dimethyldiaminomethyl group was seen at 3.18 ppm, and the N-methyl signal at 2.07 ppm. However, when 2,4(5)-disopropylimidazole (Ib: R¹=R²=Pr<sup>iso</sup>) was treated in the same way, the corresponding dimethyldiaminomethyl derivative was not obtained, and another Mannich reaction product (IIIb), derived from two imidazole molecules and two formaldehyde molecules, was isolated in poor yield.

When Ib was heated with formalin, without added base in a sealed tube at 110—120° for 3 hr, IIIb was obtained in good yield (ca. 80%). The infra red spectrum of IIIb showed no NH absorption, the mass spectrum had a molecular ion peak at m/e 328, and the NMR spectrum had a methylene proton signal of the dihydropyrazine ring at 4.96 ppm (singlet). Strangely, Ia and other imidazoles (R¹=CH<sub>3</sub>, R²=Pr<sup>iso</sup>; R¹=Pr<sup>iso</sup>, R²=CH<sub>3</sub>; R¹=Pr<sup>iso</sup>, R²=Et) gave a trace of III under similar conditions and also under other conditions tested.

Formation of a similar ring system, but with two carbonyls instead of two methylenes, was reported by Godefroi, et al.<sup>6</sup> in the reaction of 4-methyl-5-imidazolecarboxylic acid with acetic anhydride. Oxidation of IIIb with potassium permanganate or chromic acid (Collins oxidation<sup>7</sup>) gave many products which were not identified.

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Active Peptides on Smooth Muscle in the Skin of Bombina orientalis
Boulenzer and Characterization of a New Bradykinin Analogue

We have examined some biological active peptides in the skin of korean frog, Bombina orientalis Boulenzer, and obtained a new bradykinin analogue in addition to bombesin and bradykinin. This communication deals with the separation and chemical characterization of these peptides.

Separation of Active Peptides

Skin of 5 frogs was extracted with 300 ml of methanol containing 3 ml of 6% trichloroacetic acid and the extract was evaporated in reduced pressure. After the removal of fatty material by extraction with 200 ml of n-hexane and 200 ml of ether, the residue was dissolved in 5 ml of 10% acetic acid and centrifuged. The supernatant was used for further purification. Separatory process of active peptides was summarized in Chart 1. Activity in each fraction was assayed by contraction of rat uterus,<sup>11</sup> guinea pig ileum,<sup>29</sup> and guinea pig gall bladder<sup>30</sup> respectively. Smooth muscle contracting activity was found in three fractions (Bo-I, Bo-II, and Bo-III) in the first chromatography. In further purification of Bo-II, the active fraction

was separated into two peaks (Bo-II-1, and Bo-II-2). When the activity of these fractions was expressed as microgram equivalent to oxytocin action of bradykinin, 240 µg, 5000 µg, 2900 µg, and 2000 µg of active principles were obtained in Bo-I, Bo-II-1, Bo-II-2, and Bo-III. The peptides in Bo-II-1, Bo-II-2 and Bo-III except Bo-I were proved to be homogeneous respectively.

**Characterization of the Peptide in Bo-II-1**

The peptide was inactivated by chymotrypsin, but neither by trypsin nor by carboxypeptidase A and B. Amino acid composition in the acid hydrolysate determined by an amino acid analyser was as follows; Asp₂, Gln₉, Gly₉, Ala₁, Val₁, Met₁, Leu₁, His₁, Arg₁ and Trp₁ (determined by ultraviolet absorbance at 280 nm). N-Terminal amino acid was not detected by DNS (dimethylaminonaphthalenesulfonyl)-method. When the peptide was treated with chymotrypsin, dansylated and successively hydrolysed with 6N hydrochloric acid at 100° for 16 hr, DNS-alanine was detected by thin-layer chromatography. DNS-leucine was detec-
ted when the similar experiment was performed with trypsin. These characteristics of the peptide were the same as those of bombesin.  

**Characterization of the Peptide in Bo-II-2**

The peptide was assumed to be bradykinin by its susceptibility to chymotrypsin or its resistance to trypsin digestion, by analysis of its amino acid composition and N-terminal amino acid. Chromatographic behavior of the DNS-derivative coincided with that of bradykinin.

**Characterization of the Peptide in Bo-III**

The following amino acids were determined in the peptide: Ser₂, Pro₃, Gly₄, Phe₅, Lys₆, His₇, and Arg₈. The N-terminal amino acid was arginine. DNS-derivative of the peptide was cleaved into two fragments (T-1 and T-2) by treatment with trypsin. The DNS-derivative of fragment T-1 was identical with DNS-bradykinin, and fragment T-2 contained amino acids Gly₄, Lys₆, Phe₅, and His₇, indicating that T-2 is the C-terminal portion of the peptide in Bo-III. DNS-Edman procedure gave the sequence of glycyl-lysyl-phenylalanyl-histidine for T-2. The results of carboxypeptidase A or chymotrypsin digestion supported that of DNS-Edman degradation. The total amino acid sequence of the peptide in Bo-III was deduced as follows:

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Gly-Lys-Phe-His-OH

**Synthesis of the Peptide in Bo-III(5)**

The synthesis of the above tripeptide, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Gly-Lys-Phe-His-OH, was performed by the conventional method for peptide synthesis. Rudinger’s azide modification(6) was employed entirely for the fragment condensation. The azide derived from Z-Phe-Ser-Pro-Phe-Arg-Gly-NH₂Boc[acetate: Anal. Calcd. for C₄₉H₇O₉N₁₂H₂Boc[acetate: C, 57.81; H, 6.63; N, 15.13. Found: C, 57.54; H, 6.53; N, 14.93. mp 135–137° (decomp.); [x]D²⁰ = -31.8° (c=1.0, DMF); Rf' 0.57, Rf'' 0.76] was coupled with triethylammonium salt of H-Lys(Boc)-Phe-His-OH[acetate hydrate; Anal. Calcd. for C₃₈H₄₃O₈N₉; H₂O: C, 55.25; H, 7.29; N, 13.81. Found: C, 54.91; H, 7.22; N, 14.13. [x]D²⁰ = +27.3° (c=1.0, 10% acetic acid); Rf' 0.42, Rf'' 0.68] to give a crude protected nonapeptide which was hydrogenated. Purification of the ensuing hydrogenated material by column chromatography on CM-Sephadex gave homogeneous H-Phe-Ser-Pro-Phe-Arg-Gly-Lys(Boc)-Phe-His-OH(diacetate hexahydrate; Anal. Calcd. for C₄₉H₇O₁₇N₁₂₆H₂O: C, 52.99; H, 7.16; N, 14.48. Found: C, 53.04; H, 7.10; N, 14.10. [x]D²⁰ = -41.4° (c=0.7, 10% acetic acid); Rf' 0.30, Rf'' 0.69]. The final azide coupling of Z-Arg(NO₂)-Pro-Pro-Gly-N₂H₃ derived from the corresponding Boc hydrazide(7) with partially protected nonapeptide produced crude protected tripeptide, which was treated with trifluoroacetic acid to remove Boc function and then hydrogenated. The resulting product was purified by column chromatography on CM-Sephadex and gelfiltration on Bio-Gel P-2 to give chromatographically homogeneous H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Gly-Lys-Phe-His-OH[amino acid ratios in acid hydrolysate Lys₁₋₂, His₀₋₂, Arg₀₋₄, Ser₀₋₄, Pro₀₋₀, Gly₁₋₂, Phe₀₋₄ (peptide content 86%); Rf' 0.08, Rf'' 0.60]. The chromatographic behavior of synthetic DNS-tripeptide was identical with that of DNS-derivative of bradykinyl-Gly-Lys-Phe-His-OH of natural origin on thin-layer chromatography.

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5) The amino acid residues except glycine are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature, *Biochemistry*, 5, 2485 (1966); 6, 362 (1967). Designations of solvent systems for thin-layer chromatography on Silica gel G (Merck) are: Rf' 1-BuOH–AcOH–H₂O (4:1:5) upper layer and Rf'' 1-BuOH–pyridine–AcOH–H₂O (30:20:6:24).
Detailed investigation on the biological activities of the peptide Bo-III are now in progress. As we have mentioned, occurrence of this peptide Bo-III in Bombina orientalis also suggests the presence of different kininogens in animal kingdom.

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Mode of Morphological Forms of Crystalline Inorganic Component in Plants: Silicon Bodies in Wheat

I have been carrying out a series of studies on low-temperature ashed image of biological tissues, especially of plant tissues, obtained by “Low-temperature Plasma Ashing Method for Biological Materials” originated with me. Phytological studies on ashed image are especially important in observation the crystalline inorganic components, such as silicon bodies, calcium oxalate crystals, and cystoliths. My previous studies have revealed the characteristic shape, size, arrangement, and distribution of these crystalline inorganic components in various plants. In addition, my recent experiments provided numerous phenomena which suggested the presence of a regulatory mechanism in the morphological behavior of these crystalline inorganic compounds.

Crystalline inorganic components in plants have not been taken up as an attractive academic subject, other than that they are one of waste products of plants, because such components themselves do not show any physiologically interesting activity. In addition, the technique and method for observing these crystalline inorganic components are attended with unavoidable chemical and technical limitations, difficulty, and defects this study has not made much

2) K. Umemoto and K. Hozumi, Yakugaku Zasshi, 91, 828 (1971); idem, ibid., 91, 845 (1971); idem, ibid., 91, 850 (1971); idem, ibid., 91, 908 (1971); idem, ibid., 91, 1047 (1971).
5) K. Umemoto and K. Hozumi, Yakugaku Zasshi, in press.
6) K. Umemoto and K. Hozumi, Yakugaku Zasshi, in press.