Tetrahedron Vol. 49, No. 48, pp. 11183-11188, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00

Toxadocials B, C and Toxadocic acid A: Thrombin-Inhibitory Aliphatic Tetrasulfates from the Marine Sponge, *Toxadocia cylindrica*[1]

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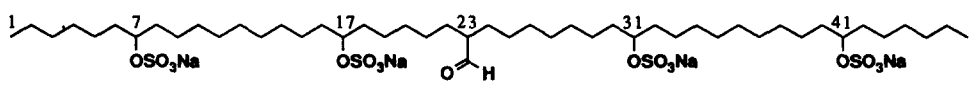
(*Received in Japan* 6 *September* 1993)

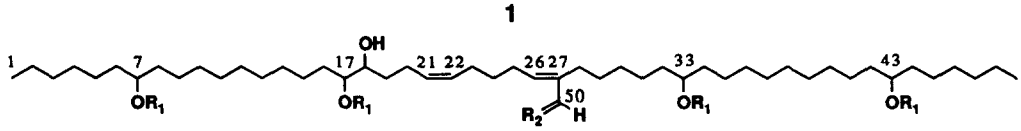
Abstract: Three new thrombin-inhibitory metabolites, toxadocial B (**2**), toxadocial C (**3**), and toxadocic acid A (**4**) have been isolated from the marine sponge *Toxadocia cylindrica*. The structures of these compounds were determined by spectroscopic and chemical methods.

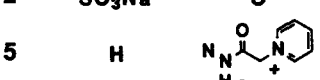
In the course of our screening program for inhibitors of serine proteases from marine invertebrates, we encountered the sponge *Toxadocia cylindrica* collected off Hachijo-jima Island. Its extract was highly inhibitory against thrombin.[2] Bioassay-guided fractionation of the MeOH extracts afforded several active substances, among which we reported the structure of toxadocial A (**1**).[3] Further fractionation led to the isolation of three related metabolites, toxadocial B (**2**), toxadocial C (**3**), and toxadocic acid A (**4**).

The water soluble portion of the MeOH extract of the frozen sponge (1.9 kg) was gel-filtered on Sephadex LH-20 followed by reverse-phase HPLC to yield toxadocial B (**2**, 4.4 x 10^{-4} % wet weight), toxadocial C (**3**, 1.9 x 10^{-3} %), toxadocic acid A (**4**, 5.8 x 10^{-4} %), along with the known toxadocial A (**1**).



1

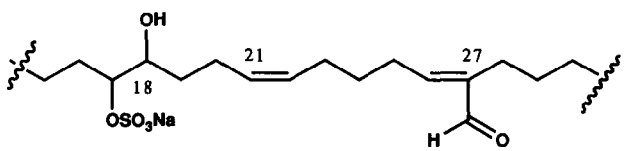


	R_1	R_2
2	SO_3Na	O
5	H	
6	H	

Toxadocial B (**2**) was obtained as a colorless amorphous solid, $[\alpha]_D^{23}$ +3.7 ° (*c* 0.2, MeOH) and had a molecular formula of $C_{50}H_{92}S_4O_{18}Na_4$ which was determined by negative ion HRFABMS [(M-Na)$^-$ *m/z*

1177.4861, Δ +0.1 mmu]. The ^{1}H NMR spectrum contained signals for two primary methyls [δ 0.77 (6H, t, *J* = 6.8 Hz)], four allylic methylenes [δ 2.16 (2H, q, *J* = 7.3 Hz), 2.29 (2H, q, *J* = 7.4 Hz), 2.30 (2H, q, *J* = 7.4 Hz), 2.39 (1H, m), and 2.55 (1H, dq, *J* = 14.3, 7.3 Hz)], five oxygenated methines [δ 4.28 (1H, dt, *J* = 10.0, 2.5 Hz), 4.86 (1H, quint, *J* = 5.9 Hz), 4.88 (2H, quint, *J* = 5.9 Hz), and 5.08 (1H, buried under solvent signal)], three olefinic protons [δ 5.44 (1H, dt, *J* = 10.5, 7.3 Hz), 5.57 (1H, dt, 10.5, *J* = 7.3 Hz), and 6.43 (1H, t, *J* = 7.4 Hz)], and an aldehyde [δH 9.50 (1H,s)], while the ^{13}C NMR spectrum revealed signals for five oxygenated methines [δC 72.60, 78.78 (2C), 78.88, and 82.90], four sp^2 carbons (δC 129.43, 131.23, 143.97, and 155.33), and an aldehyde (δC 195.31) together with methyl [δC 14.2 (2C)] and numerous methylene signals. A characteristic C23 methine in **1** (δC 53.2) was missing, suggesting the presence of an α, β-unsaturated aldehyde group in **2**, which was supported by spectral data [δC 195.31, ν_{max} 1670 cm^{-1}, λ_{max} 232 nm (ε 9900)].

Interpretation of the COSY spectrum starting from an olefinic proton on a trisubstituted double bond (δ 6.43) revealed that the two double bonds were connected through three methylene carbons. The *Z*-geometry of the second double bond was deduced from a vicinal coupling constant of 10.5 Hz. The proton at δ 5.57 was coupled to allylic methylene protons at δ 2.39 and 2.55, which were in turn correlated with methylene protons at 1.77 and 2.01. The latter methylene protons were further coupled to an oxy-methine at δ 4.28 which was correlated with another oxygenated methine at δ 5.08. This methine proton was also coupled to methylene protons at δ 1.71 and 2.02, both of which gave crosspeaks with a methylene protons at δ 1.73 and 1.55. Incidentally, *E*-geometry of the trisubstituted double bond was secured by the NOESY[4] crosspeaks δ 6.43/9.50 and 9.50/ 2.29. The methylene protons at δ 2.29 showed a COSY crosspeak with methylene protons at δ 1.39, which were correlated with a methylene in the envelope at δ 1.16. Considering HMQC[5] crosspeaks, δH 4.28/δC 72.6 and 5.08/82.9, we can assign partial structure **A**, which comprises the central moiety of the molecule.



A

The two remaining segments contained three sulfated methines, two terminal methyls, and 28 methylenes. Since further structural information could not be obtained by NMR experiments, we decided to apply MS/MS techniques. Toxadocial B was treated with dilute HCl to give rise to a pentaol which was treated consecutively with Girard's reagents P and T,[6] yielding compounds **5** and **6**. The FAB-MS/MS of **5** and **6** gave ions with 20 mu difference, revealing that fragment ions included the cationic portion in the center of the molecule. Three sets of intense ion peaks 30 mu apart in the FAB-MS/MS of **6** at *m/z* 820/790, 664/634, and 634/604 as depicted in Fig. 1 allowed us to determine the position of the five hydroxyl groups, whereas ions at *m/z* 578, 536 and 508 supported the location of the α, β-unsaturated aldehyde group. FAB-MS/MS data[7] of the corresponding derivatives prepared from tetrahydrotoxadocial B were consistent with the assigned structure.

Table 1. NMR data of toxadocial B (2)*

#C	^{13}C	^{1}H	#C	^{13}C	^{1}H
1	14.20	0.77 (t, 6.8)	26	155.33	6.43 (t, 7.4)
2	22.84	1.15 (m)	27	143.97	-
3	32.04	1.15 (m)	28	24.12	2.29 (t, 7.4)
4	29.81	1.16 (m)	29	28.99	1.39 (quint, 7.4)
5	25.47	1.53 (m)	30	~30	1.25 (m)
6	35.02	1.91 (m), 1.81 (m)	31	25.20	1.53 (m)
7	78.78	4.88 (quint, 5.9)	32	34.82	1.91 (m), 1.81 (m)
8	34.93	1.91 (m), 1.81 (m)	33	78.88	4.86 (quint, 5.9)
9	25.47	1.53 (m)	34	34.93	1.91 (m), 1.81 (m)
10	29.76	1.25 (m)	35	25.47	1.53 (m)
11	~30	1.16 (m)	36	~30	1.25 (m)
12	~30	1.16 (m)	37	~30	1.16 (m)
13	~30	1.16 (m)	38	~30	1.16 (m)
14	29.76	1.25 (m)	39	~30	1.16 (m)
15	26.07	1.73 (m), 1.55 (m)	40	29.76	1.25 (m)
16	31.56	2.02 (m), 1.71 (m)	41	25.47	1.53 (m)
17	82.90	5.08	42	34.93	1.91 (m), 1.81 (m)
18	72.60	4.28 (dt, 10.0, 2.5)	43	78.78	4.88 (quint, 5.9)
19	32.21	2.01 (m), 1.77 (m)	44	35.02	1.91 (m), 1.81 (m)
20	24.56	2.55 (dq, 14.3, 7.3), 2.39 (m)	45	25.47	1.53 (m)
21	131.23	5.57 (dt, 10.5, 7.3)	46	29.81	1.16 (m)
22	129.43	5.44 (dt, 10.5, 7.3)	47	32.04	1.15 (m)
23	27.19	2.16 (q, 7.3)	48	22.84	1.15 (m)
24	28.92	1.50 (quint, 7.3)	49	14.20	0.77 (t, 6.8)
25	28.70	2.30 (q, 7.4)	50	195.31	9.50 (s)

*solvent C_5D_5N

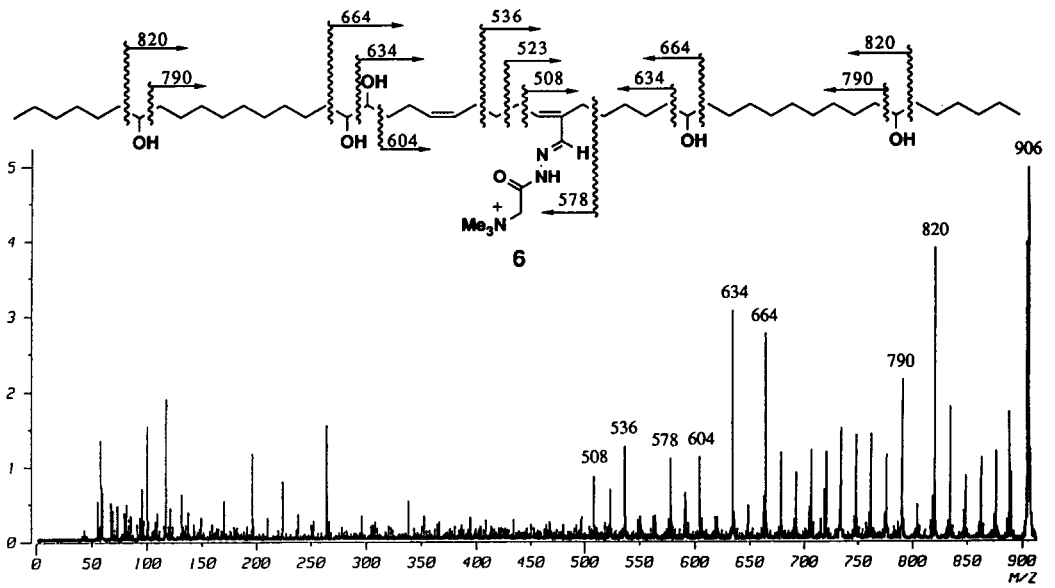
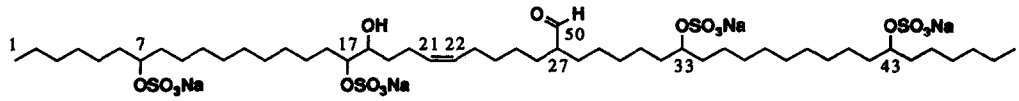


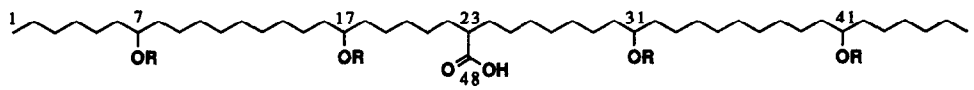
Fig. 1 Positive ion FAB-MS/MS of 6

Toxadocial C (**3**) had a molecular formula of $C_{50}H_{94}S_4O_{18}Na_4$ as determined for an $(M-Na)^-$ ion by HRFABMS. Toxadocial C had two more hydrogen than **2**, suggesting that one double bond in **2** was reduced.

The IR absorbtion at 1720 cm^{-1} suggested that the aldehyde (δ_C 205.65, δ_H 9.64) was linked to an sp^3 carbon instead of an α, β-unsaturated aldehyde as in **2**. Interpretation of the COSY, HMQC, and HMQC-HOHAHA[8] spectra led to partial structure **A** except for the C26 olefin. The position of the remaining three sulfate esters was similarly determined by FAB-MS/MS to obtain gross structure **3**.[9]



3



	R
4	SO_3Na
7	H

The molecular formula of toxadocic acid A (**4**) was determined as $C_{48}H_{92}S_4O_{18}Na_4$ by the negative ion HRFABMS. The NMR spectra were almost identical with those of **1** except for a ^{13}C signal at δ 179.15, revealing a carboxylic acid instead of an aldehyde in toxadocial A (**1**). Acid hydrolysis of **4** yielded a tetraol **7**, whose structure was elucidated by negative ion FAB-MS/MS measurements. As shown in Fig. 2 prominent ions at *m/z* 681/651 and 525/495 allowed location of hydroxyl groups, while ions at *m/z* 439 and 411 indicated the position of the carboxyl group at C23.

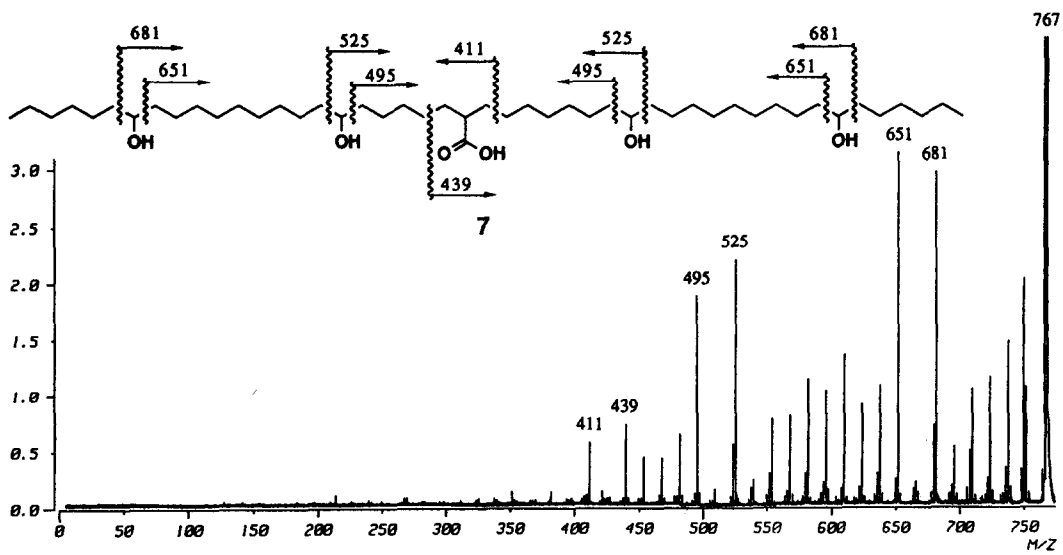
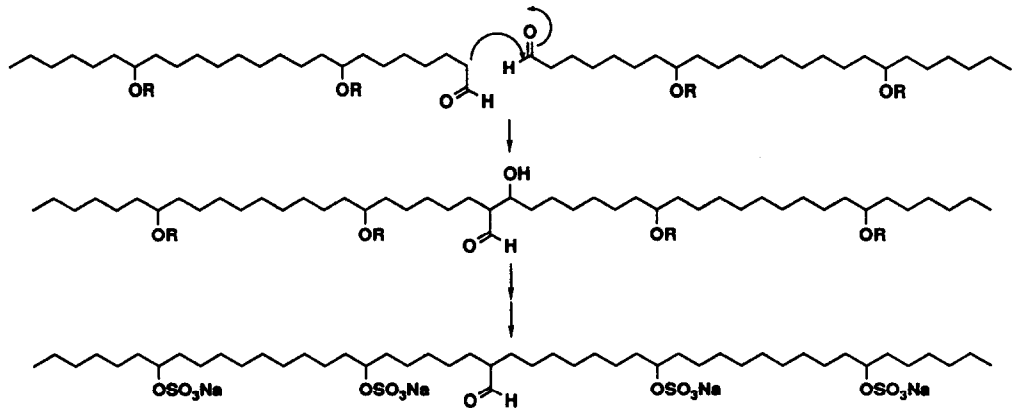


Fig. 2 Negative ion FAB-MS/MS of 7

Toxadiocials A-C and toxadocic acid A had similar activity against thrombin with IC_{50}'s of 6.5, 4.6, 3.2, and 2.7 μg/mL, respectively. This may indicate that their activity is mainly attributable to sulfate esters. Toxadocials are an unprecedented class of natural products with one carbaldehyde in the middle of a long alkyl chain. They may be biosynthesized through aldol condensation of two units of hydroxylated (or sulfated) aldehydes followed by dehydration and reduction (Scheme I).



1

Scheme I

Experimental Section

General procedure: ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 600 NMR spectrometer in C_5D_5N at 300 K. FABMS and FAB-MS/MS were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using *m*-nitrobenzyl alcohol (positive ion mode) and triethanolamine (negative ion mode) as matrices. Infrared spectra were recorded on a JASCO IR-G infrared spectrometer. Optical rotations were determined on a JASCO DIP-371 digital polarimeter in methanol.

Isolation: The concentrated MeOH extract of the sponge (1.9 kg, wet weight) collected off Hachijo-jima Island at depths of 5-20 m was extracted with Et_2O and then with *n*-BuOH. The MeOH soluble portion (16.7 g) of the *n*-BuOH extract was gel-filtered through a column of Sephadex LH-20 (5 x 80 cm) with MeOH. Fractions of 10-mL were collected and monitored by bioassay and TLC. The major active fractions (1.4 g) were separated by ODS HPLC on Cosmosil $5C_{18}AR$ (2 x 25 cm) with 52 % MeCN containing 0.25 M $NaClO_4$ to yield fractions A (220.7 mg) and B (113 mg). Fraction A was rechromatographed under the same conditions yielding fractions A1 - A6. Further purification of fractions A2 and A3 by ODS HPLC on an *L*-column (Chemicals Inspection and Testing Institute, Japan, 1 x 25 cm) with 45.5 % MeCN containing 0.1 M $NaClO_4$ afforded **2** (8.4 mg) and **4** (11.1 mg). Fraction B was purified by ODS HPLC on an *L*-column (1 x 25 cm) with 46.5 % MeCN containing 0.1 M $NaClO_4$ and desalted through an ODS short column to give 36.6 mg of **3**.

Toxadocial B (**2**): colorless amorphous solid; $[\alpha]_D^{23}$ +3.7 ° (*c* 0.2, MeOH); HRFABMS $(M\text{-}Na)^-$ *m/z* 1177.4861 ($C_{50}H_{92}S_4O_{18}Na_3$, Δ +0.1 mmu); IR (film) ν_{max} 3470, 2925, 2850, 1670, 1640, 1460, 1380, 1250, 1220, 1060, 930, 820, 770 cm^{-1}; UV (MeOH) λ_{max} 232 nm (ε 9900); ^1H and ^{13}C NMR (C_5D_5N) see Table 1.

Toxadocial C (**3**): colorless amorphous solid; $[\alpha]_D^{23}$ +2.2 ° (*c* 0.2, MeOH); HRFABMS (M-Na)$^-$ *m/z* 1179.5026 ($C_{50}H_{94}S_4O_{18}Na_3$, Δ +1.0 mmu); IR (film) ν_{max} 3470, 2925, 2850, 1720, 1640, 1460, 1380, 1350, 1220, 1060, 930, 820, 770 cm^{-1}; ^{1}H NMR (C_5D_5N) δ 9.64 (bs), 5.50 (dt, *J* = 10.5, 7.3 Hz), 5.40 (dt, *J* = 10.4, 7.3, Hz), 5.07 (m), 4.84 (quint, *J* = 4.2 Hz), 4.25 (bd, *J* = 10.1 Hz), 2.52 (m), 2.34 (m), 2.20 (m), 2.06 (q, *J* = 7.0 Hz), 2.01 (m), 1.97 (m), 1.87 (m), 1.77 (m), 1.76 (m), 1.72 (m), 1.71 (m), 1.56 (m), 1.54 (m), 1.50 (m), 1.34 (m), 1.25 (m), 1.22 (m), ~1.2 (m), 1.15 (m), 0.77 (t, *J* = 6.9 Hz); ^{13}C NMR (C_5D_5N) δ 205.65, 130.48, 130.00, 83.03, 78.94 (3C), 72.62, 51.99, 34.89 (3C), 34.82 (2C), 34.74, 32.24, 32.02 (2C), 31.45, ~30 (12C), 29.71 (2C), 28.94, 28.77, 27.26, 27.17, 26.83, 26.04, 25.40 (5C), 25.19, 24.51, 22.83 (2C), 14.20 (2C).

Toxadocic acid A (**4**): colorless amorphous solid; $[\alpha]_D^{23}$ +0.6 ° (*c* 0.36, MeOH); HRFABMS (M-Na)$^-$ *m/z* 1153.4917 ($C_{48}H_{92}S_4O_{18}Na_3$, Δ +5.7 mmu); IR (film) ν_{max} 3470, 2925, 2850, 1710, 1640, 1460, 1380, 1350, 1220, 1060, 1040, 930, 770 cm^{-1}; ^{1}H NMR (C_5D_5N) δ 4.88 (quint, *J* = 5.9 Hz), 4.87 (quint, *J* = 5.9 Hz), 2.58 (tt, *J* = 9.2, 4.4 Hz), 1.91 (m), 1.83 (m), 1.81 (m), 1.57 (m), 1.54 (m), 1.45 (m), ~1.2 (m), 1.16 (m, H-2), 1.14 (m), 0.77 (t, *J* = 7.3 Hz); ^{13}C NMR (C_5D_5N) δ 179.15, 78.83, 78.79 (3C), 46.49, 35.00 (2C), 34.95 (2C), 34.92 (3C), 34.86, ~33.0 (2C), 32.04 (2C), ~30 (14C), 29.77 (2C), 27.91 (2C), 25.46 (6C), 25.43, 25.33, 22.84 (2C), 14.20 (2C).

Preparations of Girard's reagent P and T derivatives: A 0.1 mg portion of either toxadocial B, C or toxadocic acid A was refluxed with 1 N HCl (1 mL) for 30 min., and the reaction mixture was extracted with Et_2O. To the residue of the organic layer was added 20 μL of Girard's reagents P or T in AcOH (3.3 μmol/mL) and the mixture was heated at 70 °C for 30 min.

Hydrogenation: To a solution of toxadocial B (1.0 mg) in MeOH was added 15 mg of 5 % Pd on activated carbon and stirred under 1 atm of H_2 at room temperature for 10 h. The reaction mixture was filtered through a membrane filter which was further washed with $CHCl_3$/MeOH/H_2O (6:4:1).

Acknowledgment: We thank Professor P. J. Scheuer, the University of Hawaii, for editorial comments. Thanks are also due to Mr. K. Tanaka of JEOL Co, Ltd. for valuable advice in the measurement of FAB-MS/MS. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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7. Girard's reagent T derivative of tetrahydrotoxadocial B: FAB-MS/MS *m/z* 910 [(M+H)$^+$, precursor ion], 824, 794, 668, 638, 608, 582, 510.
8. a) Gronenborn, A. M.; Bax, A.; Wingfield, P. T.; Clore, G. M. *FEBS Lett.* **1989**, *243*, 93-98.
   b) The following correlations were observed: H17/C15, C16, C18; H18/C17, C19, C20; H20/C18, C19, C21; H21/C18, C19, C20, C22, C23; H22/C19, C20, C21, C23; H23/C19, C20, C21, C22, C24, C25, C26; H26/C24, C25, C27, C28; H27/C25, C26, C28, C29.
9. a) Girard's reagent P derivative of **3**: FAB-MS/MS *m/z* 928 [(M+H)$^+$, precursor ion], 842, 812, 686, 656, 626, 600, 530.
   b) Girard's reagent T derivative of dihydrotoxadocial C gave the same FAB-MS/MS fragment patterns as those of tetrahydrotoxadocial B.