oxidase (0.2 unit/mg protein) used was a partially purified prepn from Habu snake (*Trimeresurus flaviviridis*) venom [9] and its stereospecificity was confirmed as being L-directed from the fact that a prepn of N^{δ}-benzoyl-γ-hydroxy-D-ornithine was not oxidized by the enzyme under the conditions described above.

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Phytochemistry, Vol. 21, No. 1, pp. 224–225, 1982.
Printed in Great Britain.

0031-9422/82/010224-02 $03.00/0
Pergamon Press Ltd.

L-*THREO*-γ-HYDROXYCITRULLINE FROM *VICIA PSEUDO-OROBUS*

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(*Received* 12 *June* 1981)

Key Word Index *Vicia pseudo-orobus*; Leguminosae; L-*threo*-γ-hydroxycitrulline.

Abstract—L-*Threo*-γ-hydroxycitrulline was isolated and identified from the seeds of *Vicia pseudo-orobus*. The structure was clarified from the results of elementary analysis, ^{1}H NMR spectrum, enzymic deamination and comparison of the hydrolysis product with the authentic *threo*- and *erythro*-γ-hydroxyornithine.

INTRODUCTION

Previously we reported the isolation and characterization of N^{δ}-benzoyl-L-ornithine and N^{α}-benzoyl-L-γ-hydroxyornithine from the seeds of *Vicia pseudo-orobus* [1]. Subsequently the configuration of the two asymmetric carbon atoms of the latter amino acid was unequivocally determined as L-*threo*-form by comparison with the synthetic samples [2, 3]. The seeds of *V. pseudo-orobus* contain still other ninhydrin-positive substances yielding on hydrolysis γ-hydroxyornithine. One of these proved now to be L-*threo*-γ-hydroxycitrulline.

RESULTS AND DISCUSSION

By the use of cellulose CC we obtained γ-hydroxycitrulline from the neutral and acidic amino acid fraction. The result of elementary analysis was in good agreement with the formula $C_6H_{13}N_3O_4$. It gave a brownish violet coloration with ninhydrin and the colour turned to normal violet with time. Ehrlich reagent yielded a yellow colour just as in the case of citrulline. Though on strong alkaline hydrolysis it gave a mixture of *threo*- and *erythro*-γ-hydroxyornithine, only the former was detected in the mild alkaline hydrolysis products. Also, strong acid yielded only *threo*-γ-hydroxyornithine. Epimerization of γ-hydroxyornithine is known to occur more rapidly in alkaline than in acidic solution [2]. Further, ^{1}H NMR spectrum of the natural γ-hydroxycitrulline was very similar to that of N^{α}-benzoyl-L-*threo*-γ-hydroxy ornithine and different from that of the *erythro*-form [1, 3]. An experiment with L-amino acid oxidase ascertained that our isolate belongs to the series of L-amino acids.

γ-Hydroxycitrulline was first identified as a natural product by Bell and Tirimanna from *Vicia fulgens* and *V. unijuga* but not isolated [4]. The first isolation was carried out by Inatomi *et al.* from young seeds of *Vicia faba* [5]. The stereochemical nature, however, was not studied.

EXPERIMENTAL

Plant. Seeds of *Vicia pseudo-orobus* Fisch. et Mey. were the same as previously reported [1].

Ehrlich reagent. To the solution of 1 g *p*-dimethylaminobenzaldehyde in 30 ml EtOH, 30 ml conc HCl and 180 ml *n*-BuOH were added.

Isolation. Syrup of the neutral and acidic amino acid fraction from the seeds (416 g) [1] was fractionated on a cellulose column (117 × 4.8 cm) using *n*-BuOH–HOAc–H_2O (63:10:27). γ-Hydroxycitrulline and asparagine were displaced together from the column. Asparagine crystallized first on addition with EtOH from the concentrated fractions and was removed. Me_2CO was then added dropwise to the mother liquor and γ-hydroxycitrulline was obtained (530 mg). It was purified by recrystallization × 4 from EtOH–H_2O, mp 186–189° (decomp.) [cf. lit. [5] 185–187° (decomp.)]. $[\alpha]_D^{25}$ +8.0 (2 N HCl; *c* 1) [cf. lit. [5] $[\alpha]_D^{20}$ +4.5° (2 N HCl; *c* 2)]. Found: C, 37.54; H, 7.10; N, 21.83. Calc. for $C_6H_{13}N_3O_4$: C, 37.69; H, 6.85; N, 21.98%. ^{1}H NMR (100 MHz,

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D_2O): δ 3.7–4.1 (2H, *m*, H-2, H-4), 3.1–3.4 (2H, *m*, H-5), 1.8–2.2 (2H, *m*, H-3).

Hydrolysis. Strong hydrolysis: The pure amino acid (2 mg) was dissolved in 1 M $Ba(OH)_2$ (1 ml) and heated in a sealed ampoule at 116–120° for 8 hr or in 6 M HCl (1 ml), 100°, 24 hr. Mild hydrolysis: amino acid (2 mg) in 0.5 M $Ba(OH)_2$ (1 ml), 100°, 2 hr. Hydrolysis products were separated preparatively from unchanged γ-hydroxycitrulline on cellulose thin layers ('Avicel', Funakoshi Pharmaceutical Co.) developed with *n*-BuOH–pyridine–H_2O (1:1:1). *Threo-* and *erythro-*γ-hydroxyornithine were distinguished on cellulose TLC plate developed successively ×4 with *t*-AmOH–MeCOEt–NH_4OH–H_2O (15:9:4:2).

Oxidative deamination. Crude L-amino acid oxidase prepared from Habu-snake (*Trimeresurus flavoviridis*) venom [6] in 0.05 N ammonium acetate buffer, pH 7.2 (150 μl) was added to an amino acid solution (60 μg in 50 μl H_2O). After the mixture was incubated at 37° for 24 hr, 3 μl were applied to a cellulose thin layer (DC-Alfolien, Merck) and developed with PhOH–H_2O (25:8) in the presence of NH_3.

Acknowledgements—We wish to thank Prof. S. Makisumi and Dr. K. Mizusaki, Kyushu University, for their invaluable discussion and kind gift of L-amino acid oxidase, as well as synthetic samples of L-*threo-* and L-*erythro-*γ-hydroxyornithine. Our thanks are also due to Dr. Y. Niimura, Teikyo University, for the determination of ORD. Technical assistance of Miss Y. Ishida is gratefully acknowledged.

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Phytochemistry, Vol. 21, No. 1, pp. 225–227, 1982.
Printed in Great Britain.

0031–9422/82/010225–03 $03.00/0

CHROMATOGRAPHIC SEPARATION OF SYNTHETIC NOPALINE AND ISONOPALINE AND THEIR ABSOLUTE CONFIGURATION

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(*Received* 14 *April* 1981)

Key Word Index—*Agrobacterium tumefaciens*; crown gall; *Helianthus annuus*; Compositae; sunflower; nopaline; isonopaline; absolute configuration.

Abstract—Synthetic nopaline and isonopaline were separated preparatively by anion exchange chromatography and their configurations were ascertained by an enzymic method. Nopaline prepared from crown gall of *Helianthus annuus* corresponded to authentic L-,D-form (nopaline). The strains of *Agrobacterium tumefaciens* which are known to utilize nopaline as sole nitrogen source grew also with this form, but not with L-,L-form (isonopaline).

INTRODUCTION

Nopaline, N^2-(1,3-dicarboxypropyl)-L-arginine [1], is one of the reductive conjugates produced in crown gall which are formed by the inoculation with specific strains of *Agrobacterium tumefaciens*. Interestingly, the same strains can grow on the medium containing nopaline as a sole nitrogen source. Utilization of nopaline by the bacteria, as well as its synthesis in the crown gall is specified by the genes located on plasmids of the strains [2, 3].

Jensen *et al.* [4] reported a simple and useful synthesis of a mixture of the two diastereoisomers, nopaline and isonopaline, in good yield. They determined the ORD of the mixture and presumed that the natural nopaline has the L-configuration at the α-carbon (arginine moiety) and D- at another (glutamic acid moiety). The configurations of the two asymmetric carbon atoms of isonopaline are, accordingly, both L-forms.

Independently, Cooper and Firmin [5] synthesized the mixture of the two diastereoisomers and succeeded in separating them by fractional crystallization. They described mps and $[\alpha]_D$ values of nopaline and isonopaline.

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