Sparoxomycins A1 and A2, New Inducers of the Flat Reversion of NRK Cells Transformed by Temperature Sensitive Rous Sarcoma Virus

II. Isolation, Physico-chemical Properties and Structure Elucidation

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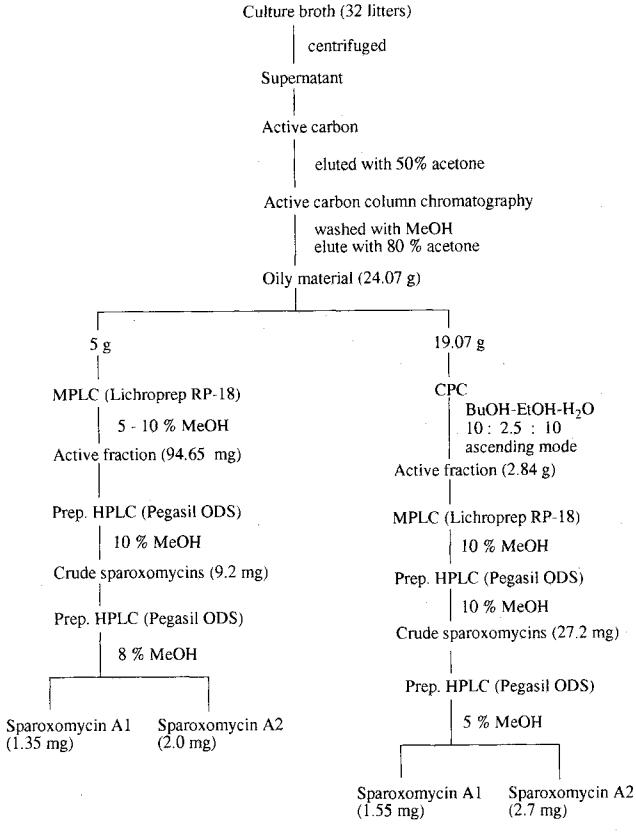
Sparoxomycins A1 and A2, isolated from the fermentation broth and mycelium of *Streptomyces sparsogenes* SN2325, are new inducers of the flat reversion of NRK cells transformed by temperature sensitive Rous sarcoma virus. Sparoxomycins A1 and A2 were isolated by active carbon chromatography, centrifugal partition chromatography (CPC), ODS column chromatography and preparative HPLC. The structure of sparoxomycins were determined by spectroscopic evidences. Stereochemical assignments of these inducers were executed from the analyses of CD spectra.

In the course of our screening for new modulators of proliferation of mammalian cells, we have isolated two new members of pyrimidinylpropenamide antibiotic, sparoxomycins A1 and A2 from a culture broth of *Streptomyces sparsogenes* SN-2325. These compounds closely related to sparsomycin, which is an inhibitor of protein biosynthesis. In this paper, we describe the isolation, physico-chemical properties and structure elucidation of sparoxomycins A1 (**1**) and A2 (**2**). The preceding paper describes the taxonomic study of the producing strain, fermentation and biological activity.[1)]

Isolation of Sparoxomycins A1 (**1**) and A2 (**2**)

As described in the preceding paper, *Streptomyces sparsogenes* SN-2325 was cultured in jar fermentors. Isolation of **1** and **2** was carried out by monitoring the flat reversion activity of *src*^{ts}-NRK cell morphology. The procedure for isolation of **1** and **2** is shown in Fig. 1. Sparoxomycins A1 (**1**) (2.9 mg) and A2 (**2**) (4.7 mg) were isolated as colorless powder from the broth filtrate (32 liters) by chromatography on active charcoal, centrifugal partition chromatography (CPC), ODS column chromatography and HPLC. Details of the isolation procedure are described in the experimental section.

Fig. 1. Extraction and isolation of sparoxomycins A1 (**1**) and A2 (**2**).



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Physico-chemical Properties of Sparoxomycins A1 (1) and A2 (2)

Physico-chemical properties of **1** and **2** are summarized in Table 1. The common molecular formula of **1** and **2** was determined to be $C_{13}H_{19}N_3O_6S_2$ by analyses of LC-FAB, ^1H NMR and ^{13}C NMR spectral data as described below. The characteristic UV spectra with λ_{max} at 303 nm and λ_{sh} 270 nm and IR spectra with 1730, 1715 and 1680 cm^{-1} of **1** and **2** suggested the presence of the pyrimidinylpropenamide moiety. Sparoxomycins A1 (**1**) and A2 (**2**) showed positive responses to anisaldehyde-H_2SO_4, 10% H_2SO_4 and I_2 reagents on TLC.

Structure Determination of Sparoxomycins A1 (1)

The molecular weights of **1** and **2** were determined to be 377 by LC-FAB spectra (column: Inertsil ODS, GL Science, solvent system: A = 0.1% AcOH/H_2O, B = 0.1% AcOH/MeCN, B = 0~95%, **1**: 2.01 minutes, Negative FAB: *m/z* 376, Positive FAB: *m/z* 378, **2**: 5.37 minutes, Negative FAB: *m/z* 376, Positive FAB: *m/z* 378). Such molecular weight and the physico-chemical data of **1** and **2** suggested that these compounds are stereoisomers with each other. Structure subunits of **1** was deduced from ^1H NMR, ^{13}C NMR, HMQC and HMBC spectra of **1** in D_2O. The C-H long-range coupling pattern of the chromophore moiety was consisted with the structure of the pyrimidinylpropenamide which was suggested by the UV and IR data as described above. The carbon signal at C-2 of the pyrimidinyl moiety was detected at 159 ppm in ^{13}C NMR of **1** in D_2O. ^1H NMR of **1** showed ^1H-^1H coupling between a pair of methylene protons at 3.57 and 3.64 ppm (H-17a, H-17b) and methine proton at 4.36 ppm which correlated with another methylene protons at 3.08 and 3.42 ppm (H-12a, H-12b). The ^1H NMR data of **1** in D_2O were resembled to the published data of sparsomycin and its synthetic stereoisomers[2,3] as shown in Table 2. The large coupling constant (J = 15.3 Hz) between 7-H and 8-H indicated the *E*-configuration at C-7. After the ^{13}C NMR measurement of **1**, isomerization of **1** to isosparoxomycin A1 (**3**) was observed. The isosparoxomycin A1 (**3**) possesses the rather small coupling constant (J = 11.8 Hz). The decrease in the coupling constant indicates that the conversion of sparsomycin to its isomer consists in isomerization of the *trans* olefin to a *cis* olefin as in the case of sparsomycin.[4] The molecular weight of **1** detected by FAB-MS is 16 unit larger than that of sparsomycin. Although the corresponding methylene protons of the dithioacetal S-oxide group could not be detected, C-H long-rang coupling between methyl protons at 2.8 ppm (16-H) and carbon signal at 68.5 ppm (C-14) were observed as shown in Fig. 2. This data suggested that the methylene exists as the dithioacetal di-S-oxide and the protons at C-14 were exchanged by deuterium in D_2O. To confirm this possibility, NMR of **1** in DMSO-d_6 were measured. The ^1H NMR of **1** in

Table 1. Physico-chemical properties of sparoxomycin A1 (**1**) and A2 (**2**).

	1	**2**
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{13}H_{19}N_3O_6S_2$	$C_{13}H_{19}N_3O_6S_2$
LC-FAB		
positive (*m/z*) :	378 $(M+H)^+$	378 $(M+H)^+$
negative (*m/z*) :	376 $(M-H)^-$	376 $(M-H)^-$
MP (°C)	169 - 173° (dec)	170 -174° (dec)
IR υ_{max} (KBr) cm^{-1}	3350, 2360, 1730	3350, 2375, 1730
	1715, 1680, 1670, 1610,	1715, 1680, 1670, 1610,
	1540, 1437, 1355, 1340,	1540, 1437, 1355, 1340,
	1305, 1210, 1085, 1025,	1305, 1220, 1085, 1025,
	980, 870, 785, 760, 710	980, 870, 785, 710
UV λ^{MeOH}_{max} nm (ε)	203 (9300), 270 (sh)	203 (8800), 270 (sh)
	303 (15200)	303 (13600)
$[\alpha]^{28}_D$	+47.8° (*c* 0.13, H_2O)	+3.62° (*c* 0.33, H_2O)

Table 2. ^1H NMR data for sparoxomycin A1 (**1**) and A2 (**2**).

	1	**2**
position	ppm (Hz)	ppm(Hz)
7	7.41 (1H, d, J =15.3)	7.28 (1H, d, J =15.6)
8	6.82 (1H, d, J = 15.3)	6.89 (1H, d, J =15.6)
11	4.36 (1H, m)	4.4 (1H, m)
12	3.08 (1H, dd, J =11.2, 13.2)	3.09 (1H, dd, J =11.2, 13.2)
	3.42 (1H, dd, J = 2.9, 13.2)	3.29 (1H, dd, J =2.9, 13.2)
14	ND	ND
16	2.80 (3H, s)	2.78 (3H, s)
17	3.57 (1H, dd, J =5.4, 11.7)	3.58 (1H, dd, J = 5.8, 11.7)
	3.64(1H, dd, J = 4.9, 11.7)	3.65 (1H, dd, J = 4.9, 11.7)
18	2.19 (3H, s)	2.21 (3H, s)

400 MHz, D_2O, δ in ppm.
ND: not detected.

Fig. 2. Long-range coupling observed in the HMBC spectra of sparoxomycin A1 (**1**).

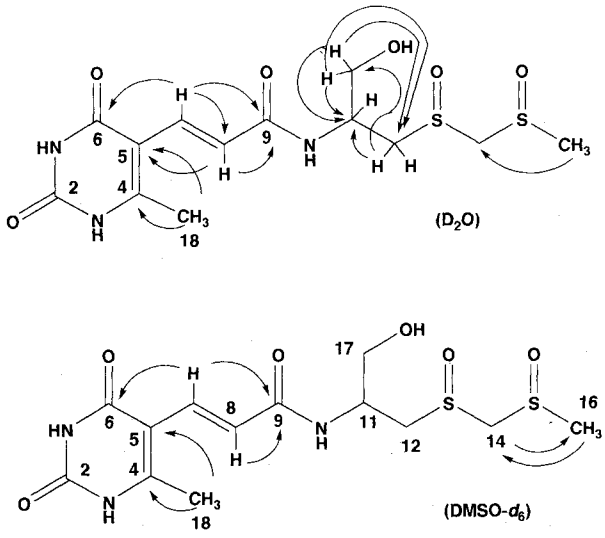


Table 3. ^{1}H NMR data of sparoxomycin A1 (**1**) and A2 (**2**).

position	1 ppm (Hz)	2 ppm(Hz)
1	10.99-11.8 (1H, br. s)	10.9-11.8 (1H, br.s)
3	10.99-11.8 (1H, br. s)	10.9-11.8 (1H, br.s)
7	7.23 (1H, d, J =15.4)	7.23 (1H, d, J =14.7)
8	7.06 (1H, d, J = 15.4)	7.05 (1H, d, J =14.7)
10	8.09 (1H, d, J = 8)	8.09 (1H, d, J = 8)
11	4.2 (1H, m)	4.24 (1H, m)
12	3.04 (1H, dd, J =10.3, 13.2)	3.05 (1H, dd, J =11, 13.2)
	3.36 (1H, dd, J = 2.9, 13.2)	3.16 (1H, dd, J =2.9, 13.2)
14	4.15 (1H, d, J = 13)	4.30 (1H, d, 11.7)
	4.47 (1H, d, J = 13)	4.35 (1H, d, 11.7)
16	2.80 (3H, s)	2.74 (3H, s)
17	3.42 (1H, ddd, J =5.5,6.6, 10.3)	3.41 (1H, m)
	3.51(1H, ddd, J = 5.1, 5.5, 10.3)	3.50 (1H, m)
17-OH	4.94 (1H, t, J = 5.5 Hz)	ND
18	2.25 (3H, s)	2.25 (3H, s)

600 MHz, DMSO-d_6, 38°C, δ in ppm.
ND: not detected because of broadening.

Table 4. ^{13}C NMR data of sparoxomycins A1 (**1**) and A2 (**2**).

position	1 ppm	2 ppm
2	ND	ND
4	155	155
5	104.5	104
6	163	163
7	130.5	130.8
8	120.8	120.8
9	166	173.8
11	46	46.3
12	54.8	55.5
14	68.5	73.6
16	38.5	39.5
17	62.8	63
18	17.5	17.2

150 MHz, DMSO-d_6, 38°C, δ in ppm, detected by HMQC and HMBC spectra.
ND: not detected.

DMSO-d_6 showed a pair of doublet of the methylene protons at 4.15 and 4.47 ppm with $J=13$ Hz. The long-range coupling between these methylene protons at C-14 and methyl carbon at C-16 as well as the methyl protons at C-16 and the methylene carbon at C-14, were observed in HMBC spectrum of **1** (Fig. 2). The ^{1}H NMR spectrum of **1** also showed the four active protons at δ 10.99~11.8 (two protons), 8.09, 4.94 ppm besides the methylene protons. The broad singlet at δ 10.99~11.8 could be assigned as 1-H and 3-H, and the doublet signal at δ 8.09 and the triplet signal at δ 4.94 are assigned as 10-H and 17-OH respectively. The complete assignments of ^{1}H NMR of **1** in DMSO-d_6 are listed in Table 3. The assignment of ^{13}C NMR of **1** were executed by the analyses of HMQC and HMBC spectra as summarized in Table 4. Thus the structure of **1** was reasonably determined as shown.

Structure Determination of Sparoxomycins A2 (**2**)

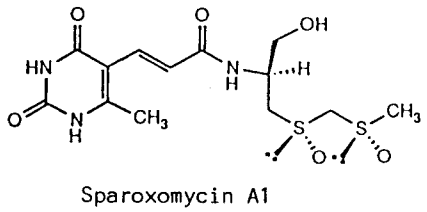
The structure of **2** was determined by HMQC and HMBC analyses as in the case of **1**. Assignments of ^{1}H NMR of **2** in D_2O are summarized in Table 2. The ^{1}H NMR data of **2** in D_2O were resembled to the data of **1** except for the chemical-shift differences of one of proton at C-12 and methyl protons at C-16. The corresponding methylene protons of the dithioacetal di-S-oxide group could not be detected, because the protons at C-14 were exchanged by deuterium in D_2O. However C-H long-rang coupling between methyl protons at 2.78 ppm (16-H) and carbon signal at 73.6 ppm (C-14) were observed as in the case of **1**. The pair of doublet of the methylene protons at C-14 was detected at 4.30 and 4.35 ppm with $J=11.7$ Hz in ^{1}H NMR of **2** in DMSO-d_6. The ^{1}H NMR spectrum of **2** showed the three active protons at δ 10.99~11.8 (1-H and 3-H), 8.09 ppm (10-H) besides the methylene protons. The

proton of 17-OH could not be detected because of broadening of the signal. The complete assignments of ^{1}H NMR of **2** in DMSO-d_6 are listed in Table 3. The assignment of ^{13}C NMR of **2** in DMSO-d_6 was executed by the analyses of HMQC and HMBC spectra as summarized in Table 4. Thus the structure of **2** was determined as a diastereomer of **1**. The large chemical-shift differences at C-14 suggested that **1** has different chirality at the sulfoxide group as mentioned in the next section.

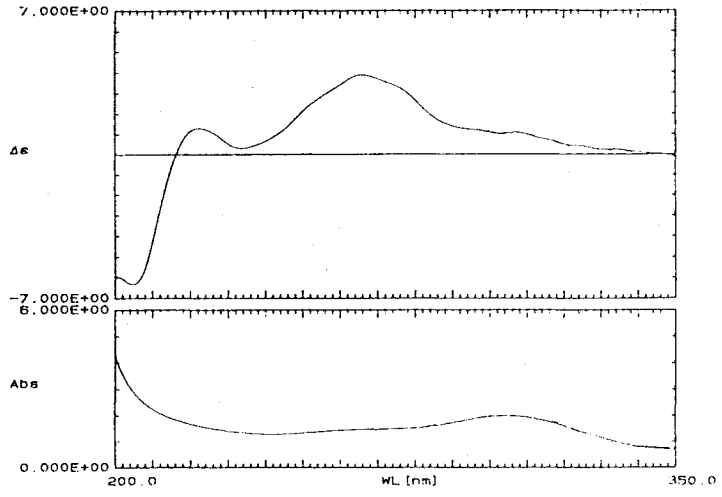
Stereochemical Assignments of Sparoxomycins A1 (**1**) and A2 (**2**)

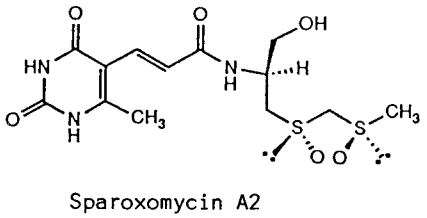
OTTENHEIJM *et al.* determined the absolute configuration of sparsomycin as S_c, R_s configuration by CD analyses and X-ray crystallographic study. The CD spectra of **1** and **2** showed positive Cotton effects at 266 and 268 nm respectively (Fig. 3). Since the Cotton effect at 260~270 nm is due to an n, π^* transition of the amide bond, the positive signs correlate with S_C configuration at C-11 as mentioned by OTTENHEIJM *et al.*[3,5] Sparoxomycin A1 (**1**) showed positive Cotton effect at 222 nm and negative Cotton effect at 205 nm, whereas sparoxomycin A2 (**2**) showed only the negative Cotton effect at 216 nm. By application of MISLOW's rule, the sulfur atoms of **1** and **2** could be assigned as R/S configurations and R/R configurations respectively.[5~7] Biosynthetic consideration of **1**, **2** and sparsomycin suggests that sparoxomycins are diastereomers at S-15, and that **1** and **2** have the S_{C-11}, R_{S-13}, S_{S-15} and S_{C-11}, R_{S-13}, R_{S-15} respectively. These assignments are consisted with the following explanation of the chemical-shifts differences (Δ 0.32 ppm for **1**, Δ 0.05 ppm for **2**) between H-14a and H-14b of sparoxomycins in ^{1}H NMR. The

Fig. 3. CD spectra of sparoxomycins A1 (**1**) and A2 (**2**) in MeOH.



Sparoxomycin A1





Sparoxomycin A2

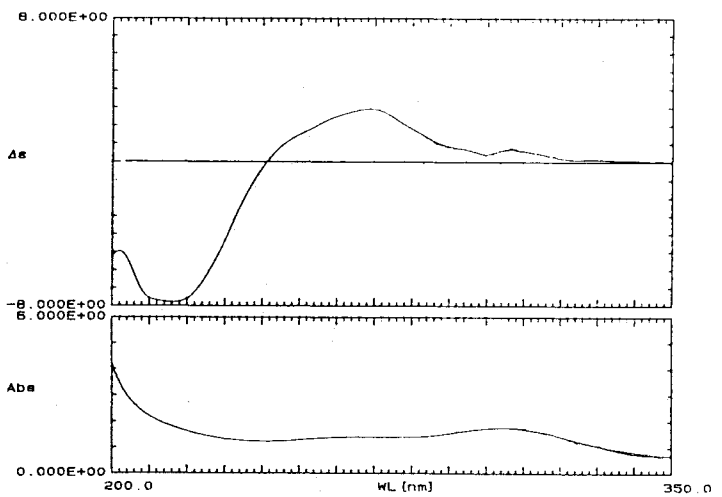
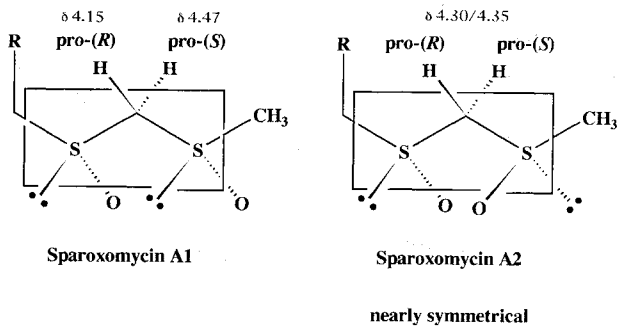


Fig. 4. Mirror planes in sparoxomycins A1 (**1**) and A2 (**2**).



proton (δ 4.15 ppm) of Pro-(*R*) at C-14 of **1** may be shielded by both S-13 sulfoxide and S-15 sulfoxide and the proton (δ 4.47 ppm) of Pro-(*S*) could be deshielded by both the sulfoxides, whereas the both protons (δ 4.30 and 4.35 ppm) of Pro-(*R*) and Pro-(*S*) at C-14 of **2** could be similarly affected by both the sulfoxides as shown in Fig. 4. These hypothetical staggered conformations of dithioacetal di-S-oxide moiety in **1** and **2** are consisted with the conformation of the methyl sulfoxide derivative determined by X-ray analysis.[5] Thus, the absolute configurations of sparoxomycins were assigned as depicted in the structural drawing.

Discussion

Sparoxomycins A1 (**1**) and A2 (**2**) produced by *S. sparsogenes* SN-2325 showed remarkable flat reversion activity on the transformed morphology of *src*^{ts}-NRK cells at the wide range of concentrations. It is noteworthy that the morphology reversion was observed by the antibiotics at concentrations of 10 to 1000 μg/ml without cytotoxicity. After the antibiotics were removed from the culture, the morphology was reversed again to the transformed phenotype. Thus **1** and **2** are reversible inducers of the flat reversion activity on *src*^{ts}-NRK cells. Moreover, it was found that **1** and **2** were specific inhibitors at G_0/G_1 phase of the cell cycle of *src*^{ts}-NRK cells. The structures of **1** and **2** are closely related to the structure of sparsomycin which is an inhibitor of protein biosynthesis. Though sparsomycin has antitumor activity, there is no report of the flat reversion and cell cycle arresting activity of *src*^{ts}-NRK cells on sparsomycin. *S. sparsogenes* SN-2325 produced tuberucidin as in the case of the isolation of sparsomycin.[8] However we could not detected sparsomycin during the isolation of **1** and **2**. This observation suggests *S. sparsogenes* SN-2325 possesses strong oxidase activity for the oxidation of sparsomycin.

Experimental

Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8100M. UV spectra were obtained by a Hitachi 220A spectrophotometer. NMR spectra were recorded on JEOL JNM-α-400 and JNM-α-600 spectrometers. Chemical shifts are reported relative to TMS in DMSO-d_6, and residual DHO (δ 4.65) for ^{1}H NMR and dioxane (δ 67.6) for ^{13}C NMR in D_2O. Directly combined HPLC mass spectrometry measurements (LC-FAB/MS) are made using a JMS-LX2000 instrument (JEOL) with HP 1050 micro liquid chromatograph and Inertsil ODS (GL Science) controlled by a JMA-DA7000 data system (JEOL). CD spectra were recorded on a Jasco J-720 spectropolarimeter.

Isolation of **1** and **2**

As described in the preceding paper, *Streptomyces sparsogenes* SN-2325 was cultured in jar fermentors. The culture broth (36 liters) was centrifuged and the supernatant (32 liters) was treated with active carbon (Shirasagi, Takeda Chemical Industries, Japan) which was eluted with 50% acetone. The 50% acetone eluate was concentrated to aqueous solution which was applied to a column of active carbon (Charcoal, Activated for chromatography, Wako Pure Chemical Industries, Ltd.). The column was washed with MeOH and eluted with 80% acetone. Acetone was removed *in vacuo* to yield 24.07 g of a brown oil.

A part of the above oil (5 g) was subjected to medium pressure liquid chromatography using ODS column (LiChroprep RP18, E Merck) with 5% MeOH and then 10% MeOH. Active fractions were combined and concentrated to aqueous solution, and lyophilized to 94.65 mg of a crude powder. The powder was subjected to preparative HPLC (Senshu Pak, Pegasil-ODS, Senshu Scientific Company, Ltd.) eluted with 10% MeOH to give 9.2 mg of a crude powder. Final purification was carried out by HPLC using the same column with 8% MeOH to yield 1.35 mg of **1** and 2 mg of **2**.

The remains of the brown oil (19.07 g) obtained by active carbon column chromatography was subjected to centrifugal partition chromatography (Model CPC-LLN, Sanki Eng., Ltd.) using a solvent system *n*-BuOH - EtOH - H_2O (10 : 2.5 : 10) of lower stationary phase and upper mobile phase in a ascending mode. The active fractions were combined, concentrated *in vacuo* and applied to a Lobar column (LiChroprep RP18, E. Merck) eluted with 10% MeOH and then MeOH. The active fractions were combined, concentrated and lyophilized to yield 433 mg of a crude powder. The crude powder was subjected to HPLC (Senshu Pak, Pegasil-ODS, Senshu Scientific Company, Ltd.) eluted with 10% MeOH. Final purification was executed by using an analytical column (Senshu Pak, Pegasil-ODS, Senshu Scientific Company, Ltd.) eluted with 5% MeOH to yield

1.55 mg of **1** and 2.7 mg of **2**. Total isolated amounts of **1** and **2** were 2.9 mg and 4.7 mg, respectively.

Acknowledgments

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