NANAOMYCINS, NEW ANTIBIOTICS PRODUCED BY A STRAIN OF *STREPTOMYCES*

II. STRUCTURE AND BIOSYNTHESIS

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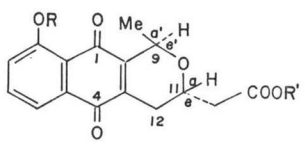
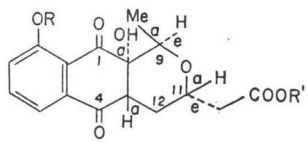
Evidence is put forward which describes the structure and stereochemistry of new antibiotics, nanaomycins A and B, as **I** and **V**, respectively. In order to study biosynthesis and to determine the position of the hydroxyl group in the naphthoquinone moiety, a feeding experiment with 1-^{13}C-acetate was effectively carried out. Nanaomycins A and B were found to be synthesized from acetate *via* a polyketide by *Streptomyces rosa* var. *notoensis*.

In the previous papers,[1,2] the isolation and preliminary characterization of nanaomycins A and B, new acidic antibiotics from *Streptomyces rosa* var. *notoensis*, were described. It was shown that the molecular formulae of nanaomycins A and B were $C_{16}H_{14}O_6$ and $C_{16}H_{16}O_7$, respectively, and nanaomycin B was readily converted to nanaomycin A in an alkaline solution. In addition, their structures were speculated from the physical and chemical properties to be closely related. We wish to report the detailed work which has led to the structures and relative stereochemistry of nanaomycins A and B as **I** and **V**, respectively, and also describe the probable biosynthetic pathway.



I: R=R'=H
II: R=Ac, R'=H
III: R=H, R'=Me
IV: R=R'=Me



V: R=R'=H
VI: R=H, R'=Me
VII: R=R'=Me

The Structure of Nanaomycin A

Results of the elementary analysis and the mass spectral analysis (M^+ *m/e* 302.084) gave the molecular formula of $C_{16}H_{14}O_6$. The pKa′ values of nanaomycin A (5.9 and 10.9 in 60% ethanol) suggested the presence of a phenolic hydroxyl group and a carboxyl group. This was confirmed by formation of a monoacetate (**II**), ν_{max}^{KBr} 1765, 1700 and 1670 cm^{-1}, and of a methyl ester (**III**), ν_{max}^{KBr} 1730, 1645 and 1615 cm^{-1}. The infrared absorption at 1640 and 1615 cm^{-1} and the ultraviolet spectra [$\lambda_{max}^{90\%\,MeOH}$ nm(ε): 250(9850), 273(12,200) and 423(4040); $\lambda_{max}^{0.1N\,NaOH\text{-}90\%\,MeOH}$ nm(ε): 279(12,700) and 528(5170)] of nanaomycin A indicated the presence of quinone carbonyl groups, one of which was hydrogen-bonded to the phenolic hydroxyl group.[3] The absorption

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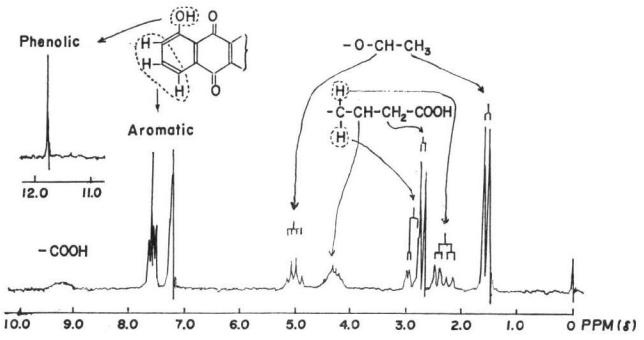
band (3010 cm^{-1}) of the chelated hydroxyl group in nanaomycin A did not change on dilution in chloroform. *O*-Methylnanaomycin A methyl ester (**IV**), ν_{max}^{KBr} 1743 and 1657 cm^{-1}, could be obtained by reaction with methyl iodide in the presence of silver oxide. One oxygen atom remaining to be accounted for was speculated to be ethereal in nature because it was inert in the above reactions.

Fig. 1. NMR spectrum of nanaomycin A in $CDCl_3$ (100 MHz)



The ultraviolet spectra of nanaomycin A in both neutral and basic media showed a striking resemblance to those of juglone (**VIII**)[4] suggesting that this moiety was present in nanaomycin A. This was also supported from the NMR spectrum of nanaomycin A (Fig. 1). The one-proton singlet at δ 11.96 and the three-proton multiplet at δ 7.15 to δ 7.75 suggest a phenolic hydroxyl group and three aromatic protons, respectively. The former disappeared by addition of deuterium oxide. In addition, the ultraviolet spectrum of *O*-methylnanaomycin A methyl ester corresponds to that of eleutherin (**X**) and isoeleutherin (**XI**).[5] This indicates that the juglone moiety and other constituent are joined at the positions of 2 and/or 3 of the naphthoquinone moiety in a similar manner to eleutherins.

The NMR spectrum of nanaomycin A was particularly useful in delineating the remaining structural details. A three-proton doublet at δ 1.58 (J=7.0 Hz) and a one-proton symmetrical quartet at δ 5.06 (J=7.0 Hz) deshielded by the ethereal oxygen atom are coupled with each other, and suggest the presence of a CH_3-$\underset{|}{C}$H-O- grouping (**i**). The presence of fragmentation ion of M^+-15 in the mass spectrum of nanaomycin A also supports the presence of this partial structure. The doublet at δ 2.71 (J=6.5 Hz) in the NMR spectrum is assigned to the signal of two equivalent methylene protons deshielded by the carboxyl group and coupled with a tertiary proton. These data suggest the presence of the partial structure of $>CHCH_2COOH$.

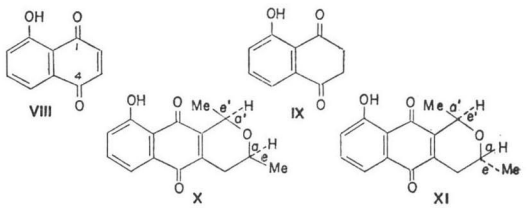
By spin decoupling experiments, a pair of doublets at δ 2.87 (J=3.2 and 19.0 Hz) and two pairs of upper field doublets at δ 2.34 (J=2.0, 10.0 and 19.0 Hz) are assigned to the signals of the geminal protons of a CH_2 group adjacent to a tertiary proton whose signal is located as a multiplet at δ 4.33. In addition, the decoupling experiments indicated that the X proton of the ABX system was the same proton which was splitting the CH_2 protons at δ 2.71 adjacent to the carboxyl group. Therefore, the partial structure of $-CH_2CH(CH_2COOH)O-$ (**ii**) is present in nanaomycin A.

To satisfy the molecular formula, $C_{16}H_{14}O_6$, the two partial structures, **i** and **ii**, must be joined through an ether bridge. Therefore, a combination of these partial structures with the established juglone system led to the complete structure for nanaomycin A as shown in **I** with the exception of the placement of the phenolic hydroxyl group. The ^{13}C-NMR spectrum of nanaomycin A (Fig. 3) also supports the structure of **I**. Signals of 16 carbon atoms in nanaomycin A were assigned as shown in Fig. 3. The phenolic hydroxyl group was revealed to be

placed at C-8 on the basis of the biosynthetic study of nanaomycin with ^{13}C-NMR spectrometry described below.

From the vicinal coupling constants between the C-12 methylene group and the C-11 tertiary proton, it can be shown that a pair of doublets at δ 2.87 ($J_{11,12\text{-a},e'}=3.5$ Hz) is associated with the pseudo-equatorial C–H bond and the corresponding upfield signal at δ 2.34 ($J_{12,11\text{-a},a'}=10.0$ Hz) with the pseudo-axial C–H bond. Thus, the configuration of the C-11 proton is axial.

The configuration of the C-9 proton relative to the C-11 proton could be determined by taking advantage of the homoallylic couplings between the C-12 geminal protons and the C-9 proton in nanaomycin A. These long-range couplings have been reported with stereochemical implications in regard to eleutherin (**X**) and isoeleutherin (**XI**).[6] Comparison of the coupling constants of the C-9, C-11 and C-12 protons in the NMR spectrum of nanaomycin A ($J_{12,12\text{-gem}}=19.0$, $J_{12,11\text{-a}',a}=10.0$, $J_{12,11\text{-e}',a}=3.2$, $J_{9,12\text{-e}',a'}=2.0$, $J_{9,12\text{-e}',e'}<1$ Hz) with those of the eleutherins showed the C-9 and C-11 protons to be pseudoequatorial and axial, respectively. Thus, the relative stereochemistry at C-9 and C-11 is as shown in **I**.



The Structure of Nanaomycin B

The molecular formula, $C_{16}H_{16}O_7$, was assigned to nanaomycin B from combusion analysis and its mass spectrum, in which the molecular ion peak is present at *m/e* 320.090. From the pKa′ values (5.8 and 10.5 in 60% methanol) nanaomycin B was speculated to be a phenolic carboxylic acid. Nanaomycin B also converted to its monoacetate, methyl ester (**VI**) and *O*-methyl methyl ester (**VII**). These derivatives could be detected by thin-layer chromatography and NMR spectra, but could not be isolated because of instability.

The UV-spectrum of nanaomycin B, λ_{max}^{MeOH} nm(ε): 231(20,800), 269(5030), and 352(4970), is identical with that of β-hydrojuglone(**IX**).[7] In alkaline media, nanaomycin B converts readily to nanaomycin A as previously reported.[2] The NMR spectrum of nanaomycin B showed the presence of a phenolic hydroxyl group (δ 11.67, 1H) and aromatic protons (δ 7.22, 1H, and δ 7.40 to δ 7.70, 2H). From the above data, it was confirmed that β-hydrojuglone moiety was present in nanaomycin B.

The formation of nanaomycin A from nanaomycin B was shown from their molecular formula to accompany the loss of H_2O, and was speculated from the above data to be due to the conversion of the β-hydrojuglone moiety of nanaomycin B to a juglone one. From these considerations, it was speculated that nanaomycin B must have a hydroxyl group at either 2 or 3 position on β-hydrojuglone moiety, and a hydrogen atom at the other position. This was confirmed by the NMR spectrum of nanaomycin B (Fig. 2). A three-proton doublet at δ 1.45 ($J=7.0$ Hz) and a one-proton quartet at δ 4.40 ($J=7.0$ Hz) suggest the presence of a $>$CH–CH$_3$ grouping. The signal of the methylene protons adjacent to a carboxylic acid was observed at δ 2.62 (*d*, $J=6.0$ Hz). These signals resemble those in nanaomycin A. However, signals corresponding to the geminal protons at C-12 of nanaomycin B are different from those in nanaomycin A. The signals overlap each other at δ 2.05, and a signal which was not observed in

Fig. 2. NMR spectrum of nanaomycin B in $CDCl_3$ (100 MHz)

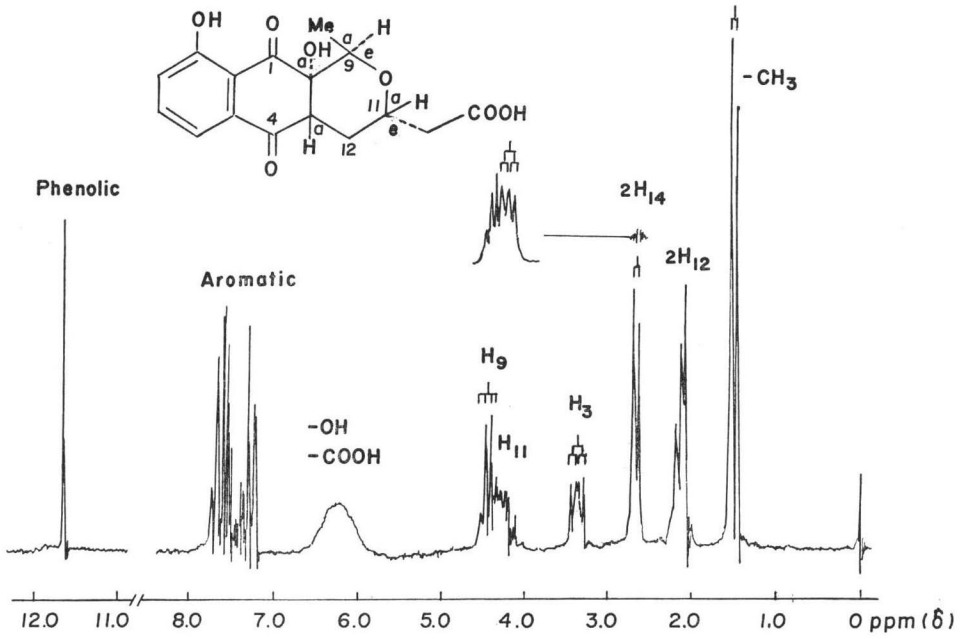
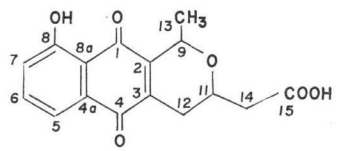
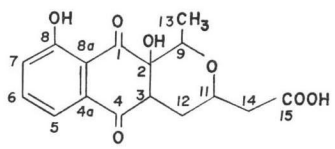
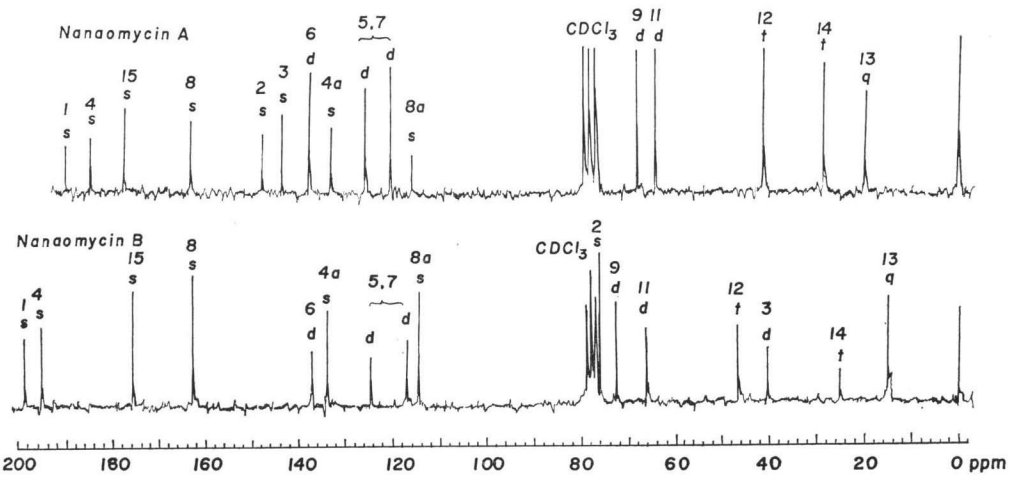


Fig. 3. Proton noise decoupled ^{13}C-NMR spectra of nanaomycins A and B

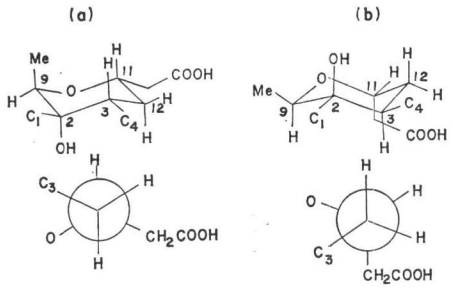
The symbols, s, d, t, and q, mean singlet, doublet, triplet and quartet, respectively, under off-resonance decoupling.







nanaomycin A appeared at δ 3.34 (1H, *dd*, J=7.0 and 9.0 Hz). Decoupling experiments indicated that the X protons of the ABXY system were the C-11 proton coupled with the methylene protons at δ 2.62 adjacent to the carboxyl group, and the Y proton was the C-3 proton at δ 3.34 on the β-hydrojuglone moiety. Thus, a hydrogen atom and a hydroxyl group must be present at C-3 and C-2 positions, respectively, and the structure for nanaomycin B was determined to be V. The structure was also supported by the ^{13}C-NMR spectrum of nanaomycin B. Assignment of signals in the ^{13}C-NMR spectrum is shown in Fig. 3.



As already mentioned above, loss of H_2O is accompanied with the transformation of nanaomycin B into nanaomycin A. This indicated that the C–OH bond at C-2 and the C–H bond at C-3 are trans-diaxial. DREIDING models showed the possibility of two chair forms (*a* or *b*), for the relative configuration of the oxiran ring of nanaomycin B as drawn aside. Further decoupling experiment was useful in determination of the remaining configuration. When the methylene protons adjacent to carboxylic acid were irradiated, $J_{11,12a}$ was 8 Hz and $J_{11,12e}$ was 5 Hz suggesting that H_{11}–H_{12a} and H_{11}–H_{12e} were trans and gauche, respectively. Thus, the C-11 proton is axial: the relative configuration of nanaomycin B is *a*.

Determination of the Position of the Phenolic Hydroxyl Group by Biosynthetic Studies of Nanaomycin with ^{13}C-NMR

There are two possibilities for the position of the phenolic hydroxyl group, C_5–OH or C_8–OH, to form an intramolecular hydrogen bond with a quinone carbonyl group of nanaomycins A and B. If nanaomycins are synthesized from acetate *via* a polyketide as speculated from the structures (Fig. 4), in the case of C_8–OH the carbon adjacent to the hydroxy group should be

Fig. 4. The probable biosynthetic pathway of nanaomycins A and B

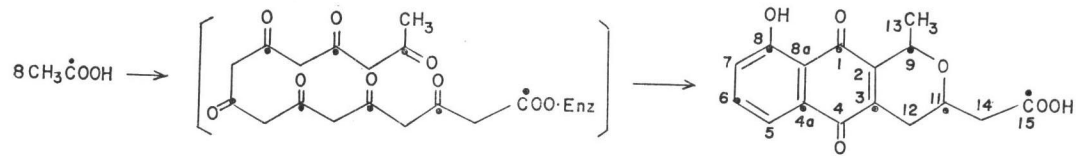
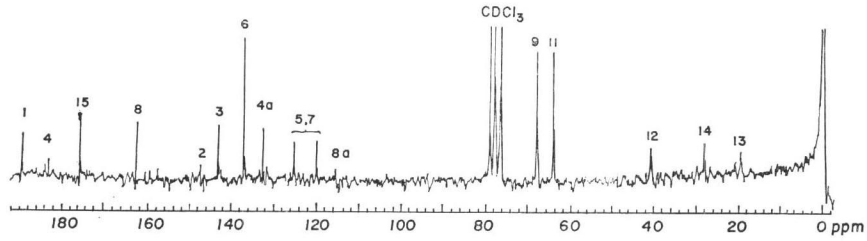


Fig. 5. Proton noise decoupled ^{13}C-NMR spectrum of nanaomycin A labeled with $CH_3{}^{13}COONa$



enriched with 1-^{13}C-acetate fed to the fermentation medium. In the other case (C_5-OH), the carbon should not be enriched with the ^{13}C-precursor. To clarify the problem, biosynthetic experiments were carried out using 1-^{13}C-acetate.

After *Steptomyces rosa* var. *notoensis* was cultivated for 1 day, $CH_3{}^{13}COONa$ was added to the culture and the cultivation was continued for 3 days. Nanaomycin labeled was isolated as nanaomycin A.

The following peaks in the ^{13}C-NMR spectra of nanaomycins A and B (Fig 3) were assigned by the aid of off-resonance and comparison with those of nanaomycins A and B and their related compounds; δ C^{TMS} for nanaomycin A: shown in Table 1, δ C^{TMS} for nanaomycin B: 14.5 (C-13), 25.6 (C-14), 40.3 (C-3), 46.5 (C-12), 65.1 (C-11), 73.1 (C-9), 75.2 (C-2), 115.4 (C-8a), 117.9 (C-5 or 7), 123.8 (C-5 or 7), 134.6 (C-4a), 137.2 (C-6), 162.3 (C-8), 175.5 (C-15), 195.1 (C-4) and 199.5 (C-1).

The ^{13}C-NMR spectrum of nanaomycin A labeled with $CH_3{}^{13}COONa$ (Fig. 5) exhibited 8 enhanced peaks assignable to C-1, 15, 8, 3, 6, 4a, 9, 11, while peaks of C-4, 2, 5, 7, 8a, 12, 14, 13 were weaker.

These results clearly indicate that nanaomycin is biosynthesized from 8 acetate units *via* a "polketide" as shown in Fig. 4. Since the carbon atom (161.4 ppm) to the phenolic hydroxyl group was enriched with $CH_3{}^{13}COONa$, the position of the phenolic hydroxyl group is not at C-5 position but must be at C-8 position.

Table 1. Estimated incorporation of $CH_3{}^{13}COONa$

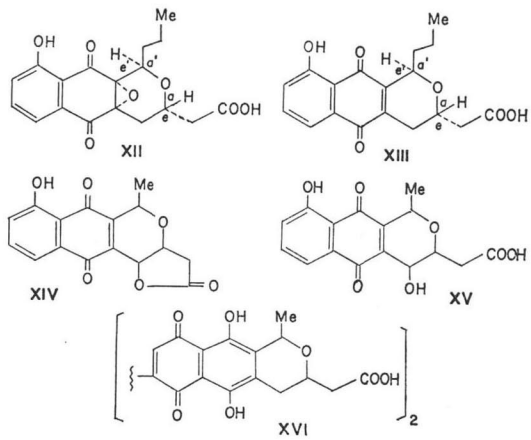
Chemical shifts	Assignment	Enrichment factor*
188.1	C-1	2.6
182.7	C-4	**
175.7	C-15	3.1
161.4	C-8	3.5
146.1	C-2	**
141.9	C-3	2.5
136.1	C-6	3.5
131.6	C-4a	3.0
124.3	C-5,7	0.9
119.0		1.0
114.6	C-3a	**
67.2	C-9	3.4
63.2	C-11	3.2
40.3	C-12	1.0
27.8	C-14	0.9
19.4	C-13	1.1

* Peak heights $\frac{\text{enriched sample}}{\text{natural abundance}}$ from spectra run under identical instrumental conditions and corrected for difference in concentration.

** Since the intensities in ^{13}C-NMR spectrum of the enriched sample were too weak, the factors were not determined.

The enrichment factors were determined to be 2.5 to 3.5 by the comparison of the signal intensities in the ^{13}C-NMR spectrum of the labeled compound with those of the unlabeled one (Table 1).

Nanaomycin A is structually analogous to deoxyfrenolicin (**XIII**) derived from frenolicin (**XII**) which was isolated from *Streptomyces fradiae*,[8,9] and to kalamycinic acid (**XV**) derived from kalafungin (**XIV**) which was isolated from *S. tanashiensis*.[10,11] To our knowledge,



nanaomycin B is the first β-hydrojuglone (2,3-dihydro-1,4-naphthoquinone) found in nature. As nanaomycins A and B were proved from the above data to be biosynthesized from acetate *via* a polyketide, actinorhodin (**XVI**) isolated from *S. coelicolor*,[12] frenolicin and kalafungin would seem to be biosynthesized in a similar manner.

Experimental Section

UV spectra were determined with a Hitachi EPS-3 spectrometer. IR spectra were determined in pressed KBr discs or chloroform with a JASCO IR-G spectrometer. NMR spectra were recorded with JEOL's JNM-4H-100. Chemical shifts are expressed in values (ppm) with tetramethylsilane as an internal standard. Mass spectra were taken on a JEOL's JMS-01S. Proton noise decoupled FT-^{13}C-NMR spectra were taken at 25.2 MHz on a Varian XL-100 spectrometer using tetramethylsilane as reference. All melting points are uncorrected.

Nanaomycins A (**I**) and B (**V**)

These compounds were prepared from culture broth of *Streptomyces rosa* var. *notoensis* as previously reported.[2]

Acetylnanaomycin A (**II**)

Nanaomycin A (200 mg) was left in a solution containing 2 ml of acetic anhydride and 4 ml of pyridine overnight at room temperature. The solution was poured onto a mixture of ice and 10 % hydrochloric acid, and extracted with chloroform. The extract was washed with water, dried, and concentrated *in vacuo* to dryness. Recrystallization from benzene gave 145 mg of pale yellow needles: mp 190~192°C; $[\alpha]_D^{22}$+32.4° (*c* 1.02, $CHCl_3$); ν_{max}^{KBr} 1765, 1700, 1670 cm^{-1}, λ_{max}^{MeOH} nm(ε): 235(20,700), 265(12,100), 270(12,300), 342(3750). Anal. Calcd. for $C_{18}H_{16}O_7$: C, 62.79; H, 4.68. Found: C, 62.89; H, 4.73.

Nanaomycin A Methyl Ester (**III**)

A solution of 200 mg of nanaomycin A in ether was treated with an excess of an ethereal solution of diazomethane for 1 hour at room temperature. Removal of the solvent *in vacuo* and subsequent column chromatography on silica gel with a chloroform-methanol system afforded the homogeneous nanaomycin A methyl ester as an orange-yellow powder (34 mg): mp 99~102°C; $[\alpha]_D^{20}$−12.7° (*c* 1.02, $CHCl_3$); ν_{max}^{KBr} 1730, 1645, 1615 cm^{-1}; λ_{max}^{MeOH} nm(ε): 248(12,400), 274(15,100), 424(5650). Anal. Calcd. for $C_{17}H_{16}O_6$: C, 64.55; H, 5.10. Found: C, 64.84; H, 5.21.

O-Methylnanaomycin A Methyl Ester (**IV**)

To a solution of nanaomycin A (200 mg) in chloroform, 310 mg of silver oxide and 0.2 ml of methyl iodide, were added and the mixture was refluxed for 4 hours. After cooling, the reaction mixture was evaporated to dryness *in vacuo*. The crude product was purified by preparative thin-layer chromatography on silica gel to yield a pale yellow powder (40 mg): mp 162~164°C; $[\alpha]_D^{22}$+23.3° (*c* 0.99, $CHCl_3$), ν_{max}^{KBr} 1743, 1657, 1585 cm^{-1}; λ_{max}^{MeOH} nm(ε): 248(15,000) 270(13,900), 402(4710). Anal. Calcd. for $C_{18}H_{18}O_6$: C, 65.45; H, 5.49. Found: C, 64.97, H, 5.58.

Nanaomycin A from Nanaomycin B in an Alkaline Medium

Nanaomycin B (200 mg) was dissolved in 60 ml of 0.1 N sodium hydroxide and the solution was allowed to stand for 10 minutes. After adjusting to pH 2.0 with 6 N hydrochloric acid, the product was extracted with ethyl acetate. The extract was evaporated and orange-yellow needles were obtained from an ethanol solution of the product. The compound was identified as nanaomycin A by mp, IR spectrum and thin-layer chromatography.

Preparation of Nanaomycin A Labeled with $CH_3{}^{13}COONa$

A two-day culture of *Streptomyces rosa* var. *notoensis* was used as a seed culture. Fermentation was carried out using a 500-ml SAKAGUCHI flask containing 125 ml of a medium for 4 days at 27°C. The composition wao 2 % glycerol, 2 % soybean meal, 0.3 % sodium chloride (pH 7.0, before sterilization).

One ml of $CH_3{}^{13}COONa$ solution (5 %) was added to the culture after 1 day of incubation. The culture broth (1,250 ml) was centrifuged. After the culture supernatant was adjusted to pH 2.0 with 6 N hydrochloric acid, nanaomycins were extracted with butyl acetate and then transferred into 0.1 N sodium hydroxide (nanaomycin B was converted to nanaomycin A by this treatment). From the aqueous solution, nanaomycin A was extracted with ethyl acetate after adjusting to pH 2.0 with 6 N hydrochloric acid. A crude powder, obtained by evaporating the solvent layer, was purified with preparative thin-layer chromatography to give a pure powder of nanaomycin A (21.5 mg).

Acknowledgements

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References

1) ŌMURA, S; H. TANAKA, Y. KOYAMA, R. ŌIWA, M. KATAGIRI, & T. HATA: Nanaomycins A and B, new antibiotics produced by a strain of *Streptomyces*. J. Antibiotics 27: 363~365, 1974
2) TANAKA, H.; Y. KOYAMA, J. AWAYA, R. ŌIWA, M. KATAGIRI, T. NAGAI, & S. ŌMURA: Nanaomycins, new antibiotics produced by a strain of *Streptomyces*. I. Taxonomy, isolation, characterization and biological properties. J. Antibiotics 28: 860~867, 1975
3) ŌMURA, S.; A. NAKAGAWA, H. YAMADA, T. HATA, A. FURUSAKI & T. WATANABE: Structures and biological properties of kinamycin A, B, C, and D. Chem. Pharm. Bull. 21: 931~940, 1973
4) MORTON, R. A.: Biochemistry of Quinones. p. 49 Academic Press Inc., New York, N. Y., 1965
5) SCHMID, H.; A. EBNOTHER & TH. M. MEIJER: Über die Konstitution des Eleutherins. Helv. Chim. Acta 33: 1751~1770, 1950
6) CAMERON, D. W.: Colouring matters of the aphididae. XXII. Nuclear magnetic resonance evidence for the structures and conformations of the naphthoquinone dimethyl ethers derived from the protoaphins, and of the erythroaphins. J. Chem. Soc. 1964: 98, 1964
7) THOMSON, R. H.: The structure of β-hydrojuglone and related compounds, keto-enols of the naphthalene series. J. Chem. Soc. 1950: 1737, 1950
8) VAN METER, J. C.; M. DANN & N. BOHONOS: Isolation and characteristics of frenolicin. Antimicrobial Agents Annual -1960: 77~80, 1961
9) ELLESTAD, G. A.; M. P. KUNSTMANN, H. A. WHALEY & E. L. PATTERSON: The structure of frenolicin. J. Am. Chem. Soc. 90: 1325~1332, 1968
10) BERGY, M. E.: Kalafungin, a new broad spectrum antibiotic, isolation and characterization. J. Antibiotics 21: 454~457, 1968
11) U. S. Patent, 3,300,382 (1967); Japan Patent, 45-26097 (1970)
12) BROCKMANN, H.; A. ZEECK, K. VAN DER MERWE & W. MULLER: Über Actinomyceten-farbstoffe. VIII. Die Konstitution der Actinorhodins. Ann. Chem. 698: 209~229, 1966