Synthesis of α Substance-I_B in *Saccharomyces cerevisiae*[1,2]

Haruhiko AOYAGI and Nobuo IZUMIYA

Laboratory of Biochemistry, Faculty of Science 33, Kyushu University, Higashi-ku, Fukuoka 812

(Received April 18, 1977)

Two hexapeptides, H–Arg–Gly–Pro–Phe–Pro–Ile–OH (**1a**) and H–Arg–Pro–Gly–Phe–Pro–Ile–OH (**1b**), have been proposed for a factor inducing sexual agglutination in the yeast and were synthesized by a conventional method. Compound **1a** was completely identical with the natural peptide in thin-layer chromatography, mass spectrometric measurements, and biological assay on agglutination. Compound **1b** showed the same biological activity as **1a** though distinct differences in the chemical data were observed. Strong bitterness of the **1a** and **1b** was discovered.

Sakurai *et al.*[3] reported the isolation of a peptidyl factor named α substance-I from α type cells (H15 strain) of heterothallic yeast *Saccharomyces cerevisiae*. The active principle induces sexual agglutinability in opposite a (ei) type cells (H22 strain) prior to the mating reaction. It was later found that the factor could be separated into two components by further purification and one of them was designated as α substance-I_B (abbreviated as I_B).[4] Sakurai *et al.* intended to determine the amino acid sequence of I_B by means of mass spectrometry and postulated a tentative structure as H–Arg–Gly–Pro–Phe–Pro–Ile–OH (**1a**). In the beginning, however, an alternate structure of H–Arg–Pro–Gly–Phe–Pro–Ile–OH (**1b**) for I_B could not be excuded because of presence of the unexpected fragment ions. We intended to ascertain the correct structure of the I_B by the syntheses of the supposed hexapeptides, **1a** and **1b**.

The present paper deals with the syntheses of **1a**·2AcOH and **1b**·2AcOH by a conventional method and the comparison of the synthetic peptides with the natural I_B by thin-layer chromatography, mass spectrometry and biological assays.

The syntheses of **1a**·2AcOH and **1b**·2AcOH are outlined in Figs. 1 and 2. The mixed anhydride method[5] was employed through all coupling reactions and the removal of Boc groups of the intermediates was performed by the action of hydrogen chloride in ethyl acetate. The protected hexapeptide **8a** prepared from Z–Arg(NO_2)–OH and **7a**·HCl was hydrogenated in the presence of palladium black, and the desired product (**1a**) was obtained as diacetate by lyophilization. The similar procedure was employed for the preparation of **1b**. The intermediate **8b** contained minor by-products and was purified by silica gel column chromatography, and the desired product (**1b**) was obtained as diacetate. The homogeneity of **1a**·2AcOH and **1b**·2AcOH was confirmed by paper and thin-layer chromatography, paper electrophoresis and elemental analysis.

For the comparison of the synthetic peptides with natural I_B, thin-layer chromatography was performed at first using four different systems, the result being given in Table 1. In all cases, the **1a**·2AcOH revealed com-

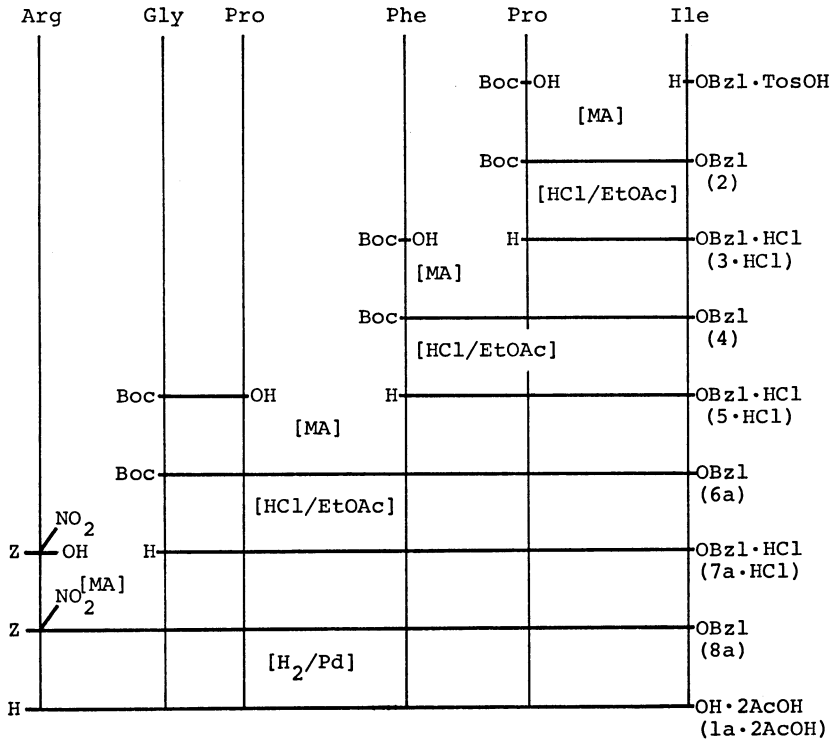

Fig. 1. Synthesis of the hexapeptide, **1a**.

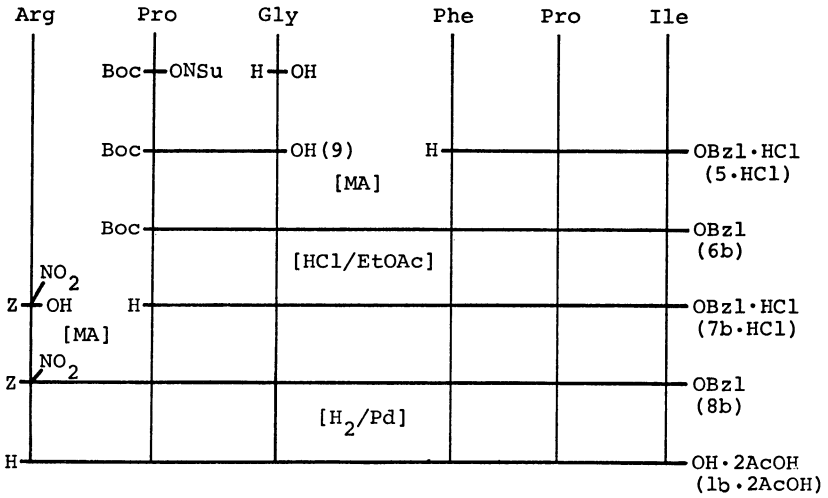

Fig. 2. Synthesis of the hexapeptide, **1b**.

Table 1. Thin-layer chromatography of natural and synthetic peptides

Solvent	Carrier	R_f[a]		
		I_B	**1a**·2AcOH	**1b**·2AcOH
1-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase)	Silica gel[b]	0.18	0.18	0.17
1-BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12)	Silica gel[b]	0.59	0.59	0.57
1-BuOH–1-PrOH–0.2 M AcOH (2 : 1 : 3, upper phase)	Silica gel[b]	0.11	0.11	0.09
1-BuOH–1-PrOH–0.2 M AcOH (2 : 1 : 3, upper phase)	Cellulose[c]	0.58	0.58	0.63

a) The chromatograms were stained with ninhydrin followed by chlorine–toluidine. b) The plates coated with siligel gel GF_{254} (Merck) in 0.25-mm thickness were used. c) The plates coated with cellulose (Merck) in 0.1-mm thickness were used.

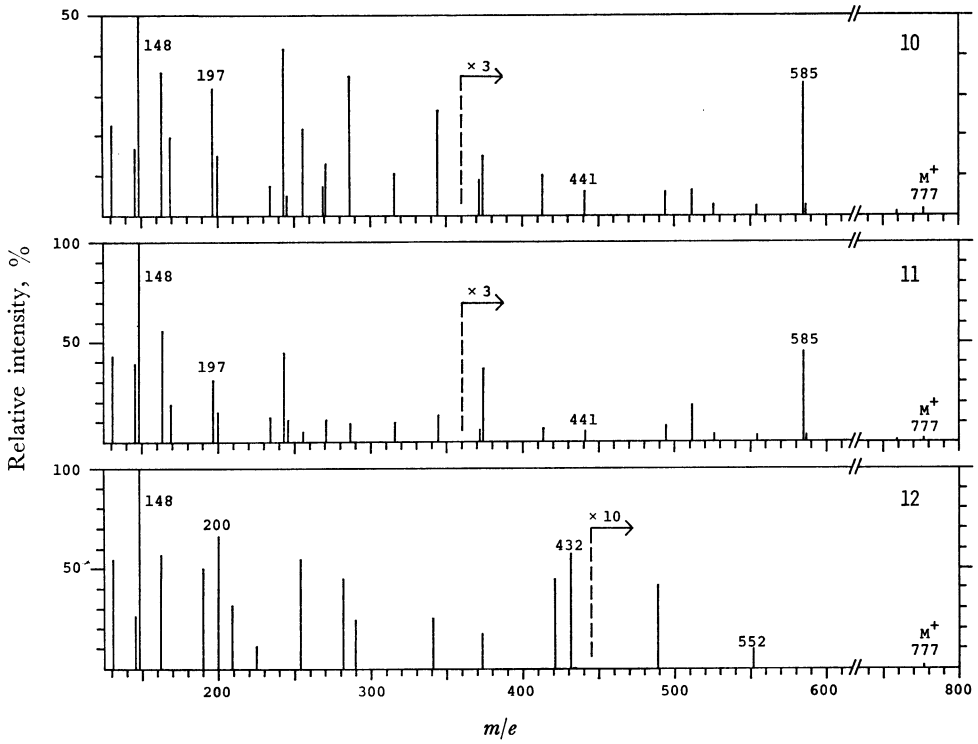

Fig. 3. Mass spectra of the derivative (**10**) of I_B and the derivatives (**11** and **12**) of the hexapeptides (**1a** and **1b**).

plete identity with I_B. The difference between R_f's of **1b**·2AcOH and I_B obvious.

Prior to comparison of mass spectra, the N-terminal arginine residues in I_B, **1a**·2AcOH, and **1b**·2AcOH were converted to α-*N*-acetyl-δ-*N*-pyrimidyl-ornithines and the C-terminus to methyl esters according to the literature.[6] The spectra of the derivatives, which named [Ac-Orn(Pyr)[1]]-I_B-OMe (**10**), [Ac-Orn(Pyr)[1]]-**1a**-OMe (**11**) and [Ac-Orn(Pyr)[1]]-**1b**-OMe (**12**), respectively, from I_B, **1a**, and **1b** are shown in Fig. 3. Compound **11** showed ion peaks at *m/e* 777 (M^+), 585, 441, 197, and 148, which were exactly the same as those observed for the corresponding **10** from I_B. On the contrary, the spectra of **12** were quite different in fragmentation pattern from those of **10** and **11**. From these results, the identity of **1a** with I_B was verified unambiguously.

The biological activities of the natural and synthetic peptides to induce agglutinability in a (ei) type cells were examined, and the I_B and **1a**·2AcOH showed the activity in the same degree at dosages at 2—20 ng/ml. Contrary to our expectation, the **1b**·2AcOH also showed almost the same activity as I_B, the finding suggesting that a strict structure is not always required for activity. This phenomenon is interesting because the replacement of amino acid residue by another in a biologically active peptide gives generally an influence for activity of the peptide.

Recently, Ribadeau Dumas *et al.* elucidated the whole primary structure of bovine β-casein,[7] which contained the partial sequence of –Arg^{202}–Gly–Pro–Phe–Pro–Ile^{207}–Ile–Val–OH^{209} in the C-terminal portion. It is noteworthy that the part of –Arg^{202}–Gly–Pro–Phe–Pro–Ile^{207}– is identical with the I_B, On the other hand, two Japanese groups isolated the peptides having the structures of H–Arg–Gly–Pro–Pro–Phe–Ile–Val–OH (**13**)[8] and H–Gly–Pro–Phe–Pro–Val–Ile–OH (**14**)[9] from the enzymatic hydrolyzate of casein as bitter principles. Neither amino acid sequence of **13** nor that of **14** can be found in any part of the primary structure of casein, but both peptides are very similar to C-terminal portion of casein and I_B. We observed that the synthetic **1a**·2AcOH and **1b**·2AcOH showed strong bitter taste in almost the same threshold value. From these results, we assume that the partial structure of a substance corresponding to **13** will be H–Arg–Gly–Pro–Phe–Pro–Ile–, and that of **14** will be H–Gly–Pro–Phe–Pro–Ile–.

Experimental

Melting points are uncorrected. The ratio in parentheses after a solvent system was indicated by vol. TLC was carried out on silica gel G (Merck) with the solvent systems: R_f^1, $CHCl_3$–MeOH (5 : 1); R_f^2, 1-BuOH–AcOH–pyridine–H_2O (15 : 3 : 10 : 12). Paper chromatography was carried out on Toyo Roshi No. 52 paper with the solvent system: R_f^3, 1-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2). Optical rotations were measured on a Union high sensitivity polarimeter PM-71. Amino acid analyses were performed with a Hitachi amino acid analyzer, KLA-5.

*Boc–Pro–Ile–OBzl (**2**).* To a chilled solution of Boc–Pro–OH (3.23 g, 15 mmol) and TEA (2.10 ml, 15 mmol) in THF (30 ml) was added isobutyl chloroformate (1.97 ml, 15 mmol) at −5 °C. After 15 min, a chilled solution of H–Ile–OBzl·TosOH (5.90 g, 15 mmol) and TEA (15 mmol) in $CHCl_3$ (30 ml) was added. The mixture was left to stand overnight at room temperature, evaporated *in vacuo*, and the oily residue was dissolved in EtOAc. The solution was washed successively with 4% $NaHCO_3$, 10% citric acid and water, and then dried (Na_2SO_4). The solvent was evaporated *in vacuo*; yield of an oil, 6.06 g (96%); R_f^1 0.91.

*Boc–Phe–Pro–Ile–OBzl (**4**).* Compound **2** (5.86 g, 14 mmol) was dissolved in 3.0 M hydrogen chloride in EtOAc (50 ml). The solution was allowed to stand for 40 min at room temperature and then evaporated; yield of oily H–Pro–Ile–OBzl·HCl (**3**·HCl), 4.87 g (98%). Then, **4** was prepared from Boc–Phe–OH (3.58 g, 13.5 mmol) and **3**·HCl (4.79 g, 13.5 mmol) as described for **2**; the yield of oily **4**; 7.20 g (94%); R_f^1 0.90.

*Boc–Gly–Pro–Phe–Pro–Ile–OBzl (**6a**).* Compound **4** (5.66 g, 10 mmol) was treated with 3.0 M hydrogen chloride in EtOAc (35 ml) as described for **3**·HCl; yield of oily H–Phe–Pro–Ile–OBzl·HCl (**5**·HCl), 4.75 g (95%). Then, **6a** was prepared from Boc–Gly–Pro·OH[10] (1.22 g, 4.5 mmol) and **5**·HCl (2.26 g, 4.5 mmol) as described for **2**; yield of oily **6a**, 3.00 g (93%); R_f^1 0.69.

*H–Gly–Pro–Phe–Pro–Ile–OBzl·HCl (**7a**·HCl).* Compound **6a** (2.88 g, 4 mmol) was treated as described for **3**·HCl. The oily product was solidified by the addition of ether; yield, 2.13 g (80%); mp 145—148 °C; $[\alpha]_D^{20}$ −69.8° (*c* 1, DMF); R_f^1 0.35, R_f^2 0.85.

Found: C, 61.28; H, 6.94; N, 10.48%. Calcd for $C_{34}H_{46}O_6N_5Cl\cdot 1/2H_2O$: C, 61.38; H, 7.12; N, 10.53%.

*Z–Arg(NO_2)–Gly–Pro–Phe–Pro–Ile–OBzl (**8a**).* This compound was prepared from Z–Arg(NO_2)–OH (1.06 g, 3 mmol) and **7a**·HCl (1.99 g, 3 mmol) as described for **2**. The product was recrystallized from EtOAc–ether; yield, 2.09 g (72%); mp 99—103 °C; $[\alpha]_D^{20}$ −58.0° (*c* 1, DMF); R_f^1 0.71, R_f^2 0.92.

Found: C, 59.31; H, 6.44; N, 14.41%. Calcd for $C_{48}H_{62}O_{11}N_{10}\cdot H_2O$: C, 59.24; H, 6.63; N, 14.40%.

*H–Arg–Gly–Pro–Phe–Pro–Ile–OH·2AcOH (**1a**·2AcOH).* Compound **8a** (389 mg, 0.4 mmol) was dissolved in a mixture of MeOH (5 ml), AcOH (2 ml) and water (3 ml), and treated with hydrogen in the presence of Pd black overnight. The filtrate of the reaction mixture was evaporated and the evaporation was repeated several times on the addition of water. The residue was dissolved in water and lyophilized to leave a solid; yield, 312 mg (95%); mp 102—108 °C; $[\alpha]_D^{21}$ −87.6° (*c* 1, H_2O). The homogeneity of **1a** was confirmed by ninhydrin and Sakaguchi reagent on paper electrophoresis and paper chromatography; R_f^3 0.88. Amino acid ratios in an acid hydrolyzate; Pro 2.12, Gly 1.00, Ile 0.98, Phe 0.96, Arg 0.97.

Found: C, 54.18; H, 7.53; N, 15.55%. Calcd for $C_{33}H_{51}O_7N_9\cdot 2CH_3COOH\cdot H_2O$: C, 53.93; H, 7.46; N, 15.30%.

*Boc–Pro–Gly–OH (**9**).* To a solution of glycine (1.13 g, 15 mmol) and TEA (15 mmol) in water (70 ml) at 5 °C was added Boc–Pro–ONSu[11] (3.89 g, 12.5 mmol) in dioxane (50 ml). The mixture was stirred for 30 min at 5 °C and overnight at room temperature. After the dioxane was evaporated, the aqueous solution was acidified with 10% citric acid and extracted with EtOAc. The organic layer was washed with water, dried and evaporated to leave a solid. The product was recrystallized from EtOAc–ether; yield, 2.37 g (70%); mp 159—160 °C; $[\alpha]_D^{20}$ −60.8° (*c* 1, DMF); R_f^1 0.22.

Found: C, 52.78; H, 7.40; N, 10.14%. Calcd for $C_{12}H_{20}O_5N_2$: C, 52.93; H, 7.40; N, 10.29%.

*Boc–Pro–Gly–Phe–Pro–Ile–OBzl (**6b**).* This compound was prepared from **9** (0.95 g, 3.5 mmol) and **5**·HCl (1.76 g,

3.5 mmol) as described for **2**; yield of an oil, 2.47 g (98%); R_f^1 0.72.

*H–Pro–Gly–Phe–Pro–Ile–OBzl·HCl (**7b**·HCl).* Compound **6b** (2.16 g, 3 mmol) was treated as described for **3**·HCl. The oily product was solidified by the addition of ether; yield, 1.70 g (84%); mp 91—94 °C; $[\alpha]_D^{20}$ −39.6° (*c* 1, DMF); R_f^1 0.34, R^2 0.82.

Found: C, 60.38; H, 7.07; N, 10.34%. Calcd for $C_{34}H_{46}O_6N_5Cl\cdot H_2O$: C, 60.56; H, 7.18; N, 10.39%.

*Z–Arg(NO$_2$)–Pro–Gly–Phe–Pro–Ile–OBzl (**8b**).* This compound was prepared from Z–Arg(NO$_2$)–OH (0.88 g, 2.5 mmol) and **7b**·HCl (1.68 g, 2.5 mmol) as described for **2**. The oily product was obtained in 2.30 g. For purification, a half (1.15 g) of the product was dissolved in a solvent of $CHCl_3$–MeOH–AcOH (95 : 5 : 1), applied on a column (2.4 × 84 cm) with silica gel, and eluted with the same solvent. The fractions with the desired product were evaporated to leave an oil which was solidified by the addition of ether, the remainder (1.15 g of the product) being purified by the same procedure. Recrystallization from EtOAc–ether gave 0.92 g (38%) of pure **8b**; mp 98—105 °C; $[\alpha]_D^{20}$ −33.8° (*c* 1, DMF); R_f^1 0.68; R_f^2 0.91.

Found: C, 59.79; H, 6.59; N, 14.40%. Calcd for $C_{48}H_{62}O_{11}N_{10}\cdot 1/2H_2O$: C, 59.80; H, 6.59; N, 14.53%.

*H–Arg–Pro–Gly–Phe–Pro–Ile–OH·2AcOH (**1b**·2AcOH).* Compound **8b** (386 mg, 0.4 mmol) was treated as described for **1a**·2AcOH; yield of a solid, 314 mg (95%); mp 107—112 °C; $[\alpha]_D^{21}$ −56.2° (*c* 1, H_2O); R_f^3 0.89. Amino acid ratios in an acid hydrozate: Pro 2.11, Gly 1.00, Ile 0.97, Phe 0.96, Arg 0.95.

Found: C, 53.67; H, 7.45; N, 15.52%. Calcd for $C_{33}H_{51}O_7N_9\cdot 2CH_3COOH\cdot H_2O$: C, 53.93; H, 7.46; N, 15.30%.

*Comparison of the Natural Peptide (I_B) and Synthetic Peptides (**1a** and **1b**).* *TLC*: TLC was carried out using four different systems, the results being shown in Table 1.

Mass Spectrometric Measurements: The I_B, **1a**·2AcOH, and **1b**·2AcOH were modified according to the literature,[6] and the measurements were performed on a Hirachi RMU-6L at 70 eV. The results are shown in Fig. 3.

Agglutinability Assays: The activities of I_B, **1a**·2AcOH, and **1b**·2AcOH to induce agglutinability in a (ei) type cells (H 22 strain) were assayed according to the literature.[3] The I_B and **1a**·2AcOH showed the activity in the same degree at 2—20 ng/ml. Compound **1b**·2AcOH also showed the same activity.

Bitterness Evaluation. Each synthetic peptide (**1a**·2AcOH or **1b**·2AcOH) was dissolved in water and a series of solution of decreasing concentration were prepared. The degree of bitterness was organoleptically determined by a panel of five men in our laboratory according to the literature.[12] Compounds **1a**·2AcOH and **1b**·2AcOH showed the bitter taste in the same degree at 0.13—0.25 mM (0.1—0.2 mg/ml).

We wish to express our thanks to Professor S. Tamura, University of Tokyo, for his helpful discussion, and to Dr. A. Sakurai, Institute of Physical and Chemical Research, Saitama, for TLC and agglutinability assays for the comparision of the synthetic peptides with I_B. We also thank to Drs. A. Isogai and K. Aizawa, University of Tokyo, for the measurements of mass spectra.

References

1) Part of this work has been briefly communicated: H. Aoyagi, N. Izumiya, A. Sakurai, K. Sakata, and S. Tamura, *Experientia*, in press.

2) The abbreviations recommended by the IUPAC-IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.*, **247,** 977 (1972)) have been used throughout. Additional abbreviations: AcOH, acetic acid; 1-BuOH, 1-butanol; DMF, *N,N*-dimethylformamide; MA, mixed anhydride method; 1-PrOH, 1-propanol; Pyr, pyrimidyl; TEA, triethylamine; THF, tetrahydrolyfuran; TosOH, *p*-toluenesulfonic acid. Amino acid symbols except Gly denote the L-configuration.

3) A. Sakurai, S. Tamura, N. Yanagishima, and C. Shimoda, *Proc. Jpn. Acad.*, **51**, 291 (1975).

4) A. Sakurai, K. Sakata, S. Tamura, K. Aizawa, N. Yanagishima, and C. Shimoda, *Agric. Biol. Chem.*, **40**, 1451 (1976).

5) T. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

6) M. M. Shemyakin, E. I. Vinogradova, Yu. A. Ovchinnikov, A. A. Kiryushkin, M. Yu. Feigina, N. A. Aldanova, Yu. B. Alakhov, V. M. Lipkin, B. V. Rosinov, and L. A. Fonina, *Tetrahedron*, **25**, 5785 (1969).

7) B. Ribadeau Dumas, G. Bringnon, F. Grosclaude, and J. C. Mercier, *Eur. J. Biochem.*, **25**, 505 (1972).

8) N. Minamiura, Y. Matsuura, F. Fukumoto, and T. Yamamoto, *Agric. Biol. Chem.*, **36**, 588 (1972).

9) T. Matoba, R. Hayashi, and T. Hata, *Agric. Biol. Chem.*, **34**, 1235 (1970).

10) J. C. Anderson, M. A. Barton, P. M. Hardy, G. W. Kenner, and R. C. Sheppard, *J. Chem. Soc.*, **1967**, 108.

11) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

12) T. Matoba and T. Hata, *Agric. Biol. Chem.*, **36**, 1423 (1972).