

ITURIN A_L — A NEW LONG CHAIN ITURIN A POSSESSING AN UNUSUAL HIGH CONTENT OF C₁₆-β-AMINO ACIDS

G. WINKELMANN

Institut für Biologie I, Mikrobiologie I, Universität Tübingen,
Auf der Morgenstelle 1, D-7400 Tübingen, FRG

H. ALLGAIER, R. LUPP and G. JUNG

Institut für Organische Chemie, Universität Tübingen,
Auf der Morgenstelle 18, D-7400 Tübingen, FRG

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From a strain of *Bacillus subtilis* a new antifungal peptidolipid complex of the iturin group was isolated. This antibiotic complex contained six lipophilic β-amino acids with 3-amino-14-methylpentanoic acid as the predominant component. Iturin A_L contains: 2 D-Asp, 1 L-Asp, 1 L-Glu, 1 L-Pro, 1 L-Ser, 1 D-Tyr and a mixture of 2.9% *iso*-C₁₄-β-amino acid, 30.7% *n*-C₁₄-β-amino acid, 15% *iso*-C₁₅-β-amino acid, 9% *anteiso*-C₁₅-β-amino acid, 35.3% *iso*-C₁₆-β-amino acid and 4.5% *n*-C₁₆-β-amino acid. The structures of the β-amino acids were determined by combined GLC/MS. FAB mass spectroscopy revealed three M+H⁺ peaks (1,043, 1,057, 1,071). Iturin A_L could be resolved into six components by HPLC whose structures confirm the high amount of long chain β-amino acids.

Cyclic peptidolipidic antibiotics of the iturin group, such as iturin A and iturin C, have been isolated and described by PEYPOUX, DELCAMBE and co-workers^{1,2,3}. Recently the structures of the β-amino acids of iturin A isolated from two different *Bacillus subtilis* strains were compared by mass spectroscopy and ¹³C NMR⁴. These data revealed that iturin A is composed of a constant set of α-amino acids and a collection of β-amino acids in which the C₁₄-β-amino acids and C₁₆-β-amino acids predominate. The present investigation, however, shows that there exist *B. subtilis* strains, which, under identical media and growth conditions, biosynthesize a mixture of homologous peptides in which the C₁₆-β-amino acids prevail.

Fermentation

The strain *B. subtilis* (A114), isolated from a lake in Tübingen, was maintained as an agar slant culture on YMG-agar, containing per liter: 4 g yeast extract, 10 g malt extract and 4 g glucose (autoclaved separately). Fermentation of iturin A_L was carried out in Landy medium, containing per liter: 5 g L-glutamic acid, 1 g K₂HPO₄, 0.5 g MgSO₄·H₂O, 0.5 g KCl, 0.15 mg Fe₂(SO₄)₃·6H₂O, 5 mg MnSO₄·H₂O, 0.16 mg Cu₂SO₄·5H₂O, pH 6.

The fermentation broth (20 liters) was obtained by inoculating 8 shake flasks containing 2.5 liters medium each with 100 ml of a 24-hour preculture. The culture flasks were incubated on a rotary shaker at 110 rpm for 5 days at 30°C.

Assay Procedure

Iturin A_L production was followed from 100 ml samples of the fermentation broth. After binding to an Amberlite XAD-2 column (Serva, Heidelberg) and washing with two volumes of distilled water, iturin A_L was desorbed with one volume of methanol, evaporated under reduced pressure, dissolved in 1 ml methanol and analyzed by thin-layer chromatography on silica gel plates (Merck, Darmstadt) us-

ing chloroform - methanol - water (65: 25: 4) as solvent system. Iturin A_L spots were detected by spraying with water and TDM reagent as described earlier⁵⁾. The antifungal activity was determined by the plate diffusion test on YMG-agar seeded with *Saccharomyces cerevisiae*, *Candida albicans* or conidiospores from *Neurospora crassa*. The antifungal activity could also be detected directly on the TLC plates by spraying with YMG-agar and subsequently with a suspension of spores or yeast cells, preincubated for 24 hours in YMG-medium at 27°C on a rotary shaker. This bioautogram could be read after 24 hours of incubation at 27°C in a moist incubation chamber.

Isolation of Iturin A_L

The fermentation broth containing iturin A_L was slowly passed through an Amberlite XAD-2 column (8 × 100 cm) and washed with 5 liters of distilled water to remove cells and polar medium constituents. Iturin A_L was desorbed with 3 liters of methanol and evaporated under reduced pressure. The dry residue was dissolved in 200 ml of methanol and purified by gel filtration on Sephadex LH-20 (Deutsche Pharmacia, Freiburg; 7 × 100 cm) using methanol as solvent. A further purification was achieved on silica gel columns (5 × 20 cm) using chloroform - methanol - water (65: 25: 4) and chloroform - methanol - acetic acid (65: 40: 5). Alternatively the fermentation broth was adjusted to pH 2 with conc. HCl and centrifuged according to the method of PEYPOUX *et al.*¹⁾. The sediment was dissolved in chloroform - methanol (1: 1), evaporated to dryness and further purified as described above. Both isolation procedures revealed identical results with regard to the composition of iturin A_L.

Chromatographic and Spectroscopic Characterization

Iturin A_L is a neutral, white amorphous powder, soluble in methanol, ethanol, propanol and butanol in decreasing order. The solubility in water is low, but sufficient to enable its detection by the agar plate diffusion test. Separation by thin-layer chromatography on silica gel was performed with the following solvent systems: chloroform - methanol - water (65: 25: 4), R_f 0.18, chloroform - dimethylformamide - water (50: 44: 6), R_f 0.34 and 1-butanol - acetic acid - water (4: 1: 1), R_f 0.31.

The UV absorption spectrum of iturin A_L revealed a maximum at 277 nm, which is typical for all tyrosyl peptide antibiotics, such as iturin, bacillomycin and mycosubtilin. The IR spectrum is dominated by the amide I and amide II bands (1650, 1520 cm⁻¹), a broad O-H stretching band (3320 cm⁻¹) and two C-H stretching bands (2920, 2850 cm⁻¹) arising from the alkanoyl chains of iturin A_L.

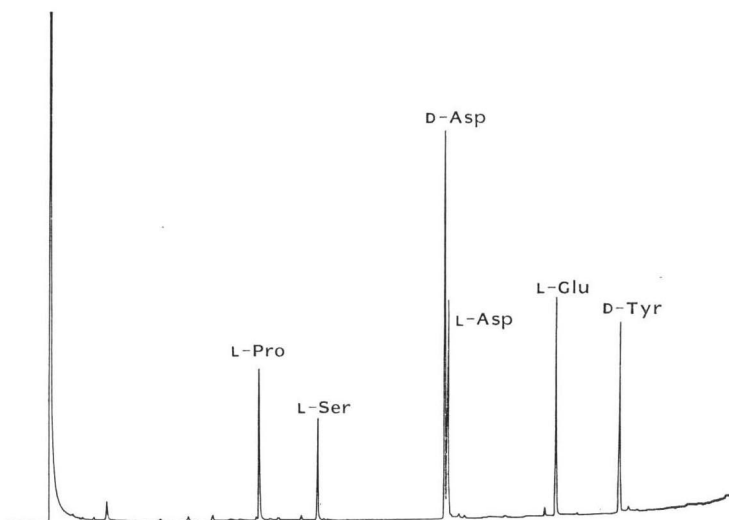
Amino Acid Analysis

Iturin A_L spots on TLC plates are ninhydrin negative but TDM positive, indicating the absence of free amino groups and the presence of peptide bonds. The hydrolysate of iturin A_L (6 N HCl, 150°C, 8 hours) was analyzed in an amino acid analyzer (Biotronic, System LC 6000E) resulting in: Asp, Glu, Pro, Ser, and Tyr in a molar ratio of 3: 1: 1: 1: 1. The absolute configuration was determined on the chiral phase *N*-propionyl-L-valine-*tert*-butylamide polysiloxan by gas chromatography, as described earlier⁵⁾. The dried hydrolysate was esterified with 200 μl water-free 2 N HCl in 2-propanol at 110°C within 1 hour. After evaporating with nitrogen, the residual amino acid isopropyl esters were acylated with 50 μl pentafluoropropionic acid anhydride in 250 μl dichloromethane for 1 hour, dried and analyzed by gas chromatography (Fig. 1).

Analysis of the β-Amino Acids

The long chain β-amino acids were analyzed by combined gas chromatography-mass spectrometry. Samples of iturin A_L were hydrolyzed with 6 N HCl for 8 hours at 150°C, evaporated to dryness, treated

Fig. 1. Gas chromatogram of *N*-pentafluoropropionyl α -amino acid isopropyl esters of the iturin A_L hydrolysate on a chiral phase (*N*-propionyl-L-valine-*tert*-butylamide polysiloxan), carrier gas hydrogen, FID, temperature program 87~200°C.



with diazomethane followed by trifluoroacetic acid anhydride to yield the *N*-trifluoroacetylated methyl esters of the β -amino acids. These derivatives were analyzed on a packed EGSS-X column (Applied Science, Europe EV) at 180°C (Fig. 2). Identification of the peaks was performed by GLC/MS. The first two peaks appearing during gas chromatographic separation revealed identical molecular ions (M^+ 353) and a largely identical fragmentation pattern (M^+ - 31, M^+ - 69, M^+ - 73, m/z 198 $F_3C-CO-NH-\dot{C}H^+-CH_2-COOCH_3$, 198-31, 198-32, 198-42, 198-59). The two peaks are derivatives of β -amino-12-methyl tridecanoic acid and β -aminotetradecanoic acid. The following two peaks (M^+ 367) represented the two derivatives of C_{15} - β -amino acids: β -amino-13-methyl tetradecanoic acid and β -amino-12-methyl tetradecanoic acid.

The last two peaks (M^+ 381) corresponded to the isomers of the C_{16} - β -amino acid ester: β -amino-14-methyl pentanoic acid and β -aminohexadecanoic acid. A mass spectrum of the derivative of the β -amino-*iso*- C_{16} acid is shown in Fig. 3.

Determination of the lipid-peptide linkage was carried out by partial hydrolysis of iturin A_L (6 N HCl, 110°C, 18 hours), extraction with chloroform and separation of the lipidic dipeptide from β -amino acids by silica gel chromatography. The dipeptide fragment revealed an R_f 0.11 on silica gel plates using chloroform - methanol - water (65:25:4). Dansylation with 5-dimethylaminonaphthalene-1-sulfonyl chloride, subsequent hydrolysis and two dimensional chromatography on polyamide with formic

Fig. 2. Gas chromatogram of *N*-trifluoroacetyl β -amino acid methyl ester on a packed EGSS-X column.

Carrier gas nitrogen, FID, 180°C

1 = *iso*- C_{14} , 2 = *n*- C_{14} , 3 = *iso*- C_{15} , 4 = *anteiso*- C_{15} ,
5 = *iso*- C_{16} , 6 = *n*- C_{16} .

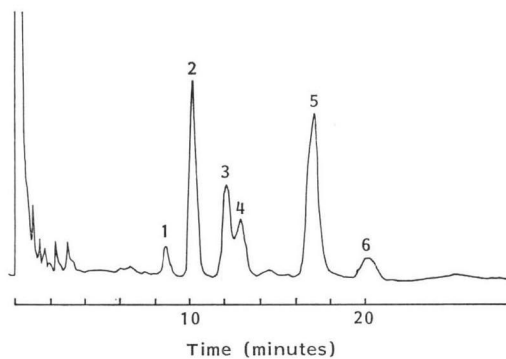
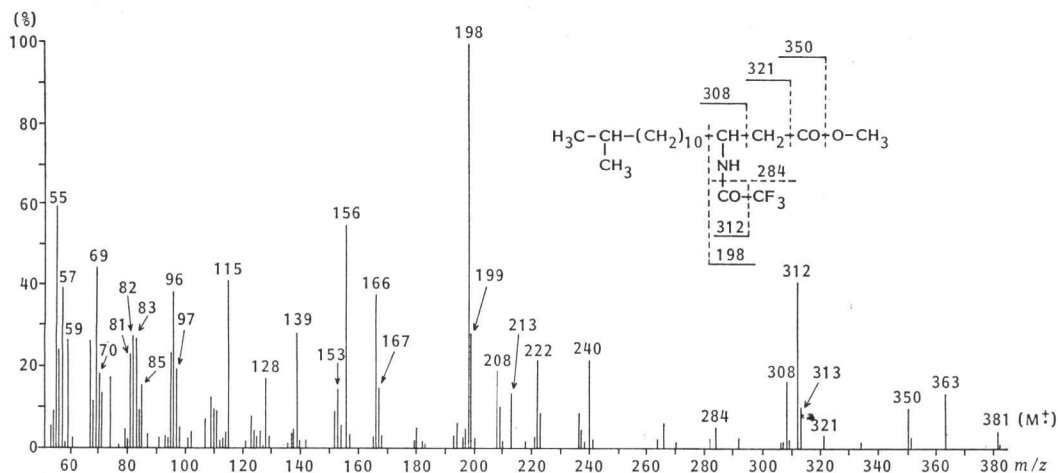
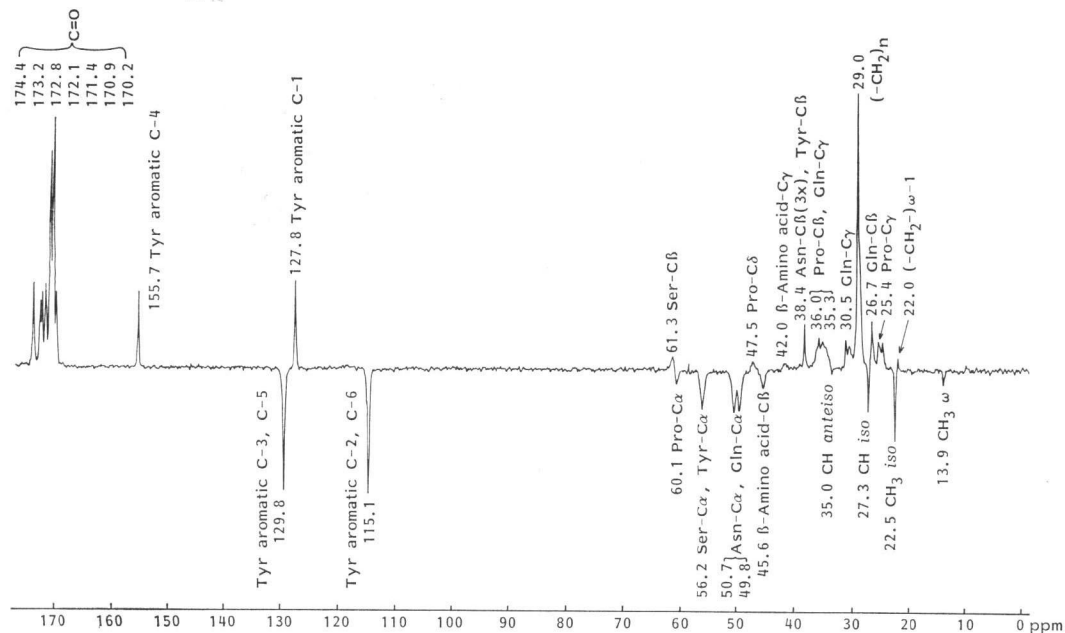


Fig. 3. Mass spectrum of *N*-trifluoroacetyl- β -amino-*iso*-hexadecanoic acid methyl ester.Fig. 4. ^{13}C NMR spectrum of iturin A_L (*J*-modulated spin echo, $T=1/J$, 22.6 MHz, in ^{12}C , ^2H]DMSO).

acid - water (4: 96) and acetic acid - toluol (20: 80) confirmed the *N*-terminal position of the serine residue.

^{13}C NMR Spectroscopy

The *J*-modulated spin echo ^{13}C NMR spectrum at 22.6 MHz of iturin A_L (Fig. 4) shows carbonyl resonances at 170.2~174.4 ppm, due to the peptide and amide carbon atoms of the cyclic peptide ring. The signal at 155.7 is assigned to the aromatic C-4 of tyrosine and C-3/C-5, C-1, C-2/C-6 absorb at 129.8, 127.8 and 115.1. The aliphatic region (61.3~24.6 ppm) containing the α -, β -, γ -, δ -carbon signals of the α - and β -amino acids was assigned with known data. As the present investigation was primarily intend-

ed to analyze the different β -amino acyl species of iturin A_L , a comparison of ppm values from model compounds with those of iturin A_L was performed:

<i>n</i> -Hexane	$H_3C - CH_2 - CH_2 - CH_2 - CH_2 - CH_3$
δ (ppm)	13.6 22.8 31.9 31.9 22.8 13.6
	22.4 CH_3
2-Methylpentane	$H_3C - \underset{\substack{ \\ CH_3}}{CH} - CH_2 - CH_2 - CH_3$
δ (ppm)	22.4 27.6 41.6 20.5 14.0
	18.4 CH_3
3-Methylpentane	$H_3C - CH_2 - \underset{\substack{ \\ CH_3}}{CH} - CH_2 - CH_3$
δ (ppm)	11.1 29.1 36.5 29.1 11.1

Using the data from *n*-hexane, 2-methylpentane and 3-methylpentane we were able to assign the observed resonances of the aliphatic region of iturin A_L . Thus the signals at 13.9 ppm and 22.5 ppm could be assigned to the terminal methyl and the branched methyl of *n*- and *iso*- β -amino acids. The signals at 27.3 ppm and 35.0 ppm could be assigned to the methine group of the *iso*- and *anteiso*- β -amino acids. The corresponding ppm values for the subterminal CH_2 -groups were assigned as follows: 45.6 ppm (*C*- β), 42.0 ppm (*C*- γ). The methylene carbons of the middle part of the chain absorb at 29.0 ppm.

HPLC — Separation and FAB Spectrum

The individual compounds of iturin A_L , containing different β -amino acids, could be separated by HPLC on a Ultrasphere column (ODS, 25 cm, 2.6 mm ϕ) using acetonitrile - ammonium acetate (10 mM) as an eluting solvent system (Fig. 5). The elution profile was similar to that observed during GLC separation of the isolated β -amino acids, with the exception of the C_{15} - β -amino acids. A small amount (0.4%) of *n*- C_{15} - β -amino iturin A_L could also be detected during HPLC analysis.

The FAB spectrum (Fig. 6) of iturin A_L yielded three $M+H^+$ peaks (1,043, 1,057, 1,071), corresponding to the homologous compounds with an equivalent chain length.

Fig. 5. HPLC separation of iturin A_L in acetonitrile-ammonium acetate (10 mM) 2:3 on Ultrasphere ODS, 25 cm, 2.6 mm ϕ , 1.2 ml/minute, measured at 230 nm.

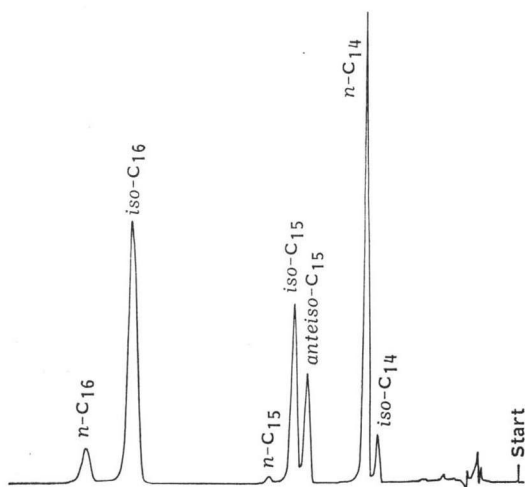
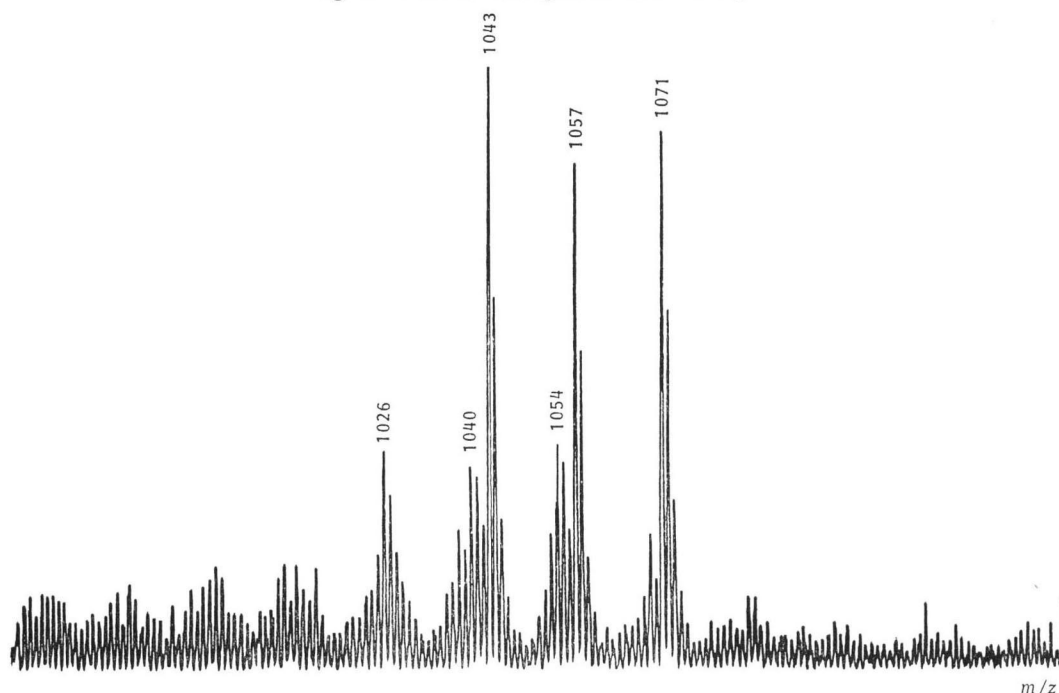


Table 1. MIC values of iturin A_L .

Fungal strain	MIC (μ g/ml)
<i>Mucor miehei</i>	2
<i>Saccharomyces cerevisiae</i>	4
<i>Neurospora crassa</i>	4
<i>Trichoderma viride</i>	6
<i>Microbotryum violaceum</i>	6
<i>Penicillium chrysogenum</i>	8
<i>Rhodotorula glutinis</i> var. <i>glutinis</i>	8
<i>Ustilago perennans</i>	10
<i>Schizonella melanogramma</i>	12
<i>Candida albicans</i>	12
<i>Coleosporium tussilaginis</i>	12

Fig. 6. Positive FAB spectrum of iturin A_L.

Biological Properties

The antifungal activity of iturin A_L was determined in YMG-medium containing increasing amounts (2~20 μg) of iturin A_L. The resulting MIC values are given in Table 1. The values observed for iturin A_L were generally lower than those reported by BESSON and coworkers⁶⁾ for iturin A, which may possibly be due to the altered β-amino acid composition. Myristic acid and cholesterol revealed a significant antagonistic effect. The hemolytic activity was assayed with human blood, washed with 3.8% Na-citrate and diluted 1:10 with phosphate buffer (10 mM phosphate, 154 mM NaCl, pH 7.2). Lysis was determined in a buffer containing 2.5 mM phosphate, 141 mM NaCl, 1 mM KCN, 0.6 mM K₃Fe(CN)₆ and increasing amounts of iturin A_L (methanolic solution). Fifty %-hemolysis occurred at 10 μg/ml.

Several derivatives of iturin A_L were prepared, which will be described in detail elsewhere.

Acetylation and methylation of the tyrosine completely abolished the antifungal activity. However the methylated iturin A_L revealed a slight synergistic effect on the antifungal activity of iturin A_L. The nitro- and amino-derivatives were also inactive. After iodination of the tyrosine residue, the antifungal activity was decreased, but still detectable.

Discussion

Several cyclic peptidolipidic antibiotics which have been isolated from *B. subtilis* strains are known as iturin, bacillomycin, mycosubtilin and subsporin⁷⁾. These antibiotics differ in their α-amino acid composition. If variations of the α-amino acids occur, without alteration of the main characteristics, the compounds are subgrouped by capital letters, e.g. iturin A, iturin C or bacillomycins L, F, D. Iturin A_L, described in the present investigation, contains an unusual β-amino acid composition, which has not been observed in other iturins. Contrary to the iturins isolated from other strains, in which C₁₄- and C₁₅-β-amino acids predominate, iturin A_L contains mainly C₁₈-β-amino acids. The Landy medium

used for iturin production represents a chemically defined medium with glutamic acid as a nitrogen source and glucose as a carbon source, allowing a direct comparison of the iturins and bacillomycins produced by different *Bacillus* strains. The long chain β -amino acids found in the peptidolipidic antibiotics resemble the long chain fatty acids found in the lipids of *Bacillus* strains, containing high amounts of *iso*- and *anteiso*-branched chains. As pointed out by KANEDA⁸⁾, the fatty acid pattern of a given species of *Bacillus* can act as a fingerprint if the organism is grown under culture conditions where the exogenous supply of the precursors of chain initiators is insignificant. Although variations of the chain length may not alter the mode of action of membrane modifying antibiotics, they still may have an effect on orientation and integration into the cytoplasmic membrane, which in turn may result in different MIC values.

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