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Research Article

The Isolation of a New *S*-Methyl Benzothioate Compound from a Marine-Derived *Streptomyces* sp.

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The application of an HPLC bioactivity profiling/microtiter plate technique in conjunction with microprobe NMR instrumentation and access to the AntiMarin database has led to the isolation of a new **1**. In this example, **1** was isolated from a cytotoxic fraction of an extract obtained from marine-derived *Streptomyces* sp. cultured on Starch Casein Agar (SCA) medium. The 1D and 2D ^{1}H NMR and ESIMS data obtained from 20 μg of compound **1** fully defined the structure. The known **2** was also isolated and readily dereplicated using this approach.

1. Introduction

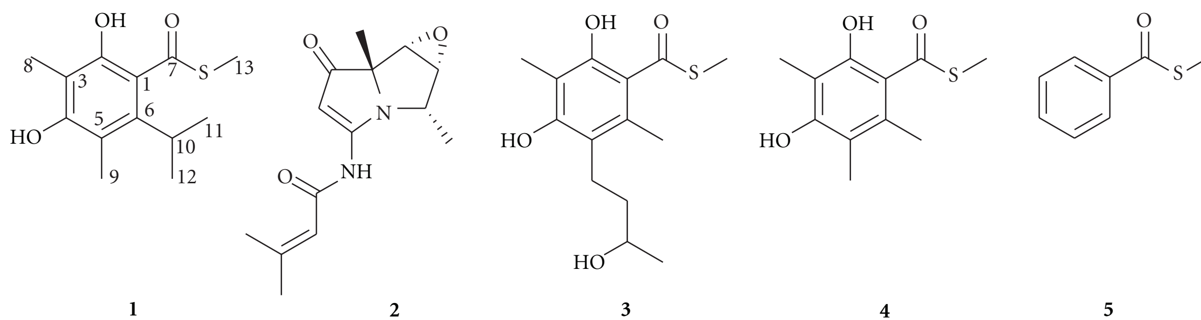
The HPLC bioactivity profiling/microtiter plate technique in conjunction with microprobe NMR instrumentation and access to the AntiMarin database [1] has been utilized by our group as a tool to enhance dereplication as well as to obtain a rapid NMR data acquisition for characterization of new metabolites by using less than 50 μg of purified material. The sensitivity of the technique to enhance structural elucidation by using only small amounts of a natural product has been described [2–10]. In our continuing efforts to rapidly characterize new bioactive metabolites, a marine-derived *Streptomyces* sp. was investigated for its bioactivity and chemical properties. Herein we report the structure of a new **1** by using 20 μg of material.

The first *S*-methyl benzothioate group of metabolites, **3**, was produced in a broth culture of *S. collinus* [11, 12]. *S. collinus* remained the only reported producer of this unusual structural type until a comparable structure, **4**, was identified from a sclerotium-colonizing isolate of the fungus, *Mortierella vinacea* [13]. The production of **5** was also reported from a marine *Streptomyces* sp. [14] and, recently, from *Phaeobacter gallaeciensis* and *Oceanibulbus indolifex* [15]. To date, **3**, **4**, and **5** are the only secondary metabolites reported for the *S*-methyl benzothioate group of metabolites (see Scheme 1).

2. Materials and Methods

2.1. General Experimental Procedures. NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, resp.), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.3 and δ_C 49.3 ppm for CD_3OD). A Protasis CapNMR microprobe was used for the microplate dereplication studies. HRESIMS were acquired using a Micromass LCT TOF mass spectrometer. MS/MS experiments were performed on a Bruker Daltonics Esquire 4000 system. Solvents used for extraction and isolation were distilled prior to use. Bioactivity assays were made using standard protocols [16, 17].

2.2. Isolation and Cultivation of Isolate. Streptomyces sp. was isolated from an unidentified tunicate collected from Lyttelton Harbour, New Zealand, in May 2004, using adapted



Scheme 1

isolation techniques [18] on SCA medium. Fermentations were carried out on 60 plates of SCA for 30 days at 28°C. The isolate (LA3L2, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand) was identified by its cultural and microscopic characteristics. For the chemical study, the isolate was grown on SCA medium for 30 days at 28°C (60 plates; 20 mL). The combined agar was macerated with EtOAc and the EtOAc removed and concentrated under reduced pressure to give the crude extract (45.7 mg).

2.3. Evaluation of Extracts. The crude extract was fractionated to isolate the compounds. The extract was initially defatted with petroleum ether (Pet. Ether) yielding 21.6 mg of Pet. Ether layer (Fraction 1), and further partitioning with H_2O and EtOAc (1 : 1) resulted in 1.4 mg H_2O layer (Fraction 2) and 22.7 mg EtOAc layer (Fraction 3). Fraction 3 was further chromatographed on HPLC to obtain pure **2** and a subfraction 3a. Aliquots of subfraction 3a ($1 \times 750\,\mu$g; $2 \times 500\,\mu$g) were analyzed by HPLC (RP-18, solvents: (A) H_2O + 0.05% TFA, (B) MeCN; gradient: 0 min 10% B, 2 min 10% B, 14 min 75% B, 24 min 75% B, and 26 min 100% B; flow: 1 mL/min; 40°C. The eluent from the DAD was split in a 1 : 10 ratio between the ELSD and the fraction collector configured to collect into a 96-well microtiter plate (15 s/well). A total of 88 wells were collected (2.5– 24.5 min). A daughter plate was prepared by transferring an aliquot (5 μL) from each well of the master plate. After complete evaporation of the solvent, the wells in the daughter plate were analyzed for activity against P388 murine leukemia cells as described previously [16, 17].

The assay established that cytotoxicity was correlated with the peak observed by HPLC/ELSD/UV. The well F10 of the dried master plate, containing the bioactive **1** was analyzed using capillary probe NMR spectroscopy. The content of well F10 was dissolved in CD_3OD (7 μL) and transferred into the Protasis CapNMR microprobe. Calibrations have shown that this effectively transfers 6 μL of sample into the probe. Standard operating conditions were used to acquire 1D and 2D NMR spectra. The quantity of the compound was estimated according to the formula:

$$\left(\frac{\mathrm{MW}}{^{\#}\mathrm{H}}\right) \times \frac{(\text{total integral for }^{\#}\mathrm{H})}{(\text{integral for }\mathrm{CHD_2OD})} \times \mathrm{CF}, \qquad (1)$$

where MW is the actual molecular weight of the compound (ESMS), or an estimated value, #H is the number of protons included in the integration of the ^{1}H NMR spectrum, and CF is the calibration factor that had previously been determined from a standard solution containing quinine (30 μg in 6 μL) in the same CD_3OD solvent.

*2.4. Isolation of **1**.* The crude extract (45.7 mg) was subjected to a reversed phase semipreparative column chromatography (Phenomenex Luna C18, 10×250 mm, 5 μm, 1 mL/min, 40°C, gradient: 20–40% acetonitrile in 0.05% TFA in H_2O over 40 min, monitored by UV absorption at 215 nm) to yield 1.0 mg of **2** and 2.3 mg of the cytotoxic fraction, containing **1**. **2** was readily dereplicated as bohemamine using the described technique [2].

An aliquot (750 μg) of the cytotoxic fraction was injected on to the HPLC, and the fractions were collected into a microtitre plate to yield **1** (4 μg; R_t 15.5 min). In the second attempt, an aliquot (up to 1000 μg) of the cytotoxic fraction was injected on to the HPLC and the fractions were collected into a microtitre plate to yield reasonably pure **1** (20 μg; R_t 15.5 min).

S-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate, **1**: light brown solid; UV (MeOH) λ_{max} 207, 227, 283; for ^{1}H NMR data and 1D and 2D spectra, see Table 1 and supporting information; HREIMS obsd, $[M+H]^+$ at *m/z* 255.1043 (calcd for $C_{13}H_{19}O_3S$, 255.1055).

3. Results and Discussion

Streptomyces sp. was obtained from liquid portions of a New Zealand marine tunicate and grown on Starch Casein Agar medium (60 plates) for 30 days at 28°C. Extraction with EtOAc yielded 45.7 mg of crude extract. This extract showed cytotoxic activity in a P388 assay (IC_{50} 383 μg/mL). Analysis by reverse-phase C_{18} analytical HPLC revealed three main peaks, one major and two minor. The result from the HPLC MTT plate assay indicated that cytotoxicity was correlated with one of the minor peaks eluted over R_t 15.0–18.5 min. In the first attempt, an aliquot of 750 μg of the cytotoxic fraction from the crude extract was chromatographed with collection of fractions into a microtitre plate. Well F10 of the microtitre plate, containing 4 μg of **1**, was analysed using the CapNMR microprobe technique and ESIMS. The ESIMS

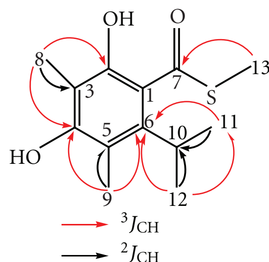


Figure 1: HMBC correlations of **1**.

spectrum indicated the mass of **1** to be 255 Da ($[M+H]^+$), and the presence of an ion at *m/z* 207, which corresponded to the loss of 47 mass units ($[M\text{-}SCH_3]^+$), suggested a thiomethyl group. Confirmation of the presence of S was supported by the HRESIMS$^+$ spectrum yielding the formula $C_{13}H_{19}O_3S$ ($M+H^+$ 255.1043 Da, calc. 255.1055 Da). The ^1H NMR spectrum of **1** indicated the presence of three singlet methyl signals at δ_H 2.07, 2.18, and 2.41 and an isopropyl group, represented by one doublet signal at δ_H 1.29 and a multiplet methine signal at δ_H 3.1. As the data from the ^1H NMR spectrum and ESIMS of **1** showed no match with those of any reported compound in the AntiMarin database [1], an additional 20 μg of **1** from the cytotoxic fraction of the crude extract was obtained for further spectroscopic analysis. Although some minor impurities contributed to the ^1H NMR spectroscopic data, HSQC-DEPT, HMBC, and NOE spectroscopic data were sufficient to elucidate the structure of **1**.

From the HSQC-DEPT spectrum, the chemical shifts of the protons at δ_H 1.29, 2.07, 2.18, and 2.41 were correlated with the chemical shifts of their directly bonded carbons ($^1J_{CH}$ couplings). The HMBC spectrum clearly illustrated the presence of a hexasubstituted benzenoid system. The position of one aryl methyl group at δ_H 2.07 was established by strong HMBC correlations with two oxygen-bearing carbons (C-2, δ_C 149.2 and C-4, δ_C 155.5) and with one higher field carbon (C-3, δ_C 110.2), thus, placing this group between two oxygenated aromatic carbons. The position of the other aryl methyl group was further established by strong HMBC connections of the signal at δ_H 2.18 with one oxygen-bearing carbon (C-4, δ_C 155.5) and two carbons (C5, δ_C 115.5 and C-6, δ_C 139.9). One of the two remaining aromatic carbons was substituted by an isopropyl group, proven by a long-range correlation of two methyl groups (δ_H 1.29) with the carbon (C-6, δ_C 139.9), leaving the C-1 position to be substituted by the carbonyl group (C-7, δ_C 198.4), which had a long-range HMBC correlation to the methyl group (δ_H 2.41). The long range couplings of this compound are shown schematically in Figure 1.

The positions of the isopropyl and the carbonyl group were further confirmed by an NOE experiment. When the methine proton (δ_H 3.1, m) and methyl proton (δ_H 2.18, s) signals were irradiated, the signal for $C\underline{H}_3$ (δ_H 1.29, d) was enhanced. Irradiation of the methyl protons (δ_H 1.29, d) enhanced the methine proton (δ_H 3.1, m) and methyl proton (δ_H 2.18, s) signals. No signals were enhanced when the two methyl groups $C\underline{H}_3$ (δ_H 2.07, s) and $C\underline{H}_3$ (δ_H 2.41, s) were irradiated. These data from the NOE experiment confirmed the relationship between the methine group (C-10, δ_C 31.6) and the two methyls (C-11, δ_C 20.7; C-12, δ_C 20.7) and the attachment overall of the isopropyl group at C-6 (δ_C 139.9). The ^1H and ^{13}C chemical shifts for the thiomethyl group of **1** were comparable to those reported for **3** [12] and **4** [13].

Table 1: NMR data of **1**.

Position	$\delta^{13}C$, ppm		δ^1H, ppm, multiplicity (J_{HH} Hz)
1	122.2	(C)	
2	149.2	(C)	
3	110.2	(C)	
4	155.5	(C)	
5	115.5	(C)	
6	139.9	(C)	
7	198.4	(C)	
8	7.2	(CH_3)	2.07, s
9	12.1	(CH_3)	2.18, s
10	31.6	(CH)	3.10, m
11	20.7	(CH_3)	1.29, d (7.2)
12	20.7	(CH_3)	1.29, d (7.2)
13	11.4	(CH_3)	2.41, s

^1H: chemical shifts recorded at 500 MHz in CD_3OD. ^{13}C: chemical shifts obtained from HSQC and HMBC spectra.

A complete list of the ^1H and ^{13}C chemical shifts for **1** is presented in Table 1. As there were no absolute matches for this structure found, the designated **1** was therefore considered a new structure and named *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate.

The major peak identified in the HPLC analysis of the crude extract was characterized as **2** as the ^1H NMR, UV, and MS data obtained matched those previously reported for bohemamine [19, 20].

4. Conclusions

A new metabolite, *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate, **1**, and a known metabolite bohemamine, **2**, were isolated and identified from *Streptomyces* sp. using the CapNMR technique. The structure of **1** was fully characterized by ^1H, HSQC, HMBC, and NOE NMR experiments. This new compound is only the fourth natural product reported to contain the *S*-methyl benzothioate group.

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References

[1] J. W. Blunt, M. H. G. Munro, and H. Laatsch, Eds., *AntiMarin Database*, University of Canterbury, Christchurch, New Zealand and University of Göttingen, Göttingen, Germany, 2006.

[2] G. Lang, N. A. Mayhudin, M. I. Mitova et al., "Evolving trends in the dereplication of natural product extracts: new methodology for rapid, small-scale investigation of natural product extracts," *Journal of Natural Products*, vol. 71, no. 9, pp. 1595–1599, 2008.

[3] M. I. Mitova, A. C. Murphy, G. Lang et al., "Evolving trends in the dereplication of natural product extracts. 2. The isolation of chrysaibol, an antibiotic peptaibol from a New Zealand sample of the mycoparasitic fungus *Sepedonium chrysospermum*," *Journal of Natural Products*, vol. 71, no. 9, pp. 1600–1603, 2008.

[4] J. W. Blunt, A. L. J. Cole, D. de Silva et al., "Simplifying complexity: the dereplication of natural products," in *Proceedings of the 4th European Conference on Marine Natural Products*, Paris, France, September 2005.

[5] M. H. G. Munro, "Simplifying complexity: the gentle art of dereplication," in *Proceedings of the International Chemical Congress of Pacific Basin Societies*, Honolulu, Hawaii, USA, December 2005.

[6] G. Lang, M. I. Mitova, S. van der Sar et al., "Dereplication, databases and discovery: the probing of fungal chemodiversity," in *Proceedings of the ICOB-5 & ISCNP-25 IUPAC International Conference on Biodiversity and Natural Products*, Kyoto, Japan, July 2006.

[7] J. W. Blunt, "Dereplication to discovery: new methodology for rapid, small-scale investigation of fungal metabolites," in *Proceedings of the 47th Annual Meeting of the American Society of Pharmacognosy*, Arlington, Va, USA, August 2006.

[8] J. W. Blunt and M. H. G. Munro, "Moving on: the future scale and scope of marine natural products research," in *Proceedings of the 5th European Conference on Marine Natural Products*, Ischia, Italy, September 2007.

[9] J. W. Blunt and M. H. G. Munro, "How much is enough?" in *Proceedings of the 2nd CMDD Symposium on Marine Natural Products*, Seoul, Korea, November 2007.

[10] J. W. Blunt, S. Chamyuang, A. L. J. Cole et al., "Simplifying complexity: the HPLC-bioactivity-NMR approach to the dereplication of natural product extracts," in *Proceedings of the 10th International Marine and Freshwater Symposium and Asian Mycology Congress*, Penang, Malaysia, December 2007.

[11] M. Tahara, T. Okabe, K. Furihata et al., "Resorthiomycin, a novel antitumor antibiotic. II. Physico-chemical properties and structure elucidation," *Journal of Antibiotics*, vol. 43, no. 2, pp. 135–137, 1990.

[12] M. Tahara, T. Okabe, K. Furihata et al., "Revised structure of resorthiomycin," *Journal of Antibiotics*, vol. 44, no. 2, p. 255, 1991.

[13] A. G. Soman, J. B. Gloer, and D. T. Wicklow, "Antifungal and antibacterial metabolites from a sclerotium-colonizing isolate of *Mortierella vinacea*," *Journal of Natural Products*, vol. 62, no. 2, pp. 386–388, 1999.

[14] J. S. Dickschat, T. Martens, T. Brinkhoff, M. Simon, and S. Schulz, "Volatiles released by a *Streptomyces* species isolated from the North Sea," *Chemistry and Biodiversity*, vol. 2, no. 7, pp. 837–865, 2005.

[15] V. Thiel, T. Brinkhoff, J. S. Dickschat et al., "Identification and biosynthesis of tropone derivatives and sulfur volatiles produced by bacteria of the marine *Roseobacter* clade," *Organic and Biomolecular Chemistry*, vol. 8, no. 1, pp. 234–246, 2010.

[16] G. Lang, M. I. Mitova, G. Ellis et al., "Bioactivity profiling using HPLC/microtiter-plate analysis: application to a New Zealand marine alga-derived fungus, *Gliocladium* sp.," *Journal of Natural Products*, vol. 69, no. 4, pp. 621–624, 2006.

[17] M. I. Mitova, B. G. Stuart, G. H. Cao, J. W. Blunt, A. L. J. Cole, and M. H. G. Munro, "Chrysosporide, a cyclic pentapeptide from a New Zealand sample of the fungus *Sepedonium chrysospermum*," *Journal of Natural Products*, vol. 69, no. 10, pp. 1481–1484, 2006.

[18] M. Namikoshi, K. Akano, H. Kobayashi et al., "Distribution of marine filamentous fungi associated with marine sponges in coral reefs of palau and bunaken Island, Indonesia," *Journal of the Tokyo University of Fisheries*, vol. 88, pp. 15–20, 2002.

[19] T. S. Bugni, M. Woolery, C. A. Kauffman, P. R. Jensen, and W. Fenical, "Bohemamines from a marine-derived *Streptomyces* sp.," *Journal of Natural Products*, vol. 69, no. 11, pp. 1626–1628, 2006.

[20] T. W. Doyle, D. E. Nettleton, D. M. Balitz et al., "Isolation and structure of bohemamine, (1aβ,2α,6aβ,6bβ)-3-Methyl-*N*-(1a, 6,6a,6b-tetrahydro-2,6a-dimethyl-6-oxo-*2H*-oxireno[a]pyrrolizin-4-yl)-2-butenamide," *Journal of Organic Chemistry*, vol. 45, no. 7, pp. 1324–1326, 1980.