4.6, 3.2)	2, 4a, 5, 6
8	135.5, CH	5.48, dd (15.4, 1.7)	2, 3, 10
9	125.0, CH	5.73, dq (15.4, 6.6)	2, 3, 10
10	17.9, CH_3	1.67, dd (6.6, 1.7)	8, 9
11	170.6, C		
12	176.1, C		
13	24.3, CH_3	1.27, s	2, 3, 8
14	21.1, CH_3	1.11, d (6.9)	4a, 5, 6

[a] Recorded at 125 MHz (reference δ_C 49.0).
[b] Recorded at 500 MHz (reference δ_H 3.31).
[c] HMBC correlations are from proton(s) stated to the indicated carbon.

COSY and HSQC data defined two main partial structures: a 1-propenyl moiety (H-8/H-9/H_3-10) with a large *trans* vicinal coupling (J = 15.4 Hz), consistent with an *E* configuration across C-8/C-9, and an aliphatic spin system (H-4a/H-5/H_2-6/H-7/H-7a) bearing methyl group (Me-14) at C-5 (Fig. 1). Key HMBC correlations established the connectivity of these fragments and located the two carboxyl groups. Correlations from H-8 and H-9 to C-2 and C-3, together with the correlation from H_3-13 to C-2/C-3/C-8, supported the connectivity of the 1-propenyl substituent and Me-13 at C-2. A carboxyl group at C-4 was assigned by correlations from H-3 and H-4a to the carbonyl carbon C-11, and by correlations from H-3 and H-4a to C-4. Likewise, correlations from H-6α/H-6β and H-7 to C-12 positioned another carboxyl group at C-7. The linkage from C-5 to C-6 and the placement of Me-14 at C-5 were corroborated by correlations from H-6α/H-6β to C-5 and from H_3-14 to C-6. In addition, reciprocal correlations between H-3 and C-4a and between H-4a and C-3, as well as the correlation from H-7a to C-2, supported the fusion of the cyclopentane and pyran rings, completing the planar structure of **1**.

The relative configuration of **1** was determined by analysis of vicinal coupling constants around the bicyclic junction (H-4a, H-5, H-7a, H-7) and NOESY correlations (Fig. 2). The large $^3J_{H\text{-}4a,H\text{-}5}$ = 8.6 Hz supports an antiperiplanar alignment, whereas the small $^3J_{H\text{-}4a,H\text{-}7a}$ = 3.2 Hz is consistent with a gauche relationship. In addition, H-7a showed a moderate coupling to H-7 (J = 4.6 Hz), consistent with a gauche relationship. At C-6, H-6α showed two relatively large couplings to H-5 and H-7 ($J_{5,6\alpha}$ = 9.1 Hz and $J_{6\alpha,7}$ = 9.1 Hz), whereas H-6β exhibited a moderate coupling to H-5 (J = 6.2 Hz) and a large coupling to H-7 (J = 9.6 Hz). The pattern of vicinal couplings observed for the C-6 methylene protons is consistent with precedents for cyclopentane-containing natural products and conformationally restricted cyclopentane-based systems, in which diastereotopic methylene protons have been reported to exhibit multiple relatively large vicinal couplings, commonly attributed to ring puckering and conformational restriction [11–15]. In the NOESY spectrum, correlations among H-4a/H-7/H-7a and between H-7a and Me-13, indicated that these protons are located on the same face of the fused bicyclic framework. Diagnostic NOESY correlations further differentiated the C-6 protons: H-6β correlated with H-7, while H-6α correlated with H-5. An additional correlation between Me-14 and H-4a (and H-6β) supported the location of Me-14 on the same face. Thus, the relative configuration of rhodopyran (**1**) was established as shown in Fig. 2. Attempts to determine the absolute configuration by ECD analysis and single-crystal X-ray diffraction were unsuccessful because the ECD spectrum was not sufficiently diagnostic and crystals suitable for diffraction could not be obtained; therefore, the absolute configuration remains unassigned.

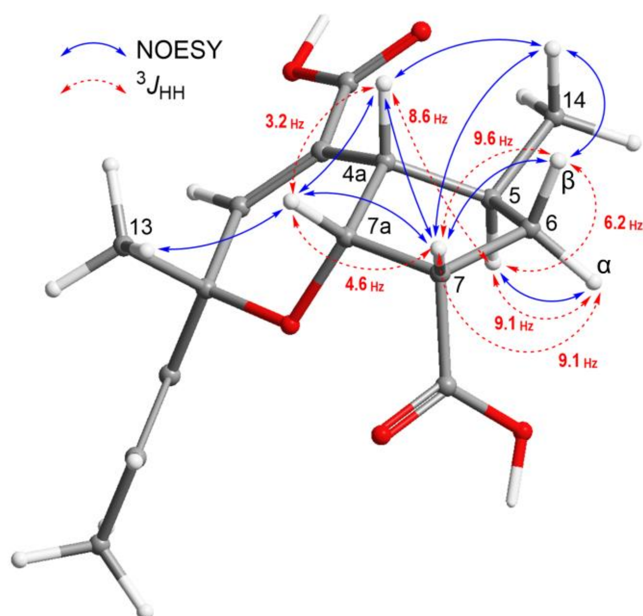

Figure 2: Selected vicinal $^{3}J_{HH}$ values and key NOESY correlations used to assign the relative configuration of **1**.

Compound **1** showed no measurable antioxidant activity in DPPH and superoxide radical-scavenging assays, no cytotoxicity toward P388 murine leukemia cells, and no antimicrobial activity against Gram-positive bacteria (*Kocuria rhizophila*, *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Ralstonia solanacearum*, *Rhizobium radiobacter*), or the yeast *Candida albicans*. Experimental details and full spectra are provided in the Supporting Information.

To evaluate the structural uniqueness of compound **1**, we performed a structure-based similarity search in CAS SciFinder^{n}. The substructure search returned no matches (0 hits). We then conducted a similarity search; the highest similarity score obtained was 77 out of 100, and among compounds bearing a cyclopenta[b]pyran framework the top score was only 68, supporting the lack of close structural analogues. To the best of our knowledge, more than 400 natural products containing a cyclopenta[c]pyran skeleton—predominantly iridoids—have been reported [14], whereas natural products featuring a cyclopenta[b]pyran skeleton are exceedingly rare [16–21]. Moreover, the few reported examples differ substantially from compound **1** in that they are more highly oxidized (e.g., ketone-containing derivatives) and possess distinct architectures such as spirocyclic motifs or three-membered rings. Collectively, these observations suggest that the natural product chemical space of this structural class remains largely unexplored, and underscore that pursuing genome mining in parallel with cultivation-based screening of underexplored microbial taxa can directly contribute to expanding structural diversity.

Conclusion

In summary, rhodopyran (**1**) is a structurally distinctive carboxylated hexahydrocyclopenta[b]pyran isolated from *Rhodococcus* sp. RD066637. Its planar structure and relative configuration were established by HRMS and detailed NMR analysis, whereas the absolute configuration remains

unassigned. This bicyclic scaffold expands the structural diversity of natural products and underscores the importance of *Rhodococcus* as a source of uncommon chemical architectures.

Experimental

General experimental procedures

Optical rotations were measured using a DIP-3000 polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a UV-1900 spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured by a spectrum 100 spectrometer (PerkinElmer, MA, USA). NMR spectra were obtained on AVANCE NEO 500 spectrometer (Bruker, MA, USA) in CD_3OD and referenced to the residual solvent signals (δ_H 3.31, δ_C 49.0). HRESITOFMS spectra were recorded on a compact QTOF mass spectrometer (Bruker). HPLC separations were performed using a COSMOSIL $5C_{18}$-AR-II Packed Column (10 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan).

Microorganism

The strain RD066637 was obtained from Biological Resource Center, National Institute of Technology and Evaluation, Japan. The RD strain refers to a collection of strains from various domestic and international sources provided by NBRC for screening purposes. The 16S rRNA gene sequence of the strain showed 99.3% homology with the closely related *Rhodococcus cerastii* $C5^T$ (FR714842), leading to its identification as belonging to the genus *Rhodococcus* (1466 nucleotides; DDBJ accession number LC688269).

Fermentation

Strain RD066637 growing on an ISP 2 agar medium consisting of 0.4% yeast extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), 1.0% malt extract (Becton Dickinson, NJ, USA), 0.4% glucose (pH 7.2) was inoculated into 500 mL K-1 flasks (custom-designed cylindrical flasks, cylindrical section: 75 mm inner diameter × 80 mm height; opening: 25 mm inner diameter) each containing 100 mL of the ISP 2 seed medium. The flasks were placed on a rotary shaker (200 rpm) at 30 °C for 2 days. Then, the seed culture (3 mL) was transferred into 500 mL K-1 flasks, each containing 100 mL of the A-3M production medium consisting of 2.0% soluble starch, 2.0% glycerol, 0.5% glucose, 1.5% Pharmamedia (Archer-Daniels-Midland Company, TX, USA), 0.3% yeast extract, and Diaion 1.0% HP-20 resin (for improvement of secondary metabolites productivity, Mitsubishi Chemical, Tokyo, Japan) in distilled water. The pH of the medium was adjusted to 7.0 before sterilization. All the media were sterilized by autoclaving at 121 °C for 20 min. The inoculated 20 flasks were placed on a rotary shaker (200 rpm) at 30 °C for 5 days.

Extraction and Isolation

After incubation, 100 mL of 1-BuOH was added to each flask, and the flasks were allowed to shake for 1 h. The mixture was centrifuged at 6,000 rpm for 10 min, and the organic layer was separated from the aqueous layer containing the mycelium. The BuOH layer was evaporated to give 12.6 g of extract from 2 L of culture. The extract was fractionated using silica gel column chromatography with a step gradient of $CHCl_3$–MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). The fraction 4:1

containing **1** was concentrated to give 1.2 g of yellowish black solid, which was subjected to preparative HPLC using isocratic condition of 30% of MeCN/0.1% HCO_2H solution at 4 mL/min, yielding rhodopyran (**1**, 22.7 mg) with a retention time of 11.2 min.

Rhodopyran (**1**): colorless amorphous powder; $[\alpha]^{25}_{D}$ −69 (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log *ε*) 217 nm (3.93); IR (ATR) ν_{max} 2965, 2928, 1688, 1640, 1421 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1 and Supporting Information; HRESITOFMS *m/z* 279.1237 $[M - H]^{-}$ (calcd for $C_{15}H_{19}O_5$, 279.1238).

Biological assays

Antimicrobial and cytotoxicity assays were carried out according to the method described previously [22]. Superoxide dismutase activity was determined by SOD Assay Kit - WST (Dojindo Molecular Technologies, Inc. Kumamoto, Japan). DPPH radical scavenging activity was determined by DPPH Antioxidant Assay Kit (Dojindo Molecular Technologies, Inc.). All experiments were performed in triplicate.

Supporting Information

Supporting Information File 1
File name: SI
File format: PDF
Title: NMR and HRMS spectra for compound **1**.

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