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NOTE

Cystodienoic acid: a new diterpene isolated from the myxobacterium *Cystobacter* sp.

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DESCRIPTION

Myxobacteria are Gram-negative bacteria that are sources of a large number of compounds with unique structures that possess varying biological activities. They are known to produce secondary metabolites that have more novel structures with those produced by other microbes such as, actinomycetes and fungi. In our search for novel secondary metabolites, *Cystobacter ferrugineus* strain Cbfe23, which was isolated from a soil sample in China, garnered our attention based on our in-house LC–MS analysis. Specifically, a peak identified with an *m/z* of 352 did not produce any hits in our mass spectral library of compounds, prompting us to further investigate the strain in detail in order to determine the structural profile of the unknown metabolite.

Isolation and fermentation of strain Cbfe23

Strain Cbfe23 was isolated from a soil sample collected in 1982 in China according to the methods of Reichenbach and Dworkin.[1] The strain was deposited at the German Collection of Microorganisms (DSM) in Braunschweig (DSM 52764).

The fermentation of Cbfe23 was conducted in a 100-l fermenter with 70 liters of medium E (skimmed milk 0.4%, soy flour defatted 0.4%, yeast extract 0.2%, starch 1.0%, $MgSO_4{\cdot}7H_2O$ 0.1%, Fe-EDTA 8 mg l^{-1}, glycerin 0.5%) for 96 h at 30 °C. The pH was adjusted to 7.4 with potassium hydroxide (5.0%) and sulfuric acid (5.0%). The broth was stirred at 100 rpm and aerated with 0.05% v.v.m. by submersed fumigation for the first 20 h and then increased to 0.1% v.v.m. until the end of the process. The dissolved oxygen content within the fermentation broth was regulated by the stirrer speed to 20% pO_2. Adsorber resin (3%) was added to the fermentation broth to bind the cystodienoic acid. The fermenter was inoculated with 3 l of a 3-day old preculture (same medium plus 50 mM HEPES).

Isolation of cystodienoic acid

The XAD resin was filtered from the fermented broth and washed twice with distilled water followed by extraction with methanol (4 l × 2). The MeOH extract was then dried *in vacuo*, which yielded a crude extract of (3.6 g). A portion of this crude extract (1 g) was then partitioned between 250 ml of hexane, CH_2Cl_2 and MeOH to give 157.9, 457.9 and 352.5 mg fractions, respectively. The MeOH fraction was subsequently purified by semipreparative reverse phase HPLC to yield the UV absorbing compound **1** (λ_{max} 230 nm, $t_R = 20.7$ min; 8.2 mg).

A detailed account of the spectroscopic analysis leading to the identification of cystodienoic acid (**1**) is presented below.

HRESI(+)MS analysis of **1** revealed a pseudomolecular ion ($[M+H]^+$) indicative of a molecular formula ($C_{20}H_{32}O_5$) with five double bond equivalents (DBEs). A detailed examination of the NMR (methanol-d_4) data (Table 1; see Supplementary Figures S1–S3) revealed four sp^2 carbon resonances (δ_C 116.4–162.1) and two carbonyls (δ_C 170.1/171.5), accounting for four DBEs with the requirement that **1** be monocyclic. COSY correlations suggested two isolated spin systems, with the first system between methylene H_2-4 (δ_H 2.38, 2.21) and the olefinic methine H-3 (δ_H 6.92). The second system was suggested to start between methylene H_2-6 (δ_H 1.40, 1.48) and a second methylene, H_2-7 (δ_H 1.47, 1.76), across two methines, H-8 (δ_H 1.79) and H-9 (δ_H 1.60) and to terminate at two sets of methylenes, H_2-10 (δ_H 1.59–1.74) and H-11 (δ_H 2.29), which thereby led to the sequence from C-6 to C-11 (Figure 1). Owing to an overlap of correlations in the COSY spectrum for the second isolated spin system, HMBC correlations were used to further corroborate this sequence. HMBC correlations from the olefinic methyl H_3-15 (δ_H 1.83) to the carbonyl C-1 (δ_C 171.5), quaternary carbon C-2 (δ_C 129.8) and C-3 (δ_C 141.3) led to the extension of the first isolated spin system. Similarly, HMBC correlations from the second olefinic methyl H_3-19 (δ_C 2.15) to C-11 (δ_C 42.0), C-12 (δ_C 162.1), C-13 (δ_C 116.4) and C-14 (δ_C 170.1) extended the second isolated spin system. The connectivity of the two extended isolated spin systems was established by reasonable HMBC correlations from H_2-4 to C-5 (δ_C 46.9), C-6 (δ_C 40.4) and C-20 (δ_C 21.4) through a quaternary carbon C-5 flanked by the pendant methyl H_3-20 (δ_H 0.97), which also showed correlations to C-4,C-5, C-6 and C-9. Further HMBC correlations from the methylene H_2-4 to C-9 and from H-9 to C-4 suggested the presence of a cyclopentane unit (C-5 to C-9; Figure 1). Moreover, HMBC correlations from the tertiary methyls H_3-17 (δ_H 1.20/δ_C 29.5) and H_3-18 (δ_H 1.16/δ_C 26.4) to one another and

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Table 1 NMR (500 MHz, CD_3OD) data for (1)

Position	δ_H, *mult (J in Hz) (**1**)*	δ_C[a]	*COSY*	*HMBC*	*ROESY*
1		171.5			
2		129.8			
3	6.92, *t* (*7.8*)	141.4	4a/b	1, 2, 4, 5, 15	20
4a	2.38, dd (*14.6, 7.8*)	41.9	3	1, 2, 3, 5, 6, 9, 20	
4b	2.21, dd (*14.6*, *7.8*)		3	1, 2, 3, 5, 6, 9, 20	
5		46.9			
6a	1.48[b], m	40.4	6b, 7a	5, 7, 8, 9, 20	
6b	1.40, m		6a, 7a/b	5, 7, 8, 9, 20	
7a	1.76, m	26.7	6a/b, 7b	8, 16	
7b	1.47[b], m		6b, 7a, 8	8, 16	
8	1.79, m	57.3	7b, 9	6	
9	1.60, m	49.4	8, 10	4, 5, 8, 10, 16, 20	
10	1.59–1.74, m	32.3	8, 11		13
11	2.29, m	42	10	9, 10, 12, 13, 19	
12		162.1			
13	5.67, s	116.4		11, 12, 14, 19	13
14		170.1			
15	1.83, s	12.8		1, 2, 3	
16		73.9			
17	1.20, s	29.5		8, 16, 18	
18	1.16, s	26.4		8, 16, 17	
19	2.15, s	19.1		11, 12, 13, 14	
20	0.97, s	21.4		4, 5, 6, 9	3

[a]Assignments supported by HSQC and HMBC experiments.
[b]Overlapping signals.

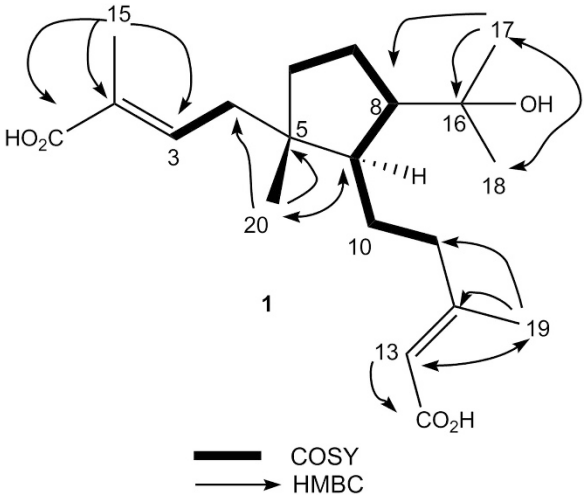

Figure 1 Key 2D NMR correlations for **1**.

to the oxy quaternary carbons C-16 (δ_C 73.9) and C-8 (δ_C 57.3) confirmed the attachment of a 2-hydroxypropan-2-yl residue to position C-8. Having taken into consideration the DBEs, the carbonyls were identified as a dicarboxylic acid moiety. ROESY correlations between H-9 and H-3 showed that they were on the same plane suggesting that the tertiary methyl H_3-20 and H-9 were in a *trans* orientation, whereas ROESY correlations between H-13 and H_2-10 and the ^{13}C chemical shifts for Me-15 and Me-19 indicated that the double bonds had an *E* configuration.

Cystodienoic acid (**1**) displayed no significant antimicrobial activity. However, it did display cytotoxic activity against HCT-116 cancer cells with a GI_{50} of 0.47 μg ml^{-1} (1.33 μM). We next carried out chemical

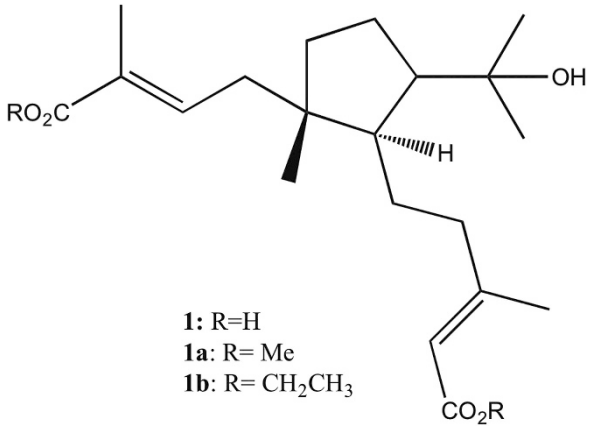

Figure 2 Structures of **1**, **1a** and **1b**.

modifications on the core structure and generated two derivatives consisting of a di-methyl ester (**1a**) and a di-ethyl ester (**1b**; Figure 2). Unfortunately, the semisynthetic derivatives **1a** and **1b** showed GI_{50} values of 32.2 and 24.5 μM, respectively. This result suggests that the carboxylic acid residue in the structure has an essential role in its cytotoxic activity.

The metabolites closest to cystodienoic acid are the dolabellane diterpenoids (Figure 3), which were first isolated from the mollusk *Dolabella californica*[2] and were later found in many brown algae mainly belonging to the family Dictoytacea.[3,4] Octocorals[5,6] have also been sources for dolabellane chemistry. Recently, these compounds have also been isolated from terrestrial plants.[7] The discovery of a similar dolabellane diterpenoid carbon skeleton, cystodienoic acid from the myxobacterium *Cystobacter* sp. suggests the possibility that these diterpenoids are produced by a microbe, perhaps an as-yet unidentified myxobacterium, mainly owing to the similar chemistry that is observed among a wide variety of marine and terrestrial organisms. The increasing number of observations of similar chemistry occurring in both microbes and marine/terrestrial organisms is rather intriguing and deserves further investigation and understanding of the role of the microbial community within an organism.

EXPERIMENTAL METHODS

NMR spectra were obtained on a Bruker Ascend 500 MHz spectrometer equipped with a cryoprobe (Bruker Biospin GmbH, Waldbronn, Germany), in the solvents indicated and referenced to residual ^1H signals in deuterated solvents. ESI–MS were acquired using an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) separations module equipped with an Agilent 1100 Series LC/MSD mass detector in both positive and negative ion modes under the following conditions: Zorbax C_8 column, 150 × 4.6 mm, eluting with 95% H_2O/MeCN to 5% H_2O/MeCN (with isocratic 0.01% TFA) over 22 min at 0.4 ml min^{-1} and then held for 5 min. HRMS was carried out using an UltiMate 3000 rapid separation liquid chromatography system (Dionex RSLC, Thermo Fisher Scientific, Dreieich, Germany) coupled to an UHR-TOF mass spectrometer (Bruker Daltonik MaXis, Bruker Daltonik GmbH, Bremen, Germany) operating in the positive ESI mode.

Biological assay

Antimicrobial screening. All microorganisms were obtained from the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen,* DSMZ), the

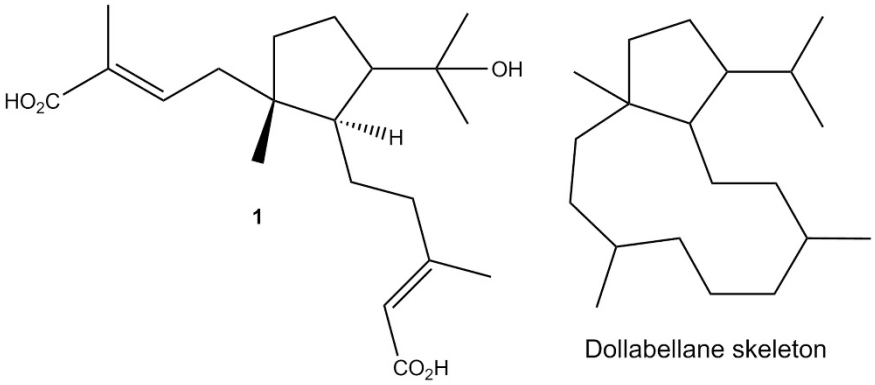

Figure 3 Structure of **1** and the dollabellane carbon skeleton.

American Type Culture Collection (ATCC) or our internal strain collection and were handled according to standard procedures. For microdilution assays overnight cultures of microorganisms were prepared in Myc medium (1% phytone peptone, 1% glucose, 50 mM HEPES, pH 7.0; yeast and fungi), M7H9 medium (Difco Middlebrook 7H9 broth supplemented with BBL Middlebrook ADC enrichment and 2 ml l^{-1} glycerol; *M. smegmatis*) or Mueller–Hinton broth (0.2% beef infusion solids, 1.75% casein hydrolysate, 0.15% starch, pH 7.4; used for all other bacteria listed). Overnight cultures were diluted in the respective growth medium to achieve a final density of 10^5–10^6 cfu ml^{-1}. Serial dilutions of cystodienoic acid and its derivatives were prepared in sterile 96-well plates. After adding the cell suspension to the wells, the microorganisms were grown on a microplate shaker (750 rpm, 30–37 °C, 16–48 h). Growth inhibition was assessed by visual inspection. The MIC values presented are the lowest concentration of antibiotic at which there was no visible growth.

Cytotoxicity assay. Human HCT-116 colon carcinoma cells (ACC--581) were obtained from the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ) and were cultured in McCoy's 5A medium supplemented with 10% FBS at 37 °C with 5% CO_2. Cells were seeded at 6×10^3 cells per well in 96-well plates (Corning CellBind, Fisher Scientific, Pittsburgh, PA, USA) in 180 µl complete medium and were directly treated with the compounds in serial dilution. Treated cells were incubated for 5 days. To assess the viability of the treated cells compared with that of the cells treated with the internal solvent control, 20 µl of 5 mg ml^{-1} MTT (thiazolyl blue tetrazolium bromide) in PBS was added per well and further incubated for 2 h at 37 °C. The medium was then discarded and cells were washed with PBS (100 µl) before adding 2-propanol/10N HCl (250:1, *v/v*; 100 µl) to dissolve the formazan granules. The absorbance at 570 nm was measured using a microplate reader (Infinite 200 PRO, Perkin Elmer, Tecan, Crailsheim, Germany), and IC_{50} values were determined by sigmoidal curve fitting.

Cystodienoic acid di-methyl ester (1a)
Cystodienoic acid (2.0 mg) was dissolved in 0.5 ml of MeOH and then mixed with 200 µl of TMS-diazomethane solution (Sigma-Aldrich). The solution was stirred at room temperature for ~6 h. The reaction mixture was then quenched with 100 µl of acetic acid and dried *in vacuo*. The reaction mixture was then resuspended in MeOH (0.5 ml) and subjected to semipreparative HPLC (Zorbax, C_8 column, 250×9.4 mm, 5 µm, 3 ml min^{-1}, 10–100% MeCN/H_2O gradient over 20 min to yield **1a** ($t_R = 17.8$ min, 1.2 mg).

Cystodienoic acid di-ethyl ester (1b)
Cystodienoic acid (2.5 mg) was dissolved in 1 ml of EtOH and DCC (0.5 mg) and DMAP (0.2 mg) were added and stirred at room temperature for 18 h. The resulting product was passed through a cotton filter, dried *in vacuo* and then resuspended in MeOH (0.5 ml). The reaction mixture was subjected to semipreparative HPLC (Zorbax, C_8 column, 250×9.4 mm, 5 µm, 3 ml min^{-1}, 10–100% MeCN/H_2O gradient over 20 min to yield **1b** ($t_R = 19.5$ min, 1.0 mg).

Cystodienoic acid (**1**): colorless oil; $[\alpha]_D^{23}$+35 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 nm (4.35); NMR data; HRESIMS *m/z* 353.2320 $[M+H]^+$ (calculated for $C_{20}H_{33}O_5$ 353.2328).

Cystodienoic acid di-methyl ester (**1a**): colorless oil; $[\alpha]_D^{23}$+30 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 nm (4.34); NMR data, see Supplementary Table S1; HRESIMS *m/z* 381.2636 $[M+H]^+$ (calculated for $C_{22}H_{37}O_5$ 381.2642).

Cystodienoic acid di-ethyl ester (**1b**): colorless oil; $[\alpha]_D^{23}$+27 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 220 nm (4.34); NMR data, see Supplementary Table S1; HRESIMS *m/z* 409.2947 $[M+H]^+$ (calculated for $C_{24}H_{41}O_5$ 409.2955).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)