Isolation, NMR spectroscopy, and conformational analysis of the antibiotic INA 2770 (cineromycin B) produced by *Streptomyces* strain

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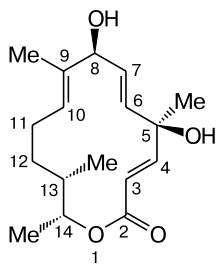
The antibiotic INA 2770 active against methicillin-resistant staphylococcus aureus (MRSA) was biotechnologically produced and isolated. This antibiotic is identical to cineromycin B. The characteristic features of the ^{1}H and ^{13}C NMR spectra of this compound were studied for the first time, and the conformational analysis was carried out by computational methods (molecular mechanics (MM3) force field) and using nuclear Overhauser effect experiments.

Key words: lactones, cineromycin B, antibiotics, macrocyclic compounds, actinomycetales, ^{1}H NMR spectra.

When searching for antibiotic producers active against MRSA, we isolated the actinomycetales strain INA 2770 of the genus *Streptomyces* producing the antibiotic INA 2770 (the registry numbers from the collection of the G. F. Gause Institute of New Antibiotics of the Russian Academy of Medical Sciences are given for the strain and the antibiotic) from a soil sample. Preliminary *in vitro* assays of the compound INA 2770 have demonstrated its high activity against a wide range of gram-positive test bacteria. In the present study, we isolated and characterized the antibiotic, investigated it by NMR spectroscopy, and carried out the experimental and theoretical conformational analysis.

Results and Discussion

To identify the antibiotic INA 2770 (its physicochemical properties are given in Table 1), we performed a search for its analogs in the Bioactive Natural Products Database (BNPD) developed by J. Bérdy (Hungary).[1] The search was carried out by putting analytical data on the molecular weights, UV spectra, and $[\alpha]^{20}_D$ into a computer. An analysis of the BNPD database showed that the antibiotic INA 2770 is identical to cineromycin B,[2—4] *viz.*, (3*E*,6*E*,9*E*,5*R*,8*S*,13*S*,14*R*)-5,8-dihydroxy-5,9,13,14-tetramethyl-1-oxacyclotetradeca-3,6,9-trien-2-one, in virtually all descriptors.



The structure of the 8-*O*-methyl derivative of cineromycin B (albocyclin) was established by X-ray diffraction[5,6] and ^{1}H and ^{13}C NMR spectroscopy.[7] However, the NMR spectroscopic data for cineromycin B were lacking in the literature. Hence, we carried out a detailed investigation of the ^{1}H and ^{13}C NMR spectra of the isolated antibiotic to confirm the configurations of all asymmetric centers in this compound.

The assignment of the signals in the ^{1}H NMR spectrum of cineromycin B in deuterochloroform was made with the use of two-dimensional homonuclear COSY ^{1}H—^{1}H spectroscopy; the assignment in the ^{13}C NMR spectrum, taking into account the correlations in heteronuclear ^{1}H—^{13}C gHSQC (signals of protonated carbon atoms) and gHMBC (signals of quaternary carbon atoms) spectra. The ^{1}H and ^{13}C NMR chemical shifts of cineromycin B are given in Table 2.

The gNOESY spectrum has a series of non-trivial correlation peaks, *viz.*, H(4)/H(7), H(4)/H(10), H(7)/H(10), H(6)/H(8), H(7)/H(8), H(8)/H(10), H(10)/H(13), H(10)/H(12´), Me(13)/H(11´), Me(5)/H(7), Me(5)/H(7), Me(9)/H(8), Me(13)/H(14), and Me(13)/Me(14), which could not be interpreted without detailed knowledge of the conformational state of the compound under study in solution. Hence, we carried out

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 4, pp. 784—787, April, 2007.

1066-5285/07/5604-0815

Table 1. Physicochemical properties of the components of the antibiotic INA 2770 and cineromycin B[2–4] *

Parameter	INA 2770	Cineromycin B
Mol. weight, EI-MS (m/z) $[M]^+$	294**	294
M.p./°C	146-148	149-150
EAS (EtOH), λ_{max}/nm	208	210
$[\alpha]^{20}_D$	−121 (*c* 0.2, MeOH)	−110 (*c* 1.0, MeOH)
TLC (SiO_2), R_f in		
chloroform—methanol (9 : 1)	0.60	—
hexane—ethyl acetate (1 : 3)	0.57	0.59
hexane—ethyl acetate (7 : 3)	0.10	0.13
benzene—ethyl acetate (1 : 1)	0.30	0.35
Solubility in		
acetone, ethyl acetate, methanol ethanol, butanol, chloroform	Good	Good
pyridine	—	Good
diethyl ether	Poor	Poor
hexane, water (pH 7.0)	Insoluble	Insoluble

* Qualitative reactions with $KMnO_4$, anisaldehyde, and Ehrlich's reagent gave positive results for both antibiotics.
** Corresponds to the molecular formula $C_{17}H_{26}O_4$.

Table 2. The ^1H and ^{13}C NMR chemical shifts of the antibiotic INA 2770 (cineromycin B)

Atom or group of atoms	δ (*J*/Hz)	
	^1H NMR	^{13}C NMR
C(2)	—	166.3
C(3)H	5.86 (d, $J_{3,4}$ = 16)	115.0
C(4)H	6.91 (d, $J_{3,4}$ = 16)	155.5
C(5)		73.4
C(6)H	5.85 (d, $J_{6,7}$ = 16)	135.3
C(7)H	5.71 (dd, $J_{6,7}$ = 16, $J_{7,8}$ = 5)	133.6
C(8)H	4.53* ($J_{7,8}$ = 5)	74.5
C(9)	—	137.9
C(10)H	5.21* (br.m)	129.4
$C(11)H_2$	2.10*, 1.84* (br.m, $J_{11,11}$ = 16.5)	25.0
$C(12)H_2$	1.22*, 1.17* ($J_{12,13}$ < 2)	34.3
C(13)H	1.40* (br.q, $J_{Me,H(13)}$ = 6, $J_{13,14}$ < 2)	39.5
C(14)H	4.56* ($J_{Me,H(14)}$ = 7)	75.6
MeC(5)	1.53 (s)	27.0
HOC(5) and HOC(8)	3.09, 2.41 (br.s)	
MeC(9)	1.71 (s)	15.0
MeC(13)	0.88 (d, $J_{Me,H(13)}$ = 6)	16.1
MeC(14)	1.21 (d, $J_{Me,H(14)}$ = 7)	18.1

* Only the reliably measured constants are given.

conformational analysis by computational methods and using nuclear Overhauser effect experiments.

The molecular mechanics calculations were performed with the use of the TINKER program package (the MM3 force field, the dielectric permeability is 1.5). The calculations of the three-dimensional structure of the cineromycin molecule by molecular mechanics methods were carried out using the data on the configurations of the asymmetric centers in albocyclin determined by X-ray diffraction.[6] The lowest-energy conformer is shown in Fig. 1, *a*. Selected pairs of protons, which are in spatial

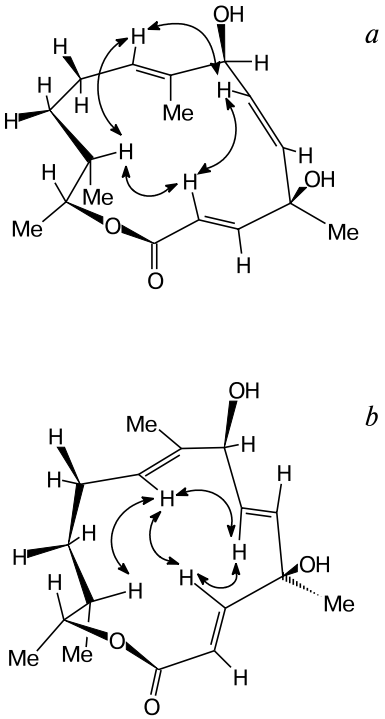


Fig. 1. Conformers of cineromycin B: (*a*) the low-energy conformer revealed by molecular mechanics calculations (the interproton distances shorter than 3 Å are indicated by arrows) and (*b*) the conformer, which is in best agreement with the experimental NOE.

proximity sufficient for NOE observations, are indicated by arrows.

As can be seen from the above data, only some of the spatial correlations found in the experiment (H(4)/H(10), H(7)/H(10), and H(10)/H(13)) correspond to the lowest-energy conformer. Moreover, contacts incompatible for individual isomers were experimentally found. For example, these are the Me(5)/H(7) and Me(5)/H(7) contacts and the H(6)/H(8), H(7)/H(8), and H(8)/H(10) contacts for the low-energy conformer. These contradictions are eliminated on the assumption that the fragment of the macrocycle including the C(6)—C(8) atoms is conformationally flexible due to rotation about the C(5)—C(6) and C(7)—C(8) bonds. The calculated energy excess for the conformers with the HO(5)—C(5)—C(6)—H(6) and H(7)—C(7)—C(8)—OH(8) dihedral angles, which differ from those in the lowest-energy conformer by ±(20—30°) (Fig. 1, *b*), is 1—2 kcal mol^{-1}.

In conclusion, the studies by molecular mechanics methods and nuclear Overhauser effect experiments demonstrated that cineromycin B exists in solution as an equilibrium mixture of conformers due to rotation about the single bonds in the macrocycle. One of the revealed conformations (see Fig. 1, *b*) is structurally similar to the conformation of the methyl derivative of cineromycin (albocyclin) in the crystal structure.[6]

Experimental

Characterization of the producer strain. The actinomycetales strain INA 2770 was isolated from a soil sample (Israel). The preliminary characterization of the antibiotic activity of the components of the corresponding culture liquid was carried out on organic agar 2 Gause by the stroke method. The antibiotic activity of the strain against the test microorganisms *Staphylococcus aureus* FDA 209P, *S. aureus* INA 00761 (MRSA), *Micrococcus luteus* NCTC 8340, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, and *Aspergillus niger* ATCC 16404, but not against the yeast *Saccharomyces cerevisiae* INA S-1, was demonstrated by the stroke method for the growth on solid media and agar diffusion for the growth in liquid nutrient media. A well-developed aerial mycelium containing helical chains of spores was formed on mineral agar 1 Gause and oat agar. The spores had a smooth surface. The strain INA 2770 had a Lechevalier type I cell wall.[8] Hydrolysates of whole cells contained LL-DAP (LL-diaminopimelic acid) and contained no diagnostic sugars. Based on the above-mentioned features, the strain INA 2770 was assigned to the genus *Streptomyces*. A pea-starch-saccharose medium proved to be the optimal medium for the antibiotic production by the strain INA 2770. Under conditions of deep cultivation, the strain synthesized the antibiotic INA 2770, which suppresses the growth of the above-listed gram-positive test bacteria. The minimum suppressing concentrations of the native culture solution and then of the crude antibiotic were determined by serial dilution of the antibiotic in agar. In all cases, organic agar 2 Gause was used.[9] The minimum suppressing concentration against MRSA of the powdered antibiotic INA 2770, which was prepared from the native solution, was 2.5 μg mL^{-1}.

Preparation of the antibiotic INA 2770. To prepare the antibiotic, the strain was cultivated in 750-mL Erlenmeyer flasks containing 150 mL of a pea-starch-saccharose medium (composition, g L^{-1}: saccharose, 21.0; pea flour, 15.0; starch, 8.5; $NaNO_3$, 5.0; NaCl, 5.0; $CaCO_3$, 5.0; pH 7.0) on a shaker at 200 rpm at 28 °C for four days. An organic agar 2 Gause medium was used as a seeding medium.[9] The culture liquid (CL) (pH 7.7—7.8) of the producer strain INA 2770 was separated by filtration into the mycelium and the native solution. The latter was extracted with ethyl acetate in 1 : 3 and 1 : 10 ratios with respect to the volume of the native solution. The extracts were combined, and the solvent was removed under vacuum on a rotary evaporator. The oily residue was dissolved in 50% EtOH, celite was added to the solution, the reaction mixture was stirred, the solvent was removed under vacuum, and the antibiotic adsorbed on celite was placed in the upper part of the column packed with Kieselgel 60 (Merck) (0.063—0.2 μm) in hexane. The elution was carried out sequentially with hexane, chloroform, and chloroform with methanol in a gradient. The elution of the active compound from the column was monitored by agar diffusion assays in Petri dishes using paper disks. The size of the growth inhibition zone for the test culture *Staphylococcus aureus* INA 00761 (MRSA) was used as the criterion of the activity of the materials under study. The antibiotic was eluted from the column using a 100 : 1 $CHCl_3$—MeOH mixture. The biologically active fractions were combined, concentrated to dryness under vacuum, dissolved in a small volume of chloroform, and chromatographed on a silica gel column using a toluene—diethyl ether gradient. After vacuum evaporation of the active fraction, the oily colorless residue was crystallized from a CH_2Cl_2—hexane mixture.

Physicochemical characterization of the antibiotic INA 2770. The purity of the antibiotic INA 2770 was monitored by TLC on Kieselgel 60 UV_{254} plates (Merck). The eluent systems and the retention times, as well as other physicochemical characteristics of the antibiotic INA 2770, are given in Table 1. The optical rotation was measured on a Perkin-Elmer-241 spectropolarimeter (UK). The UV-VIS spectra were recorded on a UV-1601 PC spectrophotometer (Shimadzu, Japan). The EI mass spectra were obtained on a Finnigan LC instrument. The ^1H and ^{13}C NMR spectra were measured on a Bruker DRX 500 instrument in $CDCl_3$ at 30 °C. The chemical shifts are given relative to Me_4Si (δ_H and δ_C are 0.0). Two-dimensional NMR experiments were performed using the standard Bruker software. In ROESY experiments, the mixing time was 300 ms. The procedure for gHMBC experiments was optimized for the constant of 8 Hz.

The present study was carried out in the framework of the Agreement on Scientific and Technological Cooperation between the N. D. Zelinsky Institute of Organic Chemistry of the Russian Academy of Sciences and the G. F. Gause Institute of New Antibiotics of the Russian Academy of Medical Sciences with partial financial support of the International Science and Technology Center (ISTC, Grant 1848).

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Received December 20, 2006;
in revised form February 1, 2007