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S-4-CHLOROTRYPTOPHAN: ITS SYNTHESIS VIA RESOLUTION, DETERMINATION OF THE ABSOLUTE STEREOCHEMISTRY AND IDENTIFICATION IN THE CRUDE SEED PROTEIN OF THE PEA, PISUM SATIVUM

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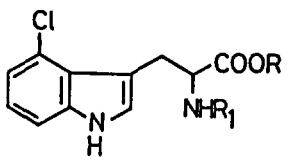
Abstract: Racemic 4-Chlorotryptophan was synthesized by an efficient route and the racemate was resolved by a chiral column into each enantiomer, the absolute stereochemistry of each was determined by reductive dehalogenation to the known chiral tryptophans. S-4-chlorotryptophan was then identified in the crude seed protein of Pisum sativum.

4-Chloroindole-3-acetic acid (4-Cl-IAA) and its methyl ester had been isolated in 1968 from immature seeds of pea, Pisum sativum, as a second natural auxin (plant hormone)[1], [2,3,4] since the discovery of indole-3-acetic acid from human urine in 1934. Its possible biosynthetic precursor, 4-chlorotryptophan (1), had also been isolated in 1970 as the methyl malonamide derivatives (2) from the same immature seeds,[5] exhibiting hypocotyl swelling activity when bioassayed with seedlings of Phaseolus mungo. 4-Chlorotryptophan is the first naturally occurring unusual amino acid to be found.



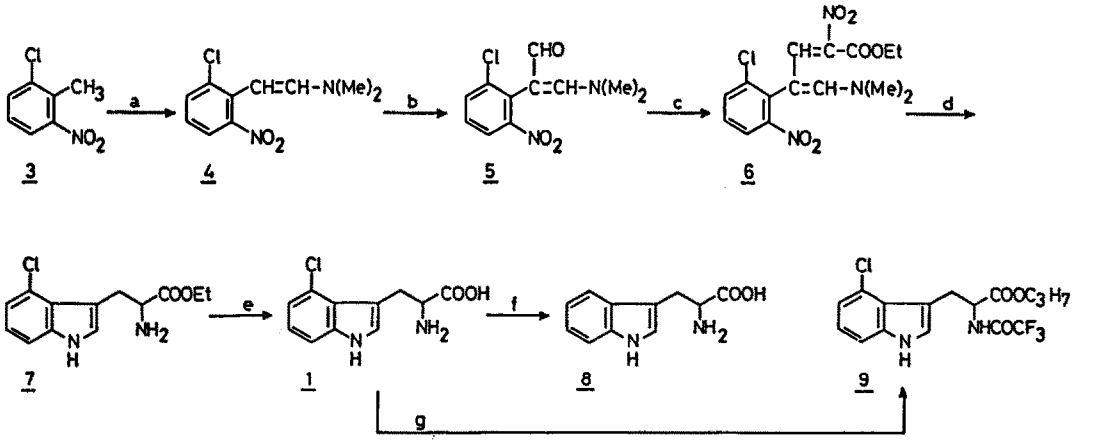
1. R = R_1 = H
2a. R = Me, R_1 = -CO-CH_2-COOMe
2b. R = Me, R_1 = -CO-CH_2-COOEt

Since pea seeds are one of the major protein sources taken by human beings , it is of extreme significance to examine whether this unusual amino acid is present in the protein fraction of pea seeds. In order to resolve this problem we have prepared chiral 4-chlorotryptophans (1a and 1b) (See Chart) as authentic samples for analysis, and achieved the identification of the S enantiomer (1b) in the crude seed protein of Pisum sativum, as described in this report.

Our aim to synthesize large amounts of 4-chlorotryptophan (1) was not only for our identification work but also for nutritional studies using rats. An efficient route to prepare the racemate (1) starting from 2-chloro-6-nitrotoluene (3) was established according to our modified method (Scheme), originally employed for the synthesis of 6-chlorotryptophan.[6] Thus, upon heating at 120°C for 36 h with N,N-dimethylformamide dimethyl acetal (DMF-DMA), 3 was readily converted to the enamine (4). Vilsmeier formylation of 4 with phosphorus oxychloride and dimethylformamide gave, after basic workup, the acrolein (5)[7] in 74% yield from 3. The reaction of 5 with ethyl nitroacetate in acetic anhydride

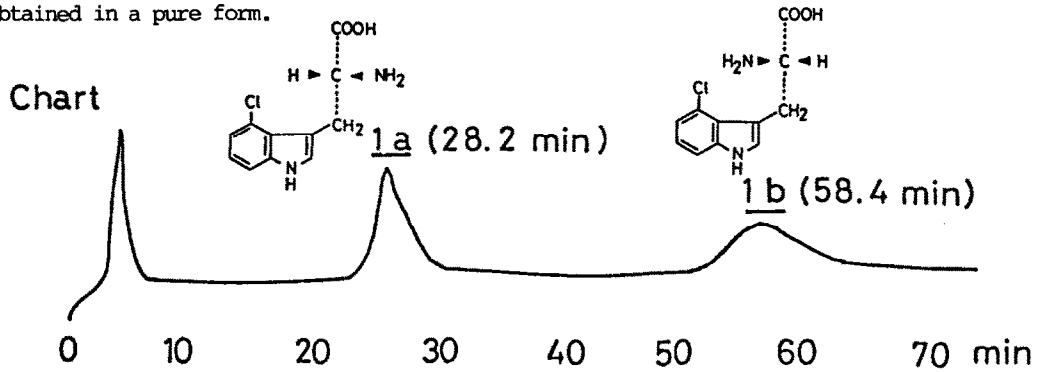
at 80-90°C yielded the orange-red crystalline diene (6)[8] in 92% yield. The conversion of 6 to the chlorotryptophan ethyl ester (7) was accomplished by catalytic hydrogenation with Raney nickel in methanol/tetrahydrofuran at 1500 psi for 24 h, and the product was purified by chromatography over silica gel. The purified ester was hydrolyzed with aqueous sodium hydroxide (20%) at room temperature for 24 h and by heating at 60°C for 3 h. The alkaline solution was acidified with dilute acetic acid, and the precipitate was crystallized from glacial acetic acid to yield 1[9] in 51% yield from 6. The present synthesis of 1 was superior in having shorter reaction steps and giving better yields than the reported method starting from 4-chloroindole via gramine.[10]

Scheme



a) DMF, DMA, 110-120°C, 36 h; b) DMF, $POCl_3$, 25°C, 5-6 h; c) Ac_2O, O_2NCH_2COOEt, 90°C, 45 min; d) Raney Ni, H_2, 1500 psi, 24 h; e) i. 20% NaOH, ii. dil. AcOH; f) Red P, HI, UV (256 nm), Hg lamp, 25°C, 72 h; g) i. CH_3COCl, n-$CH_3CH_2CH_2OH$, 110-120°C, 25 min, ii. $(CF_3CO)_2O$, CH_2Cl_2, 150°C, 5 min.

The synthesized racemic 1 was resolved over a chiral column, with each enantiomer having a retention time of 28.2 and 58.4 min respectively (Chart). These separated fractions were desalted by passing through an ODS-5 column. Thus, 1a and 1b were obtained in a pure form.



Chiral Column: Chiralpak (WH-L) (Daicel Chemical Industries, Ltd.);
Column Size: 4.0mm ID x 250mm; Solvent: 0.25 mM $CuSO_4$
Flow Rate: 1.5 ml/min; Temperature: 50°C.
Under these conditions, R- and S-tryptophan appeared at 18.5 and 62.1 min, respectively.

The dechlorination of 1 was attempted under various reaction conditions such as Pd/C, DMF, HCOOH; Pd/C, NEt_3, HCOOH; Zn/CF_3COOH; $PdCl_2$, HCOOH, NEt_3, CH_3CN; $NiCl_2$, CH_3CN; red P/HI; however, none of these were successful. Finally, reductive dehalogenation to yield tryptophan (8) in 15% yield was achieved by reaction of 1 with P/HI in the presence of UV light.

Experimental Procedure: A mixture of racemic 4-chlorotryptophan (1) (1 mM), red P (2 mM) and HI (10 mM) was stirred at room temperature for 72 h in the presence of UV (254 nm) from a Hg lamp. The reaction mixture was then neutralised with sodium acetate to pH 5 and evaporated under vacuum. The residue was dissolved in minimum amount of aqueous acetic acid (30%) and filtered. The filtrate was subjected to preparative HPLC over an ODS-5 column, and using gradient elution,[11] tryptophan (8) was separated from other products. The tryptophan fraction was evaporated at room temperature to a small volume and then lyophilized. The product thus purified was identical in all physico-chemical properties to an authentic sample of racemic tryptophan.

The enantiomer (1a) was similarly dehalogenated by the above procedure to give chiral tryptophan, which was analyzed with a chiral column. The absolute stereochemistry of the chiral tryptophan from 1a was established to be R by comparison of its retention time with that of authentic R-tryptophan. In a similar manner the absolute stereochemistry of the enantiomer 1b was established to be S.

R-4-Chlorotryptophan (1a): $[\alpha]_D^{15}$ +48°(c. 0.12, 8% aq. AcOH); $[\theta]_{280}$ -1639°(10% aq. AcOH).

S-4-Chlorotryptophan (1b): $[\alpha]_D^{15}$ -45°(c. 0.13, 8% aq. AcOH); $[\theta]_{280}$ +1645°(10% aq. AcOH).

Next, the identification of 4-chlorotryptophan in the crude protein of pea seeds was carried out as follows: Mature pea seeds (P. sativum cv. Usui) were purchased from a supermarket at Nagoya. The seeds were extracted with potassium phosphate buffer (pH 8.0) for 16 h at 4°C and the extract was centrifuged at 29000 g for 45 min to yield a supernatant liquid which was dialysed repeatedly against water and lyophilized to give the cream coloured crude protein in 12.5% yield.[12]

The crude protein (2 g) was hydrolyzed under acidic conditions with p-toluenesulfonic acid (3N aqueous solution, 20 ml) in a sealed tube for 24 h at 110°C.[13] The hydrolyzate was condensed to a small volume and the concentrate was chromatographed successively over Sephadex G-25 and cellulose and finally purified by preparative TLC over silica gel to give a pure, natural 4-chlorotryptophan.

The natural 1 (isolated from pea seeds) as well as authentic 1, were derivatized to respective N-trifluoroacetyl n-propyl esters (9) (Scheme) by a two step procedure, which involved an esterification with acetyl chloride/n-propanol (3.5 M solution) at 110°C for 25 min, followed by acylation with trifluoroacetic anhydride in methylene chloride (70%) at 150°C for 5 min.[14] A coincidence in their retention times, co-injection and mass spectra,[15] has shown that 4-chlorotryptophan is contained in the crude seed protein of Pisum sativum, and its amount was calculated as 130 ng/g dry mature seed.[16] The absolute stereochemistry of natural 4-chlorotryptophan isolated from the pea seeds was analyzed with a chiral column, and the comparison of its retention time with that of authentic S-4-chlorotryptophan demonstrated its S configuration. The malonamide derivatives of 4-chlorotryptophan (2), which we isolated earlier from immature pea seeds, had previously been assigned the R configuration;[5] however, the present finding that 4-chlorotryptophan

in the protein has the S configuration suggests that a reinvestigation is necessary.

The present investigation has revealed that S-4-chlorotryptophan is contained as a component of the protein of pea seeds. Although S-4-chlorotryptophan is only a minor constituent in the pea protein, our findings are of extreme importance because pea seeds are a major protein source in human diets. Therefore, the nutritional effect of S-4-chlorotryptophan on human health should be pursued. Further, an incorporation of a chlorinated amino acid such as 4-chlorotryptophan in the protein raises another question: how does biological chlorination proceed in the biosynthesis of 4-chlorotryptophan within the seed? We are currently pursuing this interesting question.

References and notes

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7. 3-(Dimethylamino)-2-(2-chloro-6-nitrophenyl)acrolein (5): mp 175-177°C; UV (MeOH): λ_{max} 286 (28000) nm; IR (KBr): ν_{max} 1635, 1580, 1560 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.95 (6H, br s), 7.01 (1H, s), 7.38 (1H, t, J=8.1), 7.64 (1H, d, J=8.1), 7.75 (1H, d, J=8.1), 8.97 (1H, s).

8. Ethyl 5-(dimethylamino)-2-nitro-4-(2-chloro-6-nitrophenyl)-2,4-pentadienoate (6): mp 154-156°C; UV (MeOH): λ_{max} 431 (32400), 256 (11000) nm; IR (KBr): ν_{max} 1710, 1625, 1540 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.15 (3H, t, J=7.1), 2.89 (6H, br s), 3.64 (2H, q, J=7.1), 7.21 (1H, s), 7.43 (1H, t, J=8.0), 7.65 (1H, dd, J=8.0, 1.5), 7.80 (1H, dd, J=8.0, 1.5), 7.89 (1H, s); MS (m/z): 369 (M^+, 49), 281 (17), 223 (24), 210 (87), 150 (75), 130 (100).

9. 4-Chlorotryptophan (1): UV (EtOH): λ_{max} 292 (5440), 281 (6460), 272 (6120), 223 (36400) nm; IR (KBr): ν_{max} 3400, 1640, 1580 cm^{-1}; ^1H NMR (D_2O/CF_3COOD): δ 3.14 (1H, dd, J=14.95, 10.05), 3.78 (1H, dd, J=14.95, 4.9), 4.42 (1H, dd, J=10.05, 4.9), 6.96 (1H, dd, J=6.2, 2.7), 7.17 (1H, s), 7.31 (2H, dd, J=6.2, 2.7).

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11. Column: ODS-5; Eluting solvent, A: 0.1% TFA in H_2O and B: 50% CH_3CN in 0.1% TFA; Gradient System: Solvent A (100%, 0 min), (80%, 7 min), (60%, 27 min), (0%, 32 min); Flow Rate: 1 ml/min; Retention time: 4-Chlorotryptophan 28.5 min, Tryptophan 19.5 min.

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15. Propyl (trifluoroacetyl)amino-4-chloroindole-3-propionate (9): MS (m/z): 376 (M^+, 37), 289 (17), 260 (22), 192 (20), 164 (100).

16. Detailed information regarding identification will be published elsewhere.

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