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.

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CHROMATOGRAPHIC SEPARATION OF SYNTHETIC NOPALINE AND ISONOPALINE AND THEIR ABSOLUTE CONFIGURATION

SHIN-ICHI HATANAKA, SHIGEAKI ATSUMI, KAZUHIKO FURUKAWA* and YUMIKO ISHIDA

Department of Biology, College of General Education, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan

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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.

* Present address: National Institute for Basic Biology, Myodaiji-cho, Okazaki, Japan.

Here we separate nopaline and isonopaline preparatively by simple CC and confirm the above assumption of their absolute configuration.

RESULTS AND DISCUSSION

A mixture of the two diastereoisomers was synthesized from L-arginine and α-oxoglutaric acid according to Jensen *et al.* [4]. Nopaline and isonopaline were separated from each other by fractionation on a column of Dowex 1 × 4 in acetate form. As the eluting agent, 10% HOAc gave the best result. Mps and $[\alpha]_D$ values for the purified crystals were in good agreement with the results reported by Cooper and Firmin [5].

Since L-arginine was used as starting material for the synthesis, both diastereoisomers formed should have the L-configuration at the α-carbon of the arginine moiety of the molecules. To elucidate the configuration of other asymmetric carbon atoms of nopaline and isonopaline, each purified sample was degraded by $KMnO_4$ [6]. Glutamic acid formed was then purified and subjected to decarboxylation by glutamate decarboxylase prepared from *E. coli*, which is known to be strictly specific for the L-isomer. Only the glutamic acid obtained from isonopaline was degraded, showing the L-configuration at the α-carbon of the glutamate part of isonopaline.

Crown galls which were formed on hypocotyls of *Helianthus annuus* by the inoculation with *Agrobacterium tumefaciens* 1D 135 (nopaline-type) were extracted and the nopaline-fraction purified partially by ion exchange chromatography. By comparison with the synthetic materials on TLC, the natural form proved to be the L-,D-form (nopaline).

Strains of *A. tumefaciens* which are known to utilize nopaline as sole nitrogen source also grew on synthetic nopaline, but not on isonopaline.

EXPERIMENTAL

Chromatography. Nopaline and isonopaline could be distinguished clearly on cellulose-TLC (DC-Alufolien, Merck), developed with PhOH–H_2O (25:9) in the presence of NH_3. Arginine and other monosubstituted guanidines such as above were detected by phenanthrenequinone [7]. R_f values of nopaline and isonopaline were 0.61 and 0.52, respectively, under the above conditions.

Synthesis and separation of the diastereoisomers. Mixture of two diastereoisomers was prepared according to ref. [4]. The method was modified slightly. A soln of L-Arg (18.4 g) and α-oxoglutaric acid (77.3 g) in H_2O (160 ml) was adjusted to pH 7 with conc NaOH and $NaBH_3CN$ (24.9 g) (Aldrich) was added. The mixture was stirred for 48 hr at room temp. and treated with Amberlite IR-120B (H^+) (800 ml). After the resin was washed thoroughly with H_2O, the products were eluted with 10% NH_4OH (2.9 l) and the eluate was concd to a syrup.

Each 1/10 of the above syrup was applied to a column of Dowex 1 × 4 (200–400 mesh, 3.6 × 120 cm, OAc^- -form), which had been equilibrated with 10% HOAc. The fractionation was carried out also with 10% HOAc. (10 ml/fraction). Isonopaline was displaced, e.g. in Fraction Nos. 83–92 and nopaline in 97–125. Evapn of the solvent from the combined fractions gave crude crystals of each diastereoisomer. Yield: nopaline, 12.5 g; isonopaline 10.5 g. Total 70%. Since both samples contained another phenanthrenequinone-positive substance, which moves faster than nopaline and isonopaline on cellulose-TLC, they were recrystallized ×3 from hot H_2O and Me_2CO, giving pure samples. Nopaline: mp 183°, $[\alpha]_D^{23}$ + 15.4°, +16.3° (H_2O; *c* 1). (Found: C, 42.39; H, 6.95; N, 18.37. Calc. for $C_{11}H_{20}N_4O_6 \cdot 1/2H_2O$: C, 42.17; H, 6.76; N, 17.88%.) Loss of cryst. H_2O: 85% of the calc. value at 105–110°, 4 mm Hg, 6 hr over P_2O_5. Isonopaline: mp 163°, $[\alpha]_D^{23}$ + 26.4°, 27.2° (H_2O; *c* 1). (Found: C, 40.48; H, 7.03; N, 17.55. Calc. for $C_{11}H_{20}N_4O_6 \cdot H_2O$: C, 40.99; H, 6.87; N, 17.38%.) Loss of cryst. H_2O: 84% of the calc. value at 105–110°, 4 mm Hg, 4 hr over P_2O_5.

Degradation. *Ca* 30 mg pure nopaline and isonopaline was dissolved separately and 1% $KMnO_4$ (2.5 ml) added. After 2.5 hr at room temp. the mixtures were treated with Amberlite IR-120B (H^+) (3 ml). Adsorbed amino acids were eluted with 2M NH_4OH (30 ml) and the eluates evapd to dryness. Each residue was fractionated on small columns of Dowex 1 × 4 (200–400 mesh, OAc^-) and 0.1 N HOAc. Glutamic acid-fractions from each column were concd to dryness, dissolved in 0.2 M pyridine HCl buffer, pH 4.5 (1 ml) and a few mg of glutamate decarboxylase prepared from *E. coli* (Kyowa Hakko Kogyo Co.) were added. After 9 hr the reaction was stopped by heating to 100° and treated with Amberlite IR-120B as usual. The amino acids were analysed on cellulose-TLC (BuOH–HOAc–H_2O, 63:10:27). The degradation, i.e. the formation of γ-aminobutyric acid was observed only in the case of isonopaline. A parallel expt on authentic L- and D-glutamic acid with the above enzyme prepn showed its high specificity for the L-isomer of glutamic acid.

Purification of nopaline from crown gall. *Agrobacterium tumefaciens* IDI 35 (nopaline-type) was inoculated to hypocotyls (57, each *ca* 15 cm) of *Helianthus anuus* (cv. Giant Russian) grown in a greenhouse and cultivated a further 4–6 weeks under the same conditions. Crown gall formed was collected (22.2 g fr. wt) and extrd ×4 with 80% EtOH, giving 250-ml extract. The extract was then passed through Amberlite IR-120B (H^+) (3 ml), absorbed substances were eluted with 2 M NH_4OH (33 ml), and the concd eluate (3 ml) was applied to a column of Dowex 50W × 4 (50–100 mesh, 1 × 38 cm, H^+). Neutral and acidic amino acids were displaced with 1 M pyridine (10 ml), evapd and applied to a column of Dowex 1 × 4 (200–400 mesh, 1 × 66 cm, OAc^-). The column was washed first with 0.1 M HOAc (350 ml) and fractionated with 0.5 M HOAc (3.5 ml/fraction). Glutamic acid appeared in Fraction Nos. 4–9, octopine 15–20, nopaline 31–37 and aspartic acid 43–48.

Utilization of nopaline by various strains of Agrobacterium tumefaciens. The growth of nopaline-utilizing strains IDI 35, IDI 159 (both plasmid$^+$), non-nopaline-utilizing IDI 300, IDI 329 (both plasmid$^-$) and octopine-utilizing B6, 1524, 1525 (all plasmid$^-$) was investigated in L-tubes containing N-free medium [8] (10 ml) with synthetic nopaline, isonopaline or (+)-octopine (Sigma) (each 100 μg/ml). Bacterial growth in shaking culture was followed by increase in $A_{660\,nm}$. The strains IDI 35 and IDI 159 grew only in the presence of nopaline (L-,D-form) and B6, 1524 and 1525 only with octopine. No growth was observed in other combinations.

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(−)-(*S*)-4-DIMETHYLSULFONIO-2-METHOXYBUTYRATE FROM THE RED ALGA *RYTIPHLOEA TINCTORIA*

SEBASTIANO SCIUTO, MARIO PIATTELLI and ROSA CHILLEMI

Istituto Dipartimentale di Chimica e Chimica Industriale dell'Università di Catania, Catania, Italy

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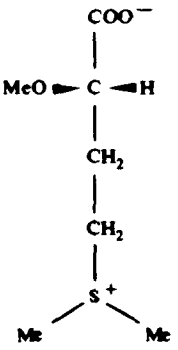
Key Word Index—*Rytiphloea tinctoria*; Rhodomelaceae; red alga; (−)-(*S*)-4-dimethylsulfonio-2-methoxybutyrate.

Abstract—An unusual compound (−)-(*S*)-4-dimethylsulfonio-2-methoxybutyrate, was identified in aqueous–ethanolic extracts of the red alga *Rytiphloea tinctoria.*

In the course of our continuing research on the constituents of Mediterranean algae we observed the presence of an unusual compound in the neutral amino acid fraction obtained from aqueous–EtOH extracts of the red alga *Rytiphloea tinctoria* (Clem.) C. Ag. by ion-exchange chromatography. This compound, which did not react with ninhydrin but gave a red-orange colour with Dragendorff's reagent, was isolated from the amino acid fraction by chromatography on a strongly acid cation exchange resin as a levorotatory syrupy liquid. The amphoteric nature of the compound was revealed by its behaviour on ion-exchange resins as well as by paper electrophoresis.

Elemental analysis and mass measurement (M^+ *m/z* 178) established the molecular formula $C_7H_{14}O_3S$. The IR spectrum included two bands at 1590 and 1400 cm^{-1} attributable to a carboxylate function, confirmed by formation of a methyl ester which showed the methyl ester absorption at δ 3.80. Two methyl resonances in the ^{13}C NMR spectrum (20.1 MHz, D_2O) at 23.43 and 23.73 ppm suggested a dimethylsulfonium group, confirmed by formation of dimethylsulfide upon treatment with base. The ^{13}C NMR also indicated the presence of two methylenes at 25.49 (*t*, C-3) and 39.03 (*t*, C-4), one methine at 78.20 (*d*, C-2), one methoxy group at 56.28 (*q*) and one carboxylate carbon at 175.74 ppm. The ^1H NMR (270 MHz), determined in D_2O after addition of CF_3COOD to pH 2, displayed a 1 H double double double doublet at δ 2.16 (J = 8, 8, 8.1, 15 Hz; H_aC–3), a 1 H double double double doublet at 2.37 (J = 3.9, 8, 8, 15 Hz; H_bC–3), two singlets at 2.85 and 2.86 (3 H each, SMe_2), a 2 H multiplet at *ca* 3.36 (H_2C–4) partially obscured by the overlapping resonance of a OMe group at 3.38, and a double doublet at 4.06 (1 H, J = 3.9 and 8.1 Hz; HC–2) assignable to a methine which might be linked to the carboxylate group since in the spectrum taken without added acid it appeared at δ 3.75. The above data established the sequence $-CH_2-CH_2-CH-COO^-$ thus leading to two alternative structures with only one, i.e. **1**, compatible with the observed chemical shifts. The magnetic non-equivalence of the methyl groups attached to the sulfur atom is possibly due to conformational preference of the $-SMe_2$ group with respect to the asymmetric center. Confirmation of structure **1** and proof of the *S*-configuration was obtained by treatment of **1** with hydroiodic acid which gave (−)-(*S*)-4-dimethylsulfonio-2-hydroxybutyrate identical (NMR, [α], TLC) with a synthetic sample prepared from (*S*)-methionine.



1

The survey of a number of Rhodomelaceous algae revealed that the new metabolite also occurs in *Halopitys*