[Chem. Pharm. Bull. 34(4)1473—1478(1986)]

Biomimetic Synthesis of Heterocyclic β-Substituted Alanines by Pyridoxal 5′-Phosphate-Catalyzed Chemical Reactions[1)]

ISAMU MURAKOSHI, FUMIO IKEGAMI,* YOSHIHIRO YONEDA, HARUMI IHARA, KUMIKO SAKATA, and CHIHARU KOIDE

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1–33, Chiba 260, Japan

(Received August 20, 1985)

Several heterocyclic β-substituted alanines were biomimetically synthesized by incubating 0.1 M acetate buffer solution containing the appropriate heterocyclic compound and *O*-acetylserine or serine in the presence of pyridoxal 5′-phosphate (PLP) and metal ions. This PLP-catalyzed chemical reaction depends upon pH, metal ions and temperature. The addition of Ga^{3+}, Fe^{3+} or Al^{3+} enhanced the rate of synthesis. The optimum of the various reaction conditions of this biomimetic method is described.

Keywords——biomimetic synthesis; amino acid synthesis; heterocyclic β-substituted alanine; non-protein amino acid; *O*-acetylserine; *N*-heterocyclic compound; pyridoxal 5′-phosphate; metal ion

Introduction

Plants produce a number of non-protein amino acids that may be regarded as heterocyclic β-substituted alanines, such as β-(pyrazol-1-yl)-L-alanine,[2)] L-mimosine,[3)] L-quisqualic acid[4)] and L-willardiine.[5)]

It has been demonstrated by Murakoshi *et al.*[6–13)] that these heterocyclic β-substituted alanines are enzymatically synthesized by condensation of the appropriate *N*-heterocyclic compound with *O*-acetyl-L-serine (OAS) as an activated form of L-serine. Furthermore, in recent studies, we found that these β-substituted alanine synthases are a group of pyridoxal 5′-phosphate-enzymes and show by far the highest activity towards OAS as a donor for the alanyl moiety, though they show marked differences in specificity for the heterocyclic substrate as an acceptor.[14,15)] In general these enzymes establish a bond between a ring-N and the β-carbon of alanine. Occasionally a ring-C is involved, resulting in a C–C bond,[9)] or an –OH group is involved, resulting in an O–C bond.[12)]

Based on the results obtained with purified β-(pyrazol-1-yl)-L-alanine synthase[14)] and L-mimosine synthase,[15)] the reaction mechanism of these β-substituted alanine synthases resembles that for β-tyrosinase established by Yamada *et al.*[16,17)]

In the course of our ongoing study on the β-substituted alanine synthases in higher plants forming heterocyclic β-substituted alanines, we have further developed a very simple biomimetic synthesis of these β-substituted alanines catalyzed by pyridoxal 5′-phosphate (PLP) and metal ions, especially gallium ion, in the light of the general mechanism for PLP-dependent reactions, as shown in Chart 1, while we have investigated the synthesis of β-(pyrazol-1-yl)alanine from α-acetamidoacrylic acid as a stable model compound of the enzyme-α-aminoacrylate complex with pyrazole.[18)]

The formation of tryptophan and β-(pyrazol-1-yl)alanine in low yield by the non-enzymatic reaction of indole or pyrazole and L-serine with pyridoxal or PLP and aluminium

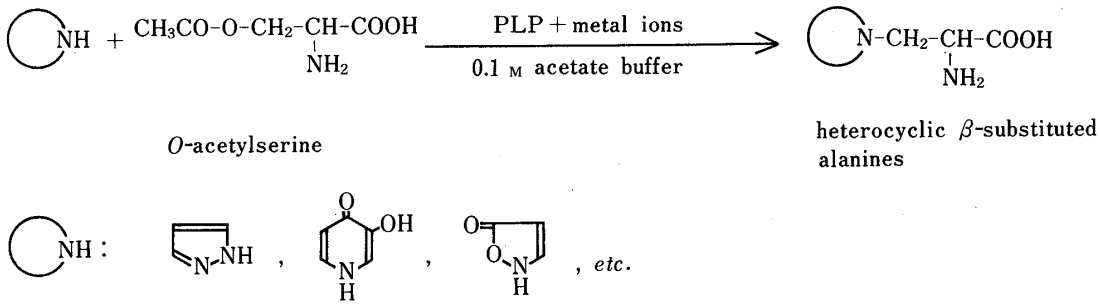

Chart 1. Schematic Representation of the Biomimetic Synthesis of Heterocyclic β-Substituted Alanines by the PLP-Catalyzed Reaction

TABLE I. Summary of the Optimal Reaction Conditions for the Formation of Heterocyclic β-Substituted Alanines in the Presence of PLP and Ga^{3+} by the PLP-Catalyzed Chemical Reaction[a)]

Reaction products	pH	Temperature (°C)	Reaction time (h)	Yield (%)
β-(Pyrazol-1-yl)alanine (**1**)	3.7	62—65	2	40—45
Mimosine (**2**)	4.5	62—65	0.5	1—2
Quisqualic acid (**3**)	5.0	62—65	2	8—10
β-(3-Isoxazolin-5-on-2-yl)alanine (**4**)	4.0	30	2	0.15
Lupinic acid (**5**)	4.0	30—33	2	1.5—1.7
Willardiine (**6**)	5.5	33—35	2	0.8—1.0
Isowillardiine (**7**)	5.5	83—85	2	0.1
β-(5-Methylisoxazolin-3-on-2-yl)alanine (**8**)	4.7	62—65	2	35—40
β-(3-Amino-1,2,4-triazol-1-yl)alanine (**9**)	4.7	62—65	2	30—35
β-(2-Furoyl)alanine[b)]	4.0	62—65	1	5—7
β-(6-Benzylaminopurin-9-yl)alanine (**11**)	4.5	62—65	2	1.5—2
Histidinoalanine (**12**)	3.5	62—65	2	0.7—0.8

a) The reaction conditions are given in Experimental. *b*) This compound was obtained by hydrolytic cleavage with 6 N HCl, providing indirect evidence of the formation of ascorbalamic acid (**10**).[25)]

ion has already been demonstrated by Metzler *et al.*[19)] and by Dunnill and Fowden.[20)]

The present work presents a general method for the biomimetic synthesis of heterocyclic β-substituted alanines with the optimum of the various reaction conditions. The effects of various metal ions in this biomimetic synthesis were also studied.

Results and Discussion

The results presented demonstrate that PLP and metal ions can catalyze the biomimetic synthesis of heterocyclic β-substituted alanines in the absence of protein, using the same substrates as are used in the enzymatic synthesis. Table I summarizes the optimal reaction conditions in the presence of PLP and Ga^{3+} for a number of products synthesized. In this biomimetic synthesis, the formation of these β-substituted alanines was greatly increased by using OAS and Ga^{3+} as compared with the previous studies.[19,20)]

The effect of pH on the reaction yield was investigated by using 0.1 M acetate buffers or citrate buffers. The biomimetic synthesis was found to be dependent upon the pH with a relatively narrow range of activity; the optimum pH value differs for every product, and varies from 3.5 to 5.5.

The reaction rates were constant for at least 1 h and decreased later, except in the case of

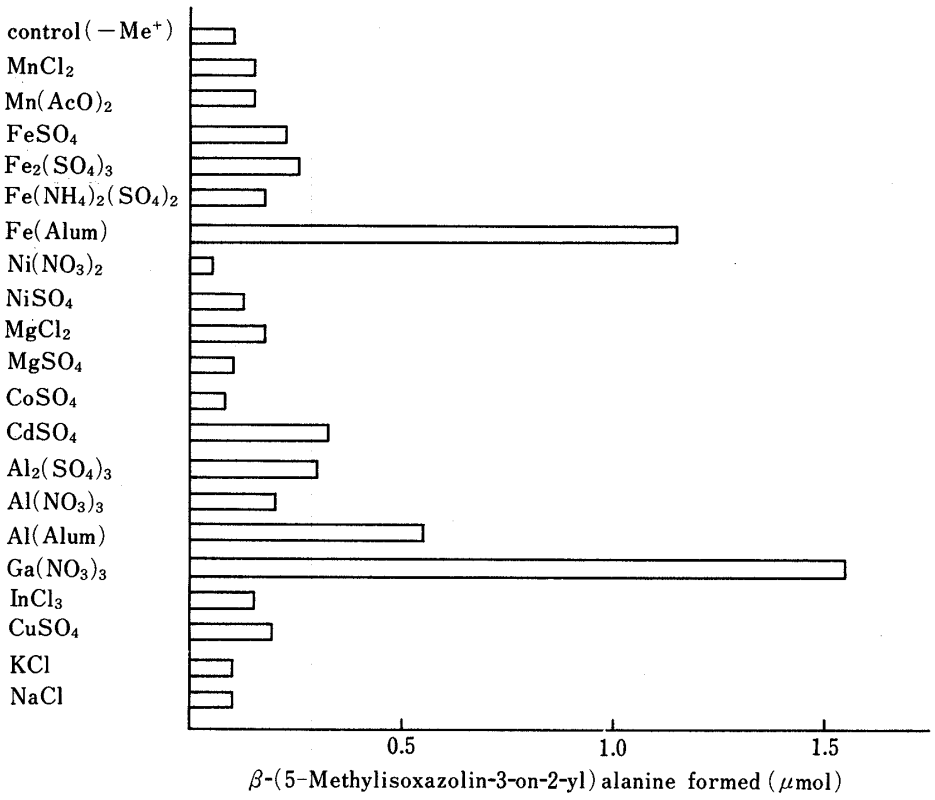

Fig. 1. Comparative Effects of Various Metal Ions[a] on the Biomimetic Synthesis of β-(5-Methylisoxazolin-3-on-2-yl)alanine (□) in the Presence of PLP under the Optimal Reaction Conditions

a) The concentration of metal ions was fixed at 1.25 mM, per 12.5 mM OAS, as described in Experimental.

mimosine (**2**), for which there is a maximum formation of soluble product after 30 min. The later decrease of soluble mimosine may be due to chelation with PLP or Ga^{3+}. The formation of lupinic acid (**5**) was decreased under the normal conditions; in this case the instability of the zeatin precursor may be the cause. The formation of isowillardiine (**7**) was minimal under the conditions used for the formation of willardiine (**6**). The very low yield of β-(3-isoxazolin-5-on-2-yl)alanine (**4**) as compared to the other compound may be explained by the low stability of the free ring under the experimental conditions.

The effect of the concentration of the heterocyclic precursor on the formation of these β-substituted alanines in the presence of 12.5 mM of OAS, 1.25 mM PLP and 1.25 mM Ga^{3+} was observed; about ten-fold excess of the heterocyclic precursor gave the optimal rate of production. Higher concentrations (200—250 mM) were slightly inhibitory. In the biosynthesis *in vitro* of heterocyclic β-substituted alanines, substrate inhibition is observed in some cases.

The effect of the concentration of PLP on these chemical reactions was also studied. A relatively low concentration (1.25 mM) of PLP gave maximum rates of synthesis, while higher concentrations (2.5—3.75 mM) severely inhibited the reaction. Pyridoxal has a catalytic effect similar to that of PLP.

In this biomimetic synthesis, the effects of various metal compounds were comparatively studied under the same conditions. The addition of metal ions, especially trivalent ions, such as Ga^{3+} ($Ga(NO_3)_3$), Fe^{3+} (iron alum) and Al^{3+} (potassium alum), to the reaction mixtures at 1.25—2.5 mM per 12.5 mM OAS enhanced the rate of heterocyclic β-substituted alanines synthesis about 15- to 20-fold compared to the controls, as shown in Fig. 1, whereas divalent ions, such as Mg^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+} enhanced it only slightly.

Addition of monovalent ions, K^+ or Na^+, caused neither stimulation nor inhibition of the formation of these β-substituted alanines. The reactions proceeded very slowly in the absence of metal ions.

Furthermore, the rate of this chemical reaction in general increased with increase in temperature: with a few exceptions, synthesis of products was maximal at temperatures of 62 to 65 °C and was very slow at 4 °C. This reaction also utilized OAS more readily than the other esters, such as *O*-phospho-L-serine and *O*-sulfo-L-serine, or L-serine itself as a donor for the alanyl moiety: the reaction mixture of OAS, PLP and Ga^{3+} might behave as a stable model compound of the enzyme-α-aminoacrylate complex, which was established to be a common key intermediate in the reaction mechanisms of the multifunctional group of PLP-enzymes.[16,17]

The chemical reaction shows no specificity for the optical form of the substrate or the product. While the β-substituted alanine synthases only use *O*-acetyl-L-serine as a donor substrate and only produce β-substituted L-alanines, in the chemical reaction both optical forms of *O*-acetylserine can be used and in both cases a mixture of D- and L-form is produced.

A crosslinking amino acid, histidinoalanine (**12**), found in proteins was finally synthesized from histidine by application of this biomimetic method.

The above results suggest that this biomimetic method can be applied to the synthesis of heterocyclic β-substituted alanines which are relatively difficult to synthesize, or which have not been synthesized before, from OAS and corresponding heterocyclic compounds, using simple reaction conditions. This procedure might also improve the economics of synthesizing some amino acids.

Experimental

Melting points were determined with a Yanaco micro melting point apparatus (MP-S3). All melting points are uncorrected. Ultraviolet (UV)-VIS spectra were taken on a Hitachi 340 recording spectrophotometer. Optical rotations were recorded with an automatic digital polarimeter DIP-140 (Jasco). Amino acid analyses were carried out with a Hitachi 835-10 automatic amino acid analyzer (AAA). This layer chromatography (TLC) was performed on 0.25 mm Merck precoated silica gel plates (60F-254) in the following solvents: 1, *n*-BuOH–AcOH–H_2O (90 : 10 : 29, v/v); 2, 2-PrOH–HCOOH–H_2O (20 : 1 : 5, v/v); 3, *n*-BuOH–AcOH–H_2O (12 : 3 : 5, v/v). Paper chromatography (PC) was performed on Toyo No. 50 filter papers in the same solvents as used for TLC. Column chromatography was performed on Dowex 50W-X8 (100—200 mesh) obtained from the Dow Chemical Company.

Chemicals——PLP and metal compounds were purchased from Boehringer Mannheim GmbH and Wako Chemical Co., respectively. *O*-Acetyl-L-serine or [3-^{14}C] *O*-acetyl-L-serine and *O*-sulfo-L-serine were synthesized in our laboratory from L-serine and [3-^{14}C] L-serine by a modification of the method of Sheehan *et al.*[21] and that of Dodgson *et al.*,[22] respectively. Pyrazole, 3-amino-1,2,4-triazole, uracil, histidine and ascorbic acid were purchased from Wako Chemical Co., zeatin and 6-benzylaminopurine were purchased from Sigma Chemical Co., and 3,4-dihydroxypyridine, 3,5-dioxo-1,2,4-oxadiazolidine, 3-isoxazolin-5-one and 3-hydroxy-5-methylisoxazole were synthesized by methods developed in our laboratory.[6—15] All other chemicals used were of the highest commercial grade available.

Reaction Conditions——The normal reaction mixtures used for the formation of heterocyclic β-substituted alanines were heated at 62—65 °C for 2 h in a final volume of 0.4 ml of 0.1 M acetate buffer or citrate buffer containing OAS or [3-^{14}C] OAS (12.5 mM, 1.25 mCi), *N*-heterocyclic compounds (125 mM), PLP (1.25 mM) and $Ga(NO_3)_3$ (1.25 mM). Occasionally, OAS was replaced by *O*-acetyl-D-serine, *O*-phospho-L-serine, *O*-sulfo-L-serine and L- or D-serine as a donor for the alanyl moiety, and Ga^{3+} was also replaced by various metal ions. With each batch of samples, several control tubes, from which PLP and metal compounds or metal compounds alone had been omitted, were included. The pH of the reaction mixtures was normally adjusted to pH 4.0—5.5 by using acetate buffer or citrate buffer. The reaction was terminated by addition of 0.05 ml of 10% NH_4OH and the reaction mixtures were allowed to stand at pH 9.5 for 1 h at room temperature: by this procedure OAS was almost quantitatively converted into *N*-acetylserine. This addition of NH_4OH was not used for the synthesis of β-(3-isoxazolin-5-on-2-yl)alanine, which is unstable in alkaline solution.

β-(Pyrazol-1-yl)alanine (1)[2,14]——Pyrazole (136 mg, 2 mmol) and OAS (294 mg, 2 mmol) were incubated in 5 ml of 0.1 M acetate buffer solution at pH 3.7 in the presence of PLP (53 mg, 0.2 mmol) and $Ga(NO_3)_3$ (80 mg, 0.2 mmol) at 62—65 °C for 2 h. After incubation, the solvent was removed by evaporation, and the resulting solution was applied to a Dowex 50W column. The column was washed with water and then the amino acid was eluted with 3%

NH_4OH. After decolorization and evaporation, the residue was recrystallized from water–ethanol to yield pure **1** as colorless needles: 40—45% yield, mp 236—238 °C (dec.), $[\alpha]_D^{20}$ 0° ($c=1.0$, H_2O).

Mimosine (2)[3,15]——3,4-Dihydroxypyridine (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 4.5 at 62—65 °C for 30 min: 1—2% yield.

Quisqualic Acid (3)[4,7]——3,5-Dioxo-1,2,4-oxadiazolidine (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 5.0 at 62—65 °C for 2 h: 8—10% yield.

β-(3-Isoxazolin-5-on-2-yl)alanine (4)[5,6]——3-Isoxazolin-5-one (25 mM), OAS or [3-^{14}C] OAS (5 mM, 0.5 mCi), PLP (0.5 mM) and Ga^{3+} (0.5 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 4.0, at 30 °C for 2 h: 0.15% yield.

Lupinic Acid (5)[10]——Zeatin (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 4.0 at 30—33 °C for 2 h: 1.5—1.7% yield.

Willardiine (6) and Isowillardiine (7)[11]——Uracil (25 mM), OAS (25 mM), PLP (5 mM) and Ga^{3+} (10 mM) were incubated in 1.0 ml of 0.1 M acetate buffer or citrate buffer solutions, pH 3.0—7.0, at 33—85 °C for 2 h: **6**; 0.8—1.0% yield (pH 5.5, 33—35 °C), **7**; 0.1% yield (pH 5.5, 83—85 °C).

β-(5-Methylisoxazolin-3-on-2-yl)alanine (8)[23]——3-Hydroxy-5-methylisoxazole (HMI) (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 4.7, at 62—65 °C for 2 h: 35—40% yield. In this biomimetic synthesis, the effects of various metal compounds were comparatively studied under the same conditions (Fig. 1). **8** was also synthesized from HMI (198 mg, 2 mmol) and α-acetamidoacrylic acid (258 mg, 2 mmol) in 5 ml of AcOH by refluxing for 2 h. The residue, obtained after removal of the AcOH *in vacuo*, was dissolved in a small volume of methanol, and then a little ether was added to the solution to precipitate α-acetamido-β-(5-methylisoxazolin-3-on-2-yl)propionic acid. Recrystallization from water gave colorless needles: 4.5% yield, mp 174 °C. This compound was then hydrolyzed in 3 N HCl at 110 °C for 2 h. The mixture was evaporated *in vacuo*, then 5 ml of water was added to the residue, and the resulting solution was applied to a Dowex 50W column. The column was washed with water and then the amino acid was eluted with 3% NH_4OH. After decolorization and evaporation of the eluate, the residue was recrystallized from water–ethanol to yield pure **8** as colorless plates: 10.5% yield, mp 203—205 °C (dec.), $[\alpha]_D^{20}$ 0° ($c=1.0$, H_2O).

β-(3-Amino-1,2,4-triazol-1-yl)alanine (9)[8]——3-Amino-1,2,4-triazole (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 0.4 ml of 0.1 M acetate buffer solution, pH 4.7, at 62—65 °C for 2 h: 30—35% yield. **9** was also synthesized by refluxing 3-amino-1,2,4-triazole (168 mg, 2 mmol) and α-acetamidoacrylic acid (258 mg, 2 mmol) in 5 ml of AcOH for 2 h. Pure **9** was obtained by the same procedure, according to Massini[24]: colorless plates, 8.5% yield, mp 232—235 °C (dec.).

Ascorbalamic Acid (10)[25]——Ascorbic acid (300 mM), OAS (150 mM), PLP (100 mM) and Ga^{3+} (20 mM) were incubated in 5 ml of 0.1 M acetate buffer solution, pH 4.0 at 62—65 °C for 1 h. In this biomimetic synthesis β-(2-furoyl)alanine was obtained by hydrolytic cleavage with 6 N HCl, providing indirect evidence of the formation of **10**, which is unstable in solution: 5—7% yield.

β-(6-Benzylaminopurin-9-yl)alanine (11)[13,26]——6-Benzylaminopurine (125 mM), OAS (12.5 mM), PLP (1.25 mM) and Ga^{3+} (1.25 mM) were incubated in 1.0 ml of 0.1 M acetate buffer solution, pH 4.5, at 62—65 °C for 2 h: 1.5—2% yield. **11** was also synthesized by refluxing 6-benzylaminopurine (450 mg, 2 mmol) and α-acetamidoacrylic acid (258 mg, 2 mmol) in 10 ml of AcOH for 1 h. Pure **11** was obtained by the same procedure as used for **8** and also by a modification of the method of Letham *et al.*[26]: colorless needles, 8.5% yield, mp 211—215 °C (dec.). UV λ_{max} at pH 3, 7, and 11 of 265, 271 and 271 nm, respectively.

Histidinoalanine (12)[27]——Histidine (20 mM), OAS (10 mM), PLP (2 mM) and Ga^{3+} (2 mM) were incubated in 0.5 ml of 0.1 M acetate buffer solution, pH 3.0—5.0 at 30—85 °C for 2 h: 0.7—0.8% yield (pH 3.5, 62—65 °C).

Identification of Heterocyclic β-Substituted Alanines as the Reaction Products——The formation of heterocyclic β-substituted alanines was demonstrated by subjecting the reaction mixtures to PC or TLC in three solvent systems as described previously.[6-15] The products formed were identical with authentic materials. This was also confirmed by ^{14}C-tracer experiments. Radioactivity on the chromatograms was monitored with a gas-flow 4π radiochromatogram scanner (Aloka PCS-4). Further confirmation of the identity of the products as heterocyclic β-substituted alanines was obtained by AAA under standard operating conditions (2.6 × 25 cm column, 33—68 °C, Li-citrate buffer system, pH 3.0—7.0).[28] Quantitative determinations of heterocyclic β-substituted alanines were done by using AAA and also by the method of Atfield and Morris[29]: the heterocyclic β-substituted alanines colored with Cd–ninhydrin reagent were extracted with 5 ml of methanol and estimated colorimetrically at 500 nm with β-substituted alanines as standards. The color obtained after reaction of β-(2-furoyl)alanine with Cd–ninhydrin was estimated at 354 nm.

Acknowledgements We are grateful to Prof. D. Fujimoto, Hamamatsu University School of Medicine, for providing an authentic sample of histidinoalanine. We are also indebted to Misses Y. Kuroiwa and K. Nasu for their technical assistance.

References and Notes

1) Parts of this work were reported at the 98th Annual Meeting of the Pharmaceutical Society of Japan at

Okayama, April 5, 1978 (Abstracts p. 346) and at the 9th International Congress of Heterocyclic Chemistry at Tokyo, August 24, 1983 (Abstracts p. 367), and were also included in the dissertation presented by F. Ikegami at Tokyo University 1981 (in Japanese).
2) F. F. Noe and L. Fowden, *Biochem. J.*, **77**, 543 (1960).
3) M. P. Hegarty and R. D. Court, *Aust. J. Agric. Res.*, **15**, 165 (1964).
4) T. Takemoto, T. Nakajima, S. Arihara, and K. Koike, *Yakugaku Zasshi*, **95**, 326 (1975).
5) F. Lambein and R. Van Parijs, *Biochem. Biophys. Res. Commun.*, **32**, 474 (1968).
6) I. Murakoshi, F. Kato, J. Haginiwa, and L. Fowden, *Chem. Pharm. Bull.*, **21**, 918 (1973).
7) I. Murakoshi, F. Kato, J. Haginiwa, and T. Takemoto, *Chem. Pharm. Bull.*, **22**, 473 (1974).
8) I. Murakoshi, F. Kato, and J. Haginiwa, *Chem. Pharm. Bull.*, **22**, 480 (1974).
9) I. Murakoshi, F. Ikegami, F. Kato, J. Haginiwa, F. Lambein, L. Van Rompuy, and R. Van Parijs, *Phytochemistry*, **14**, 1515 (1975).
10) I. Murakoshi, F. Ikegami, N. Ookawa, J. Haginiwa, and D. S. Letham, *Chem. Pharm. Bull.*, **25**, 520 (1977).
11) I. Murakoshi, F. Ikegami, N. Ookawa, T. Ariki, J. Haginiwa, Y.-H. Kuo, and F. Lambein, *Phytochemistry*, **17**, 1571 (1978).
12) I. Murakoshi, F. Ikegami, K. Harada, and J. Haginiwa, *Chem. Pharm. Bull.*, **26**, 1942 (1978).
13) I. Murakoshi, C. Koide, F. Ikegami, and K. Nasu, *Chem. Pharm. Bull.*, **31**, 1777 (1983).
14) I. Murakoshi, F. Ikegami, Y. Hinuma, and Y. Hanma, *Phytochemistry*, **23**, 973 (1984).
15) I. Murakoshi, F. Ikegami, Y. Hinuma, and Y. Hanma, *Phytochemistry*, **23**, 1905 (1984).
16) H. Yamada, H. Kumagai, H. Kashima, and H. Torii, *Biochem. Biophys. Res. Commun.*, **46**, 370 (1972).
17) H. Yamada and H. Kumagai, "Advances in Applied Microbiology," Vol. 19, ed. by D. Perlman, Academic Press, New York, 1975, p. 249.
18) I. Murakoshi, S. Ohmiya, and J. Haginiwa, *Chem. Pharm. Bull.*, **20**, 609 (1972).
19) D. E. Metzler, M. Ikawa, and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 648 (1954).
20) P. M. Dunnill and L. Fowden, *J. Exp. Botany*, **14**, 237 (1963).
21) J. C. Sheehan, M. Goodman, and G. P. Hess, *J. Am. Chem. Soc.*, **78**, 1367 (1956).
22) K. S. Dodgson, A. G. Lloyd, and N. Tudball, *Biochem. J.*, **79**, 111 (1961).
23) I. Murakoshi, F. Ikegami, T. Nishimura, and K. Tomita, *Phytochemistry*, **24**, 1693 (1985).
24) P. Massini, *Acta Bot. Neerl.*, **12**, 64 (1963).
25) R. Couchman, J. Eagles, M. P. Hegarty, W. M. Laid, R. Self, and R. L. M. Synge, *Phytochemistry*, **12**, 707 (1973).
26) D. S. Letham, R. E. Summons, C. W. Parker, and J. K. MacLeod, *Planta*, **146**, 71 (1979).
27) D. Fujimoto, M. Hirama, and T. Iwashita, *Biochem. Biophys. Res. Common.*, **104**, 1102 (1982).
28) I. Murakoshi, F. Ikegami, T. Hama, and K. Nishino, *Shoyakugaku Zasshi*, **38**, 355 (1984).
29) G. N. Atfield and C. J. O. R. Morris, *Biochem. J.*, **81**, 606 (1961).