STUDIES ON MYCOTRIENIN ANTIBIOTICS, A NOVEL CLASS OF ANSAMYCINS

II. STRUCTURE ELUCIDATION AND BIOSYNTHESIS OF MYCOTRIENINS I AND II

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The structures of mycotrienins I and II have been determined mainly by their NMR spectral analysis. Mycotrienins are unique ansamycin antibiotics containing a 21-membered macrocyclic lactam ring and a cyclohexylcarbonyl moiety. The labeling experiments with sodium [1-^{13}C]acetate and sodium [1-^{13}C]propionate revealed that six acetate units and two propionate units were incorporated into the molecule of mycotrienin I.

In the preceding paper[1], we reported the production, isolation, physicochemical properties and biological activities of mycotrienins I (MTN-I) and II (MTN-II), together with the taxonomy of the producing organism, *Streptomyces rishiriensis* T-23.

This report presents the structural elucidation and biosynthesis of the antibiotics in detail; a preliminary communication of our work has been presented[2].

Independently, ZEECK and his associates[3] reported the structures of ansatrienins A and B in his recent communication, which have the same structures as mycotrienins I and II, respectively, except for the stereochemistry of alanine.

Structural Elucidation of Mycotrienins

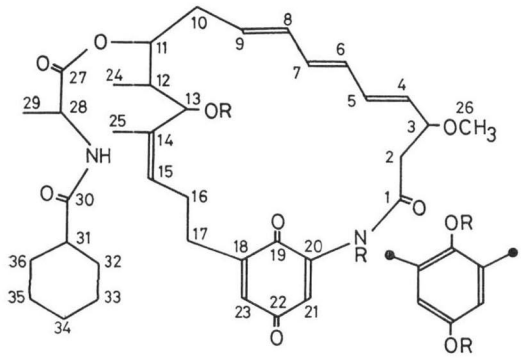
MTN-I (**1**), $C_{36}H_{48}N_2O_8$ (M^+, m/z 636), and MTN-II (**2**), $C_{36}H_{50}N_2O_8$ (M^+, m/z 638), are interconvertible *via* a redox reaction. This and ^{13}C NMR spectral considerations (see below) indicate the presence of a quinone group in the molecule. The UV spectra of both the antibiotics show absorption maxima at 260~263 nm, 270~273 nm and 280~282 nm characteristic of a triene group. Unlike ansatrienins, **1** afforded D-alanine** by acid hydrolysis[1].

Acetylation of **2** with pyridine-acetic anhydride in the presence of dimethylaminopyridine afforded a tetraacetate (**3**). The structure of this compound was established to be 13,19,22-tri-*O*-acetyl-1-(*N*-acetyl)mycotrienin II (Fig. 1). The similar *N*-acetyl derivative was reported for macbecin II[4].

The 100 MHz ^{13}C and 400 MHz ^{1}H NMR spectra of mycotrienins in pyridine-d_5 revealed the functional groups summarized in Table 1. The ^{13}C NMR spectral data are shown in Table 2. These data show that two quinone carbonyl groups of **1** were replaced by two quinone hydroxyl groups in the molecule of **2**.

** The chirality of the alanine was determined by ORD spectral data (see the preceding paper)[1]. ZEECK *et al.* reported the configuration of alanine in ansatrienins to be L.

Fig. 1. Structures of mycotrienins.



1: R=H **2**: R=H
3: R=Ac

Table 1. Functional groups of mycotrienins.

	MTN-I	MTN-II
$-CH_3$	3	3
$-OCH_3$	1	1
$-CH_2-$	9	9
$>CH-$	1	1
CH–CO–	2	2
>CHOH	1	1
>CHO–	2	2
–CH=	9	9
>C=	3	5
–CO–NH–	2	2
–CO–O–	1	1
Quinone CO	2	—
Phenolic OH	—	2

Table 2. ^{13}C NMR spectral data for mycotrienin-II.

No.	δ_c*	T_1 value**	No.	δ_c*	T_1 value**
C-1	170.3	2.21(sec)	C-19	141.7	5.19(sec)
2	43.1	0.19	20	127.7	2.67
3	80.7	0.30	21	108.1	0.27
4	131.1	0.28	22	151.3	2.21
5	135.8	0.29	23	116.4	0.27
6	130.5[a]	0.28	24	9.8	0.77
7	134.8	0.31	25	21.1	1.00
8	133.8	0.30	26	56.7	1.36
9	130.6[a]	0.30	27	173.1	2.03
10	33.6	0.15	28	49.5	0.39
11	75.4	0.25	29	17.2	0.55
12	38.9	0.30	30	176.8	2.56
13	68.1	0.32	31	44.9	0.75
14	139.8	1.43	32	30.0[b]	0.44
15	123.8	0.29	33	25.9[c]	0.38
16	27.0	0.19	34	26.0[c]	0.38
17	32.3	0.19	35	26.1[c]	0.38
18	132.9	1.45	36	29.9[b]	0.44

* δ_c in pyridine-d_5, ** T_1 values in $CDCl_3$.
[a,b,c] Assignments may be interchanged.

Since the separation of the ^1H NMR signals of **2** were much better than that of **1**, further structural elucidation of the mycotrienins was mainly based on the NMR spectral analysis of **2**. There are three carbonyl carbons present in **2** (δ_c 170.3, 173.1 and 176.8 in pyridine-d_5). Since the two nitrogen atoms contained in **2** did not show any basicity, two of the carbonyl carbons in **2** are assigned to amide functions. In agreement with this, two amide protons appeared at δ_H 8.78 (1H, bs) and 9.01 (1H, d, J=7.0 Hz), in addition to two quinone hydroxyl signals at δ_H 11.0 and 11.24 in the ^1H NMR spectrum of **2**. Furthermore, deuterium induced upfield shifts were observed *inter alia* with two carbonyl resonances at δ171.5 ($\Delta\delta_C$−0.085) and 179.2 (−0.085) in the ^{13}C NMR spectrum of **2** taken in CD_3OD/CD_3OH[5] as shown in Fig. 2. The remaining

Fig. 2. ^{13}C NMR signals of MTN-II in CD_3OD/CD_3OH (1: 1) (a) and in CD_3OD (b).

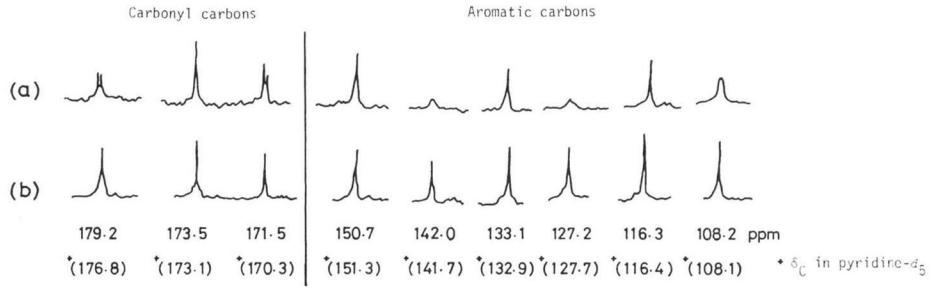
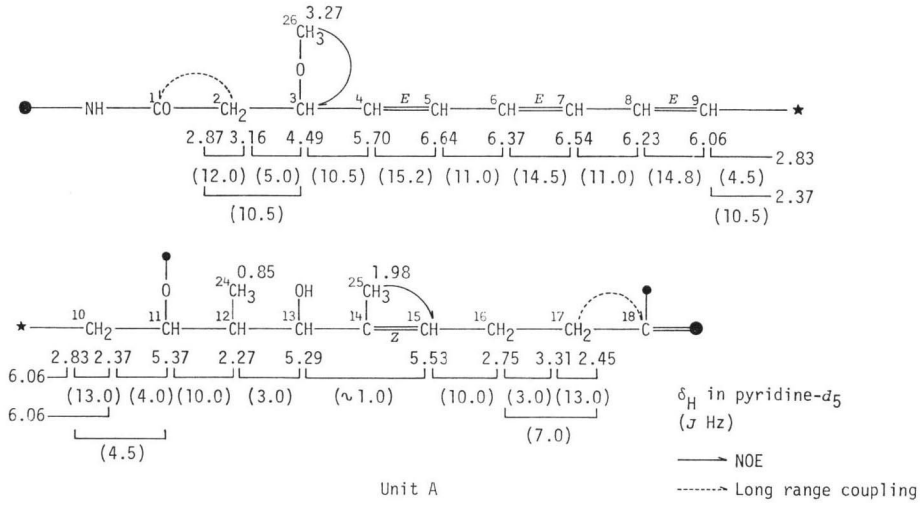


Fig. 3. Structure of Unit A.



carbonyl carbon (δ_C 173.5) is ascribed to an ester residue, which is also supported by IR absorption at 1735 cm^{-1}.

Consecutive proton spin decoupling experiments on **2** (400 MHz in pyridine-d_5 revealed the sequence from C-2 to C-17 as shown in Unit A (Fig. 3). The NOE observed with the oxymethine signal H-3 (δ_H 4.49) upon irradiation of the methoxy protons (at δ_H 3.27) indicated that C-3 must be connected to the methoxy function. This relationship is corroborated by the ^1H and ^{13}C NMR chemical shifts (δ_{H-3} 4.49 and δ_{C-3} 80.7). The hydroxyl function was located at C-13 as follows. In the ^1H NMR spectrum of **2** taken in $CDCl_3$, H-13 appeared at δ_H 4.78 (δ_H 5.29 in pyridine-d_5), while it moved downfield to δ_H 5.65 in that of tetraacetate **3**. On the other hand, the chemical shift of H-11 remained almost unchanged (δ_H 4.96 in **2** and 4.87 in **3**) showing that the oxygen at C-11 is protected by an ester linkage.

In order to extend further this partial structure, use was made of ^{13}C-{^1H} long range selective proton decoupling (in CD_3OD). Thus, irradiation at δ_H 2.91 (H-2, δ_H 3.16 in pyridine-d_5) and 2.10 (H-17, δ_H 2.45 in pyridine-d_5) collapsed sp^2 carbons at δ_C 171.5 and 133.1, respectively, affording evidences that C-2 must be combined to the amide carbon (C-1) and C-17 to a quaternary sp^2 carbon (C-18).

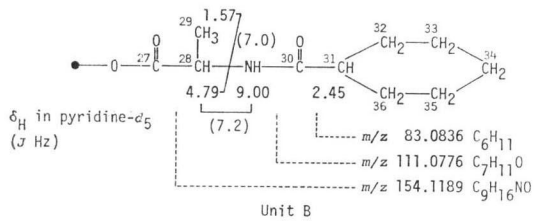
The configurations of the triene moiety were revealed to be all *E* by the coupling constants of J_{H4-H5} =15.2 Hz, J_{H6-H7}=14.5 Hz and J_{H8-H9}=14.8 Hz. The NOE observed between the methine proton at δ_H 5.53 (H-15) and the methyl protons at δ_H 1.98 (H-25) indicated the *Z* configuration of the double bond at C-14. This was confirmed by the downfield ^{13}C NMR chemical shift of the methyl carbon C-25 (δ_C 21.1).

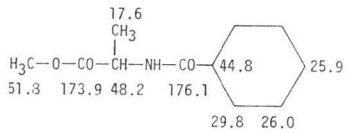
Thus, the partial structure, Unit A, has been unambiguously established as shown in Fig. 3.

The structure of Unit B was assigned by ^1H and ^{13}C NMR spectral data (Fig. 4) as well as by mass spectral analysis.

In the high resolution mass spectrum of **2**, fragment peaks were observed at *m/z* 83.0836 (C_6H_{11}, calcd. 83.0859), 111.0776 ($C_7H_{11}O$, calcd. 111.0809) and 154.1189 ($C_9H_{16}NO$, calcd. 154.1230). This implies that Unit B is a cyclohexanecarbonylalanine moiety. This structure is also supported by considerably longer relaxation times (T_1) of the relevant five methylene carbons (0.38 ~ 0.44 second) as compared to those of methylenes present in Unit A (0.15 ~ 0.19 second) as seen in Table 2. In agreement

Fig. 4. Structure of Unit B.





with this partial structure, the treatment of **2** with $NaHCO_3$ - MeOH for overnight afforded a compound (**4**), which was assigned as cyclohexanecarbonyl-D-alanine methyl ester based on the ^1H NMR and mass spectral analyses. Spectroscopic date of this compound, except for optical rotation, were completely identical with those of a synthetic sample prepared by the condensation of cyclohexanecarbonyl chloride and L-alanine in an aqueous NaOH solution followed by treatment with diazomethane. Thus, **4** was identified as cyclohexanecarbonyl-D-alanine methyl ester.

Since the carbonyl carbon in Unit B (C-27, δ_C 173.1) was assigned to an ester function by the deuterium induced upfield shift mentioned above and **4** was obtained by alkaline treatment, Unit B must be connected to C-11 through an ester linkage. It had been proved by the ^1H NMR spectral analysis of **3** that the oxygen at C-11 must be protected by an ester linkage. Thus, Unit B is connected to C-11 through an ester linkage (*vide supra*).

The remaining carbons in **2** not contained either in Unit A or B are two –C= (δ_C 127.7 and 132.9), two hydroquinone enol groups (δ_C 141.7 and 151.3, δ_H 11.0 and 11.24) and two –CH= (δ_C 108.1 and 116.4, δ_H 7.12, 2H in pyridine-d_5). The two aromatic protons were observed as an AB quartet in CD_3OD (δ_H 6.48 and 6.55, J=3.7 Hz). In the ^1H NMR spectrum of **1** in $CDCl_3$ two aromatic protons were also observed as an AB quartet (δ_H 6.52 and 7.51, J=2.4 Hz). The ^{13}C NMR spectrum of **1** in $CDCl_3$ showed two quinone carbons (δ_C 188.2 and 182.5), two –C= (δ_C 145.4 and 137.9) and two –CH= (δ_C 133.1 and 114.5). These NMR spectral data together with facile interconversion between **1** and **2** indicate that a 2,6-disubstituted *p*-benzoquinone nucleus and its hydroxy form are present in **1** and **2**, respectively, (Unit C shown in Fig. 5).

The linkage between C-17 of Unit A and the quinone nucleus was proved by the application to **2** in CD_3OD of ^{13}C-{^1H} long rang selective proton decoupling. Irradiation of the H-17 methylene signal (δ_H 2.91) collapsed the C-18 (δ_C 133.1) and C-23 (δ_C 116.3) aromatic signals. Although long range coupling between H-17 and H-23 was too small to be observed in **2**, localization of a double bond between C-18 and C-23 in the oxidized

Fig. 5. Structure of Unit C.

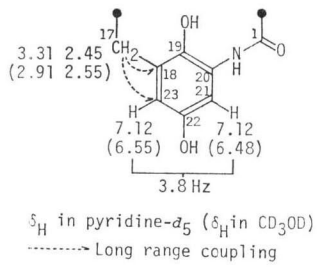
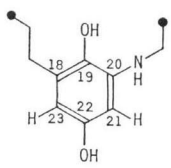


Table 3. ^{13}C NMR chemical shifts of the quinone nucleus and their calculated values in $CDCl_3$.

No.	Calcd.	Found
C-18	132.8 ppm	132.7 ppm
C-19	139.5	141.1
C-20	126.9	125.5
C-21	106.5	107.5
C-22	148.2	149.2
C-23	112.8	115.8



form **1** enabled the observation of the relationship between these protons (in $CDCl_3$, $\delta_{H\text{-}17}$ 2.31 and 2.39; $\delta_{H\text{-}23}$ 7.47, $J_{17,23}$=1.46 Hz).

In the ^{13}C NMR spectrum of **2** taken in CD_3OD/CD_3OH, deuterium induced upfield shifts (*vide supra*) were noted for C-19 (δ_C 142.0, broadening), C-20 (δ_C 127.2, $\Delta\delta$ −0.097) and C-21 (δ_C 108.2, $\Delta\delta$ −0.073) as shown in Fig. 2. Since this phenomenon is caused by a through bond effect, the only remaining NH group, at C-1 in Unit A, must be connected to C-20 of the quinone ring. The chemical shifts of these carbons are compatible with calculated values[5] (see Table 3).

Accordingly, the structures of MTN-I (**1**) and -II (**2**) have been unambiguously assigned as shown in Fig. 1.

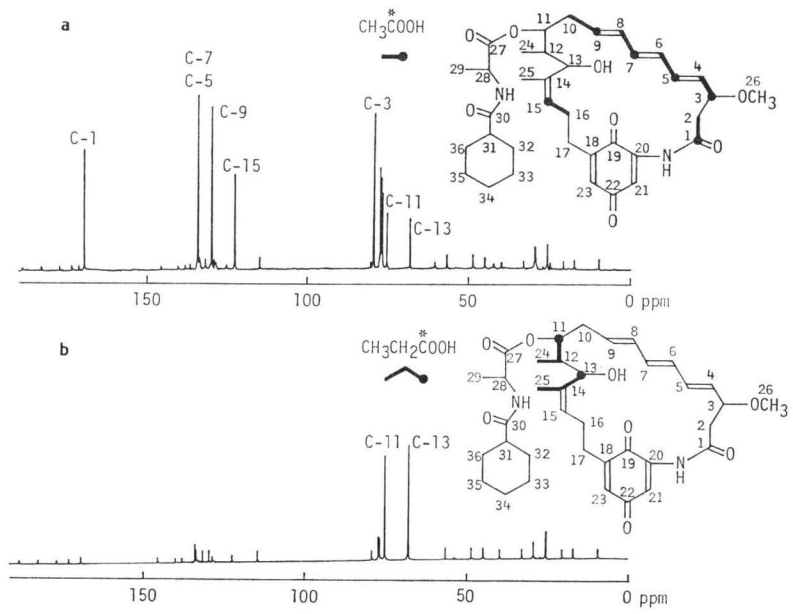
Biosynthesis of Mycotrienins

The incorporation pattern of several biosynthetic precursors was studied as described below. *Streptomyces rishiriensis* T-23 was cultured in a 500-ml Erlenmeyer flask containing 100 ml of the medium consisting of 1.0% glucose, 1.5% starch, 1.5% soybean meal, 0.2% dry yeast, 0.5% NaCl and 0.4% $CaCO_3$ (pH 7.0) on a rotary shaker.

In separate experiments sodium [1-^{13}C]acetate (50 mg) and sodium [1-^{13}C]propionate (5 mg) were added to the fermentation broth at 26, 28, 30, 32 and 34 hours after inoculation, and after a further 24 hours ^{13}C-labeled mycotrienin was isolated in the oxidized form (**1**) from the mycelium.

In the ^{13}C NMR spectrum of the sodium [1-^{13}C]acetate labeled **1**, the signal intensities of carbons 1, 3, 5, 7, 9 and 15 were increased by 10~20 fold, while [1-^{13}C]propionate was incorporated into carbons 11 and 13 by *ca.* 8 fold as shown in Fig. 6. In addition, the spectrum in Fig. 6a shows that carbons 11 and 13 are also enriched by 4~5 fold with sodium [1-^{13}C]acetate. This indicates the indirect incorporation of the precursor through conversion to succinate and propionate in a similar manner as reported by ŌMURA *et al.*[6] Thus, the mycotrienin molecule is built up from six acetate units and two propionate units as shown in Fig. 6.

Fig. 6. ^{13}C NMR spectra of MTN-I from [1-^{13}C]acetate (a) and [1-^{13}C]propionate (b) in $CDCl_3$.



Discussion

Mycotrienins I and II are closely related to macbecins I and II[4] in their structures. However, they are unique among the ansamycin group in that mycotrienin has a 21-membered macrocyclic lactam ring and in that the cyclohexanecarbonyl moiety was found for the first time in this group. ω-Cyclohexyl fatty acids have been reported as metabolites of *Curtobacterium pusillum*[7] and as a component of asukamycin[8].

The results of the incorporation experiments have shown that mycotrienin is of polyketide origin. The quinone moiety of mycotrienin may be derived from a C_7N unit in a similar manner to that suggested for rifamycin[9], geldanamycin[10] and pactamycin[11]. OSHIMA and ARIGA[12] reported that the ω-cyclohexyl group of carboxylic acids was formed from glucose, *via* the shikimate pathway. Presumably, the same mechanism may be operative in the biosynthesis of mycotrienin.

Experimental

NMR spectra were obtained on a JEOL FX-400 with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz. Mass spectra were measured on a Hitachi M-80 spectrometer. Sodium [1-^{13}C]acetate and sodium [1-^{13}C]propionate were obtained from Merck Sharp & Dohme.

Acetylation of **2**

To a solution of **2** (25 mg) in pyridine (0.5 ml) were added acetic anhydride (0.5 ml) and dimethylaminopyridine (0.3 mg) and the mixture was kept at room temperature for 16 hours. The reaction mixture was poured into cold water and extracted with ethyl acetate. The solution was washed with a saturated NaCl solution and dried over Na_2SO_4. After evaporation of the solvent, the residue was subjected to preparative TLC (benzene - chloroform - ethyl acetate, 1: 1: 1). Appropriate fractions eluted by chloroform - methanol (10: 1) were collected to give **3** as a white powder (15 mg): mp 117°C, MS (M^+) *m/z* 806.3961 (calcd. for $C_{44}H_{58}N_2O_{12}$, 806.3985), ^1H NMR 2.05 (–OAc), 2.10 (–OAc), 2.21 (–OAc) and 2.35 ppm (–$\overset{1}{N}$Ac)[4] for acetyl signals.

Alkaline Degradation of **2**

2 (100 mg) was dissolved in 5 ml of MeOH, 50 mg of $NaHCO_3$ was added, and the mixture was stirred at room temperature for 16 hours. The reaction mixture was filtered and diluted with water. The solution was extracted with ethyl acetate, and the organic extract was washed with a saturated NaCl solution and dried over Na_2SO_4. After evaporation of the solvent, the residue was subjected to silica gel column chromatography and eluted with benzene. **4** was obtained as needles (6 mg); mp 67~68°C, MS (M^+) *m/z* 213.1354 (calcd. for $C_{11}H_{19}NO_3$: 213.1363). $[\alpha]_D^{20}$ −4.0° (*c* 1.8, $CHCl_3$).

Synthesis of **4**

L-Alanine (0.9 g) and cyclohexanecarbonyl chloride (1.2 g) were dissolved in 1 N sodium hydroxide solution (20 ml), and the mixture was kept at 0°C for 1 hour. The reaction mixture was filtered and the precipitate was dissolved in MeOH. Addition of ethyl acetate and storage at room temperature gave a white powder of cyclohexanecarbonylalanine (0.32 g), mp 173~174°C. MS *m/z* 199 (M^+). ^1H NMR (pyridine-d_5) δ 1.15 (3H, m), 1.53 (1H, bs), 1.64 (3H, d), 1.75 (4H, m), 2.02 (2H, bt), 2.45 (1H, tt), 5.16 (1H, dq), 8.57 (1H, bd). ^{13}C NMR (CD_3OD) δ 17.6 (CH_3), 26.7 ($CH_2\times2$), 26.9 (CH_2), 30.49 (CH_2), 30.54 (CH_2), 46.0 (*C*H–CO–), 49.1 (CH–NH), 176.2 (C=O), 178.9 (C=O). *Anal.* Found: C 60.17, H 8.91, N 6.57, O 24.35. Calcd. for $C_{10}H_{17}NO_3$: C 60.30, H 8.54, N 7.04, O 24.12. IR ν_{max}^{Nujol} 3250, 1750, 1605, 1555, 1220, 1210, 1160 cm^{-1}.

The white powder (30 mg) was suspended in chloroform and treated with ethereal diazomethane. The reaction mixture was filtered and evaporated to dryness. **4** was obtained as needles (32 mg). mp 67~68°C. MS (M^+) *m/z* 213.1360 (calcd. for $C_{11}H_{19}NO_3$: 213.1363). ^1H NMR ($CDCl_3$) δ 1.24 (3H, m), 1.41 (3H, d), 1.45 (2H, m), 1.70 (1H, m), 1.80 (2H, m), 1.88 (2H, m), 2.14 (1H, tt), 3.68 (3H, s), 4.61 (1H, dq), 6.04 (1H, bs). ^{13}C NMR (pyridine-d_5) δ 17.6 (CH_3), 26.0 ($CH_2\times3$), 29.8 ($CH_2\times2$), 44.8 (*C*H–CO–), 48.2 (CH–NH), 51.8 (OCH_3), 173.9 (C=O), 176.1 (C=O). IR $\nu_{max}^{CHCl_3}$ 3250, 2980, 2900, 2840, 1730, 1650, 1500, 1445, 1375, 1340, 1305, 1220, 1190, 1175, 1155, 1120 cm^{-1}.

Purification of ^{13}C-Labeled **1**

After filtration of the fermentation broth (100 ml) the mycelium was suspended in 100 ml of 60% aqueous acetone and stirred for 2 hours. The supernatant was concentrated to a small volume and extracted with ethyl acetate. After drying over Na_2SO_4, the solvent was evaporated to dryness. The residue was dissolved in 1% methanolic $FeCl_3$ solution and stirred for 30 minutes. The reaction mixture was diluted with water and extracted with ethyl acetate, and the solution was evaporated to dryness and subjected to preparative TLC (Merck F_{254}, benzene - chloroform - ethyl acetate, 1 : 1 : 1). Appropriate fractions eluted by methanol were collected to give ^{13}C-labeled **1** (3~5 mg).

Acknowledgement

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