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STRUCTURE OF A BITTER PEPTIDE IN CASEIN HYDROLYZATE BY BACTERIAL PROTEINASE

Tetsuo Shiba and Ken-ichi Nunami
Department of Chemistry, Faculty of Science, Osaka University
Toyonaka, Osaka, Japan

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Numerous investigations on bitter peptides in enzymic hydrolyzate of protein have been reported during recent years[1]. Diary chemistry prompted isolations and structural studies of those bitter compounds produced particularly from casein. Recently, Yamamoto et al. isolated two bitter peptides, which they termed BP-I and BP-II, from casein hydrolyzate by alkaline proteinase of Bacillus subtilis[2,3]. A chemical structure of BP-II was proposed by the same investigators to be cyclo(L-leucyl-L-tryptophyl-L-leucyl-L-tryptophyl) (I)[3].

Our interest in this peptide was first concerned with a view that naturally originated cyclic tetrapeptide involving α-amino acids has never been found so far. However, the sample of the peptide BP-II which was offered from Professor Yamamoto showed molecular weight of about 300 by vapor pressure depression method. This new information suggested us a possibility of a diketopiperazine (II) composed of L-leucine and L-tryptophan. We attempted to synthesize both I and II to determine the structure of the natural compound.

For preparation of the cyclic tetrapeptide I, benzyloxycarbonyl-L-leucine was coupled with L-tryptophan methyl ester by 1-succinimidyl ester method to afford benzyloxycarbonyl-L-leucyl-L-tryptophan methyl ester (III). Removal of benzyloxycarbonyl group from III by hydrogenolysis gave dipeptide methyl ester acetate (IV). On the other hand, saponification of III yielded benzyloxycarbonyl dipeptide (V). Coupling of IV and V by 1-succinimidyl ester method gave a protected tetrapeptide methyl ester (VI). After conversion of VI to a corresponding 1-succinimidyl ester (VII) and removal of benzyloxycarbonyl group from VII, the tetrapeptide 1-succinimidyl ester thus obtained was cyclized in pyridine

at 60°C for 15 hr by high dilution method to secure cyclotetrapeptide I in a 8% yield. The diketopiperazine II was obtained by cyclization of IV in methanol saturated with ammonia in a 78% yield. Natural peptide BP-II was completely identical with II, but quite different from I in all respects as mentioned in Table 1. Therefore, we now decided the structure of the bitter peptide BP-II from casein hydrolyzate to be cyclo(L-leucyl-L-tryptophyl) unambiguously.

Table 1 Comparison of Natural Bitter Peptide with Synthetic Cyclic Peptides

	BP-II	Cyclo(Leu-Trp)	Cyclo(Leu-Trp-Leu-Trp)
mp	245-9°C (dec.)	261-3°C (dec.)	148°C (dec.)
TLC			
$CHCl_3$:MeOH(8:2)	0.62	0.62	0.85
$CHCl_3$:EtOH:AcOH(38:4:2)	0.49	0.49	0.57
nBuOH:AcOH:H_2O(4:1:2)	0.82	0.82	0.90
EtOAc:iPrOH:NH_4OH(45:35:20)	0.96	0.96	0.98
Toluene:$ClCH_2CH_2OH$:Pyridine :NH_4OH(50:35:15:7)	0.78	0.78	0.89
IR(cm^{-1})	1670,1470 1380	1670,1470 1380	1640,1540 1460,1380

Matoba and Hata pointed out that several dipeptides exhibited more bitterness when their amino and carboxyl groups were blocked or they were converted to diketopiperazines[4]. In order to elucidate a relationship between taste exhibition and structure of diketopiperazine, we prepared four other diketopiperazines containing either L-leucine or L-tryprophan. The result of comparison in strength of bitterness of those diketopiperazines as shown in Fig.1 apparently indicated that the diketopiperazine II was the most bitter peptide among them, although all diketopiperazines prepared showed bitterness more or less[5].

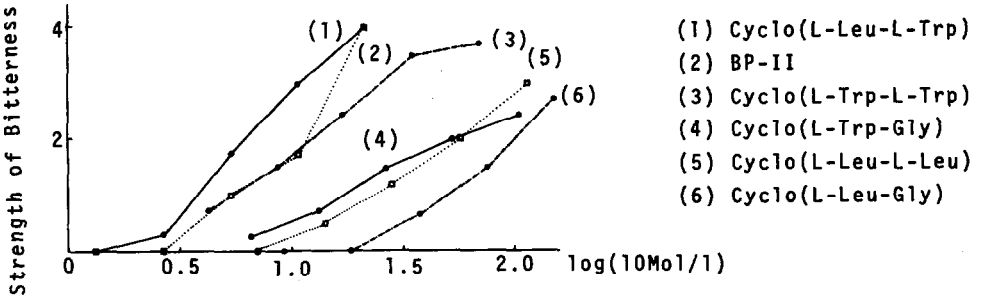

Fig.1 Strength of Bitterness of Diketopiperazines

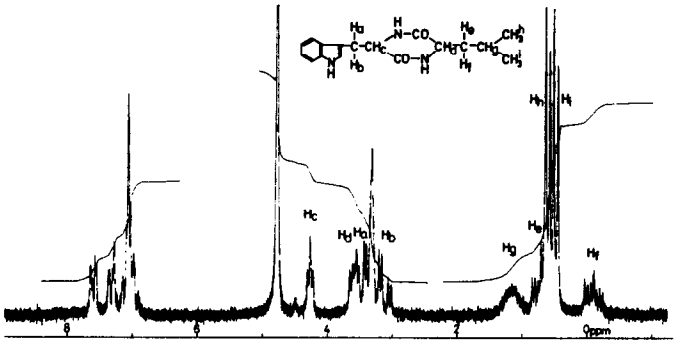

Fig.2 NMR of cyclo(L-leucyl-L-tryptophyl) in CD_3OD at 100 MHz

NMR spectra of the diketopiperazine II in CD_3OD and in d_6-DMSO at 100 MHz were essentially similar though slight solvent effect for chemical shifts was observed. Remarkable feature in the former solution is a shift of two β-methylene protons of leucine to abnormally high field, i.e., δ0.70 and -0.10 as shown in Fig.2. This may mean that side chain of leucine locates very closely to an indole ring of tryptophan at actual conformation in the solution where bitter taste is exhibited. Furthermore, coupling constants of 2.4 and 2.3 Hz due to NH protons of leucine and tryptophan respectively suggest most strongly a twist boat form for diketopiperazine ring[6]. A chair form should be neglected from values of the coupling constants of NH, and diequatorial as well as diaxial boat forms would be also impossible from positioning of side chains. If Karplus equation is applied to estimate dihedral angles for orientation of side chains of each amino acid residue and proximity of both side chains is taken into account, the most possible conformation is obtained for the whole molecule as depicted in Fig.3. However, an alternative twist boat form where both CO groups are overturned to outer side of the molecule can not be ignored. Such molecular feature as proximity of both hydrophobic groups in one side of the molecule and location of hydrogen bonding groups on the other side, may not be unconcerned to a biological mechanism of taste exhibition, since strength of bitterness and degree of high field shift of proton NMR of leucine side chain in diketopiperazines investigated seem to be parallel as shown in Fig.1 and Table 2. This tentative conclusion concerning the conformation of the bitter peptide could be one important example for discussion on a stereostructure of compound of taste.

Table 2 NMR Spectra of Diketopiperazines in d_6-DMSO

	Leucine							Tryptophan			
	β-CH_2		δ-CH_3		γ-CH	α-CH	NH	β-CH_2		α-CH	Indole NH
(Leu-Trp)	0.06	0.69	0.45	0.54	1.22	3.34	7.99	3.00	3.29	4.09	6.80 7.91 -7.69
(Leu-Leu)	1.2-2.0	1.2-2.0	0.85	0.88	1.2-2.0	3.7	8.1				
(Leu-Gly)	1.4-1.7	1.4-1.7	0.89	0.91	1.4-1.7	3.5-3.9	8.2				
(Trp-Trp)								2.15	2.75	3.85	6.63 6.60 -7.70
(Trp-Gly)								3.0-3.55	3.0-3.55	4.05	6.90 7.70 -7.65

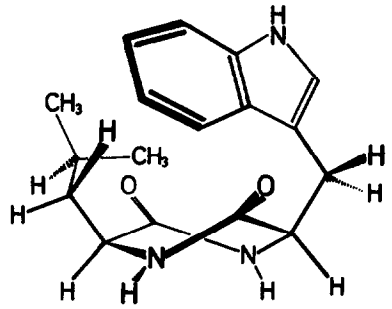

Fig.3 Possible conformation of cyclo(L-leucyl-L-tryptophyl)

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REFERENCES AND FOOTNOTES

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3) N. Minamiura, Y. Matsumura and T. Yamamoto, J. Biochem., 72, 841 (1972).

4) T. Matoba and T. Hata, Agr. Biol. Chem., 36, 1423 (1972).

5) The sensory test was carried out on aqueous solutions of the samples dissolved previously in minimum amount of methanol for a panel of six persons. The strength of bitterness was ranked as follows: 4, remarkably strong; 3, strong; 2, ordinary; 1, faint; 0, tasteless.

6) K. D. Kopple and M. Ohnishi, J. Am. Chem. Soc., 91, 962 (1969).