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BIOSYNTHESIS OF LATHYRINE IN *LATHYRUS TINGITANUS*

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Abstract—Possible mechanisms in the biosynthesis of lathyrine are discussed. An *in vitro* system for the specific 4-hydroxylation of diamino acids is reported and has been compared with the conversion of homoarginine to 4-hydroxyhomoarginine by *Lathyrus tingitanus*. The synthesis of *trans*-4,5-dehydro-D,L-homoarginine, a new amino acid is described. [guanidino-^{14}C]*trans*-4,5-Dehydro-D,L-homoarginine was not converted to [^{14}C]lathyrine when supplied to *L. tingitanus*. Lathyrine has been synthesised from 4-oxolysine via 4-oxohomoarginine. It is suggested that 4-oxohomoarginine is the direct precursor of lathyrine.

INTRODUCTION

ANALYSIS of non protein amino acids in the genus *Lathyrus* led to the isolation and identification of a new heterocyclic amino acid lathyrine (β-[2-aminopyrimid-4-yl]-alanine) (I).[1,2] By using [guanidino-^{14}C]homoarginine (III), and [guanidino-^{14}C]-4-hydroxyhomoarginine (II), Bell[3] together with Bell and Przybylska[4] have shown that both (II) and (III) are precursors of lathyrine (Scheme 1).

The hydroxylation of homoarginine at the 4-carbon atom is of interest as several naturally occurring amino acids not normally found in protein are hydroxylated at the 4-carbon atom, for example: homoserine,[5] 4-hydroxyglutamic acid,[6] 4-hydroxyvaline,[7] 4-hydroxy-L-ornithine and 4-hydroxyarginine.[8] The mechanism for the hydroxylation of these compounds is unknown.

Although 4-hydroxyhomoarginine is a precursor of lathyrine, direct cyclization to yield lathyrine is unlikely since nucleophilic displacement of a hydroxyl function by a nitrogen atom (Scheme 1, route a) is unfavourable. Reactions of this type are virtually unknown in both *in vitro* and *in vivo* systems.

One method of gaining further insight into the mechanism of lathyrine biosynthesis is to synthesise other ^{14}C-labelled homoarginine derivatives and to study their metabolism by *L. tingitanus*. Two such compounds are considered in this paper.

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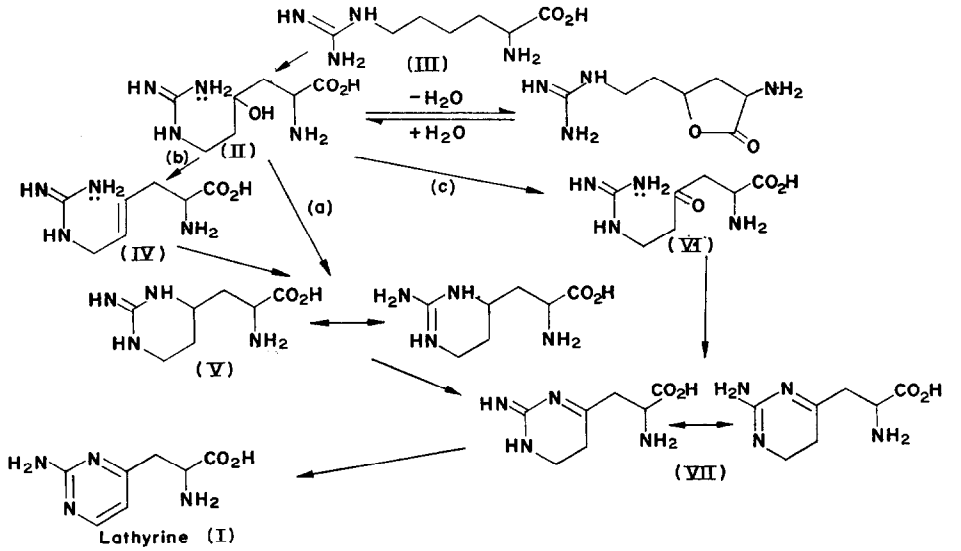
RESULTS

Possible Biosynthetic Mechanisms

4-Hydroxylation of homoarginine. Photolysis of aliphatic *N*-haloamides generates a nitrogen radical which subsequently abstracts a hydrogen atom from the 4-carbon atom of the hydrocarbon chain.[9] The probable reason for this specific radical isomerization is that the approach of the radical free valency is colinear with the axis of the σ-bond broken.[10] Thus 1,4- and 1,6-shifts are not so favourable. An analogous 1,5-hydrogen shift could occur during the enzymic 4-hydroxylation of homoarginine. The alkyl radical so formed would be trapped by peroxide formation in the presence of oxygen.[11]

Conversion of 4-hydroxyhomoarginine to lathyrine. trans-4,5-Dehydrohomoarginine (IV) could be formed from 4-hydroxyhomoarginine by dehydration, the introduction of olefinic links via hydroxylation being well documented.[12] Intramolecular nucleophilic attack by a guanidine nitrogen atom at the olefinic linkage in *trans*-4,5-dehydrohomoarginine would yield the corresponding tetrahydrolathyrine (V) (Scheme 1, route b). 4-Oxohomoarginine (VI) could be derived from 4-hydroxyhomoarginine by oxidation. Amino ketones are the known precursors of a wide variety of heterocyclic natural products[13,14] and by analogy with these biogenetic pathways, 4-oxohomoarginine (VI) could be a precursor of lathyrine. The internal condensation of amino and carbonyl groups as shown in Scheme 1, routs (c), is sterically favourable.



SCHEME 1. POSSIBLE ROUTES TO THE BIOSYNTHESIS OF LATHYRINE.

Direct hydroxylation of diamino acids. In an attempt to generate homoarginine carboxyl radicals, an aqueous solution of homoarginine (III) was irradiated with UV light and simul-

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taneously a slow stream of oxygen was passed through the solution. 4-Hydroxylation occurred in low yield. 4-Hydroxyhomoarginine was identified by comparison of its ionophoretic mobility and R_fs in two solvents, with those of an authentic sample. Arginine and lysine were also hydroxylated at the γ-carbon under these conditions. In order to establish whether the N–H bond of an amide was photochemically more labile, the ethyl ester of lysylglycine was studied. However, the yield of hydroxylated derivative was still low.

The synthesis of trans-4,5-*dehydro*-D,L-*homoarginine* (IV). The guanidation of 4,5-dehydrolysine[15] by the usual Rathke synthesis under aqueous conditions yielded 4,5-dehydrohomoarginine, contaminated with 4-hydroxyhomoarginine. These two amino acids were extremely difficult to separate, and as a consequence a modified guanidation technique was developed. Previous experience with preferential N^6-acylation of substituted lysines[16] led us to employ aprotic media for guanidation. Treatment of *trans*-4,5-dehydrolysine with 1-guanyl-3,5-dimethylpyrazole hydrochloride in dimethylsulphoxide yielded *trans*-4,5-dehydro-D,L-homoarginine (IV) in high yield in the absence of detectable quantities of 4-hydroxy-D,L-homoarginine lactone. A modification of this method was also utilized for the synthesis of [guanidino-^{14}C]-*trans*-4,5-dehydro-D,L-homoarginine (IV).

Attempted preparation of 4-*oxohomoarginine* (VI). 4-Oxolysine[15] is extremely unstable under the strongly alkaline conditions required for the Rathke synthesis. Consequently 1-guanyl-3,5-dimethylpyrazole hydrochloride in dimethyl sulphoxide was again utilised to effect guanidation. Under these mild conditions a large number of ninhydrin positive compounds were identified by ionophoresis and ion exchange chromatography. Three of these compounds were identified. Aspartic acid and β-alanine were isolated each in approximately 10% yield, and both probably resulted from fission adjacent to the carbonyl group in the parent 4-oxolysine, $NH_2.CH_2.CH_2.CO.CH_2.CH(NH_2)CO_2H$. Lathyrine was also isolated in approximately 5% yield. No amino acids were isolated containing the guanidino function, as indicated by ion exchange chromatography and ionophoretic mobility.

Amino acid analysis and ionophoresis of seed and plant extract. Neither *trans*-4,5-dehydrohomoarginine (IV) nor tetrahydro-β(2-aminopyrimid-4-yl) alanine (V) were detected in the seed extract of *L. tingitanus*, on ionophoresis of an ion exchange chromatography.

Feeding experiments with trans-4,5-*dehydro*-D,L-*homoargine.* [guanidino-^{14}C]4,5-Dehydro-D,L-homoarginine was supplied via the roots of young plants (*L. tingitanus*). The plants assimulated *trans*-4,5-dehydrohomoarginine but surprisingly failed to convert it to another amino acid. Autoradiography of the leaves and root extracts showed only 4,5-dehydrohomoarginine as being radioactively labelled.

DISCUSSION

That the *in vitro* 4-hydroxylation of dibasic amino acids reported in this paper occurred under very mild conditions, and that the majority of *in vivo* hydroxylations directly use oxygen,[17] indicate that an enzyme facilitated 1,5-hydrogen shift could be involved in the introduction of a hydroxyl group specifically at the 4-carbon atom of homoarginine. The 'feeding' experiments with [guanidino-^{14}C]4,5-dehydrohomoarginine indicate that the dehydrohomoarginine (IV) is not a lathyrine precursor. If dehydrohomoarginine (IV) is involved in the cyclization stage then the relatively stable tetrahydropyrimidine (V) would

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also be a precursor of lathyrine (Scheme 1). Bell,[3] in his survey of the *Lathyrus* genus (53 species examined), failed to find an amino acid with an ionophoretic mobility corresponding with that of tetrahydro-β(2-aminopyrimid-4-yl)alanine (V). The absence of this tetrahydro derivative in the extracts of *L. tingitanus* has been confirmed in this study by automated amino acid analysis.

In contrast to the tetrahydropyrimidine (V), 5,6-dihydro-β(2-aminopyrimid-4-yl)alanine (VII), which would result from the cyclization of 4-oxohomoarginine (VI) (Scheme 1), would be extremely susceptible to aromatization. The ability of sulphur to oxidize dihydropyrimidines to pyrimidines, leaving tetrahydropyrimidines unaffected, exemplifies this tendency towards dehydrogenation.[18] The fact that the guanidation of 4-oxolysine produced lathyrine but not 4-oxohomoarginine (VI) indicates that the cyclization of (VI) and subsequent dehydrogenation of (VII) indicated in Scheme 1 occurs readily. In a similar nitrogen heterocyclic biogenesis, only the final product, pyridine-2-carboxylic acid, and not the intermediate dihydropyridine was detected.[19]

When [guanidino-^{14}C]4-hydroxyhomoarginine was fed to the roots of *L. tingitanus*, no radioactivity was found at the same ionophoretic mobility as the stable tetrahydropyrimidine.[4] Thus it would appear that 4-hydroxyhomoarginine is converted to a labile intermediate, which is rapidly converted to lathyrine.

Thus from the results and theoretical discussion presented in this paper, we would suggest that the specific hydroxylation of homoarginine occurs by a general mechanism of a 1,5-hydrogen shift and that the resulting 4-hydroxyhomoarginine is oxidised to 4-oxohomoarginine, which in turn is cyclised to lathyrine in *L. tingitanus* (route c).

EXPERIMENTAL

Ionophoresis. Ionophoresis was conducted on Whatmann 3MM paper for 15 min at 5 kV (60 V cm^{-1}). The buffer system used was pH 3·6; HOAc–pyridine–H_2O (10:1:190). The resulting papers were developed with ninhydrin (0·2% in aq. acetone 95%), or a modified Sakaguchi reagent (2% acetone solution of 8-hydroxyquinoline).

Ion exchange chromatography. The cation exchangers, Dowex AG, 50 m W-X8 (200–400 mesh) and Amberlite IR, 120 (H^+), were used in columns of varying size. The separation of substituted lysines from the corresponding homoarginines was effected by elution of the column with a gradient of HCl (4·0 M running into a mixing chamber, containing 0·5 M acid, 500 ml). The eluate was collected in 10 ml fractions. The ninhydrin positive compounds were identified by paper ionophoresis and chromatography.

Automatic amino acid analysis. Finely ground seeds (1 g) were extracted with aq. EtOH (50%, 2 ml) at room temp. for 24 hr. After centrifugation the supernatant was evaporated under reduced pressure. The residue was dissolved in H_2O (10 ml), and the resulting solution was added to the column in 1 ml quantities. A Technicon Auto Analyser utilising a standard Dowex 50 column at 60° was eluted with sodium citrate buffer.[20] The limit of sensitivity for amino acids was 10 nmol.

Autoradiography. The autoradiographs were prepared by placing a Kodirex film (Kodak Limited, London) in contact with ionophoresis papers for 3 weeks.

Chemicals. [^{14}C]Thiourea was purchased from Amersham Radiochemical Centre (Bucks). Tetrahydro-β(2-aminopyrimid-4-yl)alanine was supplied by Prof. F. G. Strong (Department of Biochemistry, University of Wisconsin, U.S.A.). Samples of lathyrine, 4-hydroxy-L-ornithine, 4-hydroxy-L-lysine, 4-hydroxy-L-arginine and 4-hydroxy-L-homoarginine were supplied by Prof. E. A. Bell (Department of Botany, University of Texas, Austin, U.S.A.).

Synthesis of N,N-*dibenzyloxycarbonylysylglycine ethyl ester. N,N*-Dibenzyloxycarbonyllysine[21] (4·14 g, 0·01 mol), glycine ethyl ester hydrochloride (1·39 g, 0·01 mol) and dicyclohexylcarbodiimide (3·0 g, 0·015 mol) were dissolved in dimethylsulphoxide (DMSO) (15 ml). Immediate precipitation of *N,N*-dicyclohexylurea resulted on addition of NEt_3 (1·01 g, 0·01 mol). The reaction mixture was allowed to stand at room

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temp. for 12 hr in the absence of light. HOAc (0·25 ml) was added and after a further 10 min the reaction mixture was filtered. The filtrate was diluted with H_2O (200 ml) and extracted with benzene (2 × 100 ml). The organic extract was washed successively with H_2O (100 ml), 0·1 M HCl (100 ml), H_2O (100 ml) and 5% Na_2CO_3 (100 ml) and dried (magnesium sulphate). The solvent was removed, yielding a thick oil which solidified on standing. This product was dissolved in benzene, absorbed on alumina (25 × 3 cm) and eluted with benzene–light petrol. (1:1, 100 ml) to yield unreacted dicyclohexycarbodiimide (0·2 g); and subsequently with benzene (500 ml) to yield N,N-*dibenzyloxycarbonyllysylglysine ethyl ester* (2·43 g, 47%), a white solid. The analytical sample, recrystallized from benzene and light petrol., had m.p. 114–115° (Found: C, 61·6; H, 6·5; N, 8·4. $C_{26}H_{33}N_3O_7$ requires: C, 61·3; H, 6·6; N, 8·3%); ν_{max}, 1745, (ester, carbonyl); 1655, 1550, (amide bands I and II); 1695 $cm,^{-1}$ (Ph.CH_2O.CO.NH.). τ, 2·57, (Ph, 10H): 4·85, (Ph.CH_2.O, 4H); 2·7 (O.CONH.CH_2, 2H); 6·85, (CO.NH.CH_2.C, 2H); 8·6, (C.CH_2.C, 6H); 3·9, (CO.CH.C, 1H); 6·05, (CO.NH.CH_2.CO, 2H) 4·55, (CO.NH.CHR.CO, 1H); 8·85, (3H); 5·84 (2H), (ethyl group).

Lysylglycine ethyl ester. N,N-Benzyloxycarbonyllysylglycine ethyl ester (0·255 g, 0·005 mol) was dissolved in MeOH (10 ml) and shaken with Pd catalyst (50 mg) in H_2 for 3 hr, after which time the theoretical volume of H_2 (23 ml) had been absorbed. After filtration, the methanol was removed under reduced pressure leaving a residual oil. This product was chromatographically homogeneous and possessed an identical ionophoretic running time to that of the methyl ester of lysine.

Direct 4-hydroxylation of amino acids. The amino acid dihydrochloride (1 mmol) was dissolved in H_2O (2 ml). A slow current of O_2 was passed through the solution which was simultaneously irradiated with UV light. The progress of the reaction was followed by ionophoresis, samples being periodically removed from the solution. Irradiation was stopped after 48 hr. The hydroxylated product was separated from the starting material by preparative ionophoresis. In each case the yield of the substituted 4-lactone was *ca.* 5%, as judged by the relative spot sizes of the starting material and product, on ionophoresis. Irradiation of arginine at 40° for 1 week led to the formation of a wide range of ninhydrin positive compounds, as shown by ionophoresis. After irradiation of lysylglycine ethyl ester for 48 hr, the resulting solution was acidified with HCl and refluxed for 6 hr. The resulting mixture of amino acids was shown to contain lysine, glycine and 4-hydroxylysine in the ratio 1:1:0·1. This ratio was based on the comparative amino acid spot sizes obtained by ionophoresis of the mixture.

trans-4,5-*Dehydro*-D,L-*homoarginine* (*aqueous conditions*). *trans*-4,5-Dehydro-D,L-lysine dihydrochloride (0·543 g, 0·0025 mol) was dissolved in H_2O (5 ml). To this solution was added basic copper carbonate (0·42 g, 0·0018 mol) and the mixture was refluxed for 20 min. Excess copper carbonate was separated by filtration and the solution was cooled to 0°. *O*-Methylisouronium sulphate (0·625 g, 0·0025 mol) was added to the solution and the pH adjusted to 10·5 with 2 M NaOH. The reaction mixtures was allowed to stand at room temp. for 1 week, and then brought to pH 2–3 with conc. HCl. The cupric ions were removed by treatment with H_2S. The filtrate from the CuS was evaporated under reduced pressure to give a crystalline residue. This solid was dissolved in H_2O and added to an Amberlite column (IR 120, $H,^+$ 40 × 2 cm) and eluted as previously described. Fractions 55–59 contained *trans*-4,5-dehydro-D,L-lysine as shown by ionophoresis. Fractions 65–90 yielded after evaporation under reduced pressure a solid residue. Recrystallisation from H_2O and EtOH (1:9) gave trans-4,5-*dehydro*-D,L-*homoarginine dihydrochloride* (IV) (0·31 g, 57%); m.p. 172–175° (Found: C, 32·7; H, 6·3; N, 21·9. $C_7H_{16}N_4O_2Cl_2$ requires; C, 32·4; H, 6·2; N, 21·6%) ν_{max}, 3200–2500, 1940, (NH_3^+); 1740, (CO_2H); 980 cm^{-1}, (*trans*-olefin); τ, D_2O; 5·95, (RNH_2^+.CH_2.C, 2H); 4·0, (*olefin*, 2H); 6·95, (C.CH_2.C, 2H); 5·5, (NH_2^+.CH_2H, 1H). IV gave a grey colour with ninhydrin and an orange colour with Sakaguchi's reagent.

Method B, aprotic conditions. trans-4,5-Dehydro-D,L-lysine dihydrochloride (0·543 g, 0·0025 mol), 1-guanyl-3,5-dimethylpyrazole hydrochloride (0·5 g, 0·0025 mol) and NEt_3 (0·14 ml, 0·011 mol) were dissolved in DMSO (10 ml) and allowed to stand at room temp. for 4 days. The reaction mixture was added to a column of Amberlite IR 120 resin (30 × 2 cm). The solution was recycled 4× and the column was then eluted with H_2O (200 ml). Elution with 4 M HCl (250 ml), and subsequent evaporation yielded an oil (0·65 g) which was added to a Dowex 50 column, and eluted as described above. Fractions 210–270 were combined and evaporation under reduced pressure to yield an oil. Trituration with EtOH at 0° gave *trans*-4,5-dehydro-D,L-homoarginine (IV) (0·48 g, 74%).

[*guanidino-*^{14}*C*]trans-4,5-*Dehydro*-D,L-*homoarginine*. [^{14}C]Thiourea (2 mg, 50 μCi, 0·26 × 10^{-5} mol) was dissolved in EtOH (2 ml) containing MeI (0·1 ml). The solvent was removed under reduced pressure after 12 hr, and the resulting [^{14}C]*S*-methylisothiouronium iodide was dissolved in DMSO (0·5 ml). To this solution was added *trans*-4,5-dehydro-D,L-lysine dihydrochloride (6 mg, 0·26 × 10^{-5} mol) and NEt_3 (0·004 ml, 0·26 × 10^{-5} mol) in DMSO (0·5 ml). After standing at room temp. for 4 days, the solution was added to a Dowex 50 column (H^+, 6 × 0·6 cm). The solution was recycled 4× and the column was then eluted with H_2O (50 ml) followed by 6 M HCl and collected in 5 ml fractions. Fractions 3–10 contained *trans*-4,5-dehydro-D,L-lysine as shown by ionophoresis. Fractions 10–20 contained [guanidino-^{14}C]*trans*-4,5-dehydro-D,L-homoarginine. This labelled amino acid was further purified by preparative ionophoresis, and was homogeneous on chromatography in *n*-BuOH–HOAc–H_2O (3:1:1), and MeCOEt–Pr CO_2H–H_2O (2:1:2).

Attempted synthesis of 4-*oxohomoarginine*. Thiourea (0·7 mg, 9 × 10^{-7} mol) was dissolved in EtOH

(2 ml) containing MeI (0·1 ml). The solvent was removed under reduced pressure after 12 hr, and the resulting *S*-methylisothiouronium iodide was dissolved in DMSO (0·5 ml). To this solution was added 4-oxo-L-lysine dihydrochloride (2 mg, 9×10^{-7} mol) and NEt_3 (1·4 μl, 1×10^{-6} mol) in DMSO (0·5 l). After standing at room temp. for 4 days, the solution was studied for amino acid content by ionophoresis, TLC and automated amino acid analysis.

'Feeding' experiments with L. tingitanus. The seeds of *L. tingitanus* (Kew) were grown under glass on moist filter paper. 1 week after germination a solution of [guanidino-^{14}C]*trans*-4,5-dehydro-D,L-homoarginine (0·25 ml) buffered to pH 7·4 with Na_2CO_3 was added to the filter paper. Distilled H_2O was added daily in order to maintain a moist atmosphere under the glass. The plants were removed from the solution after 1, 4 and 7 days. The roots were dissected from the stem and cut into fine sections, which were homogenized in a 50% aq. EtOH. After 18 hr the homogenate was centrifuged and the supernatant applied to an ionophoretic paper. After ionophoresis at pH 3·6 the labelled amino acids were detected by autoradiography.