Rigidins B−D, New Pyrrolopyrimidine Alkaloids from a Tunicate *Cystodytes* Species

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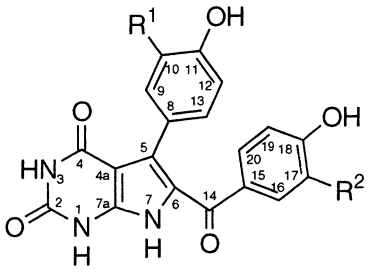
Three new pyrrolopyrimidine alkaloids, rigidins B−D (**1**−**3**), have been isolated from an Okinawan marine tunicate *Cystodytes* sp., and the structures were elucidated on the basis of spectroscopic data.

Rigidin (**4**) is a pyrrolopyrimidine alkaloid with calmodulin antagonistic activity isolated from an Okinawan marine tunicate *Eudistoma* cf. *rigida*.[1] In our continuing search for unique secondary metabolites from marine tunicates,[2,3] investigation of another Okinawan tunicate *Cystodytes* sp. resulted in the isolation of three new rigidin congeners, rigidins B−D (**1**−**3**). Here we describe the isolation and structure elucidation of **1**−**3**.



1: $R^1 = H$, $R^2 = OCH_3$
2: $R^1 = OCH_3$, $R^2 = H$
3: $R^1 = R^2 = OCH_3$
4: $R^1 = R^2 = H$

The tunicate *Cystodytes* sp. (TN-514) (1.6 kg, wet weight) collected off Ie Island, Okinawa, was extracted with MeOH. The EtOAc-soluble materials of the MeOH extract were subjected to silica gel and C_{18} column chromatographies and C_{18} HPLC to afford rigidins B (**1**, 4.9 mg, 0.00031%, wet weight), C (**2**, 1.3 mg, 0.00008%), and D (**3**, 0.6 mg, 0.00004%) together with known compounds rigidin (**4**, 12 mg, 0.00075%), iejimalides A−D,[4,5] and dytesinins A and B.[2]

Rigidin B (**1**) was shown to have the molecular formula $C_{20}H_{16}N_3O_6$ by HRFABMS [*m*/*z* 394.1029 $(M + H)^+$, Δ −1.0 mmu], which was larger than that of rigidin (**4**) by a OCH_2 unit. IR absorption bands at 3420 and 1666 cm^{-1} were attributed to OH/NH and carbonyl group(s), respectively. UV absorptions at 364 (ϵ 9400), 319 (9600), 304 (sh), 278 (13200), 236 (sh), and 206 (31400) nm were suggestive of the presence of conjugated phenol chromophore(s). The ^1H NMR (Table 1) spectrum of **1** contained 13 proton signals, five of which were D_2O-exchangeable, and the others were due to seven sp^2 methines and one methoxy group. In the ^{13}C NMR (Table 1) spectrum, all 19 carbon resonances except that of a methoxy carbon (δ_C 55.19) were observed between δ_C 95 and 190. Seven of 19 low-field carbon signals were assigned as methines by analysis of a methine-selected editing-HSQC spectrum.[5] The presence of 1,4-di- and 1,3,4-trisubstituted benzene rings was deduced from $^1H-^1H$ COSY correlations (Figure 1). $^1H-^{13}C$ HMBC correlations for H-9 and H-13 to C-11, H-10 and H-12 to C-8, and OH-11 to C-10, C-11, and C-12 indicated the presence of an 11-hydroxyphenyl group (C-8−C-13). On the other hand, the existence of an 18-hydroxy-17-methoxybenzoyl moiety (C-14-C-20) was inferred by $^1H-^{13}C$ HMBC correlations for H-16 to C-14, C-18, and C-20, H-19 to C-15 and C-17, H-20 to C-18, 17-OCH_3 to C-17, and 18-OH to C-17, C-18, and C-19. A pyrrolo[2,3-*d*]pyrimidine-2,4-dione core of **1** was assigned by detailed analysis of the $^1H-^{13}C$ HMBC and $^1H-^{15}N$ HSQC spectra and NOE experiments. The $^1H-^{15}N$ HSQC spectrum revealed three resonances at δ_H 11.87 (NH-3), 11.41 (NH-1), and 10.60 (NH-7), which were attributed to nitrogen-bearing protons. The NH-7 showed correlations for C-4a, C-5, C-6, and C-7a, indicating the presence of a tetrasubstituted pyrrole ring. Long-range $^1H-^{13}C$ correlations for NH-1 to C-2, C-4a, and C-7a and NH-3 to C-2, C-4, and C-4a suggested the presence of a disubstituted 2,4-dioxopyrimidine ring. The $^1H-^1H$ COSY cross-peak due to a *W*-type coupling was observed for NH-1/NH-3. Irradiation of NH-7 resulted in 9% NOE for NH-1, suggesting that N-1 was neighboring to N-7 through C-7a. $^1H-^{13}C$ HMBC correlations for NH-7 to C-14 and H-9 and H-13 to C-5 revealed that the 11-hydroxyphenyl and the 18-hydroxy-17-methoxybenzoyl moieties were bound to C-5 and C-6, respectively. Thus, the structure of rigidin B was concluded to be **1**, corresponding to the 17-methoxy form of rigidin (**4**).

The molecular formula of rigidin C (**2**) was established as $C_{20}H_{16}N_3O_6$ by HRFABMS data [*m*/*z* 394.1024 $(M + H)^+$, Δ −1.5 mmu], suggesting that **2** had the same molecular formula as that of **1**. Comparison of ^1H and ^{13}C NMR data (Table 1) of **2** with those of **1** or **4** indicated that **2** had the same pyrrolo[2,3d]pyrimidine-2,4-dione core as those of **1** and **4** as well as 1,4-di- and 1,3,4-trisubstituted benzene rings, a methoxy, and two hydroxy groups. The proton signals for 1,4-disubstituted benzene ring resonated at δ_H 7.30 (2H, d, $J = 8.4$ Hz) and 6.47 (2H, d, $J = 8.4$ Hz), which corresponded to signals for H-16 and H-20 [δ_H 7.28 (2H, d, $J = 8.4$ Hz)] and H-17 and H-19 [6.48 (2H, d, $J = 8.4$ Hz)] of **4**, indicating the presence of an 18-hydroxybenzoyl moiety. Proton signals due to a 1,3,4-trisubstituted benzene ring were observed for **2**, in place of those of the 10-hydroxyphenyl moiety in **4**. $^1H-^1H$ COSY cross-peaks for H-9/H-13 and H-12/H-13 and the ROESY correlation for OCH_3 to H-9 indicated that the methoxy group was located at C-10. Thus, rigidin C (**2**) was assigned as the 10-methoxy form of rigidin (**4**).

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Table 1. ^{1}H and ^{13}C NMR Data of Rigidins B−D (**1**−**3**) and Rigidin **4** in DMSO-d_6

	1		**2**		**3**		**4**	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	11.21 br		11.50 br		*a*		11.41 br	
2		150.83		150.64		151.08		150.82
3	11.65 br		10.61 br		*a*		11.87 br	
4		159.91		159.64		159.95		159.82
4a		98.10		98.10		98.16		98.13
5		128.00		128.15		128.28		128.06
6		124.73		124.03		124.85		124.89
7	10.62 brs		10.61 brs		10.60 brs		10.60 brs	
7a		141.80		141.37		141.60		141.25
8		122.82		123.00		123.12		122.74
9	6.98 d 8.4	132.37	6.69 brs	116.00	6.71 brs	116.03	6.94 d 8.4	132.29
10	6.45 d 8.4	113.83		145.82		145.84	6.45 d 8.4	113.82
11		156.50		146.11		146.10		156.44
12	6.45 d 8.4	113.83	6.48 d 8.0	116.00	6.48 d 8.0	114.55	6.45 d 8.4	113.82
13	6.98 d 8.4	132.37	6.61 d 8.0	124.22	6.64 d 8.0	124.30	6.94 d 8.4	132.29
14		185.85		185.26		185.35		185.23
15		128.85		128.81		129.14		128.62
16	6.91 brs	113.15	7.30 d 8.4	131.45	6.92 brs	113.25	7.28 d 8.4	131.51
17		146.42	6.47 d 8.4	114.27		146.45	6.48 d 8.4	114.30
18		150.32		160.74		150.33		160.71
19	6.57 d 8.0	114.50	6.47 d 8.4	114.27	6.56 d 8.0	114.26	6.48 d 8.4	114.30
20	7.00 d 8.0	123.62	7.30 d 8.4	131.45	7.27 d 8.0	123.58	7.28 d 8.4	131.51
10-OCH_3			3.56[b] s	55.43	3.51[b] s	55.36		
11-OH	9.29 brs		8.86 brs		8.89 brs		9.29 brs	
17-OCH_3	3.55[b] s	55.19			3.53[b] s	55.23		
18-OH	9.60 brs		10.06 brs		9.70 br		10.04 brs	

[a] Not observed. [b] 3H.

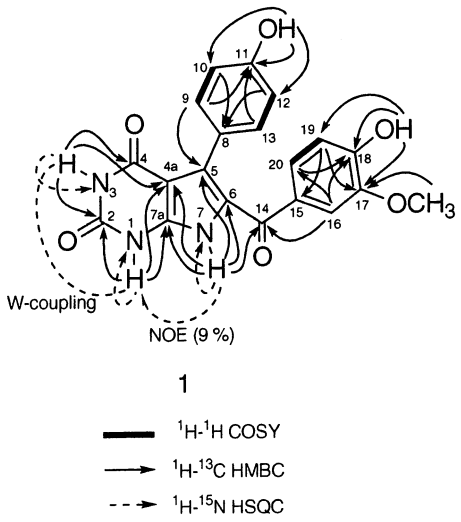

Figure 1. Selected 2D NMR correlations for rigidin B (**1**).

HRFABMS data of rigidin D (**3**) [*m*/*z* 424.1147 $(M + H)^+$, Δ +0.2 mmu] indicated the molecular formula $C_{20}H_{18}N_3O_7$, which was larger than that of rigidin (**4**) by two OCH_2 units. ^{1}H and ^{13}C NMR data of **3** disclosed the presence of the same pyrrolo[2,3-*d*]pyrimidine-2,4-dione core as those of **1**, **2**, and **4** as well as two 1,3,4-trisubstituted benzene rings, two methoxy, and two hydroxyl groups. Proton and carbon chemical shifts for two 1,3,4-trisubstituted benzene rings in **3** were close to those for the C-15−C-20 part of **1** and the C-8−C-13 part of **2**, respectively. Thus, rigidin D (**3**) was elucidated to be the 10,17-dimethoxy form of rigidin (**4**), which was supported by analysis of 2D NMR data of **3**.

The structure of rigidin (**4**) has been elucidated on the basis of 2D NMR data of its pentamethyl derivative[1] and was confirmed by the total synthesis.[6] In this study, the structure of the pyrrolo[2,3-*d*]pyrimidine-2,4-dione core in rigidin B (**1**) was assigned on the basis of ^{1}H, ^{13}C, and ^{15}N NMR data of the natural specimen. Biological activities of **1**−**3** are now under investigation.[7]

Experimental Section

General Experimental Procedures. The IR and UV spectra were recorded on a JASCO FT/IR-5300 and a Shimadzu UV-1600PC spectrophotometer, respectively. NMR spectra were recorded on a Bruker AMX-600 spectrometer in DMSO-d_6 at 300 K. For ^{1}H−^{13}C HMBC experiments, the spectral width in F_1 was 90−200 ppm, and a total of 128 increments of 1K data points were collected. A 50 ms delay time was used for long-range C−H coupling. For ^{1}H−^{15}N HMBC experiments, 95% formamide in $CDCl_3$ was used as an external reference (δ_N 112.4) of ^{15}N NMR. The spectral width in F_1 for ^{1}H−^{15}N HSQC experiments was 100−180 ppm, and a total of 128 increments of 1K data points were collected. ^{1}H−^{15}N HSQC was measured using a 5.56 ms delay for one-bond N−H coupling. The ESI mass spectrum was recorded on a Shimadzu LCMS QP-8000 spectrometer. The FAB mass spectrum was obtained on a JEOL HX-110 spectrometer using glycerol as a matrix.

Animal Material. The tunicate *Cystodytes* sp. (order Enterogona, family Polycitoridae) was collected off Ie Island, Okinawa. The tunicate was identified by Dr. T. Nishikawa, Nagoya University. The voucher specimen (TN-514) was deposited at Graduate School of Pharmaceutical Sciences, Hokkaido University.

Extraction and Isolation. The tunicate (1.6 kg, wet weight) was extracted with MeOH (1 L × 3), and the extract was partitioned between EtOAc (500 mL × 4) and 1 N aqueous NaCl (500 mL). The EtOAc-soluble materials (1.2 g) were subjected to column chromatography on silica gel (MeOH/$CHCl_3$). The fraction eluted with $CHCl_3$/MeOH (80:20) was passed through a Sep-Pak C_{18} cartridge (CH_3CN/H_2O, 1:1, and then MeOH/H_2O, 1:1). The fraction was subjected to C_{18} HPLC [Mightysil RP-18, Kanto Chemical Co., Inc., 10 × 250 mm; eluent, $CH_3CN/H_2O/CF_3CO_2H$, 15:85:0.1; flow rate, 3.5 mL/min; UV detection at 254 nm] to afford rigidin B (**1**, 4.9 mg, 0.00031%, t_R 25 min), rigidin (**4**, 12 mg, 0.00075%, t_r 27 min), and a mixture of rigidins C (**2**) and D (**3**). The mixture was subjected to C_{18} HPLC [under the same conditions, except for

eluent MeOH/H_2O/CF_3CO_2H, 38:62:0.1] to afford rigidins C (**2**, 1.3 mg, 0.00008%, t_R 20 min) and D (**3**, 0.6 mg, 0.00004%, t_R 17 min).

Rigidin B (1): yellow amorphous solid; UV(MeOH) λ_{max} 364 (ϵ 9400), 319 (9600), 304 (sh), 278 (13200), and 236 (sh) nm; IR (KBr) ν_{max} 3420 and 1666 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS (pos.) *m*/*z* 394 $(M + H)^+$ and 416 $(M + Na)^+$; HRFABMS (pos.) *m*/*z* 394.1029 [calcd for $C_{20}H_{16}N_3O_6$, $(M + H)^+$, 394.1039].

Rigidin C (2): yellow amorphous solid; UV (MeOH) λ_{max} 352 (ϵ 9400), 317 (11600), 304 (sh), 272 (14400), and 236 (sh) nm; IR (KBr) ν_{max} 3429 and 1683 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS (pos.) *m*/*z* 394 $(M + H)^+$ and 416 $(M + Na)^+$; HRFABMS (pos.) *m*/*z* 394.1024 [calcd for $C_{20}H_{16}N_3O_6$, $(M + H)^+$, 394.1039].

Rigidin D (3): yellow amorphous solid; UV (MeOH) λ_{max} 352 (ϵ 9300), 322 (11000), 304 (sh), 270 (12000), and 238 (sh) nm; IR (KBr) ν_{max} 3432 and 1637 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS (pos.) *m*/*z* 424 $(M + H)^+$ and 448 $(M + Na)^+$; HRFABMS (pos.) *m*/*z* 424.1147 [calcd for $C_{20}H_{18}N_3O_7$, $(M + H)^+$, 424.1145].

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(7) Rigidins B (**1**), C (**2**), and D (**3**) exhibited cytotoxicity against murine leukemia L1210 cells (40%, 40%, and 20% inhibition, respectively) at 10 μg/mL.

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