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STRUCTURE AND SYNTHESIS OF PUTREANINE

A NEW AMINO ACID ISOLATED FROM MAMMALIAN BRAIN*

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Abstract—The structure of putreanine, a new amino acid isolated from mammalian brain, was studied by its dissociation constants, mass spectrum, NMR spectrum and ORD. The structure N-(4-aminobutyl)-3-aminopropionic acid was assigned to it from the results obtained. This amino acid was synthesized by condensation of 4-phthalimino-1-bromobutane and ethyl β-alaninate followed by acid hydrolysis. The identity of the synthetic compound with the natural amino acid confirmed the structural assignment.

A NEW amino acid has been found in a protein-free extract of bovine brain by Kakimoto, *et al.*[1] They isolated it from a centrifuged homogenate by means of column chromatography on Amberlite IR-120 and Dowex-50. Occurrence of this amino acid is limited to the brain of mammals and birds. It is concentrated in the caudal portion of the brain and is found in higher concentration in the white matter than in the grey matter. This amino acid was named putreanine due to the structural feature described below.

This investigation was undertaken to obtain the chemical structure of putreanine mainly from mass spectra and NMR spectra, and confirm it by synthesis. A molecular formula of $C_7H_{16}N_2O_2$ was given to the free amino acid from the results of elementary analysis of its dihydrochloride, monosulfate and mono-*p*-hydroxyazobenzene-*p'*-sulfonate, and from the molecular weight peak in the mass spectrum of its ethyl ester. The measured pK_a values of 3·2, 9·4 and 11·2 suggested that putreanine is a diamino monocarboxylic acid, in which a β-amino acid grouping is present and the second amino group is located away from carboxyl group.‡

In the mass spectrum of ethyl putreaninate (Fig 1) the peak of *m/e* 102 characteristic of α-amino acid ethyl ester was not observed, whereas peaks of 70 and 88, both of which are attributable to β-amino acid structure, were present. Furthermore, the amine fragments resulting from β-amino acid structure, *i.e.* molecular weight $-$ $CH_2COOC_2H_5 = 101$ was observed and that corresponding to α-amino acid (*m/e* 115) was absent. All these data support the existence of a β-amino acid moiety in the molecule of putreanine, and are compatible with the pK_1 value.

* Preliminary report was published as an addendum to Dr. Kakimoto's paper.[1]

† This paper is dedicated to Emeritus Professor Munio Kotake in commemoration of his 75th birthday.

‡ Empirical formulas have been proposed for a dependence of dissociation constants on a distance between the amino group and carboxyl group in amino acid.[2–4] Comparison of pK_a values obtained in our investigation with that calculated according to empirical formulas and with many known values in literature indicates that the pK_1 of 3·2 corresponds to a β-amino acid. Although pK_3 of 11·2 is not so accurate, such a high value compared with usual γ, δ or ε-amino acid suggests a presence of a straight chain of more than six carbon atoms separating the second amino group and the carboxyl group.

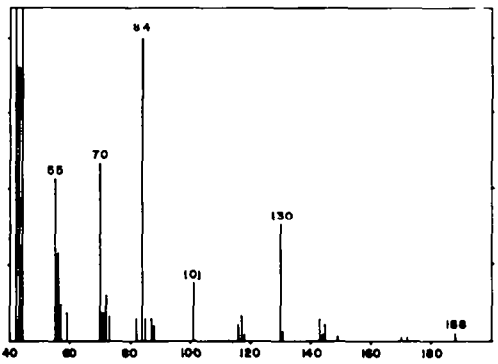

FIG. 1. Mass spectrum of ethyl putreaninate.

The most prominent peak *m/e* 84 of ethyl putreaninate seemed to be as suggestive as in the case of ethyl lysinate, where it also appeared as the most intense peak. Biemann *et al* assigned this peak of ethyl lysinate to a six-membered cyclic immonium ion, formed from the amine fragment by elimination of ammonia.[5] Ornithine also gives as a fragment a similar cyclic ion (*m/e* 70). The use of such a prominent peak in diaminocarboxylic acids for structural elucidation has been demonstrated in studies on lysopine[6, 7] and N^{ε}-methyllysine[8]. In these cases, formation of two kinds of cyclic ions, either by loss of ammonia or of alkylamine, were considered.

Since the fragment of *m/e* 84 in the mass spectrum of ethyl putreaninate was assumed to be derived from the amine fragment (*m/e* 101) by loss of ammonia, it is probable that the formation of the cyclic ion occurs in a similar way. Furthermore, the fact that neither peaks at *m/e* 173 nor 159 were present, excludes C-methyl or C-ethyl structure from the structure of putreanine. Therefore, the two nitrogen atoms in putreanine are probably linked by a straight chain of four or five carbon atoms.

Esters of diaminoacids such as α, δ-diaminobutyric acid, ornithine and lysine are converted to the corresponding lactam at an inlet temperature of 140° in the mass spectrometer, and no molecular weight peak is found. In contrast to these amino acid esters, ethyl putreaninate afforded the molecular weight peak (*m/e* 188) and the lactam peak was never observed at this temperature. This fact indicates that no amino group is found between the δ and ε positions with respect to the carboxyl group.

The NMR spectrum of putreanine in trifluoroacetic acid showed peaks at τ 7·93 (4 H), 6·84 (2 H), 6·54 (6 H), and 2·5 to 2·9 (5 H, broad). These signals can be assigned to $C-CH_2-C$, $C-CH_2-COOH$, $C-CH_2-N$ and NH_2 or NH groups respectively. The NMR spectrum in deuterium oxide had a quintet (4 H) at τ 8·22 and signals (8 H) centered at 6·91 of A_2B_2 type overlapping with another multiplet. From all the results obtained above, it is deduced that the molecule consists of two parts, $NCH_2CH_2CH_2CH_2N$ and NCH_2CH_2COOH respectively.

The Optical Rotatory Dispersion of a solution of this amino acid in 0·1 N HCl indicated that it is an optically inactive substance. Therefore, only one way remains in which link the two partial structures mentioned above, the structure thus proposed being consistent with all of the results obtained. The structure of N-(4-aminobutyl)-3-aminopropionic acid should be assigned to putreanine.* The significant peaks in the

* The nomenclature of putreanine is based on the structure, in which putrescine and β-alanine moieties are combined.

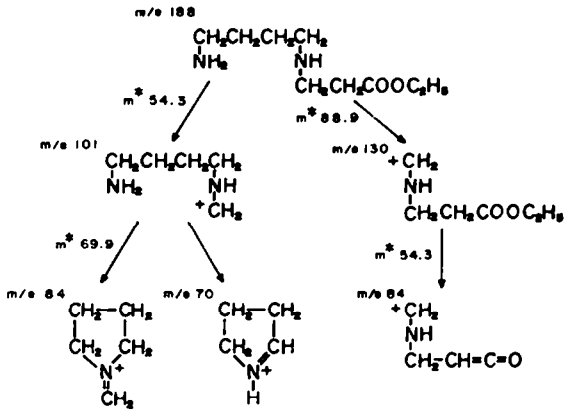

FIG. 2. Major pathway of fragmentations of ethyl putreaninate in mass spectrum.

mass spectrum of its ethyl ester can be assigned as in Fig 2. Splitting of the peak *m/e* 84 into two of comparable strength in high resolution mass spectrum indicates that it is composed of $C_5H_{10}N$ and C_4H_6NO together.

The synthesis of N-(4-aminobutyl)-3-aminopropionic acid was performed by condensation of 4-phthalimino-1-bromobutane and ethyl β-alaninate followed by hydrolysis of the product. For the preparation of 4-phthalimino-1-bromobutane from potassium phthalimide and 1,4-dibromobutane, we applied the method of Sheehan *et al.*,[9] in which dimethylformamide was used as solvent to increase the solubility of potassium phthalimide, although many other procedures for the preparation of this compound without solvent and at higher temperature had been reported.[10–13] The modified method improved somewhat the yield of the product compared with the other methods.

For the condensation reaction, 1 equivalent of 4-phthalimino-1-bromobutane and 2 equivalents of ethyl β-alaninate were used. Direct hydrolysis of the reaction product was carried out by heating with a mixture of hydrobromic acid and acetic acid. The hydrolysate was passed through a column of Dowex-50 x8, NH_4^+ type, and eluted with 0·2 N NH_4OH. From combined fractions expected to contain the product, N-(4-aminobutyl)-3-aminopropionic acid was obtained as its crystalline monosulfate. This synthetic compound gave the same melting point and the same R_f values on thin-layer chromatography as those of putreanine. All physical data including IR, NMR, mass spectrum and pK_a values confirmed the identity of the synthetic substance with the natural amino acid. Therefore, the structure of putreanine can be conclusively represented as N-(4-aminobutyl)-3-aminopropionic acid.

Although this amino acid occurs in tissues of bovine brain in a relatively large amount comparable to the usual amino acids, *i.e.* histidine, ornithine, branched and aromatic amino acids in the same tissue, no biological activity specific to the nervous system has been reported as yet. A study on the biogenesis of this amino acid is now proceeding at Dr. Kakimoto's laboratory and they postulate spermidine as the most probable precursor for putreanine.[1]

EXPERIMENTAL

All m.ps are uncorrected. The mass spectra were obtained with a Hitachi RUM 7HR. About 1 mg of the amino acid was esterified in 5 ml of EtOH saturated with HCl by heating under reflux for 3 hr. After

evaporation, the residue obtained was dried *in vacuo* over KOH overnight. Through the suspension of the residue in 2 ml of CH_2Cl_2, NH_3 gas was bubbled. NH_4Cl formed, was filtered, and the filtrate was placed in the bulb of the mass spectrometer and evaporated *in vacuo* below 30°. Ionization current: 80 A, electron energy: 70 eV, temperature of inlet system: 80° (unless otherwise stated). The NMR spectra were determined with a Varian A-60 spectrometer in CF_3COOH and D_2O, using TMS and sodium dimethylsilapentanesulfate as internal standards respectively. For ORD measurement of the soln of the amino acid in 0·1 N HCl in the region from 350 to 210 mμ, a Yanagimoto SPR 183 polarimeter was used. The pK_a values were obtained with a model G Beckman pH meter with glass electrode. A solution of putreanine dihydrochloride (4·6 mg) in 1 N HCl (0·05 ml) was titrated with 0·5 N NaOH at 27°. The TLC chromatography was carried out on silica gel G according to Stahl (Merck) developed with 1-butanol—acetic acid—water (4:1:2) and phenol—water (4:1), and stained with ninhydrin.

Derivation of putreanine

Kakimoto *et al* isolated 20·4 mg of putreanine dihydrochloride from 58 kg of bovine brain. From the mother liquor of crystallization of the dihydrochloride, they obtained the mono-*p*-hydroxyazobenzene-*p'*-sulfonate of the amino acid (222 mg), m.p. 200–205°. This was recrystallized by us until a constant m.p. 205·0–206·5° (dec) was obtained. (Found: C, 52·12; H, 6·19; N, 12·76; S, 7·03. Calc for $C_7H_{16}N_2O_2 \cdot C_{12}H_{10}N_2O_4S$: C, 52·04; H, 5·98; N, 12·78; S, 7·31%).

In another experiment, 475 mg of putreanine monosulfate was obtained from 82·0 kg of bovine brain in Dr. Kakimoto's laboratory, m.p. 250–251° (dec). (Found: C, 31·64; H, 7·24; N, 10·14; S, 11·74. Calc for $C_7H_{16}N_2O_2 \cdot H_2SO_4 \cdot 1/2H_2O$: C, 31·46; H, 7·17; N, 10·48; S, 11·97%).

4-Phthalimino-1-bromobutane

To a soln of 1,4-dibromobutane* (208 g, 0·96 moles) in distilled DMF (400 ml), potassium phthalimide (60 g, 0·32 moles) was added. The reaction mixture was vigorously stirred on a boiling water bath for 1 hr and then poured into water (800 ml). The mixture was extracted with chloroform three times (500 ml each). The combined extracts were washed with 0·1 N NaOH twice (200 ml each), and then washed with water. After drying with Na_2SO_4, CCl_4 was removed by evaporation *in vacuo*. After the residual soln had been allowed to stand overnight, crystals were formed. They were filtered off and washed with EtOH. m.p. 214–216° (4·1 g). After recrystallization from CCl_4—light petroleum, the melting point increased to 222–223° (lit,[12] 224–226° in AcOH). This compound was found to be N,N-bisphthalimino-1,4-diaminobutane. (Found: C, 68·67; H, 4·50; N, 7·96. Calc for $C_{20}H_{16}N_2O_4$: C, 68·96; H, 4·63; N, 8·04%).

The remaining soln was distilled under reduced pressure (25 mm Hg) in an oil bath at 120–130° to remove 1,4-dibromobutane and DMF. The residue obtained was heated with CS_2 under reflux for 30 min, and an undissolved substance was removed by filtration. The filtrate was condensed *in vacuo* and crystals (55·7 g) were obtained on standing, m.p. 74–77°. These were combined with the recovered crystals from the mother liquor, and recrystallized from EtOH to give 4-phthalimino-1-bromobutane (47·2 g, 70%), m.p. 79·0–80·5° (lit[10] 79·5–80°; lit[11] 80–81°; lit[12] m.p. 79–80·5°).

Putreanine monosulfate

β-Alanine was synthesized starting from acrylonitrile by the method of Buc[15] and Ford.[16] Esterification of this amino acid was carried out according to the method of Mandell *et al.*[17]

A soln of a mixture of 1-phthalimino-4-bromobutane (3·7 g, 13 mmoles) and ethyl β-alaninate (3·1 g, 26 mmoles) in benzene (80 ml) was heated under reflux for 10 hr. The ppt formed was filtered off, and the filtrate was evaporated *in vacuo*. Since an attempt to isolate the pure ester of the condensed product failed, direct hydrolysis of the product was performed.† The residue obtained above was heated with a mixture of HBr and AcOH (1:1, 60 ml) under reflux in the presence of a small amount of anisole. After evaporation, water

* 1,4-Dibromobutane was prepared from THF, NaBr and conc H_2SO_4 according to the method of Kaluszyner.[14]

† In another run of the same experiment, ethyl phthalimino-β-alaninate was obtained as a crystalline substance from the reaction mixture in a yield of 33%. (Found: C, 63·00; H, 5·21; N, 5·60. Calc for $C_{13}H_{13}NO_4$: C, 63·15; H, 5·30; N, 5·67%). NMR ($CDCl_3$) τ 2·16 (4 H), 5·81 (2 H, quartet J = 7 c/s), 5·95 (2 H, triplet, J = 7 c/s), 7·24 (2 H, triplet, J = 7 c/s), 8·73 (3 H, triplet, J = 7 c/s). It is assumed that this compound is formed by ring opening of 1-phthalimino-4-bromobutane by attack of ethyl β-alaninate, followed by ring closure with splitting of the 4-bromotetramethyleneimino group.

was added to the residue obtained and the evaporation repeated. Crystals of phthalic anhydride, insoluble in water, were filtered off. The filtrate was condensed *in vacuo*, and subjected to Dowex-50 × 8 (NH_4^+ form) column (1·8 × 48 cm) chromatography. Elution was carried out with 0·2 N NH_4OH. Fractions Nos 50 to 139 (each 10 g of eluate) were combined and condensed *in vacuo*. After treatment with decolorizing charcoal, they were evaporated *in vacuo*. The residue obtained was dissolved in water and dil H_2SO_4 was added to pH 2. Upon addition of EtOH, crystals were formed. They were filtered after being kept in refrigerator overnight, m.p. 249–250° (dec) (wt 1·04 g, 31% of theoretical). No depression of the melting point was observed when this was mixed with natural putreanine monosulfate. (Found: C, 31·88; H, 7·31; N, 10·47; S, 11·95. Calc for $C_7H_{16}N_2O_2 \cdot H_2SO_4 \cdot 1/2H_2O$; C, 31·46; H, 7·17; N, 10·48; S, 11·97%).

The synthetic sample gave pK_a values of 3·1, 9·5 and 11·1. R_f values of the synthetic sample on thin layer plates of silica gel G in butanol–acetic acid–water (4:1:2) and phenol–water (4:1) were 0·10 and 0·11, respectively. These are indistinguishable from those of the natural compound. IR, NMR and mass spectra of the synthetic compound were all completely identical with those of natural putreanine.

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