Structure, ^{1}H and ^{13}C NMR spectra, and biological activity of the antibiotic INA-1278 related to irumamycin and produced by the experimental *Streptomyces sp.* strain No. 1278*

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The screening for novel antibiotic producers by selection methods revealed the *Streptomyces sp.* strain No. 1278 producing the antibiotic INA-1278, which exhibits antimicrobial and antifungal activities. This compound was isolated from the liquid culture by extraction with organic solvents and purified by chromatographic methods. The structure of this antibiotic was studied by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy. An analysis of the results of the investigation indicate that the compound INA-1278 is structurally related to the antibiotic irumamycin, which has been studied earlier. However, the novel antibiotic may differ from irumamycin in the configurations of some asymmetric centers, which were not established at the present stage of our research.

Key words: HPLC, ^{1}H and ^{13}C NMR, ROESY, HMBC, antibiotic, INA-1278, irumamycin.

Liquid cultures produced by the cultivation of actinomycetes are promising sources of antibiotics and other biologically active compounds. Earlier, we have reported the results of structural and biological studies of cineromycin B,[1] eremomycin B,[2] and the dalbaheptide antibiotic INA-9301 related to vancomycin (for the producer strain and the antibiotic, the registry numbers from the collection of the G. F. Gause Institute of New Antibiotics of the Russian Academy of Medical Sciences are given),[3] which were isolated from different producer strains. During the screening for producers of antibiotics by selection and genetic engineering methods, we produced the *Streptomyces sp.* strain No. 1278 from the actinomycete, which has been isolated earlier. The investigation of the antibiotic activity of the liquid culture by the bioautography assay[4] showed that it contained a component exhibiting high antifungal activity. In the present study, we isolated the antibiotic INA-1278, structurally and spectroscopically characterized it by NMR spectroscopy and mass spectrometry, and investigated its biological activity.

Results and Discussion

A liquid culture was extracted with organic solvents. Flash chromatography of the extracts furnished an antibiotic, to which the number INA-1278 was assigned. Analytical reversed-phase HPLC showed that the purity of the antibiotic INA-1278 was not higher than 80% (Fig. 1; the content was estimated by integrating the ELSD (evaporative light scattering detector) signal. Because of the low solubility of the extract in the eluent (see the Experimental section), the conditions found for the analytical separation were unsuitable for the purification of preparative amounts of the component responsible for biological activity with a retention time of ~20 min (see Fig. 1). Hence, to purify this component, we elaborated the conditions of the chromatographic separation on unmodified silica gel using elution with an acetonitrile gradient in CH_2Cl_2. An example of the analytical separation under these conditions is presented in Fig. 2. In this eluent, the extract is readily soluble, which allowed us to produce the antibiotic INA-1278 with ~96% purity (Fig. 3) in amounts sufficient for investigation by NMR spectroscopy and biological assays.

The complete assignment of signals in the ^{1}H and ^{13}C NMR spectra was made for a solution of INA-1278 in DMSO-d_6 based on two-dimensional homonuclear (^{1}H-^{1}H COSY and ROESY) and heteronuclear (^{1}H—^{13}C gHSQC and gHMBC) experiments (Table 1). The ^{1}H NMR spectrum shows signals for four olefinic protons as two doublets of doublets at δ 5.62 and 5.23 and a multiplet at δ 5.40, as well as signals for protons of aliphatic groups. In particular, the spectrum has signals of nine methyl groups:

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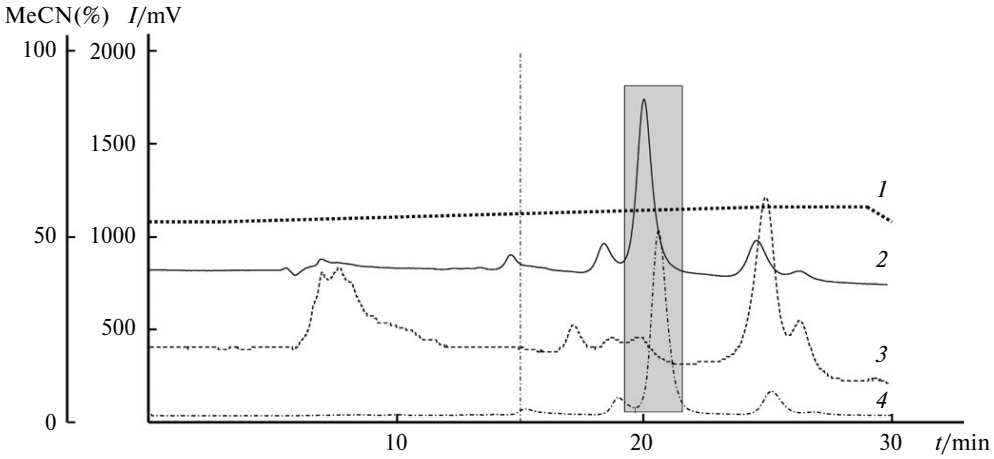


Fig. 1. Analysis of the crude antibiotic INA-1278 by reversed-phase HPLC and the plots of the MeCN content (*1*) in the eluent and the intensity of detector response (*I*) *versus* the elution time: *2*, photometer at 205 nm; *3*, photometer at 254 nm; *4*, ELSD. The fraction exhibiting the highest antimicrobial activity is indicated by a dashed line.

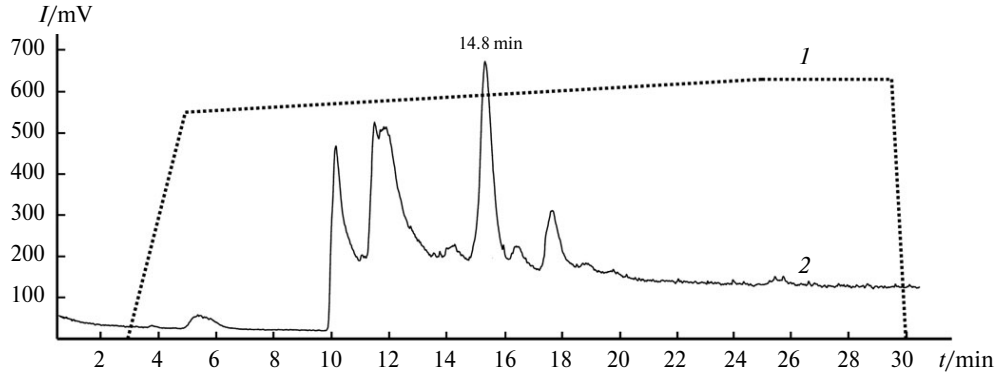


Fig. 2. Chromatogram of the crude antibiotic INA-1278 under normal-phase HPLC conditions (elution with a MeCN gradient from a column packed with unmodified silica gel equilibrated with CH_2Cl_2). The plots of the MeCN content in the eluent (*1*) and the intensity of the detector response (*2*) *versus* the time. The retention time of the compound corresponding to a substance with a retention time of ~20 min in the reversed-phase HPLC chromatogram (see Fig. 1) is marked.

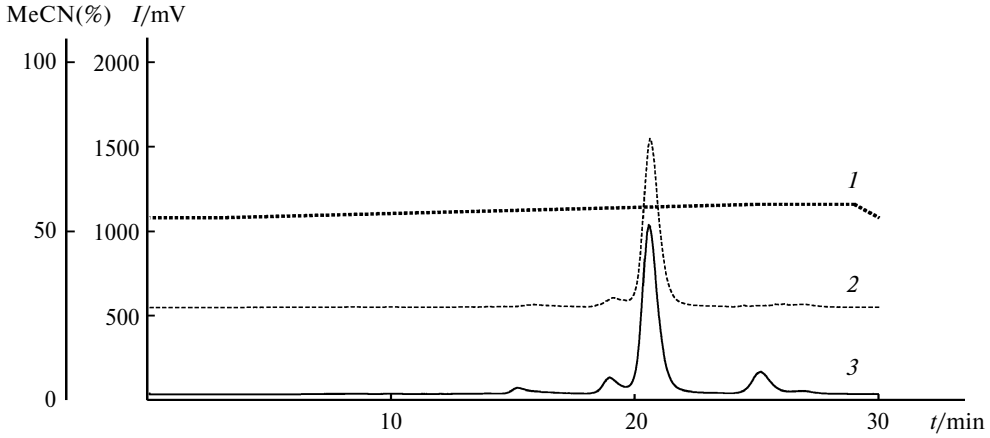


Fig. 3. Plot of the MeCN content in the eluent (*1*) *versus* the time and a comparison of the reversed-phase HPLC chromatogram (ELSD as the detector) of samples of the antibiotic INA-1278 after the purification (*2*) and before the purification (*3*).

Table 1. Chemical shifts (δ) of the signals in the ^{1}H and ^{13}C NMR spectra of the antibiotic INA-1278 and irumamycin

Atom serial number	INA-1278						Irumamycin[c] $CDCl_3$	
	DMSO-d_6		$CDCl_3$		HMBC[a]	ROESY[b]		
	δ_H	δ_C	δ_H	δ_C			δ_H	δ_C
1	—	171.8	—	173.2	—	—	—	173.3
2	2.66, 2.53	44.8	2.57, 2.66	43.4	C(1), C(3), C(4)	—	2.67, 2.58	43.4
3	—	93.7	—	93.9	—	—	—	94.0
4	2.20	34.7	2.09, 2.13	35.1	C(5), C(6)	H(2)	H.o.	35.1
5	5.44	117.5	5.47	116.8	—	—	5.50	116.8
6	—	131.8	—	132.9	—	—	—	132.9
7	4.27	79.2	4.45	80.0	C(5), C(6), C(8), C(9); Me—C(8)	—	4.46	80.1
8	—	133.4	—	134.8	—	—	—	134.9
9	5.40	129.1	5.43	129.2	C(7), C(10); Me—C(8)	—	5.45	129.3
10	2.03, 1.73	27.3	1.89, 2.11	26.9	C(8), C(9), C(11), C(12)	Me—C(8)	H.o.[d]	28.8
11	1.22 1.14	25.5	1.47, 1.23	25.9	—	H(17); Me—C(16)	1.30—1.20	27.0
12	2.18, 1.68	34.3	1.57, 1.42	35.2	C(11), C(13), C(14)	—	H.o.	35.3
13	3.91	80.8	3.92	82.3	C(1′), C(11), C(12), C(14), C(15)	—	3.92	82.4
14	5.23	130.3	5.56	134.2	C(16)	—	5.56	134.3
15	5.62	134.7	5.22	134.1	C(13), C(16); Me—C(16)	H(17)	5.24	134.1
16	2.12	43.3	2.11	42.1	C(14), C(15), C(17); Me—C(16)	H(15); Me—C(18)	H.o.	42.1
17	3.47	72.0	3.29	77.5	C(15), C(19); Me—C(18)	H(16), H(19)	H.o.	77.5
18	1.66	39.8	1.96	34.6	C(16), C(17); Me—C(18)	Me—C(16), H(20)	H.o.	34.6
19	4.92	80.6	4.84	81.5	C(1), C(17), C(18), C(20), C(21); Me—C(18), C(20)	—	4.82	81.6
20	1.97	29.0	1.78	31.9	C(19), C(21); Me—C(20)	—	H.o.	31.9
21	1.29, 0.95	31.8	1.16, 0.96	35.9	C(19), C(20), C(22); Me—C(22)	—	1.10	36.7
22	1.40	29.7	1.47	30.5	C(21); Me—C(22)	—	H.o.	30.5
23	2.97	65.4	2.69	66.1	C(22), C(25); Me—C(22)	—	3.40—3.18	66.3
24	—	63.6	—	64.3	—	—	—	64.4
25	—	210.4	—	210.7	—	—	—	210.9
26	2.37	28.0	2.33, 2.43	28.7	C(25), C(27)	—	H.o.	36.9
27	0.86	7.2	1.01	7.3	C(25), C(26)	—	—	7.3
1′	4.53	97.0	4.54	98.3	C(13), C(2′)	—	4.58	98.4
2′	1.99, 1.33	37.5	2.25, 1.66	36.8	C(1′), C(2′), C(3′), C(4′)	—	H.o.	35.9
3′	4.48	72.7	4.63	75.2	C(4′); C=O C(3′)	—	4.63	75.2
4′	2.92	73.5	3.20	75.0	C(5′), C(6′)	—	3.40—3.18	75.0
5′	3.14	71.6	3.25	72.0	C(4′), C(6′)	—	3.40—3.18	72.0
6′	1.16	17.9	1.30	17.8	C(4′),C(5′)	—	1.28	17.8
C=O(3′)	—	156.4	—	157.4	—	—	—	157.6
6-Me	1.40	18.7	1.47	19.1	C(5), C(6), C(7)	—	1.48	19.2
8-Me	1.36	10.6	1.38	10.7	C(7), C(8), C(9)	—	1.38	10.8
16-Me	1.00	21.1	0.92	17.1	C(15), C(16), C(17)	H(17); Me—C(18)	0.88	17.2
18-Me	0,74	11.3	0.88	5.4	C(17), C(18), C(19)	—	0.84	5.5
20-Me	0.77	16.7	0.84	15.9	C(19), C(20), C(21)	—	0.80	17.0

(*to be continued*)

Table 1 (*continued*)

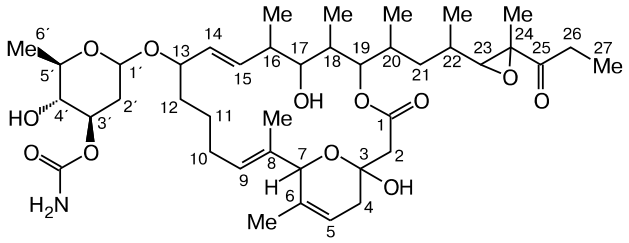
Atom serial number	INA-1278						Irumamycin[c] $CDCl_3$	
	DMSO-d_6		$CDCl_3$		HMBC[a]	ROESY[b]		
	δ_H	δ_C	δ_H	δ_C			δ_H	δ_C
22-Me	1.03	16.4	1.07	16.9	C(21), C(22), C(23)	—	1.10	15.9
24-Me	1.34	12.5	1.43	12.9	C(23), C(24), C(25)	—	1.42	12.9
3-OH	5.21	—	5.59	—	C(2), C(3)	—	1.00	—
4′-OH	4.99	—	N.d.	—	—	—	—	—
17-OH	4.36	—	N.d.	—	—	—	—	—

[a] Correlation peaks with carbon atoms in the HMBC spectrum.
[b] Nontrivial correlations in the ROESY spectrum.
[c] Literature data.[5,6]
[d] N.d., data on the assignment of the signal are not reported in the studies.[5,6]

a triplet at δ 0.86, five doublets at δ 1.16 1.03, 1.00, 0.77, and 0.74, and three singlets at δ 1.40 1.36, and 1.34. Two doublets at δ 4.99 and 4.36 and a singlet at δ 5.21 can be assigned to protons of OH groups.

The ^{13}C NMR spectrum shows 41 signals (see Table 1), including nine signals of methyl groups, eight signals of methylene groups, and thirteen signals of methine groups, nine of which are bound to oxygen atoms. The spectrum shows also signals of the acetal group of the glycosyl residue, three ethylene groups, and one carbamoyl group. In addition, there are signals of one ketone carbonyl group and the second carbonyl group, which can be assigned to lactone or the carboxylic acid residue.

An analysis of the COSY, HSQC, and HMBC spectroscopic data revealed coupled spin systems corresponding to spin systems of irumamycin.



Irumamycin

However, the chemical shifts δ_C for the carbon atoms 13, 14, 17, 18, 20, 21, 26, 3′, 16-Me, and 18-Me (see Table 1) determined in the present study substantially differ from those published earlier for a solution of irumamycin[5,7] in $CDCl_3$. Hence, for the proper comparison of the chemical shifts, we recorded spectra of INA-1278 in $CDCl_3$. The comparison of our results with the data published in the literature[5] showed that the chemical shifts of a large number of carbon atoms in $CDCl_3$ are identical to those observed in DMSO-d_6, but there are certain reliable differences in the chemical shifts. In most cases, these differences are about 1 ppm, but this difference for the signal of the C(26) atom is 8.2 ppm. The above data suggest that the skeletons and sequences of functional substituents in the structure of INA-1278 are identical to those in irumamycin (see Table 1) and that the configurations of some asymmetric centers in these compounds are different. To definitely confirm the identity of the structures or their difference, it is necessary to establish the configurations of eleven asymmetric centers in the INA-1278 molecule, which will be done in the future.

The antibiotic INA-1278 is a white powder soluble in acetone, ethyl acetate, lower alcohols, and toluene, but insoluble in water and hexane. The antibiotic INA-1278 gives a positive qualitative reaction with the reagent for saccharides (phenol—sulfuric acid method) and shows a negative reaction with ninhydrin.[8] An analysis of all data, including the fungicidal and antibacterial activity spectrum (see below), data on the solubility in organic solvents, chromatographic mobility, UV and IR spectra, and the data published in the handbook[9] shows that this compound belongs to the irumamycin group.[10]

A comparison of the activity of INA-1278 with that of amphotericin B widely used in the clinic (Table 2) was performed in a single assay and allowed us to conclude that the antibiotic INA-1278 has pronounced activity against micellar fungi. Due to the low minimum inhibitory concentrations (MIC), this antibiotic can be considered as a potent antifungal drug.

Irumamycin is known primarily as the agent having high activity against many phytopathogenic fungi, in particular, against *Piricularia oryzae, Scleotinia cinerea, Botrytis cinerea, Alternaria kikuchiana, Dictyosteliurm discoideum*, *etc.*[6,12,13] Our results can be considered as a substantial expansion of knowledge about the antifungal activity of irumamycin. The antibiotic INA-1278, which is identical or similar in the chemical structure to irumamycin, can act on yeasts and exhibits high activity against *Aspergillus niger* and *Fusarium oxysporum*.

Table 2. Comparison of the antifungal activity of the antibiotic INA-1278, irumamycin, and amphotericin B

Organism	MIC/μg mL^{-1}		
	INA-1278	Irumamycin[a]	Amphotericin B
Candida albicans ATCC 14053	8(>16)[b]	>100	0.50
Cryptococcus humicolus ATCC 9949	8(>16)[b]	—	0.25
Aspergillus niger ATCC 16404	1	25	1.00
Fusarium oxysporum VKM F-140	4	>100	2.00

[a] Literature data.[10,11]

[b] The MIC values are determined as the lowest concentration of the agent that completely inhibits the growth of the test organism after cultivation for 24 h. The values obtained by assessing the growth after cultivation for 96—120 h are given in parentheses.

A change in the duration of the incubation period of the test cultures with the antibiotic revealed the difference in the action of the agent on micellar fungi and yeasts. An increase in the duration of the incubation period to 96—120 h has no substantial effect on the activity of the agent against micellar fungi. At the same time, the long-term cultivation results in a substantial loss of the activity of the agent against yeasts. Apparently, the antibiotic INA-1278 acts on yeast cultures as a fungistatic.

An investigation of the activity of the antibiotic INA-1278 against the halophilic bacterium *Halobacterium salinarum* revealed its ability to suppress the sterol biosynthesis compared to the known inhibitor lovastatine. Even at a low concentration, the antibiotic INA-1278 (0.4 μg mL^{-1}) caused the partial inhibition of the growth of the bacterial culture *H. salinarum*. The complete suppression of the growth occurred when the agent was used at a concentration of 4 μg mL^{-1}. At higher concentrations, the agent caused lysis of the microbial test culture. It is known[14] that the formation of phytanyl phosphatidyl glycerolphosphate diester, which is the main steroidal component of the membrane *H. salinarum* follows the mevalonate biosynthesis pathway. Agents inhibiting the sterol biosynthesis can cause the destruction of the membrane integrity.[15] Under hypertonic conditions of a nutrient medium necessary for the growth of *H. salinarum*, the destruction of the membrane integrity generally leads to the cell lysis.

The suppression of the growth of *H. salinarum* induced by the antibiotic INA-1278, as opposed to the inhibition initiated by lovastatine, is not eliminated in the presence of exogenous mevalonic acid, which suggests considerable differences in the mechanism of action of both agents. The antibiotic INA-1278, like the antibiotic chlorotricin studied earlier,[16] cannot suppress early steps in the sterol biosynthesis preceding the formation of mevalonic acid and, apparently, acts in later steps of the biosynthesis. These data additionally suggest that the novel antibiotic deserves further structural and microbiological study because of its biological activity.

Experimental

The NMR spectra were recorded at 25 °C on Bruker DRX-500 and Bruker Avance 600 instruments. The high-resolution electrospray ionization (ESI) mass spectra were obtained on a Bruker micrOTOF II instrument using positive (capillary voltage was 4500 W) and negative (capillary voltage was 3200 W) ion modes. The mass scan range, m/z, was 50—3000 D; the internal calibration (Electrospray Calibrant Solution, Fluka) was used. A syringe pump was employed to introduce samples, the flow rate was 3 μL min^{-1}; acetonitrile was used as the solvent; nitrogen served as the nebulizer (4 L min^{-1}); the interface temperature was 180 °C.

The HPLC analysis was performed on a C-18 silica gel (5 μm) column (Alltima 4×250 mm, Alltech, USA) with a water—acetonitrile system as the eluent at a flow rate of 1 mL min^{-1} or on a ZorbaxSil silica gel (10 μm) column (4×250 mm, DuPont, USA) with a CH_2Cl_2—acetonitrile system as the eluent at a flow rate of 1 mL min^{-1}. The plots of the eluent composition *versus* the time of the analysis are shown in Figs 1 and 2. Compounds in the eluate were detected by a combined method: by spectrophotometry at 225 and 280 nm (Gilson 155, France) and using an ELSD detector (Alltech 2000, USA). Flash chromatography was performed with the use of Kieselgel 60 silica gel (40—63 μm) (Merck, Germany). The TLC analysis was performed on Kieselgel 60 plates (Merck, Germany). Antibiotics were detected by bioautography[4] with the use of *Aspergillus niger* INA 00760, *Fusarium oxysporum,* and *Candida albicans* INA 00763 as test organisms. Compounds were visualized on plates using general and carbohydrate- and double bond-specific reagents.[8]

Isolation of the antibiotic INA-1278. To prepare a liquid culture, the producer strain No. 1278 was grown on a pea agar at 37 °C for 7—10 days. The two-step fermentation was performed in 750 mL Erlenmeyer flasks filled with 100 mL of a nutrient medium using a rotary shaker (220 rpm) at 28 °C. The flasks containing the seed medium (consisted of the following components (wt.%): Hottinger´s broth, 3.0; peptone, 0.5; glucose, 1.0; NaCl, 0.5, pH 7.0—7.2) was inoculated with culture agar blocks (0.5 cm^2) and grown for 48 h. The specially designed fermentation medium consisted of the following components (wt.%): glycerol, 2.0; soy flour, 1.0; NaCl, 0.5; $CaCO_3$, 0.5, pH 7.0—7.2. The fermentation medium was inoculated with 10 mL of a seed culture grown for 48 h. The duration of the cultivation was 144 h. After the completion of the fermentation, the micellar phase was separated by centrifugation at 4000 rpm, suspended in acetone, and kept for 17 h. After the filtration, acetone was removed from the solution on a rotary evaporator and twice extracted with ethyl acetate (1/3 and 1/10 of the volume of the aqueous residue). The organic phase was concentrated on a rotary evaporator and precipitated with hexane.

The further purification was performed by flash chromatography on silica gel in chloroform by eluting with a chloroform—methanol mixture in successive ratios of 100 : 1, 50 : 1,

and 20 : 1. The elution of the antibiotic from the column was monitored by bioautography using paper discs in Petri dishes containing a medium inoculated with the test culture *Aspergillus. niger*. The fractions, which suppressed the culture growth and which were eluted with a chloroform—methanol mixture (20 : 1), were combined, the solvent was removed on a rotary evaporator, the residue was dissolved in ethyl acetate, and the antibiotic was precipitated with hexane. The active substance (2.5 g), which was obtained from 3.5 g of the crude product applied on the column, was rechromatographed on silica gel using successive elution with a toluene—acetone mixture in ratios of 10 : 1; 5 : 1, and 3 : 1. The antibiotic INA-1278 was eluted from the column with a toluene—acetone mixture (3 : 1), the eluate was concentrated *in vacuo*, the residue was dissolved in ethyl acetate, and the antibiotic was precipitated with hexane. The isolation from the crude product (2.5 g) afforded 1.4 g of a cream amorphous powder of the antibiotic INA-1278.

A solution of INA-1278 (19.1 mg) in CH_2Cl_2 (4 mL) was applied on a ZorbaxSil column (20×250 mm) equilibrated with CH_2Cl_2 and eluted with an increasing concentration of acetonitrile at a rate of 15 mL min^{-1}. The time dependence of the acetonitrile concentration corresponds to that shown in Fig. 2 for analytical HPLC. The fractions containing INA-1278 were combined, and the solvent was removed *in vacuo*. White amorphous INA-1278 was obtained in a yield of 7.8 mg (41%). The NMR spectroscopic data are given in Table 1.

Found: m/z 786.4392 $[M + Na]^+$. $C_{41}H_{65}NO_{12}Na$. Calculated: M + Na = 786.4399. Found: m/z 762.4435 ($[M - H]^-$. $C_{41}H_{64}NO_{12}$. Calculated: M − H = 762.4434).

Biological assay of INA-1278. The biological activity of the antibiotic INA-1278 was characterized based on the maximum tolerable concentration (MTC) against the fungi *Aspergillus niger* and *Fusarium oxysporum* and the yeasts *Candida albicans* and *Cryptococcus humiculus*, as well as against the halophilic archaebacteria *Halobacterium salinarum* as a model for investigation of the inhibition of the sterol synthesis.[16] It should be noted that MTC of the antibiotic INA-1278 was determined by the conventional method of serial dilutions during growing of the test cultures in a nutrient medium containing the antibiotic in decreasing concentrations.

The antifungal activity was determined also by the micromethod recommended by the National Committee for Clinical Laboratory Standards (NCCLS) using twofold serial dilutions of the antibiotic in the nutrient liquid medium RPMI 1640 containing L-glutamine in the absence of sodium bicarbonate.[11,16] The minimum inhibitory concentration was determined as the lowest concentration of the agent that completely inhibits the growth of the test microorganism after cultivation for 24 h in the case of *Candida albicans* and *Cryptococcus humiculus* and after cultivation for 48 h in the case of *Aspergillus niger* and *Fusarium oxysporum*. The ability to inhibit the sterol biosynthesis was determined from experiments on the inhibition of the growth of the test bacterium *Halobacterium salinarum* in nutrient liquid and agar media with a high NaCl content.[17,18]

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