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Studies on Iodinated Compounds. II.[1] High Performance Liquid Chromatographic Studies on the Synthesis and Purification of Monoiodocarnosine

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Conditions for the synthesis and purification of monoiodocarnosine (MIC) were examined by high performance liquid chromatography (HPLC). When carnosine (β-alanyl-L-histidine) was iodinated to obtain MIC, the maximum yield was attained by the equimolar reaction of carnosine with I_2 in 0.5 N NaOH solution. The synthesized MIC was separated by preparative reversed-phase HPLC with H_2O–MeOH (90 : 10) as the eluent and then purified by recrystallization. Chemically pure MIC was obtained in 72% yield in a short time.

Keywords——monoiodocarnosine; diiodocarnosine; carnosine; iodination; reversed-phase HPLC; SEP-PAK C_{18} cartridge; preparative HPLC; NMR spectra; UV detection

A physiologically active dipeptide, L-carnosine (β-alanyl-L-histidine), is known to be present in the body, and exerts an anti-inflammatory effect.[2] When carnosine is iodinated, the hypotensive effect of carnosine is intensified and the heart rate is decreased.[3] More precise studies are required in order to examine the biological properties of iodinated carnosine, and thus we investigated the optimum conditions for synthesis and purification.

When carnosine is iodinated, iodine combines with the imidazole moiety of the peptide, and two iodinated products, monoiodocarnosine (MIC) and diiodocarnosine (DIC), are obtained.[4] DIC was effectively synthesized by Ishikawa *et al.*,[3] by a modification of the method of Brunings.[5] However, MIC is hard to obtain by Brunings' method because the same problems[1] arise as are found in the synthesis of monoiodohistidine. There is no established synthetic method for obtaining pure MIC in high yield. MIC has been reported in the literature,[4] but its physical constants have not been described anywhere.

In the present study, carnosine was iodinated in a stepwise manner, and the reaction products were analyzed by HPLC at each step in order to find suitable conditions for obtaining MIC in maximum yield. The MIC synthesized under the optimal conditions was isolated by preparative HPLC and purified by recrystallization.

Results and Discussion

Optimum Conditions for MIC Synthesis

In the preceding paper,[1] we reported a study of the conditions for iodinating histidine by the use of HPLC; it was found that the yields of iodohistidines were dependent on the concentration of iodinating reagents. In the present study, the iodination of carnosine was similarly examined by using HPLC to find the optimal conditions for the synthesis of MIC. As the iodinating reagent, 0.1 M I_2 in EtOH[1] was used. Chromatograms obtained with the

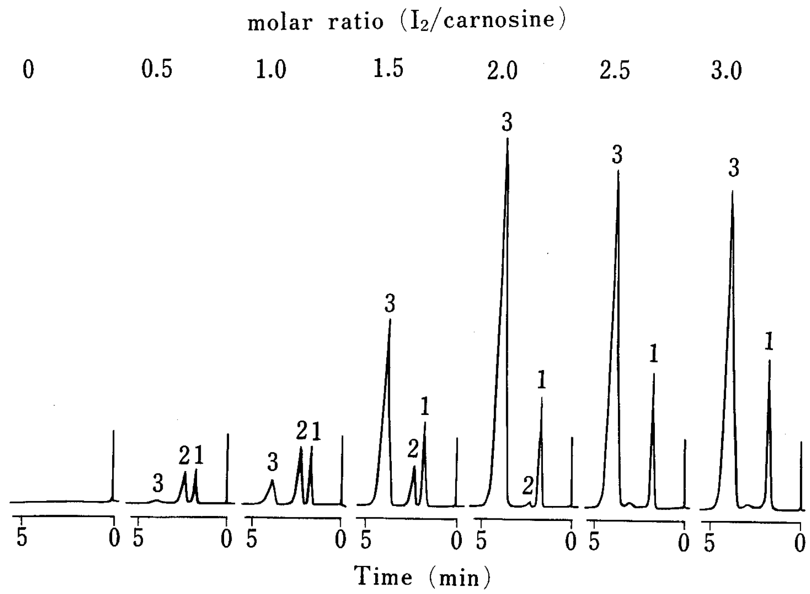

Fig. 1. Effect of the Molar Ratio of Iodine to Carnosine on the Formation of MIC and DIC

Detector sensitivity: 0.05 AUFS. Peak identity: 1, I^-; 2, MIC; 3, DIC. See "Experimental" for details.

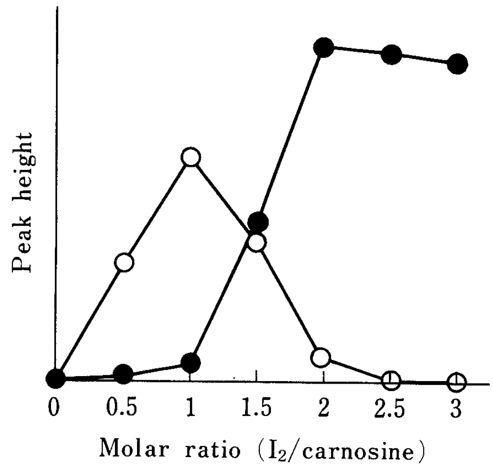

Fig. 2. Relationship between Peak Heights of Iodocarnosines and the Amount of I_2 Added

○, MIC (peak height at 0.01 AUFS); ●, DIC (peak height at 0.05 AUFS).

reaction mixtures are shown in Fig. 1.

As shown in Fig. 2, the yields of MIC and DIC were maximal when the molar ratios of iodine to carnosine were 1.0 and more than 2, respectively. The mode of iodination of carnosine was quite similar to that of histidine.[1] Thus, the iodination of the imidazole ring seemed not to be much influenced by the β-alanyl residue.

Isolation and Purification of MIC

Separation and Purification of MIC by Preparative HPLC——It was extremely difficult to obtain pure MIC in high yield by Brunings' method[5] using extraction and recrystallization. However, preparative HPLC was found to be satisfactory as shown below.

Carnosine (271 mg, 1.2 mmol) was reacted with an equimolar amount of I_2 in 75 ml of 0.5 N NaOH solution. After being neutralized with HCl, the reaction mixture was concentrated and charged onto a SEP-PAK C_{18} cartridge to remove colored and other interfering materials. The effluent from the cartridge was further concentrated to 9 ml (additional concentration resulted in the precipitation of salts) and passed through a 0.45 μm filter to

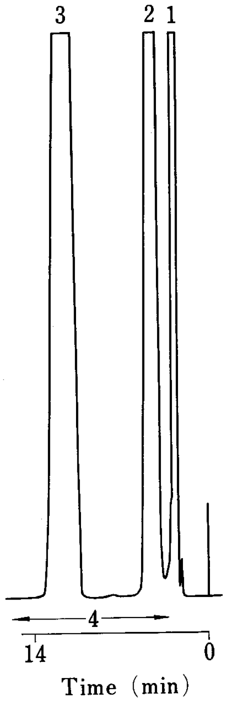

Fig. 3. Preparative HPLC of MIC

Peak identity: 1, I^-; 2, MIC; 3, DIC; 4, carnosine (detected by ninhydrin reaction).

See "Results and Discussion" and "Experimental" for details.

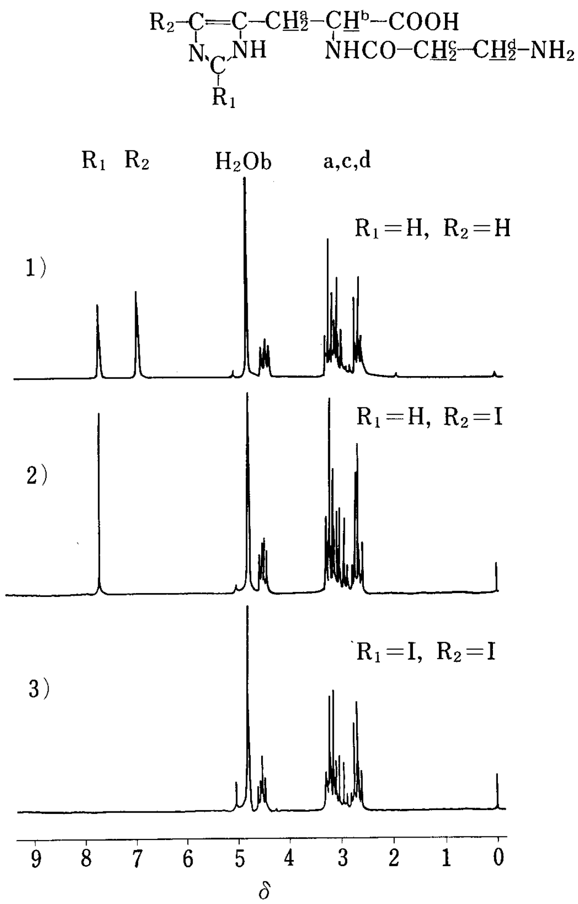

Fig. 4. NMR Spectra of 1) Carnosine, 2) MIC and 3) DIC

obtain a specimen for HPLC.

The preparative HPLC was performed on a reversed-phase μBondapak C_{18} column (7.8 mm i.d. × 30 cm) with H_2O–MeOH (90 : 10). The MIC was obtained by repeated injection of 1.8 ml aliquots of the specimen (equivalent to 54.2 mg carnosine being iodinated).

As shown in Fig. 3, MIC could be separated well from the by-products, I^- and DIC. Other inorganic ions, Na^+ and Cl^-, were co-eluted with I^- [6] and were not found in the MIC fraction. Unreacted carnosine was not detectable at 254 nm. However, when authentic carnosine was subjected to the HPLC and detected by means of the ninhydrin test, the separation of MIC from carnosine was found to be unsatisfactory.

Separation of MIC from Carnosine——When the MIC fraction was concentrated under reduced pressure, MIC crystallized easily as needles while carnosine remained in solution. Thus, carnosine contaminating the MIC fraction was removed by recrystallization of the product. The total yield of MIC thus obtained was 302 mg (72%).

Examination of Purity of MIC, and Confirmation of its Identity——Purified MIC was subjected to thin layer chromatography (TLC) together with carnosine and DIC. TLC was performed on a cellulose plate with a solvent mixture, BuOH : AcOH : H_2O = 4 : 1 : 2,[3] and the developed chromatogram was visualized by the use of ninhydrin. The MIC specimen gave a single spot at Rf = 0.28 (carnosine, Rf = 0.18; DIC, Rf = 0.63).

The identity of the product as MIC was confirmed by its nuclear magnetic resonance (NMR) spectrum (Fig. 4) as well as by elementary analyses. Judging from the known NMR spectra of iodohistidines,[7] the NMR spectrum of MIC suggested that iodine is present at the 4-position of the imidazole ring of carnosine.

Conclusion

The purity and yield of MIC synthesized by the present method are satisfactory. MIC was efficiently and rapidly separated by preparative HPLC, and the procedures for separation of MIC from the reaction mixture and purification have thus been shortened considerably.

Experimental

Reagents and Materials——L-Carnosine was obtained from the Peptide Institute, Protein Research Foundation. DIC was synthesized in this laboratory according to the method described by Ishikawa *et al.*[3] Other reagents were commercially available reagent-grade products. SEP-PAK C_{18} cartridge was the product of Waters Associates. TLC plates used were DC-Fertigplatten Cellulose (ohne Fluoreszenzindikator), Schichtdicke 0.1 mm, from Merck.

Instruments and Measurements——HPLC equipment was a product of Waters Associates, ALC/GPC 204 type (with a model 6000A pump, a U6K universal injector and a model 440UV (254 nm) detector), with a μBondapak C_{18} column for analysis (3.9 mm i.d. × 30 cm) and for preparative purposes (7.8 mm i.d. × 30 cm). Melting points were determined on a melting point apparatus (type MP-1, Yamato Scientific Co.) and are uncorrected. NMR spectra were taken on a JEOL LNM-FX-100 (100 MHz), using D_2O as the solvent and TMS as the external standard. MIC or DIC was dissolved in D_2O (2% solution in D_2O) by warming, and NMR spectra were taken immediately. Optical rotation was determined on a DIP-181 digital polarimeter, JASCO. Ultraviolet (UV) spectra were determined on a Hitachi type 100-50 double-beam spectrophotometer with a type 200 recorder. Elementary analyses were performed at the analytical laboratory of Hoshi College of Pharmacy.

Examination of the Optimal Conditions for Iodination of Carnosine by HPLC——Carnosine (27 mg, 1.2×10^{-4} mol) was dissolved in 0.5 N NaOH (7.5 ml). To this solution, 0.6 ml (0.6×10^{-4} mol) of 0.1 M I_2–EtOH was added with stirring over a period of 10 min under ice-cooling, and stirring was continued for a further 5 min. The same procedure was repeated 5 more times; the final amount of I_2 added was 3.6×10^{-4} mol, a 3-fold excess over carnosine.

An aliquot of 50 μl was withdrawn from the reaction mixture at each step, and 50 μl of 0.5 N HCl and 0.4 ml of H_2O were added. The solution was filtered through a 0.45 μm filter for HPLC. Using a solution of 0.005 M NaH_2PO_4/MeOH (80/20) as the mobile phase, 10 μl of the sample was developed at a flow rate of 1.8 ml/min. The detector sensitivity was set at 0.01 AUFS for MIC and 0.05 AUFS for DIC.

Established Method for the Synthesis of MIC——L-Carnosine ($[\alpha]_D^{27}$ +21.2° (c=1.5, H_2O)), 271 mg, was dissolved in 75 ml of 0.5 N NaOH, and 12 ml of the iodinating reagent (0.1 M I_2–EtOH) was added dropwise with stirring over 1 h under ice-cooling. After additional stirring for 30 min, the pH of the mixture was adjusted to 6.5 with conc. HCl, and the mixture was concentrated under reduced pressure to 15 ml. The concentrate was passed through a SEP-PAK C_{18} cartridge previously treated with MeOH and H_2O, and then the cartridge was washed with 5 ml of H_2O 4 times, and the washings were combined with the eluate. The SEP-PAK-treated solution was concentrated under reduced pressure to 9 ml, and passed through a 0.45 μm filter. The sample thus obtained was subjected to HPLC in 1.8 ml portions × 5 (column: μBondapak C_{18} (7.8 mm i.d. × 30 cm); eluent, H_2O : MeOH = 90 : 10; flow rate, 5.0 ml/min; detector sensitivity, 2.0 AUFS) to isolate MIC. The isolated MIC fraction was concentrated to 4 ml under reduced pressure, and the concentrate was allowed to stand at low temperature overnight. Needle-like white crystals of MIC were collected by filtration and washed with a small amount of water. Yield was 247 mg. The filtrate was further concentrated and 55 mg of crystals was obtained as a second crop. Overall yield was 302 mg (72%). Melting point, 216—218 °C (dec.). UV $\lambda_{max}^{H_2O}$ nm (ε): 188 (15105). ^{1}H-NMR (2% solution in D_2O) δ: 7.68 (1H, S). $[\alpha]_D^{27}$ +8.8° (c=1.5, 1 N HCl). *Anal.* Calcd for $C_9H_{13}IN_4O_3$: C, 30.70; H, 3.72; N, 15.91. Found: C, 30.57; H, 3.70; N, 15.86.

References and Notes

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