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L-4-Chlorotryptophan from Immature Seeds of *Pisum sativum* and Reassignment of the Absolute Stereochemistry of *N*-Malonyl-4-chlorotryptophan

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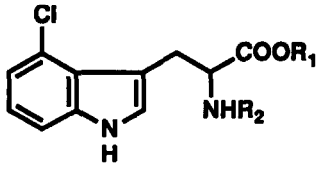
Abstract : Free 4-chlorotryptophan (4-Cl-Trp) was isolated from immature seeds of *P. sativum*, identified, and its stereochemistry determined to be L-form. Reinvestigation of the *N*-malonyl-4-Cl-Trp isolated from the same seeds showed that the stereochemistry of 4-Cl-Trp residue is L-form.

Chlorine-containing tryptophan (**1**) was first isolated from immature seeds of *Pisum sativum* as *N*-malonyl derivatives of 4-chlorotryptophan (**2a, 2b**), and its stereochemistry assigned to be D-form[1]. 4-Chloroindole-3-acetic acid (4-Cl-IAA) also was isolated from the same seeds as the second natural auxin (plant hormone)[2], exhibiting much stronger activity, e. g., more than one hundred times inhibition of lettuce seed germination, than indole-3-acetic acid (IAA), the latter is the first natural auxin, being now ubiquitously distributed in plant kingdom. Although it has passed over ca. sixty years since the discovery of IAA in nature, its biosynthetic pathway is not fully elucidated, whether it is originated from L-tryptophan(L-Trp, **3a**) or its D isomer. The immature pea seeds that produced both 4-Cl-Trp and 4-Cl-IAA, accompanying with the minor Trp and IAA, could be a good bio-system to investigate the biosynthetic pathway of auxins (4-Cl-IAA and IAA) from their possible precursors (4-Cl-Trp or Trp). Recently, we have found that 4-Cl-Trp isolated from the hydrolyzate of pea seed protein showed L-form[3]. Therefore, *N*-malonyl-4-Cl-Trp should be reinvestigated of its absolute stereochemistry, whether it has D-form as assigned previously[4], or L-form as present in the protein fraction. We describe here the isolation of free 4-Cl-Trp as well as *N*-malonyl-4-Cl-Trp from immature pea seeds, and their absolute stereochemistry determined to be both the L-form. The present findings urges us further to re-investigate the past assignment of the D-form to *N*-malonyl-Trp that was reported to be found in other plant species.

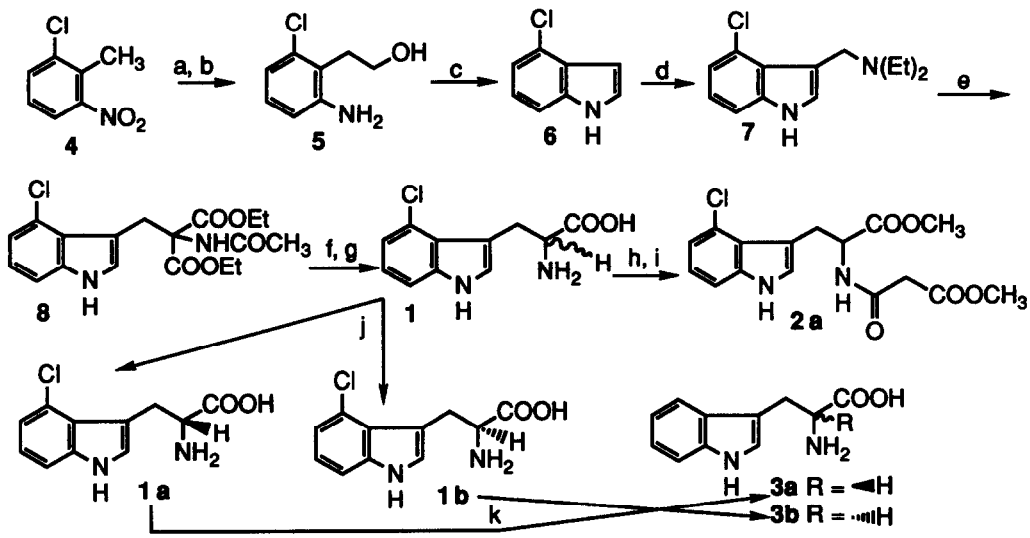


1. $R_1 = R_2 = H$
2a. $R_1 = Me$, $R_2 = CO\text{-}CH_2\text{-}COOMe$
2b. $R_1 = Me$, $R_2 = CO\text{-}CH_2\text{-}COOEt$

Our synthesis of D- and L-4-Cl-Trp (**1a** and **1b**) (Scheme) was devised to provide authentic samples used for the present identification and to establish a small scale synthesis of the labeled compounds used for our next biosynthetic study. 2-Chloro-6-nitrotoluen(**4**) was condensed with cold (or hot) paraformaldehyde followed by reduction gave 2-amino-5-chlorophenethyl alcohol

(5)(84% yield). The alcohol (5) was converted by dehydrogenation with ruthenium catalyst to 4-chloroindole (6)(80%)[5], which was derivatized to racemic 4-Cl-Trp (1) *via* gramine by a slight modification of the original synthesis[6]. The indole (6) was reacted with aqueous cold(or hot) formaldehyde (1%) and diethylamine to give gramine (7)(82%), which was condensed with diethyl acetoamidomalonate to afford 8(87%). The amido-ester (8) was saponified followed by decarboxylation, giving crude racemic 4-Cl-Trp(1). The crude product (1) was purified with Sep-pak (C-18) in a small scale (20mg of 6) or with an ODS open column in a large scale synthesis (2g of 6). Racemic 4-Cl-Trp(1)(37% from 4) was then resolved to each enantiomer using a chiral column(1a Rt = 24.5 min, 1b Rt = 54.6 min)(step j). The eluate of each isomer was freed from $CuSO_4$ through the Sep-pack (C-18) column, and dechlorinated to Trp by reductive dehalogenation with P/HI under UV light (step k) ; D-Trp(3a) was produced from 1a and L-Trp(3b) from 1b, thus the stereochemistry of 1a and 1b determined to be D and L, respectively. In this synthesis, the overall yield from 4 to 1a and 1b was excellent (18% and 16% respectively), the carbon isotopes being easily introduced to 2-position of the indole (step a), 3'-position of the side chain (step d) or both from labeled formaldehyde.

Scheme



a) $(HCHO)_n$, DMSO, 30% TritonB in MeOH b) 5%Pd-C, H_2, EtOAc c) $RuCl_2(PPh_3)_3$, toluene reflux d) 1%HCHO, $NH(Et)_2$, AcOH e) diethyl acetoamidomalonate, $P(C_4H_9)_3$, reflux f) 10%NaOH, reflux, 7hr g) 3N $HS(CH_2)_2SO_3H$, 110°C, 16hr h) HCl/MeOH i) monomethyl malonate, DCC j) HPLC with Chiralpak WH-L(4 x 250 mm, Daicel Chemical) solvent : 0.25mM $CuSO_4$ k) Red P, HI UV(256 nm)

During these experiments, we noticed that racemic 4-Cl-Trp gave a long elliptical spot on cellulose TLC. We therefore examined various solvent systems for resolution of the enantiomers and found that a system pyridine-methanol-water(3 : 20 : 80) was best. Using this simple procedure, racemic 4-Cl-Trp was resolved to D- (Rf=0.53) and L- (Rf=0.41) isomer. Racemic Trp was similarly resolved (D: Rf=0.62, L: Rf=0.58) with the same system[7]. We thus established two methods for chromatographic identification of D- and L-4-Cl-Trp using HPLC (step j in Scheme) and cellulose TLC.

Free 4-Cl-Trp was isolated from immature seeds of *P. sativum* as follows: pea seeds were extracted with 90% aqueous acetone and the extracted acetone solution was concentrated to an aqueous residue. This residue was loaded on Sep-pak (C-18), from which the eluate with 40% CH_3CN contained 0.1% TFA was collected. This fraction was further purified by HPLC with ODS column that was developed with a gradient solvent system between H_2O and CH_3CN containing 0.1% TFA. The chromatogram was monitored at 280 nm, and the peak with the same retention time as synthetic 4-Cl-Trp was collected. 4-Cl-Trp from immature pea seeds was identified and quantified by FRIT-FAB LC-MS[8] by comparison with the synthetic compound (Fig.). The mass spectrum showed the typical pattern of one chlorine atom containing per molecule. The yield of 4-Cl-Trp was 24 μg/g dry seeds calculated from the peak area of the m/z 239($M(^{35}Cl)+H$)[9]. The stereochemistry of the natural 4-Cl-Trp was found to be L-form by means of two methods described above. Only the peak of L-4-Cl-Trp was detected, and even a trace amount of the D isomer was not. We then reinvestigated the stereochemistry of the malonyl compound by reisolating from the immature pea seeds.

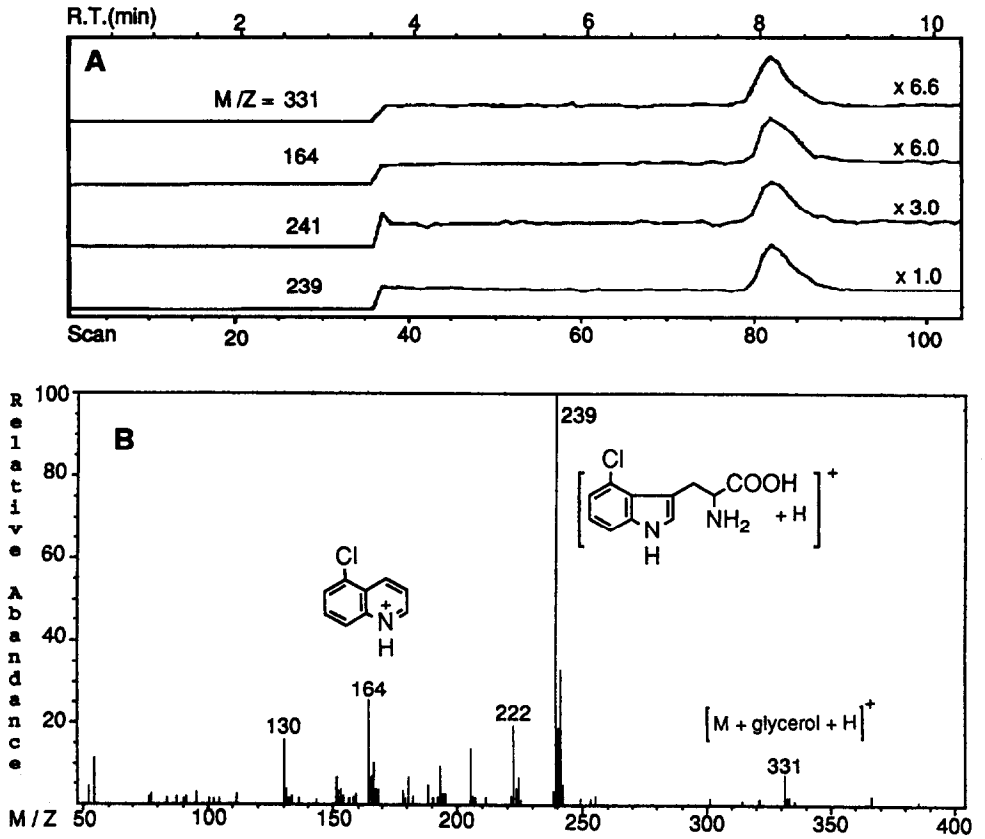


Fig. FRIT-FAB LC-MS of 4-Cl-Trp

A : Mass chromatogram of 4-Cl-Trp monitored at m/z 239, 241, 164, 331

B : Mass spectrum of 4-Cl-Trp obtained by subtracting scan No.60 from No.82

Immature seeds of *P. sativum* L cv. Nankai-Midori (FW. 72 Kg) was purchased from the market in Nagoya, being treated with MeOH (100 *l*). The methanol extract was purified as in a previous report except for the final step[1]. The pure methylated compound (0.6 mg) was obtained from the final HPLC by using an ODS column with a linear gradient between H2O and CH3CN. The EI-MS, ^{1}H-NMR spectra and other spectral data of this compound[10] were identical to those of the synthetic *N*-malonyl(methyl ester)-4-Cl-tryptophan methyl ester (**2a**). This malonyl derivative of 4-Cl-Trp (100 μg) obtained from pea seeds was hydrolyzed at 110°C for 16 hr with 4*N*-methansulfonic acid containing 0.2% tryptamine. The hydrolysate then was purified with Sep-pak C-18 which gave free 4-Cl-Trp. This free 4-Cl-Trp was analyzed by HPLC with a chiral column and cellulose TLC. Only L-4-Cl-Trp was detected. The 4-Cl-Trp residue in the malonyl derivative therefore was shown to be the L-form, and the previous assignment should be revised.

N-Malonyl-Trp is found in various plants extracts, *e.g.*, *Astragalus cicer* and *Acorus calamus*[11]. The stereochemistry of Trp residue in these plants was reported to be the D-form from digestion experiments with amino acid oxidase[12]. This stereochemistry, however, should be reexamined using the chemical methods, as reported here. We now have synthesized 3'-^{14}C- labeled D- and L-4-Cl-Trp, either isomer of which is metabolized to 4-Cl-IAA is now in progress.

References and Notes

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2. S. Marumo, H. Hattori, H. Abe and K. Munakata, *Nature* (London), **1968**, 218, 959.
3. S.V. Thiruvikraman, Y. Sakagami, M. Katayama and S. Marumo, *Tetrahedron Lett.* **1988**, 29, 2339.
4. In the previous study[1], the optical rotation of **2b** from pea seeds was $[\alpha]_{400}$= - 24.6°, which was compared with those of synthetic *N*-malonyl(methyl ester)-Trp methyl ester; D isomer $[\alpha]_{400}$ = - 66.2° and L-isomer $[\alpha]_{400}$ = + 67.2°, leading to the incorrect assignment.
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6. H.N. Rydon and J.C. Tweddle, *J. Chem. Soc.*, **1955**, 3499.
7. We tried to resolve other racemic amino acids: Phe, Tyr, Leu etc., but did not succeed.
8. T. Takeuchi, S. Watanabe, N. Kondo, M. Goto and D. Ishi, *Chromatographia*, **1988**, 25, 523.
9. Instruments: DX-705L, JEOL with FRIT-FAB attachment system; Conditions: column: Develosil ODS-5 2 x 250 mm(Nomura kagaku, Seto, Japan), flow rate: 0.1 ml/min, solvent: 25% CH3CN in H2O containing 0.1% TFA and 0.5% glycerol, injection volume: 10μl of dissolved in 50% acetic acid; Quantification: The peak area monitored at m/z 239 was linear from 0.1 μg to 5 μg of sample. The amount of 4-Cl-Trp in seeds varies with the growth stage. The maximum value for seeds collected on the 14th day after flowering. Details will be reported elsewhere.
10. MS: m/z(%) ; 354(7.6), 352(23), 237(32), 235(95), 166(33), 164(100), ^{1}H-NMR(in $CDCl_3$, δ ppm): 3.27(2H s), 3.39(1H dd, *J*=13.2, 17.4), 3.45(1H dd, *J*=7.8, 17.4), 3.67(3H s), 3.73(3H s), 4.97(1H dd, *J*=7.8, 13.2), 7.07(1H d, *J*=4.5), 7.11(1H s), 7.21-7.28(2H m), 7.48(1H brd.s), 8.18(1H brd.s), $[\alpha]_D$ = - 6.72°, $[\alpha]_{400}$ = - 23.8° (c = 0.0472, MeOH).
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12. M.H. Zenk and H. Scherf, *Biochem. Biophysic. Acta*, **1963**, 71, 737.

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