REVISED STRUCTURE OF MYCOSUBTILIN, A PEPTIDOLIPID ANTIBIOTIC FROM *BACILLUS SUBTILIS*

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The structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*, was revised by FAB mass spectrometry, 2D NMR spectrometry and also by Edman degradation of the peptide resulting from the *N*-bromosuccinimide reaction. Four homologous β-amino acid components were identified by capillary gas chromatography. The cyclopeptide mycosubtilin consists of seven α-amino acids in an LDDLLDL sequence closed by a β-amino acid linkage similar to that found in other antibiotics of the iturin group.

Mycosubtilin was isolated by WALTON and WOODRUFF from *Bacillus subtilis* 370[1]. Previous structural determination of this peptide antibiotic by chemical methods gave a cyclic structure containing eight water-soluble α-amino acids and a lipid-soluble β-amino acid[2]. All other antibiotics of the same group also have a cyclic structure with a lipid-soluble β-amino acid, but only seven α-amino acids[3~6]. A reinvestigation of the structure of mycosubtilin was therefore deemed necessary.

Recent studies of bacillomycin D and bacillomycin L by fast atom bombardment (FAB) mass spectrometry led to some structural precision of these antibiotics[7]. Similar determination of the molecular weight of mycosubtilin by FAB mass spectrometry, revealed a significant difference from the values required by the previously published formula[2].

The lipophilic components of mycosubtilin were also reinvestigated by capillary gas chromatography according to the method recently used[7] for bacillomycin D and bacillomycin L.

The complete structure of mycosubtilin is disclosed in this paper.

Fast Atom Bombardment Mass Spectrometry

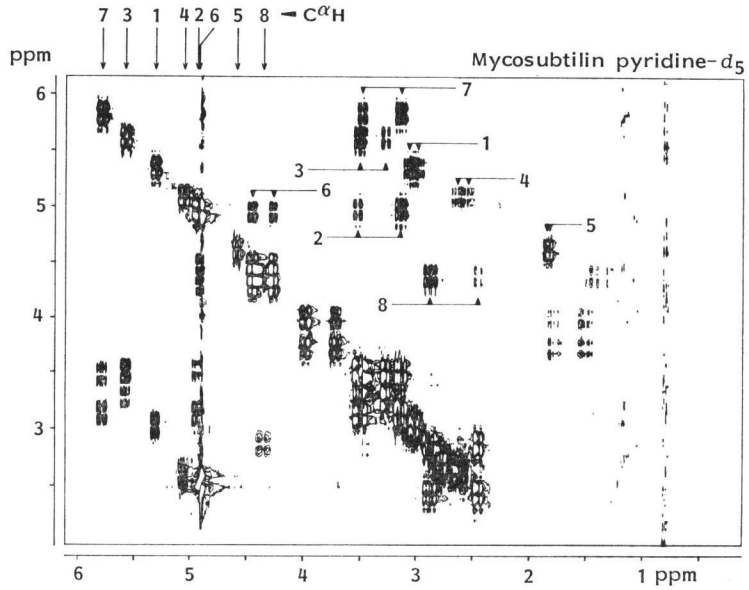
FAB mass spectrum was obtained with a Kratos MS 80 mass spectrometer. The FAB ion source was of the standard Kratos design and was equipped with an Ion Tech atom gun. The bombardment was with $6 \sim 7$ Kv xenon atoms. The peptide (*ca.* 5 μg) was placed on the copper target end of a direct insertion probe using glycerol as matrix to which a little dimethylformamide was added.

Two major homologous $[M+H]^+$ ions were observed at m/z 1,071 and 1,085 which did not agree with the previously reported formula of mycosubtilin: βAA (C_{16} or C_{17})*, L-Asn_2, D-Asn_2, L-Gln_1, L-Pro_1, D-Ser_1, D-Tyr_1. The peptide sequence was therefore reinvestigated by Edman degradation and by 2D NMR spectrometry.

* βAA is a β-amino acid having 16 or 17 carbon atoms.

Fig. 1. 2D double-quantum filter COSY map at 27°C.

The chemical shifts of the C^{α} protons (C^{β} proton for the β-amino acid) are indicated on the top. The labelings inside the correlation map point towards the cross-peaks with their own $C^{\beta}H_2$ for all residues except the β-amino acid ($C^{\alpha}H_2$). A 256×4,096 data point matrix was sampled and the phase-sensitive display of the transformed spectrum is shown.



Sequential Resonance Assignment by Two-dimensional ^1H NMR

Two-dimensional (2D) proton COSY[8,9] and rotating frame NOESY[10] NMR correlation experiments were carried out with a Bruker AM 300 WB spectrometer in pyridine-d_5 solution (4 mM).

The *J*-correlated spectrum recorded at 27°C showed eight spin systems, which can be classified in three groups according to the coupling pattern observed on the CH next to the nitrogen (see Fig. 1):

A Pro residue; the $C^{\alpha}H$ at δ 4.62 ppm is not linked to an amide proton.

A β-amino acid, where the $C^{\beta}H$ at δ 4.39 ppm is connected not only with an NH but also with two pairs of protons belonging to CH_2 groups.

Six amino acids corresponding to a four-spin system;

$$NH\text{–}C^{\alpha}H(CO)\text{–}C^{\beta}H_2\text{–}R.$$

Among these six residues, one is a tyrosine (in view of a pair of doublets around 7.15 ppm) and four bear a side-chain amide group (either Asn or Gln), each of these residues leading to a pair of coupled signals between 7.5 and 8.5 ppm.

Due to the lack of any inter-residue *J*-coupling, the nuclear Overhauser effect (NOE) offers the only alternative for sequential resonance assignment. Unfortunately, at 27°C the molecular tumbling is too fast, so that the NOE vanishes to zero. As a result, the NOESY correlation map was recorded at −20°C and two types of connectivities have been gathered:

Sequential links between any NH and either the NH or the $C^{\alpha}H$ of the preceeding residue.

Intra-residue connectivities between the $C^{\beta}H_2$ and the linked side chain (tyrosyl ring, amide group . . .).

Finally, it may be noted that, at this stage of our study, the NMR data do not provide any in-

Table 1. ^1H resonance assignment of mycosubtilin at 27°C in pyridine-d_5.

	L-Asn 1	D-Tyr 2	D-Asn 3	L-Gln 4	L-Pro 5	D-Ser 6	L-Asn 7	βAA
NH	9.01	10.27	9.31	7.83		9.68	8.55	7.93
$C^{\alpha}H$	5.35	4.92	5.62	5.01	4.62	4.89	5.82	2.94[a]
$C^{\beta}H$	3.10	3.53	3.53	2.65	1.90	4.48	3.51	4.39
	3.00	3.15	3.31	2.55	1.88	4.30	3.15	
$C^{\gamma}H$					1.84			1.43
					1.55			1.31
$C^{\delta}H$					4.02			
					3.75			
NH_2 *trans*	8.30		8.37	8.16		8.27		
cis	7.79		8.01	7.80		7.81		
$C_{2,6}H$		7.25						
$C_{3,5}H$		7.03						

[a] This residue contains two C^{α} protons.

Table 2. Result of Edman degradations on the NBS-cleaved mycosubtilin.

Degradation step	1	2	3	4	5
Recovered PTH amino acid	Asn	Gln	Pro	Ser	Asn

formation with respect to the chirality of the amino acids. The following sequence can thus be proposed on the basis of the NMR study:

$$\begin{matrix} \text{Asn} & & \text{Asn} & \text{Asn} & & & \text{Asn} & \\ \text{or} & - \text{Tyr} - & \text{or} & \text{or} & - \text{Pro} - & \text{Ser} - & \text{or} & - \beta\text{AA} \\ \text{Gln} & & \text{Gln} & \text{Gln} & & & \text{Gln} & \end{matrix}$$

The chemical shifts of the assigned resonances are reported in Table 1.

Edman Degradation of the Peptide Chain

The cyclic peptide chain was opened up with *N*-bromosuccinimide (NBS) as described previously[6]. This reagent cleaved the *C*-peptidyl bond of the tyrosyl residue and a partial *N*-terminal sequence was determined by Edman degradation according to TARR[11]. After each cycle of degradation the released amino acid was identified as its phenylthiohydantoin (PTH) derivative by thin-layer chromatography on Silica gel 60 F_{254} in chloroform - methanol (85: 15 or 95: 5). The *N*-terminal amino acid of the remaining peptide was determined as its dinitrophenyl derivative[12]. Five Edman degradations were carried out after which the reaction was stopped by the presence of a β-amino acid.

The results, summarized in Table 2, gave the following sequence:

Asn→Gln→Pro→Ser→Asn→βAA→X→Tyr

Whereas the FAB mass spectrum of mycosubtilin revealed a molecular weight of 1,070 ($M+H^+$ at 1,071) corresponding to a lower homologue of the β-amino acid, *i.e.* C_{16} β-amino acid, the known part of the above structure of the antibiotic added up to a mass of only 956 for the C_{16} β-amino acid homologue. The difference of 114 mass units (1,070 − 956) could be accounted for by the presence of an Asn residue at the position 'X' in the above structure.

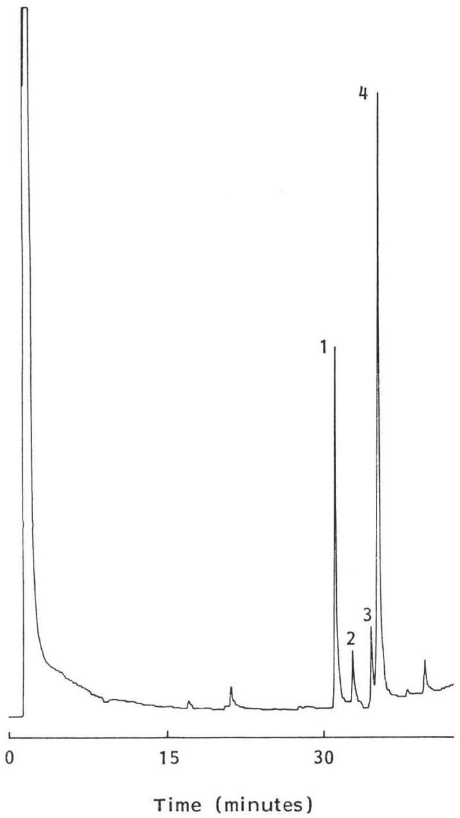
Configuration of α-Amino Acids

The configurations of α-amino acids have been previously determined by enzymatic methods[3]. For amino acids other than the Asn residues, no ambiguity should exist since mycosubtilin contains

Table 3. Configuration of Asn residues in peptides.

Peptides	Configuration of Asn residue
Asn→Gln→Pro→Ser→Asn→βAA→Asn→Tyr (oxidized) after NBS treatment of mycosubtilin	D and L
Asn→βAA→Asn→Tyr(oxidized) after 4 Edman degradations	L
βAA→Asn→Tyr(oxidized) after 5 Edman degradations	L

Fig. 2. Gas chromatographic separation of the *N*-trifluoroacetyl methyl esters of the lipid part of mycosubtilin on a fused-silica capillary column of CP WAX 57 CB from 140°C to 220°C with programmation of temperature (2°C/minute).



only one mol of each of these amino acids. As regards the three Asn units present in the antibiotic, their configuration in the form of the corresponding aspartic acid was determined with the hydrolysates of various peptides by an enzymatic method using L-glutamate-oxaloacetate transaminase as described previously[3]. The results are indicated in Table 3.

Thus, the following sequence delineated for the linear peptide resulting from the NBS treatment of mycosubtilin:

D-Asn→L-Gln→L-Pro→D-Ser→L-Asn
→βAA→L-Asn→D-Tyr (oxidized)

Identification of β-Amino Acids

Mycosubtilin was hydrolyzed with 6 N HCl at 150°C for 8 hours. The lipid moiety was derivatized to *N*-trifluoroacetyl methyl esters which were analyzed by gas chromatography on CP WAX 57 CB fused-silica capillary column (26 m×0.31 mm). The elution profile is drawn in Fig. 2.

The identification of each component was performed by comparison of the retention times with those of the β-amino acid derivatives obtained from bacillomycin F[13]; in this latter case the structures had been completely established by NMR spectrometry of the isolated derivatives. The major components of mycosubtilin are *iso* C_{16} (29%) (peak 1) and *anteiso* C_{17} (54%) (peak 4) with small amounts of *n* C_{16} (6%) (peak 2) and *iso* C_{17} (7%) (peak 3) β-amino acids.

Structure of Mycosubtilin

In conclusion, the following structure has been established for mycosubtilin:

$R(CH_2)_{10}CHCH_2CO$→L-Asn→D-Tyr→D-Asn
(CH bonded to) NH←L-Asn←D-Ser←L-Pro←L-Gln (D-Asn→L-Gln)

R = $CH_3CH_2CH_2$, $CH_3CH(CH_3)$, $CH_3CH(CH_3)CH_2$, $CH_3CH_2CH(CH_3)$

Table 4. Nature of X_1 and X_4 to X_7 residues of the uncommon part of iturin group antibiotics.

Antibiotic	L-Asx(X_1)	X_4	X_5	X_6	X_7
Iturin A	L-Asn	L-Gln	L-Pro	D-Asn	L-Ser
Iturin C	L-Asp	L-Gln	L-Pro	D-Asn	L-Ser
Bacillomycin D	L-Asn	L-Pro	L-Glu	D-Ser	L-Thr
Bacillomycin L	L-Asp	L-Ser	L-Gln	D-Ser	L-Thr
Bacillomycin F	L-Asn	L-Gln	L-Pro	D-Asn	L-Thr
Mycosubtilin	L-Asn	L-Gln	L-Pro	D-Ser	L-Asn

Until now six compounds of the iturin group are known and they possess some common structural characteristics. All these antibiotics have a cyclic structure of the following type:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad (X_1) \\ R(CH_2)_{8\sim12}CHCH_2CO \rightarrow \text{L-Asx} \rightarrow \text{D-Tyr} \rightarrow \text{D-Asn} \\ \qquad\qquad\quad | \qquad\qquad\qquad\qquad\qquad\qquad \downarrow \\ \qquad\qquad\quad NH \leftarrow X_7 \leftarrow X_6 \leftarrow X_5 \leftarrow X_4 \end{array}$$

R is a CH_3, $\underset{CH_3}{\underset{|}{CH}}$ or $CH_3CH_2\underset{CH_3}{\underset{|}{CH}}$ group in accordance with *n* C_{14}, *iso* C_{15}, *anteiso* C_{15}, *n* C_{16}, *iso* C_{16}, *iso* C_{17}, *anteiso* C_{17} β-amino acids. The nature of L-Asx (X_1) and X_4 to X_7 residues are summarized in Table 4.

Thus all antibiotics of the iturin group have the same LDDLLDL sequence with a restricted number of amino acid residues: Asx, Glx, Pro, Ser, Thr, Tyr, a common part of the cyclic peptide: βAA→L-Asx→D-Tyr→D-Asn and a variable moiety containing four amino acid residues.

Acknowledgments

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