ISOLATION AND STRUCTURE ELUCIDATION OF NEW ANTIBIOTICS RELATED TO ANGOLAMYCIN

JUDIT KÁDÁR-PAUNCZ, BENJAMIN PODÁNYI* and GYULA HORVÁTH

Institute for Drug Research,
Budapest, P.O.B. 82, H-1325, Hungary

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Angolamycin (**1**) and two novel analogues (**2** and **3**) were isolated from the culture broth of a *Streptomyces* strain. NMR and MS analysis proved that **2** is the 18-dihydro-, while **3** is the 18-deoxo-18-dihydro derivative of angolamycin. Full experimental assignment of the ^{1}H and the ^{13}C NMR spectra of these compounds was obtained from 1D and 2D chemical shift correlation measurements. Compounds **2** and **3** are less potent antibiotics than angolamycin.

In the course of our screening program of soil microorganisms producing new antibiotics angolamycin (**1**) and two of its derivatives (**2**~**3**) were isolated from fermentation broth of a *Streptomyces* strain (86-070). Angolamycin was first isolated by CORBAZ *et al.*[1] in 1955, and described as a macrolide antibiotic. Later its sugar constituents were isolated and identified after acidic hydrolysis of angolamycin[2]. The structure of angolamycin was proposed by KINUMAKI and SUZUKI based on a few assigned signals of its low field ^{1}H NMR spectrum and on the similarity of its mass spectral fragmentation to tylosin[3]. The identity of angolamycin with shincomycin A[4] was also proved in this study[3]. CI-MS data were also in agreement with the proposed structure[5], however, no detailed study on the structure of angolamycin has been published so far. 18-Dihydro-angolamycin as a new, naturally occuring antibiotic, isolated from *Streptomyces* sp. SK-62 is listed in the first table of ŌMURA's book[6] (p. 6), however, it is characterized only by its UV absorption maximum.

Fermentation and Isolation of Compounds **1**~**3**

The antibiotics were produced by *Streptomyces* sp. (86-070), fermented for 72 hours. The taxonomy and the details of fermentation of this strain will be reported elsewhere.

The bioactive substances were isolated from the broth filtrate by adsorption on Amberlite XAD-2 resin and elution with methanol. The eluate was concentrated and then fractionated on a Sephadex LH-20 column. Each fraction was monitored by TLC and fractions containing compounds **1**~**3** were collected. Preparative TLC on silica gel was used for further separation and purification of compounds **1**~**3**. Compound **2** was separated from the mixture using benzene-acetone (1:4), then

Table 1. Rf values of compounds **1**~**3** and related macrolides in TLC.

Compounds	Rf × 100			
	Solvent systems			
	I	II	III	IV
1 (Angolamycin)	48	43	53	51
2	40	33	46	52
3	45	50	56	54
Erythromycin	10	11	15	51
Carbomycin	84	75	89	66
Tylosin	30	14	40	56

Stationary phase: Silica gel 60 F_{254} (E. Merck).
Mobile phase: Solvent system I, benzene-acetone (1:4); II, chloroform-methanol (12:1); III, benzene-methanol-acetone (4:1:5); IV, *n*-butanol-acetic acid-water (3:1:1).

compound **1** was separated from compound **3** using chloroform-methanol (12:1). The purity of the compounds was checked by TLC using several eluent systems. Rf values of compounds **1**~**3** in different eluent systems are summarized in Table 1 together with the data of some related macrolides.

Characterization and Structure Elucidation of Compounds **1**~**3**

Compounds **1**~**3** are white powders readily soluble in methanol, ethyl acetate and chloroform, less soluble in benzene and insoluble in water and apolar organic solvents like hexane. The UV spectra of all three compounds are almost identical having absorption maxima at 240 nm.

The IR spectrum of compound **1** is identical with the published spectra of angolamycin[1,4]. Fig. 1 shows the double bond stretching region of the IR spectrum of compounds **1** and **2**. The bands at 1620 cm^{-1} and at 1690 cm^{-1} were assigned to the $\nu_{C=C}$ and to the $\nu_{C=O}$ stretching vibrations of the α,β-unsaturated keto group. The band at 1730 cm^{-1} in the spectrum of compound **1** consists of the $\nu_{C=O}$ stretching vibrations of the lactone and the aldehyde moieties. The intensity of this band is reduced in the spectrum of **2** suggesting that one of these functions is missing from compounds **2**. This is the only difference between the IR spectra of compounds **1** and **2**. The IR spectrum of compound **3** is nearly identical with the IR spectrum of compound **2**.

The EI mass spectrum of compound **1** showed a molecular ion at *m/z* 915 and prominent ions at *m/z* 771: $(M-(\text{L-mycarosyl}-H))^{+\cdot}$, *m/z* 696, *m/z* 580, *m/z* 405: $(\text{aglycone})^+$, *m/z* 302: (angolosaminyl−L-mycarose)$^+$, *m/z* 158: $(302-(\text{L-mycarosyl}-H))^+$. The EI mass spectrum of compound **3** showed a similar pattern with a mass shift of −14 dalton (CH_3 instead of CHO group), a molecular ion at *m/z* 901 and prominent ions at *m/z* 757, 682, 566, 391, 302, 158 (assignments see above). Good quality EI mass spectrum of compound **2** could not be taken. The DCI mass spectrum of compound **2** showed a quasi-molecular-ion $(M+H)^+$ at *m/z* 918 and prominent ions at *m/z* 774 $(M+H-(\text{L-mycarosyl}-H))^+$, *m/z* 320 (HO−angolosaminyl−L-mycarose+H)$^+$, *m/z* 302, 158 (see above).

Fig. 1. IR spectra of compounds **1** and **2**.

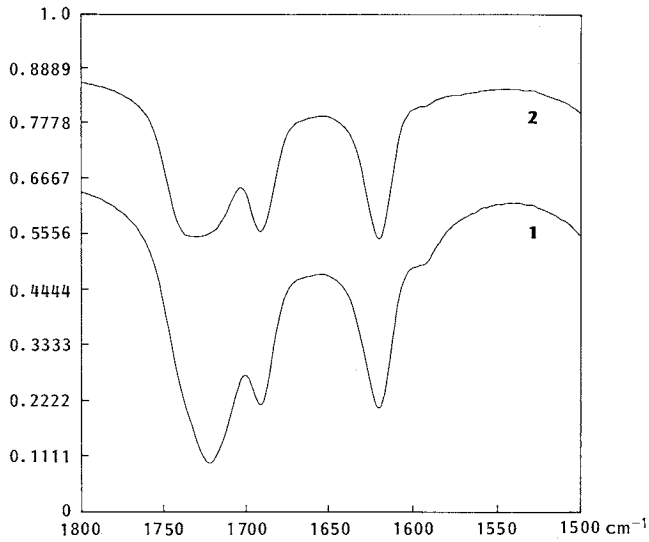
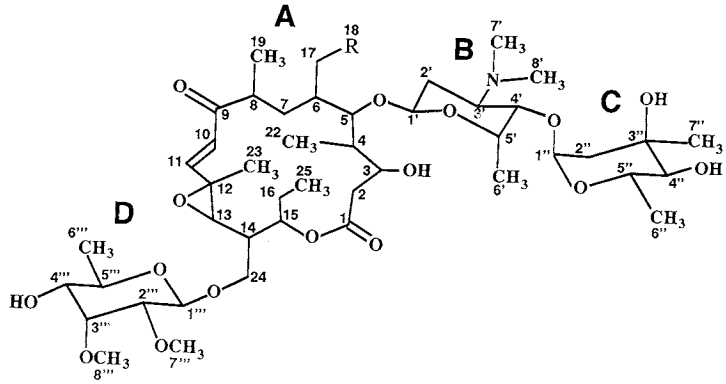


Fig. 2. The structure of compounds **1**~**3**.



1: R=CHO, **2**: R=CH_2OH, **3**: R=CH_3.
Numbering is according to ref 7.

Table 2. ^1H NMR chemical shifts of compound **2**[i].

	A	B	C	D
1-H	—	4.40 dd	5.07 dd	4.57 d
2-H_a	2.59 dd	1.48[h]	1.75[c]	3.10 dd
2-H_b	2.09[h]	2.03[h]	2.02[c]	—
3-H	3.82[h]	2.54[h]	—	3.78[h]
4-H	1.67[c]	3.27[h]	2.93 d	3.22[h]
5-H	3.75[c,h]	3.22[h]	4.13 dq	3.54[h]
6-H_x	1.7[c] (1H)	1.26[c] (3H)	1.28[c] (3H)	1.27[c] (3H)
7-H_x	1.66[c] (2H)	2.26 s (3H)	1.23 s (3H)	3.56 s (3H)
8-H_x	2.74[h] (1H)	2.26 s (3H)	—	3.62 s (3H)
10-H	6.44 d	—	—	—
11-H	6.58 d	—	—	—
13-H	3.18[h]	—	—	—
14-H	1.74[h]	—	—	—
15-H	5.32 ddd	—	—	—
16-H_a	1.92[h]	—	—	—
16-H_b	1.54[h]			
17-H_a	1.97[h]			
17-H_b	1.68[h]			
18-H_2	3.66[h]			
19-H_3	1.17 d			
22-H_3	0.98 d			
23-H_3	1.42 s			
24-H_a	4.15 dd			
24-H_b	3.66[h]			
25-H_3	0.90 t			

[i] In $CDCl_3$; $\delta_{TMS}=0.00$ ppm; characteristic coupling constants (Hz): in A (macrolide ring): $^2J_{2a,2b}=16.8$; $J_{2a,3}=10.8$; $J_{10,11}=15.7$; $J_{14,24a}=3.2$; $^2J_{24a,24b}=10.6$; in B (angolosamine ring): $J_{1,2a}=9.3$; $J_{1,2b}=1.4$; in C (mycarose ring): $J_{1,2a}=2.9$; $J_{1,2b}\simeq 0.5$; $^2J_{2a,2b}\simeq 15$; $J_{4,5}=9.6$; in D (mycinose ring): $J_{1,2}=7.7$; $J_{2,3}=2.7$.

a: Denote axial proton in the sugar rings or one of the protons of a methylene group in the macrolide ring where stereochemical assignment can not be made.

b: Denote equatorial proton in the sugar rings or one of the protons of a methylene group in the macrolide ring where stereochemical assignment can not be made.

[c] The chemical shift of the overlapping signal was determined from a COSY crosspeak.

d: Doublet; q: quartet; s: singlet; t: triplet.

[h] The chemical shift of the overlapping signal was determined from a crosspeak of the 2D heteronuclear chemical shift correlation spectrum.

Based on these results compound **1** was identified as angolamycin, **2** as dihydro-angolamycin and **3** as dihydro-deoxo-angolamycin.

The structure elucidation of compounds **1**~**3** was completed by NMR spectroscopy. ^{1}H and ^{13}C NMR spectra of all three compounds were recorded in $CDCl_3$. DEPT spectra were used to obtain the multiplicities of the ^{13}C NMR signals. ^{1}H and ^{13}C NMR spectral data of compound **1** are in agreement with the structure of angolamycin. Comparing the spectra of **2** and **3** with the spectra of **1** we observed, that the aldehyde signal is missing in the ^{1}H and the ^{13}C NMR spectra of both **2** and **3**, however a new triplet appears at 60.9 ppm in the ^{13}C NMR spectrum of **2** and a new methyl signal in the spectra of **3**. Therefore, the structure of **2** was established as 18-dihydro-angolamycin and the structure of **3** as 18-deoxo-18-dihydro-angolamycin (Fig. 2).

NMR Study of Compounds **1**~**3**

Only a few assigned signals of the low field ^{1}H NMR spectrum of angolamycin was reported in the literature so far. The ^{13}C NMR spectrum of angolamycin has not been published yet. A comprehensive study on several other 16-membered macrolide antibiotics was reported in 1975[7], however, the spectral assignment in this work is mostly based on chemical shift comparison of related compounds.

Therefore we decided to perform a detailed NMR study of compound **2**. ^{1}H and ^{13}C NMR data of this compound are summarized in Tables 2 and 3, respectively. Unambiguous assignment of the spectra was obtained from chemical shift correlation measurements. Correlations based on

Table 3. ^{13}C NMR chemical shifts of compounds **1**~**3**[a].

	1	**2**	**3**		**1**	**2**	**3**
Ring A				Ring B			
C(1)	173.3 s	173.5 s	173.8 s	C(1)	101.4 d	102.3 d	102.3 d
C(2)	39.8 t	39.7 t	39.9 t	C(2)	27.8 t	27.5 t	27.5 t
C(3)	66.4 d	66.9 d	66.7 d	C(3)	64.3 d	64.3 d	64.1 d
C(4)	41.1 d	41.0 d	45.1 d	C(4)	74.6 d	75.2 d	75.2 d
C(5)	81.8 d	81.8 d	81.1 d	C(5)	73.1 d	73.3 d	73.2 d
C(6)	31.1 d	32.9 d	38.4 d	C(6)	19.1 q	19.1 q	17.8 q
C(7)	31.5 t	32.8 t	33.0 t	C(7,8)	40.8 q	40.9 q	40.9 q
C(8)	45.0 d	44.9 d	45.1 d	Ring C			
C(9)	200.2 s	201.2 s	201.0 s	C(1)	96.6 d	96.7 q	96.6 d
C(10)	122.6 d	123.1 d	122.9 d	C(2)	41.0 t	41.0 t	40.9 t
C(11)	151.3 d	150.8 d	150.5 d	C(3)	69.8 s	69.5 s	69.6 s
C(12)	59.4 s	59.4 s	59.4 s	C(4)	76.6 d	76.5 d	76.5 d
C(13)	64.4 d	64.4 d	64.4 d	C(5)	66.4 d	66.0 d	66.0 d
C(14)	43.6 d	43.5 d	43.6 d	C(6)	18.4 q	18.4 q	18.4 q
C(15)	74.0 d	74.0 d	73.9 d	C(7)	25.6 q	25.4 q	25.4 q
C(16)	24.7 t	24.8 t	24.7 t	Ring D			
C(17)	43.6 t	31.6 t	21.1 t	C(1)	101.0 d	100.9 d	100.9 d
C(18)	202.7 d	60.9 t	12.0 q	C(2)	81.9 d	81.9 d	81.9 d
C(19)	17.4 q	17.4 q	17.5 q	C(3)	79.7 d	79.7 d	79.6 d
C(22)	9.5 q	9.8 q	9.9 q	C(4)	72.7 d	72.7 d	72.7 d
C(23)	15.0 q	15.0 q	14.9 q	C(5)	70.8 d	70.8 d	70.8 d
C(24)	67.3 t	67.4 t	67.3 t	C(6)	17.8 d	17.8 d	17.8 d
C(25)	9.2 q	9.2 q	9.2 q	C(7)	59.7 q	59.7 q	59.7 q
				C(8)	61.7 q	61.7 q	61.7 q

[a] In $CDCl_3$, $\delta_{TMS}=0.00$ ppm.
s: Singlet, d: doublet, t: triplet, q: quartet, multiplicities obtained from DEPT experiments.

^{1}H-^{1}H J couplings were detected using 2D double-quantum filtered COSY spectra[8] (first column in Table 4). Correlations transferred by two consecutive ^{1}H-^{1}H couplings were detected in 2D relayed COSY experiments[9] (second column in Table 4). Correlations based on long range ^{13}C-^{1}H couplings were detected in series of semiselective INEPT (INAPT) experiments[10] using different delay values optimized for the coupling constants (third column in Table 4). Furthermore correlations based on $^{1}J_{C,H}$ couplings were detected using 2D heteronuclear shift correlation measurements[11] for all protonated carbons.

These experiments provided correlations to all signals, then we need to know the assignment of only one signal as the starting point, and following the coupling pathways all signals can be attributed. There are several peaks which can be unambiguously assigned from the 1D spectra. Starting from these signals self consistent assignment was achieved in all cases. The ^{13}C NMR chemical shifts of compounds **1** and **3**, which were assigned based on chemical shift comparison with **2**, are summarized also in Table 3. There are couplings between atoms of different rings, this information shed light to the

Table 4. The results of chemical shift correlation experiments of compound **2**.

^{1}H NMR signal	Correlated signals		
	COSY	Rely COSY	INAPT
Ring A			
2-H_a	2-H_b, 3-H		
2-H_b	3-H		
3-H	4-H	22-H_3	
4-H	5-H, 22-H_3		
5-H	6-H		
7-H	8-H		
8-H	19-H_3		C(6), C(7), C(9), C(10), C(19)
10-H	11-H		C(9), C(12)
11-H			C(9), C(23)
13-H	14-H	15-H	
14-H	15-H, 24-H_a, 24-H_b		
15-H	16-H_a, 16-H_b	24-H_b, 25-H_3	C(1), C(13), C(14), C(25)
16-H_a	25-H_3		
16-H_b	25-H_3		
17-H_b	18-H_2		
19-H_3			C(7), C(8), C(9)
22-H_3			C(3), C(4), C(5)
23-H_3			C(11), C(12), C(13)
25-H_3			C(15), C(16)
Ring B			
1-H	2-H_a, 2-H_b	2-H_b, 3-H	C(5-Ring A), C(2)
2-H_a	2-H_b, 3-H	4-H	
2-H_b	3-H	4-H	
5-H	6-H_3		
Ring C			
1-H	2-H_a, 2-H_b		C(4-Ring B), C(3), C(5)
4-H		6-H_3	C(3), C(5), C(6), C(7)
5-H	6-H_3		
Ring D			
1-H	2-H	3-H	C(24-Ring A), C(3), C(5)
2-H	3-H		
3-H	4-H	5-H	
4-H	5-H		
5-H	6-H_3		

sequence of the sugar units, and the position of their attachment to the macrolide ring. The long range ^{13}C-^{1}H coupling between 1'-H and C(5) detected in an INAPT experiment supports evidence for the junction of the disaccharide unit to 5-oxygen (earlier it was a matter of question whether it is attached to 5-OH or to 3-OH)[3].

Biological Properties

Compounds **1**~**3** are active against Gram-positive bacteria. Compound **1** shows limited activity against some Gram-negative strains. The minimum inhibitory concentration (MIC) of compounds **1**~**3** was determined against various bacteria by agar dilution method, the results are shown in Table 5. Compound **2** was active against Gram-positive bacteria but less active than angolamycin, and compound **3** was almost inactive against the bacteria tested. The acute toxicity of compound **1** (angolamycin) was tested by intraperitonial administration to mice. It was not toxic at ten doses of 500 mg/kg.

Table 5. Antimicrobial activities of compounds **1**~**3**.

Test organisms	MIC (μg/ml)		
	1	**2**	**3**
Bacillus subtilis ATCC 6633	0.16	6.25	50
Staphylococcus aureus Smith	2.5	50	50
S. aureus 1110 pen. rez.	10	100	100
Streptococcus pneumoniae	10	50	100
S. pyogenes A 118	2.5	50	100
S. pyogenes A 115 ROBB	10	50	100
Staphylococcus epidermidis ATCC 12228	3.2	50	100
S. epidermidis ATCC 6538P	1.6	50	100
Klebsiella pneumoniae ATCC 10071	12.5	100	100
Escherichia coli 6R	100	100	100

MICs were determined by the agar dilution method using PB B243 (Oxoid).

Discussion

Our detailed NMR study fully supports the proposed structure of angolamycin[3]. The 18-dihydro and the 18-deoxo-18-dihydro derivatives of the 18-aldehyde containing macrolide antibiotics rosamicin and M-4365G_2 were known as natural products[6], while the 18-deoxo-18-dihydro derivative of angolamycin is a new antibiotic. The comparison of our unambiguously assigned ^{13}C NMR chemical shifts with the more empirical literature shift values of closely related antibiotics, carbomycin, tylosin and cirramycin[7], supports the earlier attribution at most carbons, only the assignment of C(8) and C(17) in carbomycin, and C(4) and C(8) in tylosin and in cirramycin should be reversed. However, our data suggest that the assignment of the ^{13}C NMR spectrum of rosamicin[12,13] must be in error for several carbon atoms. Our data are in full agreement with the ^{13}C chemical shifts of rosamicin and its derivatives reported after the completion of this work[14,15].

Based on the observation that the 18-dihydro derivatives of leucomycin, spiramycin and other 16-membered macrolides have much lower antibacterial activity (MIC) *in vitro* than their parent compounds Ōmura suggested[16] that the aldehyde group at C(17) is essential for the activity of 16-membered macrolide antibiotics. More recently derivatives modified at the C(18) aldehyde function have been isolated[17] and synthesized[18,19]. Some of these derivatives showed enhanced antimicrobial activity compared to the parent compounds, especially when they were administered orally[19]. Among these compounds, however, the 18-dihydro and the 18-deoxo-dihydro derivatives were less active than the parent compounds. Our findings that **2** and **3** are less active than angolamycin are in agreement with the above structure-activity relationship.

Experimental

General Procedures

UV spectra were recorded on a LKB 4050 UV/VIS spectrometer, and IR spectra on a Bruker IFS-85 instrument. Optical rotations were measured in a 10-cm tube on a Perkin-Elmer 241 MC polarimeter. Mass spectra were taken on a Finnigan MAT 8430 instrument under the following opera-

tion conditions:

EI and DCI: resolution, 1,200; ion acceleration voltage, 3 kV; ion source temperature, 250°C; EI: electron energy, 70 eV; electron current, 50 μA; evaporation temperature, 250°C; DCI: reagent gas, *i*-butane; electron energy, 180 eV; electron current, 20 μA.

NMR spectra were acquired on a Bruker AC-250 spectrometer in $CDCl_3$ solution using TMS as an internal standard. 2D NMR spectra were measured with the standard Bruker microprograms. Selective INEPT spectra were measured with delay values optimized for 7 Hz couplings ($\Delta_1 = 46$ ms, $\Delta_2 = 56$ ms), for 3 Hz couplings ($\Delta_1 = 146$ ms, $\Delta_2 = 156$ ms), or for couplings with methyl groups ($\Delta_1 = 46$ ms, $\Delta_2 = 23$ ms) using 10 ms selective 90° proton pulses.

Fermentation

The spore suspension of *Streptomyces* sp. (86-070) from agar slant culture was inoculated into a seed culture medium (50 ml). The inoculated flask was shaken on a rotary shaker at 28°C for 68 hours. The seed culture was transferred to a 10-liter fermenter containing 5 liters of the medium. The seed and production medium consisted of glucose 2%, soybean meal 2.5%, casein hydrolisate 0.2%, potato starch 2.0%, corn steep liquor 0.5% and $CaCO_3$ 0.7%. The medium was adjusted to pH 7.2, and then it was sterilized at 120°C for 45 minutes. The fermentation was carried out for 72 hours at 28°C, under aeration of 1 vol/vol/minute and agitation of 150 rpm.

TLC Method for the Detection of Compounds **1**~**3**

Suitable TLC methods were developed for the detection of compounds **1**~**3** in fermentation broth and in the course of separation. Home made and commercial silica gel thin layers (E. Merck Art. 5562) were used. The following solvent systems were applied as eluent: Solvent system I, benzene-acetone (1:4); solvent system II, chloroform-methanol (12:1); solvent system III, benzene-methanol-acetone (4:1:5); solvent system IV, *n*-butanol-acetic acid-water (3:1:1).

Bioautographic method, chemical staining or UV light were used for the detection of the substances. For bioautography *Bacillus substilis* ATCC 6633 was used as test organism[20].

Isolation of Compounds **1**~**3**

The pH of the culture broth (5 liters) was adjusted to 4 and it was filtered. The micelial cake obtained was washed with water, then with methanol (2×1,500 ml). The methanol was evaporated. The aqueous residue was combined with the broth filtrate, and mixture (I) was obtained. The pH of mixture (I) (5.5 liters) was adjusted to 7 and adsorbed on a column of Amberlite XAD-2 resin (4 i.d.×50 cm). The column was washed first with water (5 liters) and then with water containing 20% methanol (3 liters), then finally with water containing 40% methanol (5 liters) consecutively and these inactive solutions were discarded. The biologically active substances were eluted from the resin with methanol (1 liter). The eluate was concentrated under reduced pressure. After dissolving the residue in water the adsorption and elution procedure was repeated. The eluate containing biologically active substances were evaporated *in vacuo* and an oily residue was obtained.

This was dissolved in methanol (20~40 ml) and subjected to column chromatography on Sephadex LH-20 (2.5 i.d.×55 cm) and eluted with methanol, 6~8 ml fractions were collected.

The activity of each fraction was monitored by TLC on Silica gel 60 F_{254} plates with solvent system I using the bioautographic method. The active fractions (4~12) were combined and the solvent evaporated, and 300 mg crude antibiotic complex was obtained.

The crude antibiotic complex was further purified and separated into components by preparative TLC on silica gel. Compound **2** was separated from the mixture of compounds **1** and **3** using solvent system I ($Rf_{\text{compound 2}} = 0.4$; $Rf_{\text{compounds 1+3}} = 0.45 \sim 0.48$). Compounds **1** and **3** were separated using eluent system II ($Rf_{\text{compound 1}} = 0.43$; $Rf_{\text{compound 3}} = 0.5$). The compounds were detected in UV light, and the corresponding silica gel was scraped down. The compounds were eluted with methanol or methanol-ethyl acetate (1:1) from silica gel and then the solvent was evaporated to yield compound **1**: (92 mg) mp: 133~136°C; $[\alpha]_D^{20}$ −62.5° ($CHCl_3$); UV λ_{max}^{MeOH} nm (ε) 240 (15,000). Compound **2**: (128 mg) mp: 125~127°C; $[\alpha]_D^{23}$ −57.1° ($CHCl_3$); UV λ_{max}^{MeOH} nm (ε) 240 (13,000). Compound **3**: (4.8 mg) mp: 86~88°C; UV λ_{max}^{MeOH} nm (ε) 240 (13,500).

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