Thiomycalolides: New Cytotoxic Trisoxazole-Containing Macrolides Isolated from a Marine Sponge *Mycale* sp.[1]

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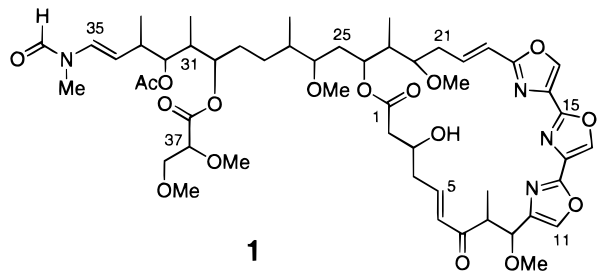
Two new cytotoxic trisoxazole macrolides, thiomycalolides A (**2**) and B (**3**), have been isolated from a marine sponge *Mycale* sp. The structures were determined to be glutathione adducts of mycalolides A and B by interpretation of spectral data and chemical transformation. Thiomycalolides A and B are highly cytotoxic against P388 murine leukemia cells.

Mycalolides A, B (**1**), and C are potent actin-depolymerizing agents isolated from a marine sponge of the genus *Mycale*.[2,3] They are closely related to ulapualides,[4] kabiramides,[5] and halichondramides[6] isolated either from eggmasses of *Hexabranchus* nudibranchs or sponges of the genus *Halichondria*. These compounds are macrocyclic lactones encompassing three contiguous oxazoles and a side chain terminating in *N*-methylformamide. During our search for further cytotoxic constituents of *Mycale* sp., which contains mycalolides, we noticed the presence of metabolites related to mycalolides in a highly polar fraction,[7] from which we have isolated two major cytotoxic components named thiomycalolides A (**2**) and B (**3**).



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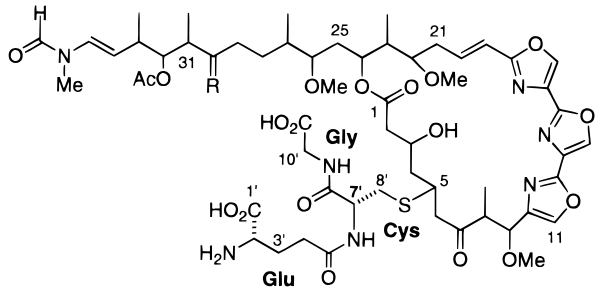

2 R= O
H

3 R= O OMe
OMe

The EtOH extract of the sponge samples (5.0 kg wet weight) collected off the Kii Peninsula, 330 km southwest of Tokyo, was partitioned between ether and water; the aqueous phase was successively fractionated by ODS and Sephadex LH-20 chromatographies, preparative TLC, and ODS HPLC to afford thiomycalolides A (**2**, 3.3 mg)[8] and B (**3**, 8.8 mg) as brownish powders.

The major constituent, thiomycalolide B, exhibited an ^{1}H NMR spectrum reminiscent of mycalolide B,[2] i.e., three oxazole singlets (δ 8.58, 8.52, and 7.95), doubled formamide signals (δ 8.29 and 8.05), five *O*-methyls (δ 3.40, 3.34 (×2), 3.32, and 3.26), doubled *N*-methyl (δ 3.06 and 2.97), an acetyl (δ 2.03), and five secondary methyls (δ 0.98, 0.97, 0.96, 0.90, and 0.83). The COSY spectrum clearly showed that the $\Delta^{5,6}$-olefin was missing, while $\Delta^{19,20}$- and $\Delta^{34,35}$-olefins were present. Detailed interpretation of the COSY and HMQC spectra revealed that the C-7−C-9 and C-19−C-35 portions in **3** were identical with those of mycalolide B, whereas the $\Delta^{5,6}$-olefin was replaced by a $CHCH_2$ unit [δ 3.57 (m; H5), 2.85 (dd, J = 8.7, 16.5 Hz; H6a), 3.14 (dd, J = 5.7, 16.9 Hz; H6b)]. The HMQC spectrum indicated that C-5 and C-6 resonated at 39.8 and 51.8 ppm, respectively, suggesting that C-5 was linked to a sulfur atom.[9] In addition to the signals attributable to the mycalolide B skeleton, there were three isolated spin systems: C-10′ [δ 3.68, d (J = 17.3 Hz); 3.70, d (J = 17.3 Hz)], C-7′,8′ [δ 4.54, dd (J = 5.0, 8.5 Hz); 3.02, dd (J = 5.2, 13.7 Hz); 2.83, dd (J = 8.8, 13.9 Hz)], and C-2′,3′,4′ [δ 3.59, dd (J = 6.2, 8.8 Hz); 2.06, (2H, m); 2.50, (2H, m)]. In addition, thiomycalolide B (**3**) showed an $(M + Na)^+$ ion at *m*/*z* 1356 in its FABMS, 307 amu larger than mycalolide B.

The gross structure of **3** was determined by connecting the above-mentioned units on the basis of HMBC data (Scheme 1 and Table 1). The three isolated units were assigned as Gly, Cys, and Glu residues on the basis of intraresidual HMBC cross-peaks between the α-methine proton and the carbonyl carbon. HMBC data allowed not only the connection of these units as a glutathionyl group (*i*-Glu-Cys-Gly) but also the placement of this peptide at C-5 via the sulfur atom.[10] Two carboxyls and one amino group must be terminal to satisfy the molecular formula.[11] Other structural features such as the presence of an *O*,*O*′-dimethylglycerate on C-31, an acetate on C-33, and *O*-methyls on C-9, C-22, and C-26 were also confirmed by HMBC data.

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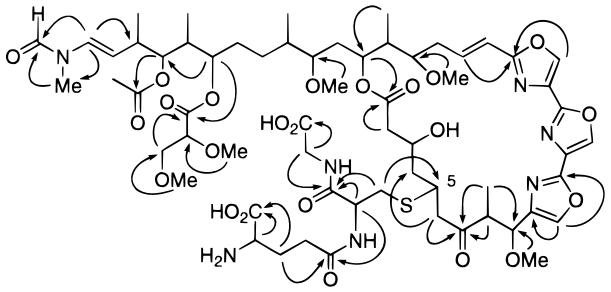
Scheme 1. Key HMBC Correlations of Thiomycalolide B



Marfey analysis[12] of the acid hydrolysate of **3** showed that both Glu and Cys residues had the L-configuration. Therefore, the gross structure of **3** was assigned as 5-glutathionyl mycalolide B.

To confirm the proposed structure, mycalolide B was treated with L-glutathione in the presence of $NaHCO_3$. Conjugate addition took place instantaneously, giving a 3:2 mixture of adducts, which were separated by ODS HPLC. The ^{1}H NMR spectrum of the minor product was identical with that of **3**, thus confirming the structure.[13,14]

Thiomycalolide A (**2**) exhibited an ^{1}H NMR spectrum similar to that of **3**. The molecular ion in the FABMS was consistent with a glutathione adduct of mycalolide A, which was supported by COSY data. Due to the paucity of the sample, the HMBC spectrum of **2** was not good enough to detect all carbon signals. Therefore, the structure of **2** was determined by preparing the glutathione adduct of mycalolide A, whose ^{1}H NMR spectrum was identical with that of **2**.

Thiomycalolides A and B exhibit cytotoxic activity against P388 cells with an IC_{50} value of 18 ng/mL each. It is not known whether thiomycalolides are produced by an enzymatic reaction in the sponge or are simple adducts of co-occurring mycalolides and glutathione.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL A600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 3.30 and δ_C 49.0 for CD_3OD. FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using 3-nitrobenzyl alcohol as matrix. Amino acid analysis was carried out with a Hitachi L 8500-A amino acid analyzer. Optical rotation was determined on a JASCO DIP-1000 digital polarimeter in CH_3OH. UV spectra were recorded on a Hitachi 330 spectrometer.

Animal Material. Sponge samples were collected by hand using snorkeling at depths of 2−5 m in the Gokasho Bay on the Kii Peninsula, immediately frozen, and kept at −20 °C until processed. A voucher specimen (S3103) was deposited at the Laboratory of Aquatic Natural Products Chemistry, University of Tokyo. The sponge was identified as *Mycale* sp. by Professor P. R. Bergquist, University of Auckland, on the occasion of the structure elucidation of mycalolides A−C.[2] The sponge has many fine green fingers of a diameter 1−2 cm, arising from a single stalk. The sponge tissue is soft and slimy. It grows on rocks or over dead stalks of brown algae and is found usually in a sunny place.

Extraction and Purification. The frozen sponge (5.0 kg wet weight) was chopped into small pieces and extracted with EtOH (3 × 2 L). The combined extracts were concentrated and partitioned between ether and water. The aqueous layer was separated by ODS flash chromatography with MeOH/H_2O (15:85) and EtOH/MeOH (1:1). The fraction eluted with EtOH/MeOH (1:1) was further subjected to flash chromatography on ODS with a MeOH/H_2O system, and the active fraction was gel-filtered on Sephadex LH-20 (80% MeOH) followed by preparative TLC on Merck Kieselgel 60 F_{254} with $CHCl_3$/MeOH/H_2O (7:3:0.5) to furnish a mixture of related compounds as a brown gum, which was finally purified by reversed-phase HPLC on Capcell Pak with 70% MeOH containing 0.1 M $NaClO_4$. Desalting of major peaks on a short column of ODS afforded thiomycalolide A (**2**; 3.3 mg) and thiomycalolide B (**3**; 8.8 mg).

Thiomycalolide A (2): FABMS (matrix: 3-nitrobenzyl alcohol, positive) [*m*/*z* 1238 $(M + Na)^+$]; ^{1}H NMR (CD_3OD) δ 0.84 (d, 3H, J = 6.9 Hz; 27-Me), 0.95 (d, 3H, J = 6.9 Hz; 23-Me), 0.99 (d, 3H, J = 6.9 Hz; 8-Me), 1.03 (d, 3H, J = 6.9 Hz; 33-Me), 1.07 (d, 3H, J = 6.9 Hz; 31-Me), 1.21 (m, 1H; H-28), 1.57 (m, 2H; H-25), 1.71 (m, 1H; H-28), 1.77 (m, 1H; H-27), 1.83 (m, 1H; H-4), 1.91 (m, 1H; H-4), 1.92 (m, 1H; H-23), 1.99 (s, 3H, 32-OAc), 2.09 (m, 2H; H-3′), 2.53 (m, 1H; H-21), 2.53 (m, 2H; H-4′), 2.54 (m, 1H; H-2), 2.56 (m, 2H; H-29), 2.58 (m, 1H; H-33), 2.66 (m, 1H; H-2), 2.68 (m, 1H; H-21), 2.87 (m, 1H; H-31), 2.88 (m, 1H; H-8′), 2.93 (m, 1H; H-6), 3.01 (3.10) (s, 3H, 35-NMe), 3.05 (m, 1H; H-8′), 3.06 (m, 1H; H-26), 3.18 (m, 1H; H-6), 3.41 (m, 1H; H-22), 3.46 (m, 1H; H-8), 3.62 (m, 1H; H-2′), 3.66 (m, 1H; H-5), 3.68 (m, 2H; H-10′), 4.50 (m, 1H; H-3), 4.55 (m, 1H; H-9), 4.57 (m, 1H; H-7′), 5.10 (m, 1H; H-32), 5.16 (5.09) (m, 1H; H-34), 5.25 (m, 1H; H-24), 6.47 (d, 1H, J = 16.6 Hz; H-19), 6.76 (7.13) (d, 1H, J = 14.2 Hz; H-35), 7.16 (m, 1H; H-20), 7.94 (s, 1H; H-11), 8.33 (8.09) (s, 1H, 35-NCHO), 8.50 (s, 1H; H-17), 8.56 (s, 1H; H-14).

Thiomycalolide B (3): $[\alpha]_D$ −3.3° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 238 nm (ϵ 33 500); HRFABMS [*m*/*z* 1356.5898 $(M + Na)^+$, $C_{62}H_{91}O_{23}N_7SNa$, Δ +11.4 mmu]; ^{13}C and ^{1}H NMR data in CD_3OD at 300 K, see Table 1.

Acid Hydrolysis of Thiomycalolide B. A 100 μg portion of **3** was dissolved in 0.5 mL of 5 N HCl and heated at 110 °C for 13 h. The solvent was evaporated in a stream of N_2, and the residue was redissolved in 1% HCl and subjected to standard amino acid analysis using a cation-exchange column with postcolumn derivatization with ninhydrin, which indicated the presence of Cys, Glu, and Gly.

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of **3** were added 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (10 mg/mL) and 100 μL of 1 M $NaHCO_3$, and the mixture was kept at 80 °C for 3 min. To the reaction mixture were added 50 μL of 2 N HCl and 200 μL of 50% MeCN, and the mixture was analyzed by reversed-phase ODS-HPLC: linear gradient elution from H_2O−TFA (100:0.1) to MeCN−H_2O−TFA (60:40:0.1) in 60 min; detection with UV absorption at 340 nm.

Table 1. NMR Data for Thiomycalolide B (1)[a] in CD_3OD

position	^1H (δ)	^{13}C (δ)	HMBC	position	^1H (δ)	^{13}C (δ)	HMBC
1		174.4		28	0.95 m	28.2	C: 27, 29
					1.42 m		C: 27, 29
2	2.53 m	43.5	C: 1, 3	29	1.40 m	31.2	C: 28, 31
	2.65 m		C: 1, 3		1.58 m		C: 28, 31
3	4.40 m	68.0		30	5.06 dd (1.1, 7.3)	74.7	C: 28, 29, 31, 32, 36
4	1.79 m	43.7	C: 3	31	1.85 m	39.0	
	1.86 m		C: 3				
5	3.57 m	39.8		31-Me	0.97 d (6.5)	10.0	C: 30, 31, 32
6	2.85 dd (8.7, 16.5)	51.8	C: 4, 5, 7	32	4.74 dd (2.3, 10.0)	78.4	C: 32-OAc
	3.14 dd (5.7, 16.9)		C: 4, 5, 7				
7		214.0		32-OAc		172.6	
8	3.45 m	48.5	C: 7, 9, 10 8-Me		2.03 s	21.0	
8-Me	0.96 d (6.5)	13.0	C: 7, 8, 9	33	2.62 m	38.0	C: 31
9	4.43 d (6.9)	79.4		33-Me	0.98 d (6.9)	19.8	C: 32, 33, 34
9-OMe	3.32 s	57.2	C: 9	34	5.06 dd (9.6, 14.2)	112.0	C: 33-Me
					(5.14) dd (9.6, 14.6)	(114.0)	C: 33-Me
10		140.7		35	6.73 d (14.2)	132.0	C: 33, 34, 35-NMe
					(7.10) d (14.6)	(126.5)	
11	7.95 s	139.1		35-NMe	2.97 s	27.6	C: 35, 35-NCHO
					(3.06) s	(33.5)	
12		157.0		35-NCHO	8.29 s	163.0	
					(8.05) s	(164.0)	
13		131.3		36		172.2	
14	8.58 s	140.4		37	3.91 dd(3.5, 6.5)	81.5	C: 36
15		158.2		37-OMe	3.40 s	59.0	C: 37
16		130.9		38	3.59 dd (4.2, 10.8)	74.0	C: 36, 38-OMe
					3.63 dd (6.9, 10.4)		C: 36, 38-OMe
17	8.52 s	140.7	C: 18	38-OMe	3.33 s	59.5	C: 38
18		164.5		Glu			
19	6.47 d (15.8)	118.2	C: 21	1′		173.6	
20	7.22 m	141.6	C: 18, 21	2′	3.59 dd (6.2, 8.8)	55.5	C: 1′, 3′, 4′
21	2.62 (2H, m)	34.0	C: 20, 22, 23	3′	2.06 (2H, m)	27.8	C: 1′, 2′, 4′, 5′
22	3.27 m	82.0		4′	2.50 (2H, m)	33.3	C: 2′, 3′, 5′
22-OMe	3.34 s	57.8	C: 22	5′		175.2	
23	1.99 m	40.8		Cys			
23-Me	0.90 d (6.9)	10.0	C: 22, 23, 24	6′		172.0	
24	5.19 dd (6.5, 10.4)	74.4	C: 1, 25	7′	4.54 dd (5.0, 8.5)	55.4	C: 5′, 6′, 8′
25	1.40 m	31.8		8′	2.83 dd (8.8, 13.9)	32.6	C: 6′, 5
	1.52 m				3.02 dd (5.2, 13.7)		
26	3.02 m	82.8		Gly			
26-OMe	3.26 s	58.2	C: 26	9′		175.6	
27	1.79 m	35.3		10′	3.68 d (17.3)	44.2	C: 6′, 9′
					3.70 d (17.3)		C: 6′, 9′
27-Me	0.83 d (6.9)	16.2	C: 26, 27, 28				

[a] All spectra referenced to residual solvent signal of CD_3OD (δ_H 3.30, δC 49.0) at 27 °C and recorded at 600 MHz.

Cytotoxicity Assay. For cytotoxicity test see ref 15.

Preparation of 3 from 1. To a solution of mycalolide B (2 mg in 2 mL of MeOH) were added L-glutathione and 4 μM $NaHCO_3$, and the mixture was stirred for 16 h. The reaction mixture was purified by ODS HPLC with MeOH/100 mM $NaClO_4$ in H_2O (7:3) followed by desalting on a short ODS column to furnish thiomycalolide B (0.7 mg) together with its C-8 epimer.

Preparation of 2 from Mycalolide A. Thiomycalolide A was prepared from mycalolide A as described above for the preparation of **3**. Two peaks in a ratio of 2:3 appeared in the HPLC, and the smaller peak coincided with **2** in the ^1H NMR spectrum. Although we did not characterize the larger peak, it was likely to be the C-8 epimer of **2**.

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(7) The FAB mass spectrum of this fraction suggested that it was a mixture of at least six related compounds. However, only two major compounds could be isolated due to the difficulty of separation and their instability.
(8) Compound **2** contaminated with significant amounts of ODS inadvertently came out from the column that was used to remove $NaClO_4$. Due to the instability of the compound, further purification was not conducted.
(9) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*, 3rd ed.; VCH: New York, 1990; pp 233−235.
(10) The presence of the three amino acid residues was confirmed by the amino acid analysis of the acid hydrolysate of **3**.
(11) The presence of three ionizable groups agreed with the high polarity of thiomycalolide B: silica gel TLC ($CHCl_3$/MeOH/H_2O 7:3:0.5) R_f value = 0.37.

(12) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(13) The major product is likely to be the C-8 epimer of **3**, which was suggested by a downfield shift of H-9 (4.85 ppm) as well as an altered $J_{8,9}$ value of 5.4 Hz. Coupling constants of H-3, H-4a, H-4b, and H-5a were almost identical for the two compounds, suggesting that the stereochemistry at C-5 of the two compounds was identical.
(14) It is interesting to note that the chemical shift of H-20 in **3** was concentration dependent. The higher the concentration, the lower the resonance of H-20.
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