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Chemical Structures of Amicoumacins Produced by *Bacillus pumilus*

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The chemical structures of amicoumacin A, B and C, which are produced by *Bacillus pumilus* BN-103, were elucidated on the basis of mass, ^{1}H-NMR and ^{13}C-NMR spectral analyses and chemical degradation. Amicoumacin A was derived from amicoumacin B *via* amicoumacin C.

As reported in our previous paper,[1,2] amicoumacins are a complex of closely related isocoumarin compounds which are produced by a strain of *Bacillus pumilus*. The amicoumacin was separated into three components, amicoumacin A (**I**), B (**II**) and C (**III**). (**I**), $C_{20}H_{29}N_3O_7 \cdot HCl$, is a carboxylic amide, and exhibited antibacterial, antiinflammatory and antiulcer activities. (**II**), $C_{20}H_{28}N_2O_8$, is a carboxylic acid, and (**III**), $C_{20}H_{26}N_2O_7$, is a γ-lactone of (**II**). (**II**) and (**III**) showed moderate activity against bacteria, but did not exhibit pharmocological activity. The present paper deals with the structural determination and chemical transformation of (**I**), (**II**) and (**III**).

RESULTS AND DISCUSSION

Acid hydrolysis of amicoumacins

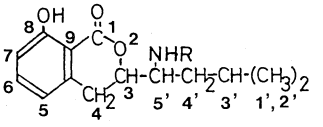
Amicoumacin A (**I**), B (**II**) and C (**III**) were isolated from the culture broth of *Bacillus pumilus* BN-103, as already described in the previous papers.[1,2] The acid hydrolysis of (**I**) yielded a chromophoric moiety (**IV**) and an acidic fragment. The latter could not be isolated, but (**IV**) was crystallized from a mixture of methanol and water to yield colorless needles. In a similar manner, (**IV**) was isolated from the hydrolysis of (**II**) and (**III**). Compound (**IV**) exhibited characteristic UV spectra in neutral and alkaline solutions, resembling 3,4-dihydro-8-hydroxy isocoumarin.[3] Acetylation of (**IV**) in methanol gave the mono-*N*-acetyl derivative (**IV′**), $C_{16}H_{21}NO_4$ (Mass, M^+ *m/z* 291), and in pyridine the *N,O*-diacetyl derivative (**IV″**), $C_{18}H_{23}NO_5$ (Mass, M^+ *m/z* 333). (**IV**) gave positive color reactions with ninhydrin ($-NH_2$) and $FeCl_3$ (phenolic OH). But (**IV′**) was negative with ninhydrin, and (**IV″**) was negative with both ninhydrin and $FeCl_3$.

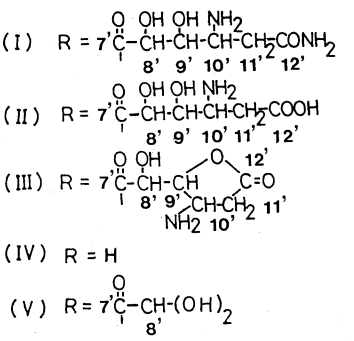
The ^{1}H-NMR spectra of (**IV**) revealed the following functional groups; $\underset{\underline{C}H_3}{\overset{\underline{C}H_3}{}}>CH-$ (δ 1.04, 6H, d), $\underset{CH_3}{\overset{CH_3}{}}>C\underline{H}-C\underline{H}_2-$ (δ 1.75, 3H, m), $-CH_2-\underset{NH_2}{C\underline{H}}-CH-$ (δ 3.65, 1H, m) and aromatic three protons (δ 6.88, 1H, d; δ 6.96, 1H, d; δ 7.57, 1H, t). The ^{13}C-chemical shifts of (**IV**) are summarized in Table I. All signals were assigned completely on the basis of chemical shifts[4] and splitting under the off-resonance. On the basis of UV, ^{1}H-NMR and ^{13}C-NMR spectra, the structure (**IV**) was proposed for the chromophoric moiety (Fig. 1), which was consistent with the chromophoric moiety of baciphelacin.[5]

*Structure of amicoumacin A (**I**)*

Acetylation of (**I**) in methanol yielded the mono-*N*-acetyl derivative (**I′**), $C_{22}H_{31}N_3O_8$, and in pyridine the *N,O*-tetraacetyl derivative (**I″**), $C_{28}H_{37}N_3O_{11}$. These results suggested

TABLE I. ^{13}C CHEMICAL SHIFTS OF AMICOUMACINS AND DEGRADATION PRODUCT





Carbon No.	Amicoumacin A (I)	Amicoumacin B (II)	Amicoumacin C (III)	(IV)	(V)
1	170.5 (s)	168.5 (s)	169.4 (s)	169.2 (s)	170.6 (s)
3	82.1 (d)	80.7 (d)	81.6 (d)	79.3 (d)	82.5 (d)
4	39.4 (t)	38.7 (t)	39.3 (t)	39.3 (t)	40.6 (t)
5	119.8 (d)	118.1 (d)	119.3 (d)	119.5 (d)	119.4 (d)
6	137.6 (d)	135.9 (d)	137.2 (d)	137.5 (d)	137.4 (d)
7	116.2 (d)	114.9 (d)	116.6 (d)	116.9 (d)	116.5 (d)
8	160.8 (s)	160.4 (s)	160.9 (s)	162.8 (s)	162.5 (s)
9	108.6 (s)	108.0 (s)	108.5 (s)	108.9 (s)	108.9 (s)
10	140.5 (s)	140.3 (s)	140.2 (s)	139.9 (s)	141.0 (s)
1′	21.7 (q)	21.4 (q)	22.2 (q)	22.0 (q)	21.9 (q)
2′	23.6 (q)	23.2 (q)	24.0 (q)	23.2 (q)	23.6 (q)
3′	25.2 (d)	23.9 (d)	25.1 (d)	25.2 (d)	25.7 (d)
4′	30.0 (t)	28.9 (t)	29.8 (t)	30.5 (t)	30.7 (t)
5′ or 10′	51.2 (d)	50.1 (d)	49.6 (d)	53.2 (d)	50.3 (d)
7′	173.8 (s)	172.2 (s)	171.1 (s)		171.5 (s)
8′ or 9′	71.2 (d)	71.2 (d)	71.9 (d)		95.1 (d)
9′ or 8′	73.2 (d)	71.2 (d)	85.6 (d)		
10′ or 5′	50.2 (d)	47.9 (d)	48.7 (d)		
11′	32.3 (t)	33.3 (t)	35.3 (t)		
12′	175.1 (s)	174.1 (s)	175.6 (s)		

the presence of one amino group and three hydroxy groups. In order to determine the structure of (**I**), all the protons were assigned by 200 MHz ^1H-NMR spin-decoupling of *N*-DNP-tri-*O*-acetyl amicoumacin A (Fig. 2). On irradiation of a multiplet at δ1.75 (1H, 3′-CH), a double doublet at δ0.95 (6H, 1′,2′-CH_3), a multiplet at δ1.35 (2H, 4′-CH_2) and a multiplet at δ4.38 (1H, 5′-CH) all collapsed to a doublet. This fact indicated the following partial structure,

$$-CH-CH_2-CH-(CH_3)_2 .$$

On irradiation of a multiplet at δ4.38 (1H, 5′-CH), a multiplet at δ4.58 (1H, 3-CH) collapsed to a double doublet and a doublet at δ7.42 (1H, 6′-NH) to a singlet. On the other hand, irradiation of a multiplet at δ4.58 (1H, 3-CH), a signal at δ2.95 (2H, 4-CH_2) collapsed to a double doublet. Therefore, the following partial structure was suggested,

$$-CH_2-CH-CH-NH- .$$
$$\quad\quad\quad\quad\quad 5'$$

On irradiation of a multiplet at δ5.05 (1H, 10′-CH), doublets at δ9.18 (1H, 10′-NH) and at δ2.82 (2H, 11′-CH_2) collapsed to a singlet, and a double doublet at δ5.62 (1H, 9′-CH) was changed to a doublet. Thus, the doublet at δ5.40 was assigned to a proton attached to C-

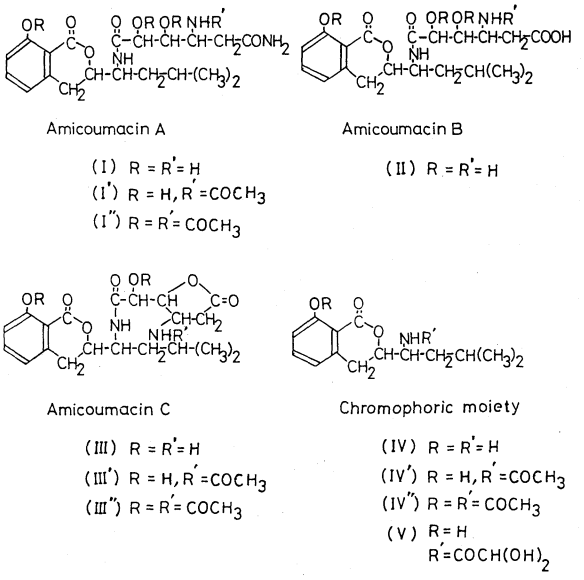


FIG. 1. Structures of Amicoumacins and Derivatives.

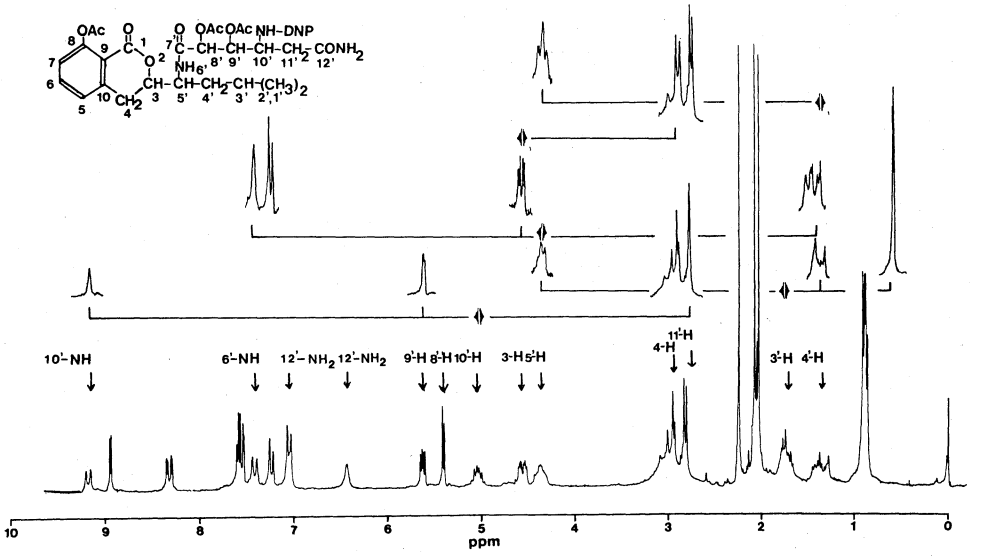


FIG. 2. ^{1}H-NMR Spectrum of *N*-DNP-tri-*O*-acetyl Amicoumacin A (200 MHz, d_6-acetone).

8′. This result suggested the following partial structure,

$$-CO-\underset{A}{CH}(OAc)-CH(OAc)-CH(NH\text{–}DNP)-CH_2-\underset{B}{CO}-.$$

A carbonyl group, A or B in the above partial structure, should attach to the chromophoric moiety (**IV**). An extra NH_2 group was considered most probably to be present as a carboxylic amide, since (**I**) only gave the mono-*N*-acetyl derivative (**I′**). The signal at δ 7.05 was assumed to be an aromatic proton of 2,4-dinitrophenol and a proton of carboxylic amide. On the addition of D_2O, the signal at δ 7.05 was changed to the doublet of one proton and the signal at δ 6.50 disappeared. Thus, the signals at δ 6.50 and δ 7.05 were

assigned to carboxylic amide. It was characteristic that the carboxylic amide protons had a different chemical shift. Attachment of the carbonyl A to (**IV**) was supported by the periodate oxidation[6] of (**I**), which yielded the derivative (**V**) generated by cleavage of the bond between C-8′ and C-9′. The ^{13}C-chemical shifts of (**V**) are summarized in Table I. From these results, the structure (**I**) was proposed for amicoumacin A (Fig. 1).

*The structures of amicoumacin B (***II***) and amicoumacin C (***III***)*

Based on the elemental analysis and IR spectrum, it was suggested that (**II**) had the same structure as (**I**) except for the replacement of the carboxylic amide residue by a carboxylic acid. In order to confirm the structure proposed, the following chemical reactions were carried out. Acetylation of (**II**) in methanol yielded mono-*N*-acetyl derivative (**III′**), $C_{22}H_{28}N_2O_8$, and in pyridine the *N,O*-triacetyl derivative (**III″**), $C_{26}H_{32}N_2O_{10}$. The IR spectra of compound **III′** and **III″** indicated the presence of a γ-lactone group (1770 cm^{-1}), which was lacking in the parent compound (**II**). Formation of the lactone under the anhydrous condition indicated the presence of carboxylic acid in (**II**). Furthermore, the mono-*N*-acetyl (**III′**) or *N,O*-triacetyl derivatives (**III″**) of (**II**) were identical with those of (**III**), prepared under similar conditions. Thus, it was concluded that (**III**) was the γ-lactone of (**II**). The ^{13}C-NMR spectra of (**II**) and (**III**) revealed six signals due to carbons constructing the side chain, and these were assigned to two carbonyl carbons, three methines and one methylene on the basis of their chemical shifts and splittings under the off-resonance condition (Table I). From all the above evidence, the structures (**II**) and (**III**) were proposed for amicoumacin B and amicoumacin C (Fig. 1). The X-ray structural analysis[7] of (**II**) (Fig. 3) and chemical transformation of the amicoumacins described below (Fig. 4) also supported those structures.

Chemical transformation of amicoumacin A, B and C

Taking into account the fact that (**I**), (**II**) and (**III**) had the same structure except for the carboxylic acid moiety, we attempted the chemical correlation of these amicoumacins.

In the first step, (**I**) was converted to (**II**) by treatment with sodium bicarbonate. In the second step, the (**II**) thus obtained was converted to (**III**) by reacting with trifluoroacetic acid at room temperature. In the last step, (**III**) was changed to (**I**) by reacting with liquid ammonia. The (**I**) obtained by this sequence of

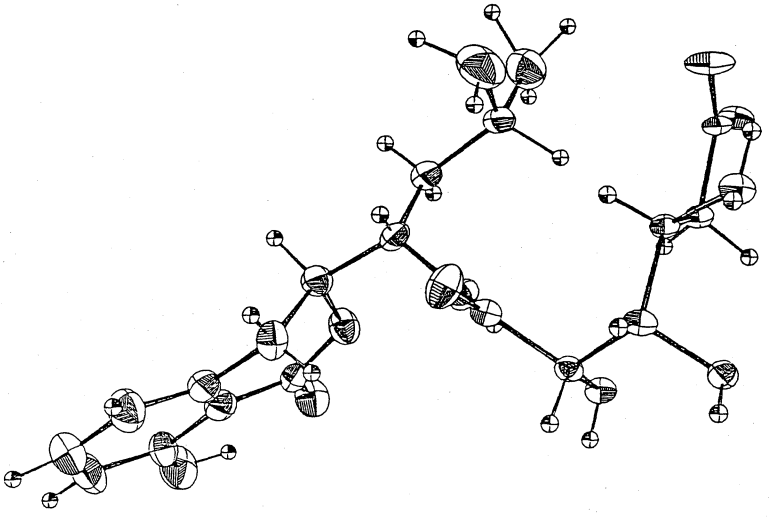


FIG. 3. Molecular Profile of Amicoumacin B.

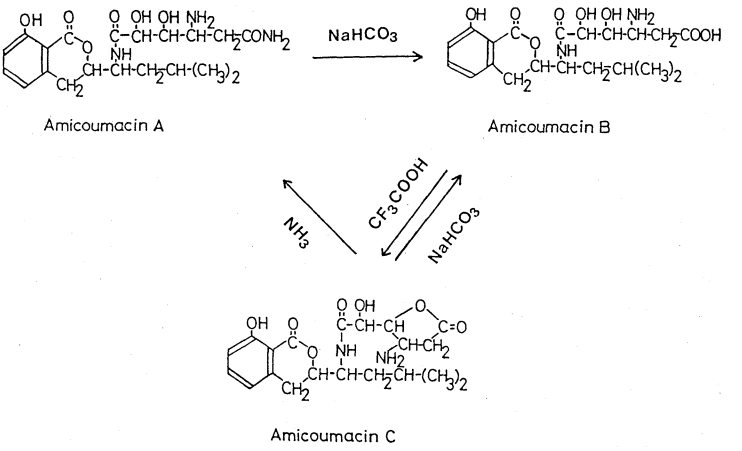


FIG. 4. Transformation between Amicoumacin A, B and C.

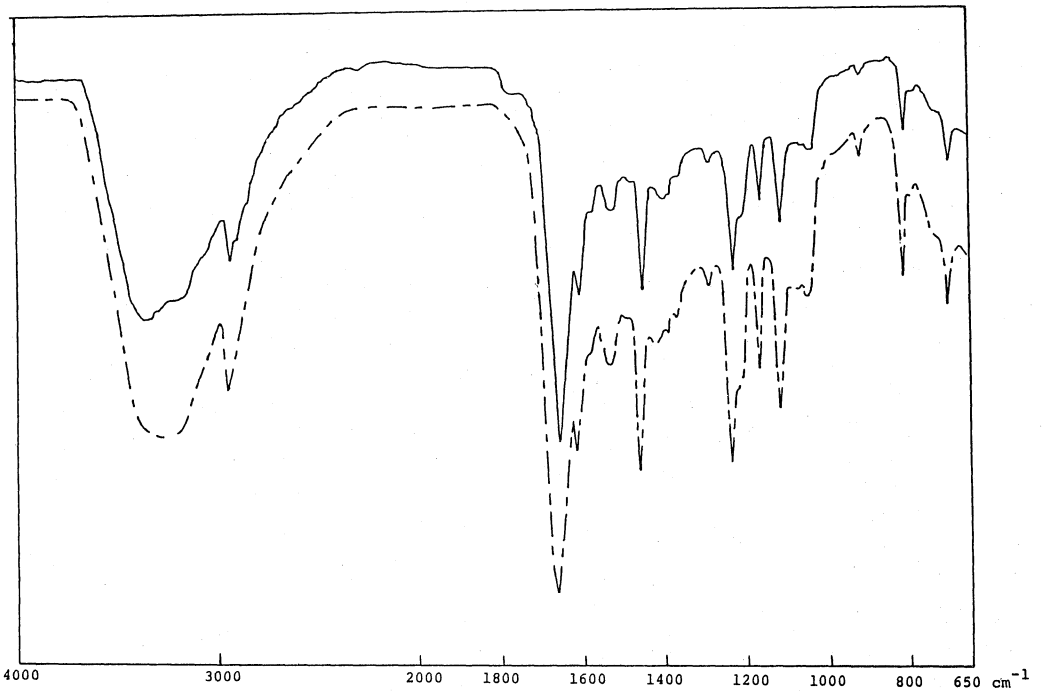


FIG. 5. IR Spectra of Natural Amicoumacin A (——) and Derived Amicoumacin A (— - —) in KBr Disk.

transformation gave the same IR spectrum (Fig. 5) and antibacterial activity as those of natural (**I**). This transformation also confirmed the terminal position of the carboxylamide group in (**I**). Treatment of (**III**) with sodium bicarbonate gave (**II**) quantitatively, as expected.

EXPERIMENTAL

Melting points were determined with a Yamato capillary apparatus, and uncorrected. Mass spectra were obtained with a Hitachi M-80 mass spectrometer. IR spectra were measured on a Hitachi 260-10 spectrophotometer, UV spectra on a Hitachi 200-20 spectrophotometer, ^{1}H-NMR spectra at 200 MHz on a JEOL JNM FX-200 spectrometer and ^{13}C-NMR spectra at 25.16 MHz on a Varian XL-100-12 spectrometer. 50 ~ 100 mg of (**I**), (**II**),

(**III**), (**IV**) and (**V**) were dissolved in 0.8～1.0 ml of D_2O, *d*-DMSO, *d*-DMSO, CD_3OD and CD_3OD, respectively. ^{13}C Chemical shifts were expressed in ppm from the ^{13}C resonance of TMS or measured relative to the ^{13}C signal of dioxane ($\delta = 67.4$ ppm). High performance liquid chromatography (HPLC) was performed with a Shimadzu LC-3A under the following conditions: column, Nucleosil $5C_{18}$, $\phi 4.6 \times 150$ mm; flow rate, 1.0 ml/min; mobile phase, 0.025 M KH_2PO_4/CH_3CN 70/30.

Isolation of the chromophoric moiety (**IV**). (**I**) (100 mg) was dissolved in 5 ml of 6 N HCl and heated at 105°C for 16 hours. The solution was concentrated to dryness. The acidic fragment could not be isolated, but the chromophoric moiety was crystallized from a mixture of methanol and water to yield colorless needles. mp 238～239°C. $[\alpha]_D^{23}$ −58.5 (*c* 0.5, MeOH). UV λ_{max}^{MeOH} nm (ε): 213 (19,550), 246 (6,600), 314 (4,180); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 213 (18,680), 247 (5,660), 316 (3,740); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 213 (34,860), 244 (sh), 324 (3,800). IR λ_{max}^{KBr} cm^{-1}: 1,690 (C=O), 810, 700 (aromatic). MS: M^+ *m/z* 249. *Anal.* Calcd for $C_{14}H_{19}NO_3 \cdot HCl$: C 58.83, H 7.07, N 4.90, Cl 12.40; Found: C 58.77, H 6.97, N 4.84, Cl 11.46.

N-Acetylation of amicoumacin A. To a solution of (**I**) (100 mg) in methanol (1 ml) was added 1 ml of acetic anhydride, and the solution was stirred for 2 hours at room temperature. The reaction mixture was concentrated to dryness. The *N*-acetyl derivative (**I′**) was crystallized from chloroform. Yield, 65 mg. mp 178～181°C $[\alpha]_D^{23}$ −79.8 (*c* 0.5, MeOH). UV λ_{max}^{MeOH} nm (ε): 206 (21,700), 246 (5,470), 315 (3,830); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 206 (21,700), 246 (5,580), 315 (3,830); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 228 (15,100), 250 (5,390), 350 (5,670). MS: $(M+1)^+$ *m/z* 466. *Anal.* Calcd for $C_{22}H_{31}N_3O_8$: C 56.76, H 6.73, N 9.02; Found: C 57.05, H 6.80, N 9.30.

N,O-Acetylation of amicoumacin A. To a solution of (**I**) (100 mg) in pyridine (2 ml) was added 2 ml of acetic anhydride. The reaction mixture was kept at room temperature for 2 hours, and concentrated to dryness. The *N,O*-acetyl derivative (**I″**) was crystallized from acetone. Yield, 110 mg. mp 227～229°C. $[\alpha]_D^{23}$ −79.0 (*c* 0.5, MeOH). UV λ_{max}^{MeOH} nm (ε): 206 (27,660), 235 (8,010), 288 (2,070); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 206 (27,660), 235 (7,860), 288 (2,000); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 229 (16550), 250 (5,670), 350 (5,910). MS: M^+ *m/z* 591. *Anal.* Calcd for $C_{28}H_{37}N_3O_{11}$: C 56.84, H 6.32, N 7.10; Found: C 57.12, H. 6.50, N 7.42.

Preparation of N-DNP-tri-O-acetyl amicoumacin A. To a solution of (**I**) (40 mg) in 1 ml of M/20 borate buffer was added 1-fluoro-2,4-dinitrobenzene (35 mg) in 1 ml of acetone. The reaction mixture was stirred at room temperature for 30 minutes. Extraction with ethyl acetate and evaporation of the solvent layer gave 30 mg of a residue. This was purified by means of column chromatography over silica gel (chloroform–methanol, 3 : 1, vol/vol) to give *N*-DNP amicoumacin A. To a solution of the *N*-DNP derivative (30 mg) in 1 ml of pyridine was added 0.5 ml of acetic anhydride. The mixture was stirred for 1 hr and concentrated to dryness. The residue was crystallized from benzene. Yield, 25 mg. mp 143°C. $[\alpha]_D^{23}$ −10.8 (*c* 0.5, MeOH). M^+ *m/z* 715. *Anal.* Calcd for $C_{32}H_{37}N_5O_{14}$: C 53.30, H 5.00, N 9.62; Found: C 53.70, H 5.22, N 9.78.

Periodate oxidation of amicoumacin A. To a solution of (**I**) (100 mg) in 3 ml of water was added 3 ml of 0.12 M $NaIO_4$. The solution was stirred for 24 hours at 5°C and the reaction mixture concentrated to dryness. The derivative (**V**) was purified by silica gel column chromatography developed with chloroform–methanol (10 : 1, vol/vol). Yield, 36 mg. mp 95°C $[\alpha]_D^{23}$ −92.4 (*c* 0.5, MeOH). UV λ_{max}^{MeOH} nm (ε): 208 (20,130), 248 (4,560), 316 (3,000); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 210 (18,300), 248 (4,700), 316 (3,100); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 250 (sh.), 350 (4,370). MS: M^+ *m/z* 323, *m/z* 305 ($M^+ - H_2O$). *Anal.* Calcd for $C_{16}H_{21}NO_6$: C 59.42, H 6.56, N 4.33; Found: C 59.07, H 6.26, N 4.24.

N-Acetylation of amicoumacin B. A solution of (**II**) (50 mg) in 1 ml of methanol and acetic anhydride (1 ml) was worked up according to the procedure employed for (**I**). The *N*-acetyl derivative (**III′**) was crystallized from chloroform. Yield, 45 mg. mp 212～214°C. $[\alpha]_D^{23}$ −51.0 (*c* 0.4, MeOH). UV λ_{max}^{MeOH} nm (ε): 210 (16,500), 246 (4,810), 315 (3,500); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 210 (16,100), 246 (3,740), 315 (3,400); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 207 (16,200), 230 (10,600), 250 (4,100), 350 (3,850). MS: M^+ *m/z* 448. *Anal.* Calcd for $C_{22}H_{28}N_2O_8$: C 58.91, H 6.41, N 6.24; Found: C 59.25, H 6.60, N 6.65.

N,O-Acetylation of amicoumacin B. A solution of (**II**) (50 mg) in 2 ml of pyridine and acetic anhydride (2 ml) was worked up according to the procedure employed for (**I**). The *N,O*-acetyl derivative (**III″**) was purified by silica gel column chromatography developed with chloroform–methanol (9 : 1, vol/vol). Yield, 40 mg. $[\alpha]_D^{23}$ −69.8 (*c* 0.6, MeOH). UV λ_{max}^{MeOH} nm (ε): 209 (11,400), 236 (5,100), 290 (1,300); UV $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε): 208 (11,700), 236 (5,270), 290 (1,380); UV $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε): 208 (11,800), 228 (8,800), 250 (4,100), 350 (3,100). MS: M^+ *m/z* 532. *Anal.* Calcd for $C_{26}H_{32}N_2O_{10}$: C 58.63, H 6.07, N 5.26; Found: C 58.90, H 6.52, N 5.48.

Chemical correlation of amicoumacin A (**I**), *B* (**II**) *and C* (**III**).

a) A solution of (**I**) (100 mg) in 5 ml of 0.1 M $NaHCO_3$ was kept at 37°C for 48 hours. The solution was passed through a column of Diaion HP-20. After washing with water, the column was eluted with 50% aqueous acetone and then evaporated to dryness to yield a white powder (85 mg). The IR spectrum and HPLC data of this com-

pound were identical with those of (**II**).

b) A solution of (**II**) (85 mg) in 2 ml of trifluoroacetic acid was kept at room temperature for 2 hours. The solution was evaporated to yield a white powder (54.5 mg), whose ^{1}H-NMR and HPLC data were identical with those of the authentic (**III**).

c) To a solution of (**III**) (54.5 mg) in methanol (5 ml) was added 1 ml of liquid ammonia, and the mixture kept at 5°C for 2 hours. The solution was evaporated to dryness. The residue was dissolved in water (1 ml) and chromatographed on a Sephadex G-10 column. The column was developed successively with water and then with 0.1 M sodium chloride. The fractions eluted with 0.1 M sodium chloride were desalted by the procedure using Diaion HP-20 as described previously.[2] The solution was concentrated and then lyophylized to give 32 mg of amicoumacin A. The semisynthesized amicoumacin A was identical with the authentic amicoumacin A (**I**) by comparison of ^{1}H-NMR, IR and HPLC data.

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