NOBORITOMYCINS A AND B, NEW POLYETHER ANTIBIOTICS

CAMILLA KELLER-JUSLÉN, HAMILTON D. KING, MAX KUHN, HANS-RUDOLF LOOSLI and ALBERT VON WARTBURG

Sandoz Ltd., Pharmaceutical Division, Chemical Research, CH-4002 Basel, Switzerland

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Noboritomycins A and B, two new polycyclic ionophoric polyethers were isolated from a strain of *Streptomyces noboritoensis*. The crystal structure and absolute configuration of noboritomycin A were established by X-ray analysis of its silver salt $C_{43}H_{63}O_{14}Ag$. Noboritomycin A is the first metabolic polyether possessing two carboxylic acid functions on the carbon backbone (C-31), namely a free acid and an additional carboxylic acid ethylester group. An unusual spiroketal system as well as a salicylic acid chromophore represent further remarkable elements. Noboritomycin A shows in this respect a structural relationship to salinomycin and lasalocid respectively. Comparison of physico-chemical data, in particular the interpretation of the ^{1}H- and ^{13}C-NMR spectra, revealed that noboritomycins A and B are structurally closely related, noboritomycin B carrying an ethyl substituent on the aromatic ring in the place of a methyl group present in noboritomycin A. Both metabolites exhibit activity against Gram-positive bacteria and against *Eimeria tenella* (chicken coccidiosis).

In the course of our screening for new antibiotics from soil actinomycetes we isolated a strain of *Streptomyces noboritoensis* (NRRL 8123) which produced two metabolites effective against Gram-positive bacteria. The active compounds designated as noboritomycins A and B were characterized as carboxylic acid ionophores and represent new members of the polyether antibiotic group.[1] This report deals with the taxonomy of the producing strain, as well as fermentation, isolation, structure and biological activities of noboritomycins A and B.

Taxonomic Study

The noboritomycin-producing strain NRRL 8123 was isolated from a soil sample collected in Ile de Lokrum, Yugoslavia, in 1971. The microorganism was identified as a strain of *Streptomyces noboritoensis*.[2] It had the fundamental characteristics of the organism, namely, it belongs to the strong chromogenic type; aerial mycelia are relatively long and wavy; aerial mycelia are hardly observed on starch-inorganic salts agar but are comparatively abundant on malt-yeast extracts agar and glycerine-asparagine agar; liquefaction of gelatin is absent; starch hydrolysis is weak, but nitrate reduction is strong and the carbon utilization pattern is quite characteristic; however, in contrast to the reference strain,[2] the strain NRRL 8123 decomposes rhamnose but not mannitol.

Fermentation

The fermentation was performed on a 1,500-liter scale in a stainless steel fermenter tank. One hundred and fifty liters of a good preculture was made by inoculating a dense spore suspension into a medium consisting per liter, 25 g Pharmamedia (Traders Protein Division, Fort Worth, Texas) and 25 g Cerelose (glucose technical grade) with a pH 6.7. The incubation of this vegetative culture was

Fig. 1. Electron micrograph showing aerial mycelia (Oatmeal agar ×1,750)

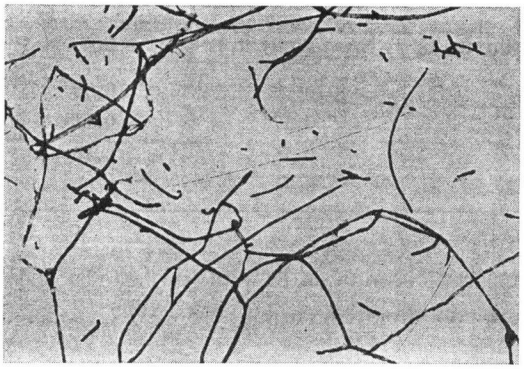
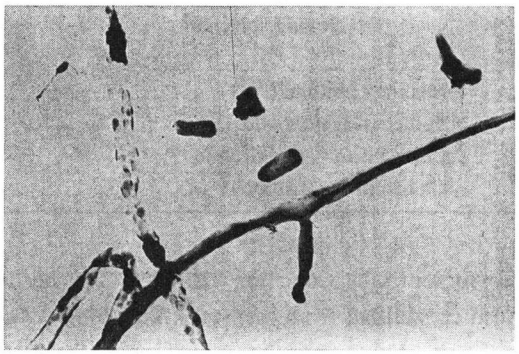


Fig. 2. Electron micrograph showing free spores (Oatmeal agar ×9,500)



made in 100-liter stainless steel fermenters, stirred at 100 rpm and aerated at 1 liter air/min./liter medium for 3 days at 27°C. This preculture was then inoculated to 1,500 liters of fermentation medium containing per liter, 30 g malt extract; 7.5 g Textrol (a type of soyabean meal); 1.0 mg $FeSO_4 \cdot 7H_2O$; 1.0 mg $MnCl_2 \cdot 4H_2O$ and 1.0 mg $ZnSO_4 \cdot 7H_2O$ with a pH 6.7. The culture was incubated, stirring at 100 rpm and aerating at 0.5 liter air/min./liter medium for 4 days at 27°C.

The fermentation broth was tested for antibiotic activity by the paper-disc-agar-diffusion assay using *Staphylococcus aureus* and *Micrococcus lysodeikticus* as test organisms. The fermentation was harvested at the time of its highest production of the antibiotic. The antibiotics were principally obtained from the mycelium.

Isolation

The fermentation broth (1,460 liters) was centrifuged yielding 260 kg mycelial cake which was homogenized three times with 350 liters of methanol - water (9 : 1) for 1 hour. After separation the combined filtrates were concentrated to 60 liters to remove methanol. The concentrate adjusted to pH 8 with 1 N NaOH was extracted five times with 60 liters of 1,2-dichloroethane. The combined organic layers were washed with water and evaporated furnishing 534 g of crude extract. The remaining aqueous concentrate was brought to pH 4 with 1 N HCl and extracted again five times with 50 liters of 1,2-dichloroethane. The organic phases were worked up in an analogous manner giving an additional 209 g of residue. A solution of the pooled extracts (743 g) in chloroform was adsorbed on silica gel (1 kg) and the resulting powder placed on a column prepared with 1 kg of silica gel (Merck, 0.06~0.2 mm). Elution with chloroform and chloroform - acetone (9 : 1) furnished a total of 141 g of crude material, which exhibits antibacterial activity against *Staphylococcus aureus*. Isolation of the antibiotics from this material was accomplished by careful silica gel column chromatography on 600 g Kieselgel Merck using chloroform and chloroform - acetone (95 : 5 to 90 : 10) as solvent mixture. Active fractions were pooled yielding 25 g of a mixture of noboritomycins A and B. The antibiotics were transformed to their sodium salt by shaking a solution of the mixture in 300 ml of chloroform with 30 ml 2 N NaOH. The organic layer was washed with water, dried over Na_2SO_4 and evaporated. Separation of the metabolites could be carried out by additional chromatography on 1 kg silica gel with chloroform containing 7.5% acetone. The first active fractions distinguished by their Rf value of 0.31 (Table 1) were combined (896 mg) and crystallized from methanol yielding 633 mg noboritomycin

Table 1. TLC of noboritomycins A and B

Developing solvents	Rf values		
	Noboritomycin A	Noboritomycin B	A 204A[3]
Hexane - acetone (3 : 1)	0.37	0.38	0.35
Toluene - acetone (9 : 1)	0.32	0.37	0.11
Chloroform - ethyl acetate (2 : 1)	0.52	0.56	0.16
Chloroform - acetone (9 : 1)	0.27	0.31	0.08

B (sodium salt) with m.p. 220~222°C. The following fractions with an Rf value of 0.27 (Table 1) also crystallized from methanol furnishing 5.7 g of pure noboritomycin A (m.p. 235~237°C; sodium salt).

Noboritomycins A and B can be distinguished from each other by thin-layer chromatography on silica gel plates (Kieselgel Merck 60, 0.25 mm). The Rf values of the sodium salts in comparison with A 204A[3] are listed in Table 1.

Detection was performed by spraying with a solution of Ceric-sulfate (0.2%) in 50% H_2SO_4 followed by heating at 110~130°C thus producing brown spots.

Physical and Chemical Properties

Noboritomycins A and B (sodium salts and free acids) are readily soluble in benzene, dichloromethane and chloroform, dissolve moderately in methanol, alcohol and acetone, are only slightly

Table 2. Physico-chemical properties of noboritomycins A and B

	Noboritomycin A Na-salt	Noboritomycin B Na-salt
Nature	white crystals	white crystals
m.p. (°C)	235~237°	220~222°
$[\alpha]_D^{20}$ (in $CHCl_3$)	−28.7° (*c* 1.029)	−29.9° (*c* 0.972)
MW (MS)	826	840
Formula	$C_{43}H_{63}NaO_{14}$ (826.96)	$C_{44}H_{65}NaO_{14}$ (840.99)
Anal. Found	C 62.4, H 7.8, Na 2.9, O 26.9	C 62.1, H 7.8, Na 2.7, O 26.8%
Calcd.	C 62.4, H 7.7, Na 2.8, O 27.1	C 62.8, H 7.8, Na 2.7, O 26.6%
UV (MeOH)	λ_{max} 208 nm log ϵ 4.53	λ_{max} 208 nm log ϵ 4.54
	300 nm log ϵ 3.56	301 nm log ϵ 3.55
	244 nm log ϵ 3.61 (sh)	244 nm log ϵ 3.56 (sh)
IR (CH_2Cl_2) (Figs. 3 and 4)	3330 cm^{-1} OH	3330 cm^{-1} OH
	1737 cm^{-1} Ester	1739 cm^{-1} Ester
	1713 cm^{-1} C=O	1715 cm^{-1} C=O
	1593 cm^{-1} Carboxylate-ion	1590 cm^{-1} Carboxylate-ion
^{1}H-NMR ($CDCl_3$) (Figs. 5 and 6)	δ0.6~1.25 9 C-CH_3	δ 0.5~1.30 10 C-CH_3
	2.00 (s, 3 H) CH_3	2.00 (s, 3 H) CH_3
	2.20 (s, 3 H) CH_3 (arom.)	missing
	3.44 (s, 3 H) OCH_3	3.44 (s, 3 H) OCH_3
	5.9~6.2 2 olefin. H	5.9~6.2 2 olefin. H
	6.60 (d, 1 H) J=8 Hz arom. H	6.63 (d, 1 H) J=8 Hz arom. H
	7.01 (d, 1 H) 〃 〃	7.03 (d, 1 H) 〃 〃

Fig. 3. IR-Spectrum of noboritomycin A (Na-salt in CH_2Cl_2)

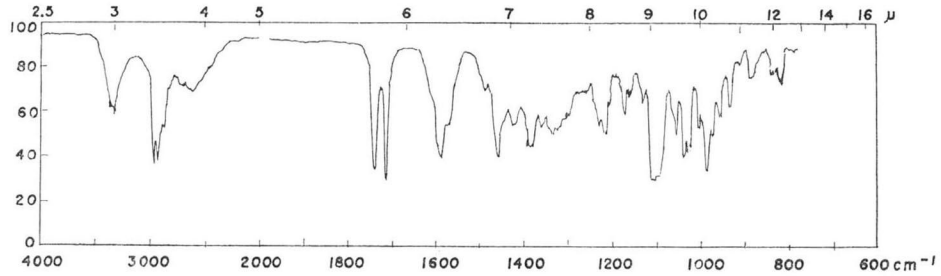


Fig. 4. IR-Spectrum of noboritomycin B (Na-salt in CH_2Cl_2)

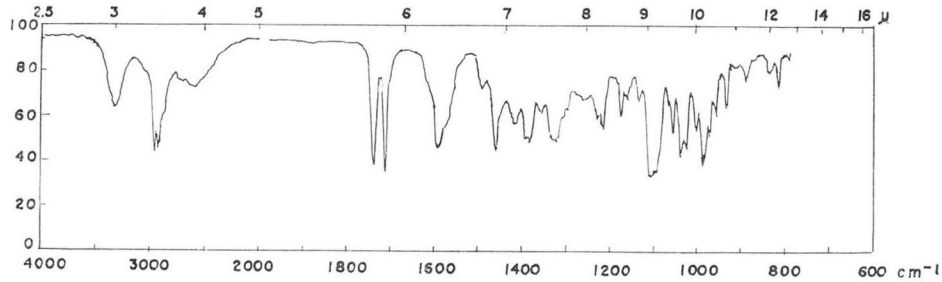
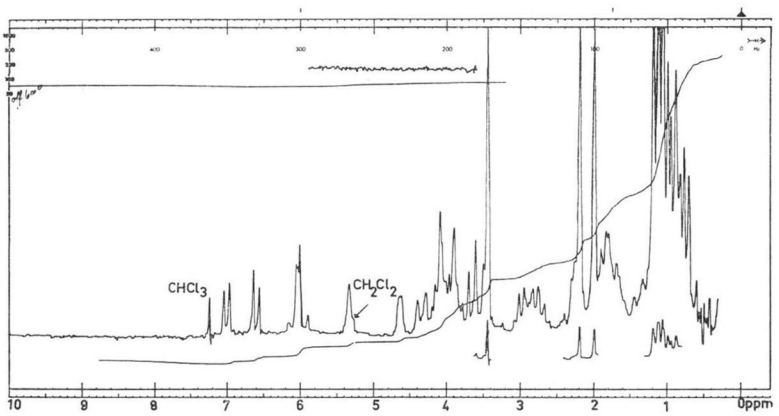


Fig. 5. ^{1}H-NMR-Spectrum of noboritomycin A (Na-salt in $CDCl_3$)

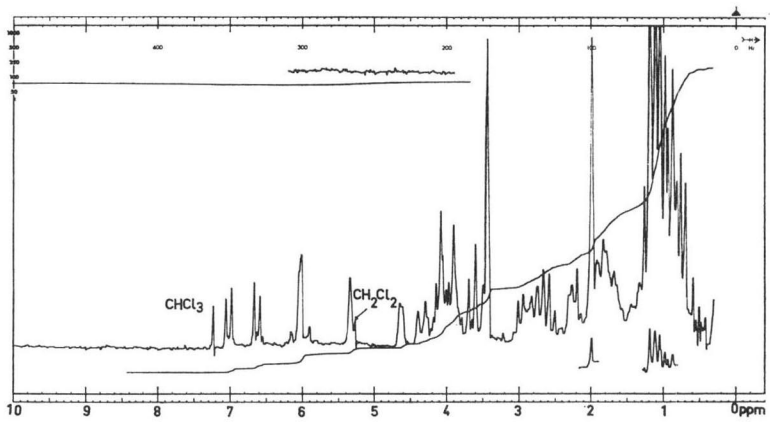


soluble in ether and practically insoluble in water. Physico-chemical data listed in Table 2 furnished basic information about several prominent structural features of noboritomycins A and B.

Both antibiotics contain the following structural elements: a free carboxylic acid group, an ester and a ketone function, one methoxyl, several OH-groups, an isolated double bond, an aromatic ring and 9~10 C-methyl groups.

These observations, together with the molecular formulae, suggested plainly that noboritomycins A and B belong to the class of polycyclic, ionophoric polyethers which include septamycin[4] and mutalo-

Fig. 6. ^{1}H-NMR-Spectrum of noboritomycin B (Na-salt in $CDCl_3$)



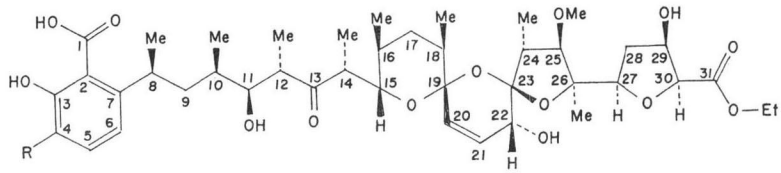
mycin,[5] two antibiotics isolated earlier in our laboratories. Several reviews of the ionophores have appeared recently.[1]

Structure of Noboritomycin A

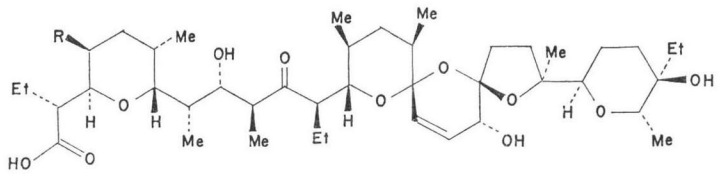
The molecular formula $C_{43}H_{68}NaO_{14}$ of noboritomycin A was established on the basis of elemental analysis and its mass spectrum.

In order to elucidate the structure of noboritomycin A we prepared its crystalline silver complex by treatment of the sodium salt with $AgNO_3$. Noboritomycin A proved to be a molecule large enough to surround an Ag-cation completely, forming a 1 : 1 complex. X-ray crystallographic analysis of the silver salt by WEBER and PETCHER[6] revealed the complete structure and the absolute configuration of noboritomycin A as indicated in Fig. 7.

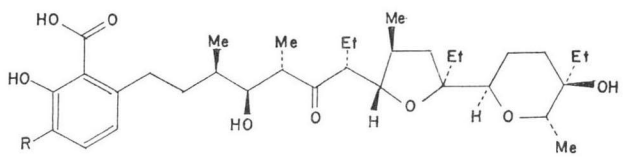
Fig. 7. Structural formulae



R = Me Noboritomycin A
R = Et Noboritomycin B



R = H Salinomycin
R = Me Narasin A



R = Me Lasalocid A
R = Et Lasalocid B

Noboritomycin A is distinguished by some remarkable features: It is the first polyether antibiotic so far report-

ed to possess two carboxylic acid functions, namely a free acid group on an aromatic ring and in addition a carboxylic acid ethylester group marking the opposite end of the 31-carbon backbone. The tetra-substituted aromatic ring represents a partial structure which has also been found in lasalocid.[7] The observed bathochromic shift in the UV ranging from λ_{max} 300 nm (noboritomycin A sodium salt) to λ_{max} 315 nm (free acid) is characteristic for a salicylic acid chromophore.[7] The unusual spiroketal system comprising three rings is another interesting structural detail. In this regard noboritomycin A is related to salinomycin[8] and narasin A.[9]

Structure of Noboritomycin B

Comparison of the physico-chemical parameters (Table 2), in particular the spectral analytical data, clearly demonstrated a close structural relationship between noboritomycins A and B. The molecular peak in the MS of noboritomycin B and elemental analysis agreed with the formula $C_{44}H_{65}NaO_{14}$ indicating an additional methylene group compared with noboritomycin A. The tetrasubstituted aromatic nucleus characteristic for noboritomycin A could also be demonstrated in noboritomycin B by the significant λ_{max} 301 nm as well as by chemical shifts in the ^{1}H-NMR spectrum consistent with two aromatic protons. A major difference in the NMR pattern of noboritomycin B consisted in the absence of the signal at 2.2 ppm, assigned to a methyl group on the aromatic ring. This suggested a variation in the substitution at carbon atom 4. Spin decoupling experiments showed that the multiplet at 2.6 ppm observed in the ^{1}H-NMR spectrum of noboritomycin B collapsed to a singlet upon irradiation in the region of the methyl resonances (at 1.15 ppm). Supported by this evidence we postulated that noboritomycin B (Fig. 7) contained an ethyl group on the aromatic ring in the place of the methyl group present in noboritomycin A. An analogous difference exists also between lasalocids A and B.[10]

^{13}C-NMR Spectra of Noboritomycins A and B

A detailed analysis of the ^{13}C-NMR spectra of both metabolites was performed to confirm our conclusion regarding the different substituents on the aromatic ring as well as to prove that no additional constitutional and/or configurational discrepancies exist between noboritomycins A and B (a possibility which could not be excluded on the basis of ^{1}H-NMR spectra).

Thanks to the publication of DORMAN *et al.*[11] on the biosynthesis of narasin a tentative assignment for the ^{13}C signals of ionophorous antibiotics is possible. A complete assignment assisted by ^{13}C-^{13}C coupling has recently been reported for salinomycin[12] and for lasalocid by SETO *et al.*[13]

In the ^{13}C-NMR spectra of the sodium salts of noboritomycins A and B all 43, respectively 44 carbon atoms were clearly resolved.

Based on the signal multiplicity in off-resonance spectra and on the chemical shifts noboritomycin A exhibited the following features: A ketone (C_{13} 216.7), a carboxylic acid and an ester group (C_1 170.5 and C_{31} 173.9), six aromatic carbon atoms (115 to 156.4), two olefinic carbons (C_{20} 122.5 and C_{21} 131.2), two ketal carbon atoms (C_{19} 98.9 and C_{23} 104.7), ten oxygenated carbons (59.6 to 95.0) of which one is tetra-, seven are tri-, and one each is di- and monosubstituted, seven further methines (29.6 to 48.5), three methylenic carbon atoms (35.1 to 43.8) and ten methyls (7.6 to 28.7).

Supported by the assignment of the ^{13}C signals of salinomycin[12] and by the use of shift comparisons and additivity increments[14,15] we were able tentatively to assign the signals of the noborito-

Table 3. ^{13}C-NMR-spectra[a] of noboritomycins A and B (sodium salts). Tentative spectral assignments

Carbon number	δ Noboritomycin A	δ Noboritomycin B	Mult.[b]	Δδ[c]	Carbon number	δ Noboritomycin A	δ Noboritomycin B	Mult.[b]	Δδ[c]
1	170.5	170.4	s	−0.1	17	38.6	38.6	t	0
2	119.6	119.7	s	+0.1	18	40.5	40.5	d	0
3	156.4	155.9	s	−0.5	18-CH_3	17.4	17.4	q	0
4	121.4	127.5	s	+6.1	19	98.9	98.9	s	0
4-CH_3	15.9	13.9	q	−2.0	20	122.5	122.5	d	0
4-CH_2	—	23.0	t	—	21	131.2	131.2	d	0
5	130.7	129.0	d	−1.7	22	71.5	71.4	d	−0.1
6	115.0	115.0	d	0	23	104.7	104.7	s	0
7	146.0	145.8	s	−0.2	24	45.0	44.9	d	−0.1
8	29.6	29.6	d	0	24-CH_3	13.1	13.1	q	0
8-CH_3	28.7	28.7	q	0	25	95.0	94.9	d	−0.1
9	43.8	43.8	t	0	25-OCH_3	59.6	59.6	q	0
10	30.4	30.3	d	−0.1	26	85.4	85.4	s	0
10-CH_3	7.6	7.5	q	−0.1	26-CH_3	18.9	18.9	q	0
11	75.9	75.9	d	0	27	68.7	68.7	d	0
12	48.5	48.4	d	−0.1	28	35.1	35.0	t	−0.1
12-CH_3	12.7	12.6	q	−0.1	29	64.1	64.1	d	0
13	216.7	216.6	s	−0.1	30	82.0	81.9	d	−0.1
14	46.7	46.7	d	0	31	173.9	173.9	s	0
14-CH_3	13.8	13.7	q	−0.1	31-OCH_2-CH_3	61.4	61.4	t	0
15	81.4	81.4	d	0					
16	32.0	32.0	d	0	31-OCH_2-$\underline{C}H_3$	12.7	12.6	q	−0.1
16-CH_3	15.6	15.6	q	0					

[a] δ chemical shifts in ppm; $\delta_{TMS}=0$, $CDCl_3$ solution.

[b] Multiplicities from off-resonance spectra; s=singlet, d=doublet, t=triplet, q=quadruplet.

[c] $\Delta\delta=\delta_{\text{noboritomycin B}}-\delta_{\text{noboritomycin A}}$

mycins (Table 3). The spectrum of noboritomycin B exhibited one extra methylenic carbon atom (23.0) and displayed the expected significant differences in the signals of the aromatic carbon atoms. A comparison of the shifts $\Delta\delta=\delta_{\text{ethyl benzene}}-\delta_{\text{toluene}}$ showed good agreement (Table 4).

Table 4. Comparison of expected and measured Δδ's.

Carbon number	Expected Δδ (ppm)[a]	Measured Δδ (ppm)[b]
2	−0.1	+0.1
3	−1.2	−0.5
4	+6.4	+6.1
5	−1.2	−1.7
6	−0.1	0
7	+0.3	−0.2

[a] $\Delta\delta=\delta_{\text{ethyl benzene}}-\delta_{\text{toluene}}$

[b] $\Delta\delta=\delta_{\text{noboritomycin B}}-\delta_{\text{noboritomycin A}}$

In summary, the ^{13}C-NMR spectra confirmed that the methyl group in noboritomycin A is replaced in noboritomycin B by an ethyl substituent; since all other differences in chemical shift outside the aromatic region are less than or equal to 0.1 ppm the remaining constitution and configuration of noboritomycin B must be the same as that of noboritomycin A proven by X-ray analysis of the silver salt.

Biological Properties

Noboritomycins A and B had activity against a wide range of Gram-positive bacteria but were inactive against Gram-negative organisms, yeasts and hyphomycetes. No cross resistance could be observed with aminoglycosides, tetracyclines and β-lactams. Noboritomycin A exhibited only weak anticoccidial activity (*Eimeria tenella*) in chicken. The antimicrobial spectra *in vitro*, obtained by broth-dilution assay (brain heart infusion broth; temperature 37°C, time 24 hours; inoculum 10^5 CFU/ml) is given in Table 5.

Table 5. Antimicrobial spectra of noboritomycins A and B

Organisms	MIC (mcg/ml)	
	Noboritomycin A	Noboritomycin B
Staphylococcus aureus res. penicillin	0.01	0.01
Micrococcus lysodeikticus	0.01	0.01
Micrococcus sp., res. tetracycline	0.01	0.01
Bacillus subtilis	0.01	0.01
Streptococcus faecalis res. aminoglycosides	0.01	0.01
Sarcina lutea res. macrolides	0.01	0.01
Neisseria pharyngis	0.01	0.01
Clostridium pasteurianum	0.01	0.01
Mycoplasma laidlawii	0.01	0.01

Acute toxicity of noboritomycins A and B: $LD_{50}=5$ mg/kg i.p. (mouse), 0.5 mg/kg i.v. (mouse).

Experimental

^{1}H-NMR spectra were run on a HA-100 at 100 MHz, ^{13}C-NMR spectra on a Bruker HX-90-E spectrometer at 22.6 MHz using TMS = 0 ppm as internal standard.

Noboritomycin A silver salt:

To 331 mg noboritomycin A sodium salt dissolved in 60 ml of dichloromethane - methanol (5 : 1) a solution of 2.64 g $AgNO_3$ in 4 ml of water was added and the mixture kept in the dark for 3 days. The solvents were removed *in vacuo* and the residue was taken up in 200 ml of dichloromethane. This solution was washed 5 times with 10 ml of water and the organic layer evaporated to dryness. The resulting solid dissolved in 1 ml of dichloromethane spontaneously crystallized by adding 10 ml of methanol. Yield 293 mg of noboritomycin A silver salt with m.p. 218~219°C.

Anal. Calcd. for $C_{43}H_{63}O_{14}Ag$: C 56.6; H 7.0; O 24.6; Ag 11.8
Found: C 56.9; H 6.6; O 24.7; Ag 12.4

For the X-ray analysis the silver salt was recrystallized from acetonitrile.

Noboritomycin A free acid:

A solution of 342 mg of noboritomycin A sodium salt in 200 ml of chloroform was shaken with a mixture of 10 ml of water and 5 ml 1 N HCl at room temperature. The organic layer was washed with 20 ml of water and evaporated *in vacuo*. The free acid of noboritomycin A was obtained as white amorphous powder (325 mg) showing m.p. 110~113°C.

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