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# Enzymic Synthesis of Quisqualic Acid from O-Acetylserine and 3,5-Dioxo-1,2,4-oxadiazolidine by Extracts of Higher Plants

Takemoto, *et al.*[1] reported the isolation of β-(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine(III) from the seeds of *Quisqualis indica* L. and *Q. indica* L. var. *villosa* CLARKE (Combretaceae) and named it quisqualic acid. The chemical structure was confirmed as III on the basis of chemical and spectroscopic studies and by comparison of the isolated amino acid with synthetic DL-quisqualic acid. Quisqualic acid is the only naturally-occurring amino acid containing a 1,2,4-oxadiazolidine ring reported to date.

Murakoshi, *et al.*[2,3] have described enzyme systems from plants which catalyze the synthesis of β-substituted alanines; serine is first converted to an O-acetyl derivative, and an alanyl-moiety derived from this activated intermediate then undergoes condensation with the appropriate heterocyclic compound to yield mimosine,[2] β-pyrazol-1-ylalanine[2] and β-(3-isoxazolin-5-on-2-yl)alanine[3] in *Leucaena* and *Mimosa*, *Citrullus* and *Pisum* seedlings, respectively. Neither serine, O-phosphoserine or O-sulfoserine[3,4] served as donors of the alanyl-moiety.

In this communication we report an analogous reaction for the synthesis of quisqualic acid; crude extracts of *Pisum sativum* and *Q. indica* L. var. *villosa* catalyzed the synthesis of quisqualic acid (III) from O-acetylserine (II) and 3,5-dioxo-1,2,4-oxadiazolidine (I).[5]

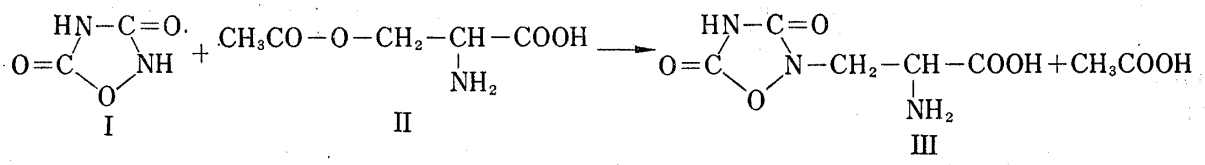


Fig. 1

Seedlings of *P. sativum* were grown in the dark for 3—5 days at 30° and plants of *Q. indica* L. var. *villosa* were raised in a glasshouse. *Pisum* seedlings, with testas removed, and leaves and stems of *Quisqualis* were used as the material for enzyme extraction. Plant tissue was macerated in 0.1M potassium phosphate buffer, pH 7.4, containing 65 mM 2-mercaptoethanol (0.35 ml/g of tissue) at 4°. The clear suppernatant solution recovered by centrifugation at 25000 ***g*** for 30 min was passed through a column of Sephadex G-25 (fine) to obtain an enzyme solution free from low mol. wt. substances as previously described.[2,3] The eluted protein was used as the source of enzyme activity.

Standard incubation mixtures for the synthesis of quisqualic acid were performed at 30° for 90 min and contained O-acetylserine (5 μmoles), 3,5-dioxo-1,2,4-oxadiazolidine (30 μmoles) and 0.2 ml of enzyme solution (containing the soluble protein from 0.3 g fresh weight of plant tissue) in a final volume of 0.4 ml. The pH of the incubation mixture was normally at pH 7.4 by 0.1M potassium phsophate buffer. Unless otherwise specified, pea seedling ex-

1) This work was presented at the 16th Symposium on the Chemistry of Natural Products at Osaka, October 20, 1972, by T. Takemoto, N. Takagi, T. Nakajima, S. Arihara, and K. Koike (Symposium Papers, p. 256).
2) I. Murakoshi, H. Kuramoto, J. Haginiwa, and L. Fowden, *Phytochem.*, **11**, 177 (1972).
3) I. Murakoshi, F. Kato, J. Haginiwa, and L. Fowden, *Chem. Pharm. Bull.* (Tokyo), **21**, 918 (1973).
4) O-Sulfoserine was a substrate for S-alkyl-cysteine synthetase in a number of higher plants (I. Murakoshi, A. Yamazaki, and J. Haginiwa, presented at the Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 7, 1972 (Meeting Papers, II. p. 260).
5) 3,5-Dioxo-1,2,4-oxadiazolidine was synthesized by the method of G. Zinner and R. Stiffel (*Arch. Pharmaz.* **302**, 691 (1969)).

tracts were used as the source of enzyme activity. Incubations were terminated by the addition of 3 volumes of ethanol. Precipitated protein was removed by centrifugation and samples of the supernatant solution were examined for the formation of III by paper chromatography in the following solvents: 1, propan-2-ol–formic acid–water (80: 4: 20, by vol.); 2, pyridine–butan-1-ol–acetic acid (1: 1: 1, by vol.). The *Rf* values for III obtained in these solvents were 0.16 and 0.21, respectively, whilst O-acetylserine exhibited the following *Rf* data; 0.43 and 0.44, respectively. Under the same conditions, serine moved at *Rf*'s of 0.29 and 0.31, respectively.

Analysis of terminated reaction mixtures by chromatography indicated the formation of a product which reacted with ninhydrin and ran with the same *Rf* as III in solvents 1 and 2. The product of the reaction with ninhydrin was a pale yellow brown, which subsequently turned to violet. The product was not formed in reaction mixtures lacking I or II, nor was the product formed when the enzyme extract was pretreated at 100°. When unlabelled O-acetylserine was replaced in the incubation mixture with O-acetylserine-3-$^{14}$C(5 μmoles, 0.5 μCi), radioactivity was associated with the ninhydrin positive product with the same *Rf* as III. Radioactivity on the chromatograms was monitored with a gas-flow $4\pi$ radiochromatogram scanner (Aloka model PCB-2B, Tokyo).

The reaction product was examined in an automatic amino acid analyzer (Shibata model AA-500, Tokyo). Under standard operating conditions,[2,3] the product eluted from the column at about 201 min (103—104 ml). These conditions were the same as the conditions for the elution of authentic quisqualic acid: III eluted from the column at a position between the peaks due to S-methylcysteine sulphoxide and S-carboxymethylcysteine.

Hydrogenation of the reaction product ($PtO_2$ catalyst) yielded albizzine. On a subsequent acid hydrolysis, α-β-diaminopropionic acid was amongst the degradation products. This behaviour is in general agreement with the results of Takemoto, *et al.*[1] for authentic III.

Some properties of the enzyme-dependent synthesis of III were studied; III was estimated quantitatively by the method of Atfield and Morris.[6] Addition of pyridoxal phosphate (up to 20 μg/ml) had no effect on the formation of III but higher concentrations (100 μg/ml) caused 25—30% inhibition. The rate of synthesis of III was constant for at least 60 min but the rate then decreased; after 100 min the formation of III ceased. The yield of III after 100 min was approximately 6% with respect to the initial substrate concentration of II. The optimum pH for the enzyme-dependent synthesis of III was 7.4 using 0.1 M potassium phosphate buffer. The enzyme was active only over a narrow pH range. The optimum concentration of I for the synthesis of III was 75 mM; higher concentrations were inhibitory. The activity of enzyme extracts was unstable; activity decreased by 50—55% after storage of the extracts at 0° for 26 hr.

Enzyme extracts from other plant species were examined for their activity to catalyze the formation of III from I and II; the specific activity of extacts from *Q. indica* L var. *Villosa* was approximately 5—6 fold greater than extracts from *Pisum*. Extracts from *Leucaena* and *Citrullus* did not catalyze the synthesis of III although analogous reactions for the synthesis of mimosine and β-pyrazol-1-ylalanine have been described in *Leucaena* and *Citrullus*, respectively.[2] This suggests that the enzymes from different plant species which catalyze the synthesis of β-substituted alanines from O-acetylserine have different substrate specificities.

The non-enzymic synthesis of III was demonstrated by a simulation of the enzyme-dependent system using pyridoxal phosphate as the catalyst. Non-enzymic incubations were conducted at 45—50° for 60—100 min and contained I (30 μmoles), II (5 μmoles) and pyridoxal phosphate (100 μg) in 0.5 ml of 0.1 M acetate buffer, pH 5.5. III was also synthesized when

6) G.N. Atfield and C.J.O.R. Morris, *Biochem. J.*, **81**, 606 (1961).

II was replaced with O-phosphoserine or serine.

A more detailed study of the enzyme(s) catalyzing the condensation reaction to form quisqualic acid is in progress in our laboratory. The biosynthetic pathway leading to the synthesis of 3,5-dioxo-1,2,4-oxadiazolidine ring has not been reported.

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## Chemical Structure and Sweet Taste of Isocoumarins and Related Compounds

Phyllodulcin (the sweet principle of leaves of *Hydrangea serrata* Seringe var. *thunbergii*: Japanese name is Amacha) and its related compounds change into bitter or tasteless substances by a slight modification of its molecular structure.[1] There should be, therefore, a correlation between the sweet taste and the chemical structure of these compounds.

We have examined to elucidate the relationship between structure and appearance of sweet taste in this series of compounds and found that $\beta$-(3-hydroxy-4-methoxyphenyl) ethylbenzene (I, R, R′=H), which constitutes a partial structure of phyllodulcin indicated by a dotted line in the phyllodulcin structural formula is essential for the appearence of sweet taste[2] and that the number of methylene group between the two phenyl group is an important factor to the sweet taste. The existence of two methylene group brought the sweet taste strongly and the sweet taste has not appeared with either less or more methylene groups than that.[3] We have already reported the effect of substituents R and R′ in $\beta$-(3-hydroxy-4-methoxyphenyl) ethylbenzene derivatives I.[4]

On the basis of these observations, we synthesized N-(3-hydroxy-4-methoxybenzyl)-aniline (II), a nitrogen analog of $\beta$-(3-hydroxy-4-methoxyphenyl) ethylbenzene from the point

1) Part I: M. Yamato, T. Kitamura, K. Hashigaki, Y. Kuwano, N. Yoshida, and T. Koyama, *Yakugaku Zasshi*, **92**, 367 (1972).
2) Part II: M. Yamato, K. Hashigaki, Y. Kuwano, and T. Koyama, *Yakugaku Zasshi*, **92**, 535 (1972).
3) Part III: M. Yamato, T. Kitamura, K. Hashigaki, Y. Kuwano, S. Murakami, and T. Koyama, *Yakugaku Zasshi*, **92**, 850 (1972).
4) Part IV: M. Yamato, K. Sato, K. Hashigaki, T. Ishikawa, and T. Koyama, *Yakugaku Zasshi*, **93**, 1639 (1973).