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Synthesis and analysis of oxidation and carbonyl condensation compounds of tryptophan

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ABSTRACT

Modified methods of synthesizing the oxidative degradation products N-formylkynurenine, oxyindolylalanine diastereomers and dioxyindolylalanine diastereomers (DiOia) and the carbonyl condensation products 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and 1-pentyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid are described. These methods produce compounds with purities sufficiently high to allow them to be used as reference substances for analytical purposes and as samples for toxicological investigations. The obtained substances were characterized by ^{1}H-NMR, ^{13}C-NMR, IR and UV spectroscopy. The purities of the substances were verified by RP-HPLC and UV detection. An RP-HPLC method was developed which allowed the separation of the synthesized products, 5-hydroxytryptophan, 3-hydroxykynurenine and tryptophan. As an application, a sample of eosinophilia–myalgia syndrome (EMS)-related tryptophan was examined. Low contents of the oxidation products were found, together with the known peaks A–E.

INTRODUCTION

The essential amino acid tryptophan (Trp) is exceptional in its diversity of biological functions. In particular, it is the precursor of the neurotransmitter serotonin. The formation of Trp-derived antinutritional and potentially toxic compounds occurs during processing and storage of food and feedstuffs (Fig. 1). Elucidation of the degradation conditions is important for the evaluation of pharmaceuticals, food and feedstuffs.

The *oxidative degradation* of Trp reduces the nutritional value of proteins. *Carbonyl condensation* reactions of Trp with aliphatic aldehydes (Pictet–Spengler reaction) produce 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (THCC), which have neurophysiological activity [1,2].

The occurrence of eosinophilia–myalgia syndrome (EMS; generalized myalgia and increased eosinophil count) in 1989 was correlated to the intake of L-Trp as antidepressant which contained 0.01% 1,1′-ethylidenebis(L-tryptophan). This L-Trp dimer was determined as the causative agent of this autoimmune disease, which afflicted 1500 people [3] and caused 27 fatalities in the USA [4]. Hitherto, most investigations have focused on the decrease in Trp or the formation of only single degradation products.

The aims of our studies were:

(1) The synthesis of high-purity compounds for use as reference substances.

(2) The development of an HPLC separation of the major known Trp oxidation and carbonyl condensation derivatives that might be formed from free or peptide-bound Trp.

MATERIALS AND METHODS

Chemicals

3-Hydroxykynurenine (3-OH-Kyn, Sigma), kynurenine (Kyn, Fluka) and 5-hydroxytryptophan (5-OH-Trp, Merck) were purchased

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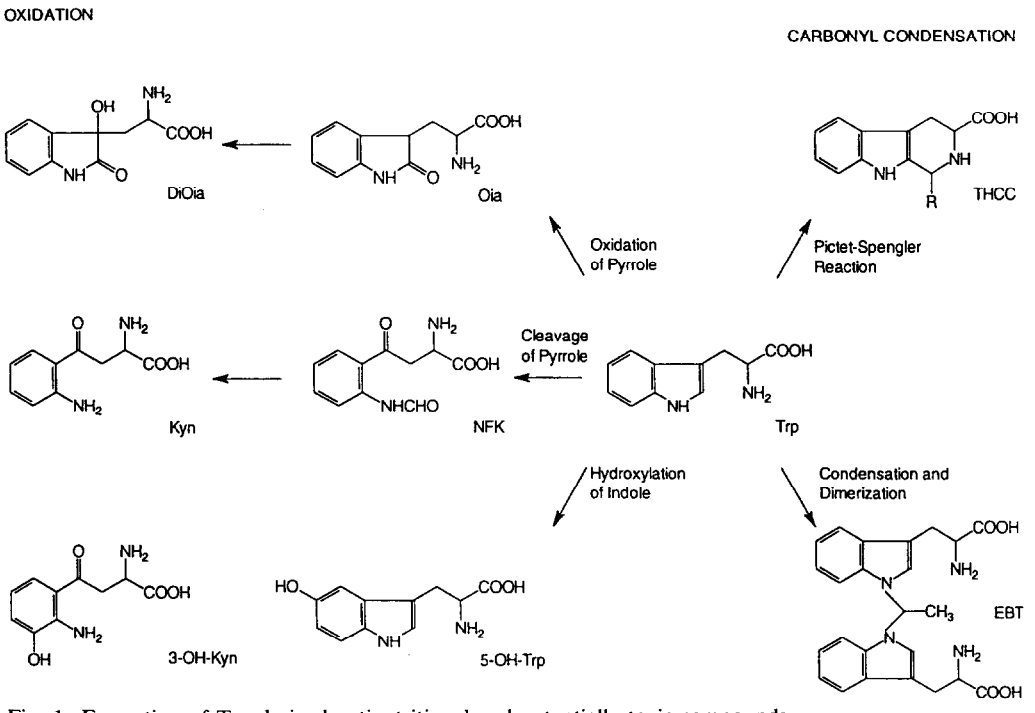

Fig. 1. Formation of Trp-derived antinutritional and potentially toxic compounds.

from commercial distributors. L-Tryptophan was donated by Degussa (Hanau, Germany). A sample of an EMS case-related L-Trp lot was donated by the Centers for Disease Control (Atlanta, GA, USA).

Synthesis of reference substances

Oxindolylalanine was synthesized as described in refs. 5 and 6 by treating 5 g of Trp suspended in 25 ml of acetic acid with a mixture of 4.5 ml of dimethylsulphoxide and 12.5 ml of concentrated HCl. The mixture was stirred for 70 min at room temperature. Oxyindolylalanine diastereomers (Oia) precipitate after adjusting the pH to 5.8 (isoelectric point), yielding 4.05 g of Oia (75%). A higher purity was obtained by ion-exchange chromatography. Conditions were as follows: column, DOWEX 1 × 2-200 (100 × 26 mm I.D.); eluent A, water (200 ml); eluent B, 2 *M* acetic acid (200 ml); linear gradient 0–100% eluent B; flow-rate 2 ml/min.

^{1}H-NMR (400 MHz, ^2H_2O): Diastereomer A: 7.47 (mc, 2H, H-5 and H-6), 7.29 (mc, 1H, H-4 or H-7), 7.14 (mc, 1H, H-4 or H-7), 4.62 (t, 1H, α-H), 2.78 (dd, 1H, β-H_a), 2.54 (dd, 1H, β-H_b). $J_{\alpha,\beta} = 6.4$, $J_{\beta a,\beta b} = 15.1$ Hz. Diastereomer B: 7.47 (mc, 2H, H-5 and H-6), 7.29 (mc, 1H, H-4 or H-7), 7.14 (mc, 1H, H-4 or H-7), 4.50 (t, 1H, α-H), 2.83 (dd, 1H, β-H_a), 2.45 (dd, 1H, β-H_b). $J_{\alpha,\beta} = 7.1$, $J_{\beta a,\beta b} = 15.1$ Hz. The H-3 protons were exchanged by deuterium and therefore they were not detected.

Dioxindolylalanine was prepared by aerating a solution of Oia adjusted to pH 10–12 with triethylamine [6] for several hours. The product was isolated by ion-exchange chromatography (conditions as above), followed by the removal of the solvent yielding a mixture of diastereomers in a 70:30 ratio.

^{1}H-NMR (400 MHz, ^2H_2O): Diastereomer A, 70%: 7.63 (d, 1H, H-4 or H-7), 7.52 (t, 1H, H-5 or H-6), 7.32 (t, 1H, H-5 or H-6), 7.18 (d, 1H, H-4 or H-7), 4.33 (dd, 1H, α-H), 2.62 (dd, 1H, β-H_a), 2.40 (dd, 1H, β-H_b). $J_{4,5} = 7.4$, $J_{5,6} = 7.4$, $J_{6,7} = 7.4$, $J_{\alpha,\beta a} = 3.7$, $J_{\alpha,\beta b} = 9.6$, $J_{\beta a,\beta b} = 15.7$ Hz. Diastereomer B, 30%: 7.58 (d, 1H, H-4 or H-7), 7.50 (t, 1H, H-5 or H-6), 7.29 (t, 1H, H-5 or H-6), 7.15 (d, 1H, H-4 or H-7), 4.43 (dd,

1H, α-H), 2.60 (dd, 1H, β-H_a), 2.46 (dd, 1H, β-H_b). $J_{4,5}=7.4$, $J_{5,6}=7.4$, $J_{6,7}=7.4$, $J_{\alpha,\beta a}=3.4$, $J_{\alpha,\beta b}=9.8$, $J_{\beta a,\beta b}=15.7$ Hz.

N-Formylkynurenine (NFK) was synthesized by formylation of a solution of 0.75 g of Kyn in 1.65 ml of formic acid using a mixture of 0.72 ml of formic acid and 0.36 ml of acetic anhydride [7]. After 2 h the mixture was poured into diethyl ether and the precipitate was isolated and recrystallized from ethanol yielding 0.56 g of NFK (66%).

^{1}H-NMR (250 MHz, ^2H_2O): 8.64 (bs, 1H, Aryl-NH), 8.19 (s, 1H, Formyl-H), 8.02 (d, 1H, H-3), 7.63 (t, 1H, H-5), 7.30 (t, 1H, H-4), 7.24 (d, 1H, H-6), 4.46 (t, 1H, α-H), 3.88 (d, 2H, β-H). $J_{3,4}=8.0$, $J_{3,5}=1.0$, $J_{4,5}=7.7$, $J_{4,6}=1.0$, $J_{5,6}=8.2$, $J_{\alpha,\beta}=4.4$ Hz.

1-Methyl- (MeTHCC) and *1-pentyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (*PeTHCC*)* [8,9] were obtained by acid-catalysed cyclization of Trp with acetaldehyde and hexanal, respectively. For MeTHCC 2 g of Trp were suspended in 9 ml of 0.00625 *M* H_2SO_4 and 2 ml of acetaldehyde were added. The mixture was stirred for 6 h at room temperature, then the precipitate was filtered and recrystallized from water yielding 1.4 g of MeTHCC (62%). *cis*-Isomer can be separated by fractionated crystallization from water.

^{1}H-NMR (400 MHz, MeO^{2}H + 1% TFA): 7.95 (bs, 1H, H-9), 7.58 (d, 1H, H-5), 7.46 (d, 1H, H-8), 7.25 (t, 1H, H-7), 7.16 (t, 1H, H-6), 4.88 (q, 1H, H-1), 4.49 (dd, 1H, H-3), 3.56 (ddd, 1H, H-4a), 3.22 (ddd, 1H, H-4b), 1.87 (d, 3H, 1-Me). $J_{1,1\text{-Me}}=6.6$, $J_{1,4a}=1.4$, $J_{1,4b}=1.5$, $J_{3,4a}=5.7$, $J_{3,4b}=12.2$, $J_{4a,4b}=16.4$, $J_{5,6}=8.1$, $J_{5,7}=1.4$, $J_{6,7}=7.7$, $J_{6,8}=1.0$, $J_{7,8}=8.0$ Hz.

For PeTHCC 1 g of Trp and 0.67 ml of hexanal were suspended in 20 ml of 0.0125 *M* H_2SO_4 and 10 ml of methanol. The mixture was heated for 4 h and than neutralized with 25% ammonia. The precipitate was collected, yielding 0.35 g of PeTHCC (25%).

^{1}H-NMR (400 MHz, MeO^{2}H + 1% TFA): 7.91 (bs, 1H, H-9), 7.53 (d, 1H, H-5), 7.40 (d, 1H, H-8), 7.15 (t, 1H, H-7), 7.07 (t, 1H, H-6), 4.80 (q, 1H, H-1), 4.40 (dd, 1H, H-3), 3.55 (ddd, 1H, H-4a), 3.20 (ddd, 1H, H-4b), 2.40 (mc, 1H, H-10a), 2.05 (mc, 1H, H-10b), 1.65 (mc, 2H, H-11), 1.50 (mc, 4H, H-12 and H-13), 1.00 (t, 3H, H-14). $J_{1,4a}=1.2$, $J_{1,4b}=2.5$, $J_{1,10a}=1.7$, $J_{1,10b}=7.2$, $J_{3,4a}=5.1$, $J_{3,4b}=12.3$, $J_{4a,4b}=16.4$, $J_{5,6}=7.8$, $J_{5,7}=1.0$, $J_{6,7}=7.5$, $J_{6,8}=1.0$, $J_{7,8}=8.2$, $J_{13,14}=7.1$ Hz.

The structures of the synthesized reference substances were confirmed by UV, IR, ^{1}H and ^{13}C-NMR spectroscopy. Their purity as determined by RP-HPLC and UV detection (260 nm) was higher than 98%.

The synthesis of *1,1'-ethylidenebis(L-tryptophan)* according to the only published method, that of Smith *et al.* [10], failed. The formation conditions of EBT and a new simple procedure for synthesis will be published shortly.

RESULTS AND DISCUSSION

The desired compounds were obtained in sufficient amounts by using previously published methods of synthesis, which were varied and optimized in order to yield reference substances in high purities. With these compounds it was possible to establish an HPLC separation method (for conditions, see Table I) giving high resolution of all substances of interest (Figs. 2 and 3). Using this method it was possible to determine the oxidation and carbonyl condensation compounds of Trp in a single-step analysis.

In an EMS-related Trp sample (Figs. 4 and 5), we identified the oxidation products dioxindolylalanine diastereomers (DiOia), Oia, Kyn and, confirming the findings of Toyo'oka *et al.* [11], 5-OH-Trp. NFK could not be identified in this Trp sample. In addition, we detected MeTHCC and the known peaks A–E as described in ref. 12. The identification of the detected compounds was achieved by standard addition of synthesized authentic material as well as by comparison of UV–VIS spectra (200–400 nm) obtained by diode-array detection.

The concentrations of DiOia (25 ppm for each diastereomer), Oia (90 ppm in total for both diastereomers), Kyn (20 ppm) and 5-OH-Trp (120 ppm) were determined by external standard and related to Trp. The concentrations of MeTHCC (40 ppm) and peak E (110 ppm) were similar to those found by Müller *et al.* [12].

In comparison with the described material, a

TABLE I

HPLC CONDITIONS

Stationary phase		Nucleosil 120 3-C_{18}, 250 × 4 mm		
Mobile phase		0.1% TFA	MeOH	MeCN
Gradient	0 min	95%	5%	0%
	−10 min	86%	14%	0%
	−30 min	46%	14%	40%
Flow		1 ml/min		
Temperature		35°C		
Injection volume		20 μl		
Detection		UV, 260 nm		
		Fluorescence, excitation 290 nm, emission 356 nm		
		Diode-array detection, 260 nm; UV–VIS spectra, 200–400 nm		
Standards		10 μg/ml (Oia, DiOia 20 μg/ml)		

control sample of Trp donated by Degussa was similarly analysed. The pattern of the detected oxidation products did not differ (Figs. 6 and 7). Their amounts, however, were smaller than in the EMS-related sample, in particular the concentration of 5-OH-Trp (<5 ppm) showed a considerable difference. Furthermore, peak E was absent in this Trp sample.

During the RP-HPLC monitoring of the syntheses much information about the conditions of formation and the stability of the described compounds was collected. This information will influence in further analytical work:

(1) NFK is deformylated with the formation of Kyn in alkaline and acidic solutions.

(2) Oia is oxidized in alkaline solutions of pH > 12, mainly to Kyn, and at pH 10–12 to DiOia.

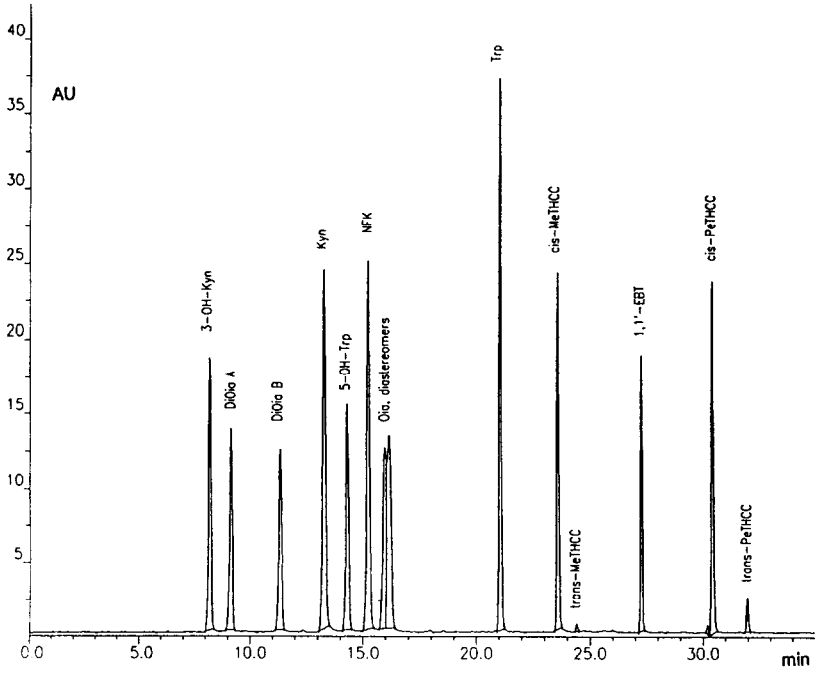

Fig. 2. HPLC chromatogram of a standard mixture (UV detection).

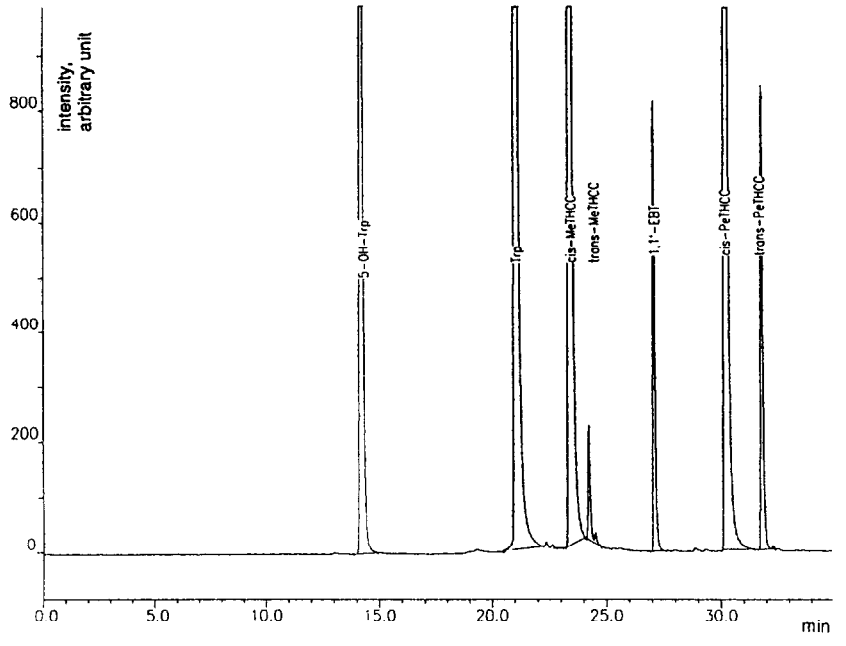

Fig. 3. HPLC chromatogram of a standard mixture (fluorescence detection).

These results imply that the formation of different oxidation products depends not on the oxidizing agent [O_2, $h * \nu_{(sens)}$, irradiation, peroxides] but on the pH value. It seems that the formation and degradation of EBT is fundamentally dependent on the reaction conditions, espe-

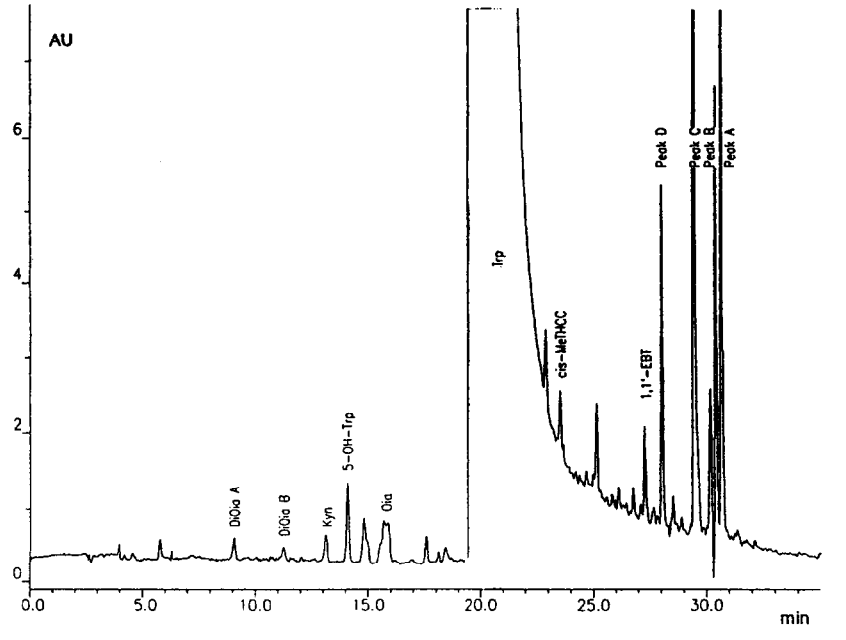

Fig. 4. HPLC chromatogram of an EMS-related Trp sample (UV detection), overloaded, 10 mg/ml.

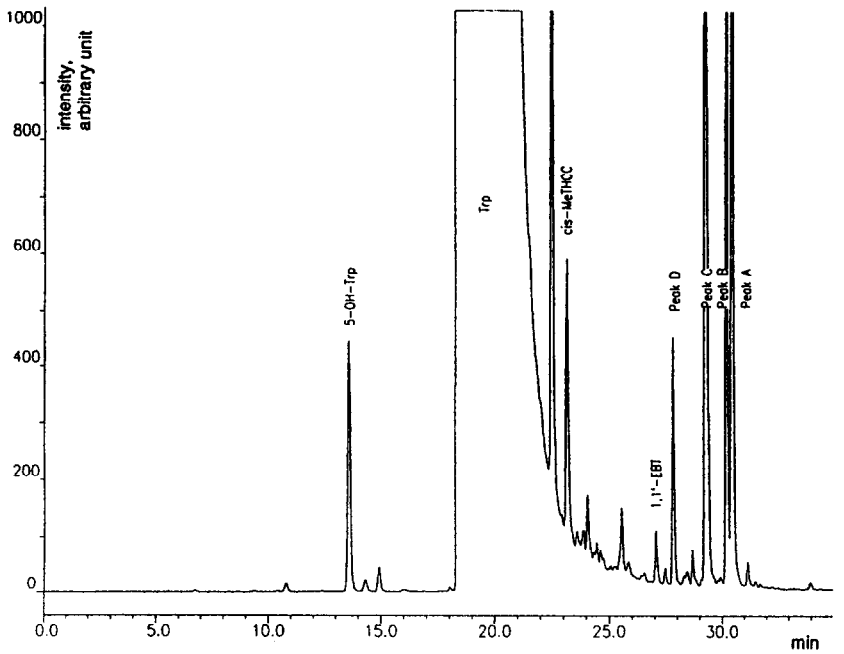

Fig. 5. HPLC chromatogram of an EMS-related Trp sample (fluorescence detection), overloaded, 10 mg/ml.

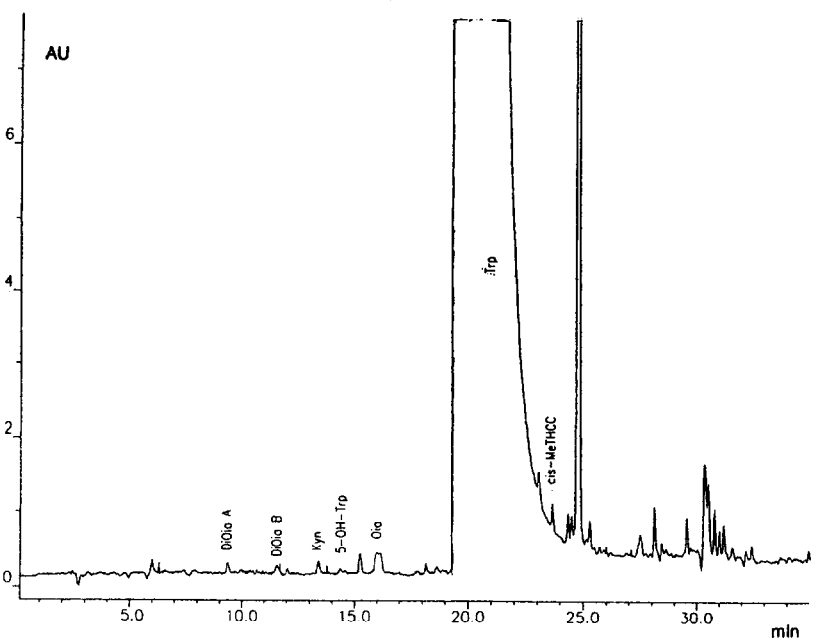

Fig. 6. HPLC chromatogram of a Trp control sample (UV detection), overloaded, 10 mg/ml.

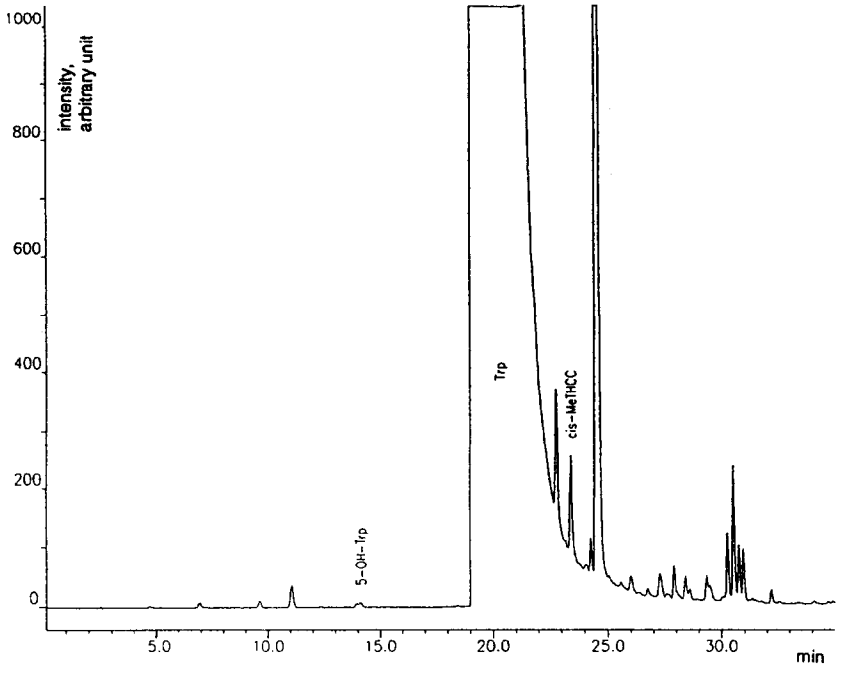

Fig. 7. HPLC chromatogram of a Trp control sample (fluorescence detection), overloaded, 10 mg/ml.

cially temperature and acid concentration. By varying these parameters it was possible to obtain EBT by a simple procedure.

FUTURE PROSPECTS

(1) Further examination of the conditions for the formation and degradation of EBT.

(2) Synthesis and determination of the stability of further oxidation products, for example 5-OH-Trp analogues.

(3) Determination of degradation products of peptide-bound Trp in different model systems (oxidation and carbonyl condensation model systems).

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