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TOTAL SYNTHESIS OF PEPTIDE ANTIBIOTIC NISIN

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Summary: Total synthesis of a lanthionine peptide nisin was successfully achieved for the first time by application of new methods for preparations of dehydroalanine and lanthionine moieties, resulting in a confirmation of the proposed structure.

A peptide antibiotic nisin has been used as a food preservative, since it shows a remarkable antibacterial activity particularly against Clostridium botulinum. Nisin was first found in 1928[1] but isolated by Mattick et al. in 1947[2] from Streptococcus lactis. Its unique structure composed of thirty four amino acid residues including three dehydroamino acids and five cyclic sulfide parts was proposed by Gross et al. in 1971.[3](Fig. 1) We aimed the total synthesis of nisin not only from a view point of the synthetic interest but also for a confirmation of the proposed structure as well as an elucidation of structure-activity relationship.

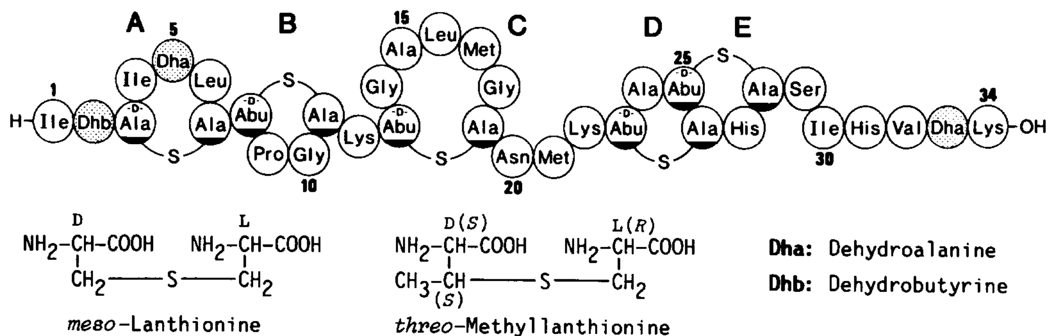

Fig. 1. Structure of nisin.

We first exploited novel methods for a preparation of dehydroalanine (Dha) peptide based on the Hofmann degradation of 2,3-diaminopropionic acid (A_2pr) residue[4] and for a formation of lanthionine peptide from disulfide peptide by desulfurization reaction[5] with hexaethylphosphorous triamide [$P(NEt_2)_3$]. By utilization of these methods, we could synthesize each cyclic part in nisin respectively.[6] (2R,2'S,3'S)-Methyllanthionine parts of threo form in the rings B to E were solely obtained by desulfurization of peptides obtained from threo-3-methyl-D-cysteine and L-cysteine.

Total synthesis of nisin was carried out by fragment condensation of each lanthionine peptide part. For this purpose, four fragments including respective ring parts and one C-terminal linear fragment were prepared according to schemes mentioned in Fig. 2. So far as we tested, Dha residue, which is generally labile in acidic media, was actually stable to hydrogen fluoride (HF) as well as trifluoroacetic acid (TFA) under anhydrous condition. The fact made possible, for us to use both t-butyl and benzyl-type protecting groups throughout this

study.

Thus, N-terminal fragment containing ring A part was prepared as shown in Fig. 2a. Dehydrobutyrine (Dhb) residue next to the N-terminal was introduced by dehydration of Thr residue in the peptide **1** using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCI·HCl) and copper(I) chloride.[7] The presence of doublet methyl signal of Dhb at 1.7ppm on ^{1}H-NMR of the peptide **2** thus obtained clearly indicated a sole formation of the desired Z-isomer of Dhb in this reaction.[8] Saponification of methyl ester in the peptide **2** gave

(a)
Boc·D-Ala·Ile·Dha·Leu·Ala·OMe 6a) (S-bridge 3–7)
i)TFA ii)HCl/MeOH
Z·Ile·Thr·OH + HCl·H·D-Ala·Ile·Dha·Leu·Ala·OMe
WSCI/HOBt 83%
Z·Ile·Thr·D-Ala·Ile·Dha·Leu·Ala·OMe (1)
WSCI·HCl–CuCl 74%
Z·Ile·Dhb·D-Ala·Ile·Dha·Leu·Ala·OMe (2)
1M NaOH(dioxane) 50%
Z·Ile·Dhb·D-Ala·Ile·Dha·Leu·Ala·OH (3) (1–7)

(b)
Boc·D-Abu·Pro·Gly·Ala·OBzl (4) 6b) (8–11)
i)HF–anisole (9:1) ii)TrocOSu 74%
Troc·D-Abu·Pro·Gly·Ala·OH (5) + HCl·H·Lys(Z)·OBu^t (6)
WSCI/HOBt 90%
Troc·D-Abu·Pro·Gly·Ala·Lys(Z)·OBu^t (7)
i)Zn/AcOH ii)HCl 86%
HCl·H·D-Abu·Pro·Gly·Ala·Lys(Z)·OBu^t (8) (8–12)

(c)
Boc·D-Abu·Gly·Ala·Leu·Met·Gly·Ala·OBzl 6c) (13–19)   Nps·Asn(Mbh)·Met·OBu^t
i)HF–anisole (9:1) ii)TrocOSu 80%   HCl 96%
Troc·D-Abu·Gly·Ala·Leu·Met·Gly·Ala·OH + HCl·H·Asn(Mbh)·Met·OBu^t
WSCI/HOBt 90%
Troc·D-Abu·Gly·Ala·Leu·Met·Gly·Ala·Asn(Mbh)·Met·OBu^t
i)Zn/AcOH ii)HCl 58%
HCl·H·D-Abu·Gly·Ala·Leu·Met·Gly·Ala·Asn(Mbh)·Met·OBu^t (9) (13–21)

(d)
Boc·D-Abu·Ala·D-Abu·Ala·His(Ts)·Ala·OMe 6d) (23–28)
i)TFA ii)HCl/MeOH
Boc·Lys(Z)·OH + HCl·H·D-Abu·Ala·D-Abu·Ala·His(Ts)·Ala·OMe (10)
WSCI/HOBt 69%
Boc·Lys(Z)·D-Abu·Ala·D-Abu·Ala·His·Ala·OMe (11)
$NH_2NH_2·H_2O$ 89%
Boc·Lys(Z)·D-Abu·Ala·D-Abu·Ala·His·Ala·NHNH$_2$ (12) (22–28)

(e)
Ser(29) Ile His Val Dha Lys(34)
Boc — *1 75% — A$_2$pr(Troc) — *1 82% — Lys(Z)-OBzl
Zn/AcOH: Boc — Lys(Z)-OBzl
CH$_3$I–KHCO$_3$ 73%: Boc — Dha — Lys(Z)-OBzl (13)
i)TFA ii)NaHCO$_3$
Boc-His(Ts)-OH H — Lys(Z)-OBzl
i)DCC/HOBt ii)TsCl/TEA 72%: Boc-His(Ts) — Lys(Z)-OBzl
Boc-Ser(Bzl) — *2 62% — *2 80% — His(Ts) — Lys(Z)-OBzl (14)
HOBt (6eq) 81%: Boc-Ser(Bzl) — Lys(Z)-OBzl (15)
*1: DCC/HOBt method
*2: i) DCC/HOBt method; ii) TsCl/TEA

Abbreviations:
Boc: t-butoxycarbonyl
Z: benzyloxycarbonyl
WSCI: 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide
HOBt: 1-hydroxybenzotriazole
Troc: trichloroethoxycarbonyl
OSu: succinimido-oxy
Nps: 2-nitrophenylthio
Mbh: 4,4'-dimethoxybenzhydryl[9]
DCC: dicyclohexylcarbodiimide
Ts: tosyl
Bu^t: t-butyl

Fig. 2. Preparation of five fragments for the total synthesis of nisin.

N-terminal fragment **3** as a carboxyl component. Second fragment corresponding to the residue (8-12) in nisin was prepared as shown in Fig. 2b. Synthetic ring B derivative **4**[6b] was once converted into N-trichloroethoxycarbonyl(Troc)-ring B (**5**) which was coupled with Lys residue **6**. Troc group of resulting peptide **7** was then selectively removed to give an amine component **8**. Third fragment **9** containing ring C part was prepared in a similar manner as in the fragment **8**. In this case, amide group of Asn residue was protected with 4,4'-dimethoxybenzhydryl (Mbh) group to prevent dehydration or imide formation during the coupling reaction.[9](Fig. 2c) A preparation of the fourth fragment including conjunctive ring D-E moiety was carried out as shown in Fig. 2d. Boc-Lys(Z)-OH was coupled with bicyclic peptide **10** and then tosyl (Ts) group of His residue was removed with HOBt. The resulting peptide **11** was converted to peptide hydrazide **12**. Fifth fragment corresponding to C-terminal linear peptide **15** was prepared as shown in Fig. 2e. Dha residue in this fragment was introduced by the Hofmann degradation of A_2pr residue at rather early stage. A C-terminal tripeptide **13** containing Dha was prepared and then elongated to hexapeptide **14** by DCC/HOBt method as shown in Fig 2e. N^{im}-Ts group of His residue should be reintroduced after each coupling step for purification of the product, but was finally removed with HOBt to afford the desired fragment **15**.

The coupling procedures of five peptide fragments thus obtained were shown in Fig. 3. After coupling of fragment **3** with fragment **8** by WSCI/HOBt method, Bu^t ester was removed with TFA to give dodecapeptide **16**. The structure of **16** was confirmed by comparison with the authentic N-benzyloxycarbonylated trypsin digestive peptide (1-12) derived from the natural nisin. Peptide **16** was then coupled with ring C fragment **9**. Bu^t ester and Mbh group were removed with TFA to give the carboxyl component **17**. On the other hand, hydrazide derivative of ring D-E moiety **12** was coupled with de-t-butoxycarbonylated linear peptide prepared from **15** by azide method. After the coupling, imidazole groups of two His residues were blocked with Boc group for purification of the product. In order to confirm the structure of this coupling product, the fully protected peptide **18** was treated with anhydrous HF in the presence of anisole. The resulting free peptide was completely identical with authentic peptide (22-34) obtained by BrCN degradation of natural nisin. Protected nisin was finally obtained by condensation of carboxyl component **17** with amine component **19** derived from **18** by removal of all Boc groups.

All the protecting groups in the final coupling product were removed with anhydrous HF in

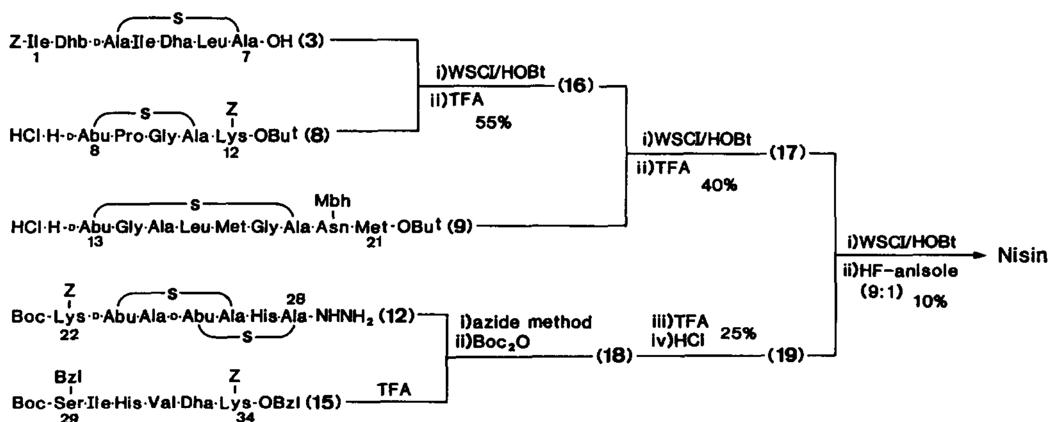

Fig. 3. Synthetic scheme to nisin.

Table 1. Antibacterial activities of nisin

Test Organism	Natural	Synthetic
Staphylococcus aureus ATCC 6538P	12.5	12.5
Staphylococcus epidermidis sp-al-1	12.5	12.5
Staphylococcus epidermidis ATCC 12228	>100	>100
Streptococcus pyogenes A089	1.6	1.6
Streptococcus faecalis 030021	50	100
Micrococcus luteus ATCC 10240	<0.2	0.4
Micrococcus luteus IFO 3333	6.3	3.1
Corynebacterium diphteriae IID 527	12.5	12.5
Bacillus subtilis ATCC 6633	25	25
Escherichia coli NIHJ-JC2	>100	>100
Salmonella typhimurium ATCC 14028	>100	>100
Shigella flexneri IID 642	>100	>100
Proteus vulgaris OX-19	>100	>100

the presence of anisole at 0°C. The crude product was purified by preparative HPLC. Synthetic nisin thus obtained was completely identical with natural product in respects of retention time on HPLC,[10] FAB-mass spectrometry,[11] ^{1}H-NMR spectrometry, as well as antibacterial activities (Table 1).

We could accomplish the total synthesis of nisin for the first time, and consequently, confirm the structure of nisin synthetically. Furthermore, we could now establish general synthetic methods not only for dehydroamino acid peptide but also for lanthionine peptide, which may be applicable to the other biologically important peptides containing these amino acid moieties.

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8) In general, the doublet methyl signal of Z-isomers of Dhb derivatives appeared at 1.7ppm whereas that of E-isomers at 2.0ppm; A. Srinivasan, R.W. Stephenson, and R.K. Olsen, J. Org. Chem., **42**, 2256 (1977).
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10) Both synthetic and natural nisin were eluted at the same retention time under following conditions. Column: Nucleosil 300-7C_{18}, 6 X 250mm; Solvent: CH_3CN-0.01M HCl[gradient: 30-50% (2%/min)], flow rate: 1.5ml/min, retention time: 8.1min. Solvent: CH_3CN-0.3M guanidine hydrochloride[gradient: 30-50% (2%/min)], flow rate: 1.5ml/min, retention time: 10.9min.
11) FAB-mass spectra of synthetic and natural nisin gave the pseudo molecular ion peak: m/z 3352.7[$(M+H)^+$] [Calcd. for $C_{143}H_{231}O_{37}N_{42}S_7$(M+H), 3352.6].

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