Derivatives of 3,4-Dihydro-β-carboline-3-carboxylic Acid from C-Terminal Tryptophan-Containing Peptides. Synthesis and Properties

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C-Terminal tryptophan containing peptides have been converted into 1-peptidyl-3,4-dihydro-β-carboline-3-carboxylic acids by acid catalyzed isomerization of their azlactone derivatives. The main properties of these abnormal peptides are a strong absorption around 360 nm an intense yellow fluorescence and ability to generate reactive carbanions when dissolved in basic media. This tryptophan conversion takes place with high yield and is of general applicability.

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Structures containing the β-carboline [1] ring have recently aroused considerable interest in biochemistry and neuropharmacology [2-4]. The β-carboline derivatives are structurally related to tryptophan which often constitutes the principal starting material both for their chemical and biological synthesis [1].

The rapidly growing interest in the β-carboline derivatives is however suffering from the limitation imposed by the few chemical methods presently available for their preparation.

This work is part of a program on synthesis and reactivity of the 3,4-dihydro-β-carboline containing peptides [5-7], especially focused on their ability to generate ambident carbanions by basic treatment under mild conditions [8]. These carbanions constitute transient species in the oxidation route to fully aromatic β-carboline derivatives as well as reactive intermediates for synthesizing complex molecules containing the β-carboline moiety.

We report in this paper that peptides containing tryptophan as *C*-terminal residue (**Ie**) and *N*-α-acyltryptophan derivatives **Ia, Ic, Id** can be easily converted into 1-substituted-3,4-dihydro-β-carboline-3-carboxylic acid (**III**) by anhydrous acids provided they are activated as azlactones **II**.

The acid-base equilibria of the 3,4-dihydro-β-carboline-3-carboxylic acids were investigated and found to be dependent on the C-1 substituent.

EXPERIMENTAL

Materials and Methods.

Apparatus.

The hplc analysis were carried out using an ultrasphere ODS (Cl8) column (0.4 × 25 cm) and eluting with by acetonitrile:buffer, *p*H 2.6 (800 mg phosphoric acid and 280 mg triethylamine per liter) as eluent at a constant flow rate of 1 mℓ/mn. Amino acid analysis was automatically performed on a 119 Beckman amino acid analyser. The nmr spectra were recorded on a CFT 20 Varian spectrometer. The uv-visible absorption spectra were recorded on a Varian electroscan spectrometer.

Materials.

N-α-Acetyltryptophan, *N*-α-propionyltryptophan, *N*-α-phenylacetyltryptophan were obtained by reacting tryptophan with suitable acid anhydride or acyl chloride by the usual Schotten-Baumann procedure. Crystalline propionyl tryptophan azlactone was prepared from tryptophan and propionic anhydride. *C*-Terminal tryptophan containing peptides were synthetized by coupling *N*-blocked amino acids and peptides with the methyl ester of tryptophan *via* *N*-hydroxysuccinimide esters [9].

Acylated peptides free acids were obtained by saponification of the corresponding peptide esters. The purity of tryptophan containing peptides was checked by hplc analysis and amino acid analysis after hydrolysis catalyzed by methane sulphonic acid [10] to prevent tryptophan destruction.

Tryptophan bounded enkephalin was a generous gift of Dr. Moroder (Max-Planck Institut, Martinsreed GFR).

1(Cbz-aminomethyl)-3,4-dihydro-β-carboline-3-carboxylic Acid.

Cbz-glycyltryptophan (2.4 g) was added to a solution of dicyclohexylcarbodiimide (1.2 g) in dichloroethane (50 mℓ). After 3 hours of stirring at 20° the dicyclohexylurea was filtered off and the filtrate was directly collected into a round bottom flask containing 50 mℓ of trifluoroacetic acid. After 15 minutes at 20° the solvent was evaporated under reduced pressure and the residue freed from acid under vacuum over potassium hydroxide pellets. 1-(Cbz-aminomethyl)-3,4-dihydro-β-carboline-3-carboxylic acid crystallized as trifluoroacetate by trituration in ethyl ether, yield, 80%. Recrystallization was effected from methanol/ethyl ether, mp 148°.

Anal. Calcd. for $C_{22}H_{20}F_3N_3O_6$: C, 55.11; H, 4.17; N, 8.76. Found: C, 55.10; H, 4.25; N, 8.73.

1(Cbz-glycylaminomethyl)-3,4-dihydro-β-carboline-3-carboxylic Acid.

Cbz-glycylglycyltryptophan was treated as described for cbz-glycyltryptophan. It was obtained in 81% yield, mp 192°.

Anal. Calcd. for $C_{24}H_{23}F_3N_4O_7$: C, 53.73; H, 4.29; N, 10.46. Found: C, 53.80; H, 4.41; N, 10.44.

3,4-Dihydro-β-carboline Derivative of Enkephalin.

Boc (*Ot*-butyl)tyrosyl glycyl glycyl phenylalanyl leucyl tryptophan (50 mg) was dissolved in 1,2-dichloroethane (0.8 mℓ) and treated with dicyclohexylcarbodiimide (100 mg) dissolved in 1,2-dichloroethane (0.2 mℓ). After 3 hours at 20°, trifluoroacetic acid (1 mℓ) was added to the reaction mixture and the formation of the 3,4-dihydro-β-carboline ring spectrophotometrically followed at 360 nm on suitable aliquots diluted in methanol. A maximum formation of 3,4-dihydro-β-carboline occurs within 10 minutes and the reaction mixture was allowed to stand for an additional hour for removing the *t*-butyl protecting groups. The solvent was evaporated under reduced pressure and the residue taken up with 1% formic acid in water. The soluble material was introduced into a column of Sephadex LH 20 (40 × 1.5 cm, flow rate 8 mℓ/hour) equilibrated with the same solvent (Figure 1). Fractions (4 mℓ) containing 3,4-dihydro-β-carboline derivatives (detected by fluorescence and absorbance at 360 nm) were combined and lyophilized. The purified product yielded a single ninhydrin-positive fluorescent spot by paper chromatography (in butanol:acetic acid:water, 40/10/50).

Amino acid analysis (4 *M* methanesulfonic acid containing 0.2% tryptamine; 110°, 24 hours) yielded (Phe = 1):Tyr (0.97); Gly (2.10): Phe (1.00).

Micro-scale General Procedure.

N-α-Acetyltryptophan or *C*-terminal tryptophan containing peptides (50 μmoles) were dissolved or suspended in 2 mℓ of 1,2-dichloroethane (dichloromethane or ethyl acetate can be used) containing dicyclohexylcarbodiimide (50 μmoles/mℓ). After 3 hours at 20° under vigourous shaking (some insoluble tryptophan derivatives dissolved during their conversion into azlactones while dicyclohexylurea separated) 2 mℓ of trifluoroacetic acid were added while the solution gradually assumed a yellow-orange colour. The course of the reaction was followed spectrophotometrically (360 nm) on suitable aliquots diluted with methanol.

Reaction of **IIIb** with 2-Formylbenzensulfonic Acid.

D,L-1-Methyl-3,4-dihydro-β-carboline-3-carboxylic acid (1.15 g) and 2-formylbenzensulfonic acid sodium salt (2.08 g) were dissolved in 2 *N* sodium hydroxide (20 mℓ). After 20 minutes at 20° the 1-methyl-3,4-dihydro-β-carboline-3-carboxylic acid completely disappeared. The solution was acidified by concentrated hydrochloric acid while an orange coloured solid mass separated. The solid material was washed with water, dissolved in methanol and precipitated by ethyl ether and dried, yield, 1.5 g; uv (p*H* 7): λ max 360 nm, ε 13300 M^{-1} cm^{-1}.

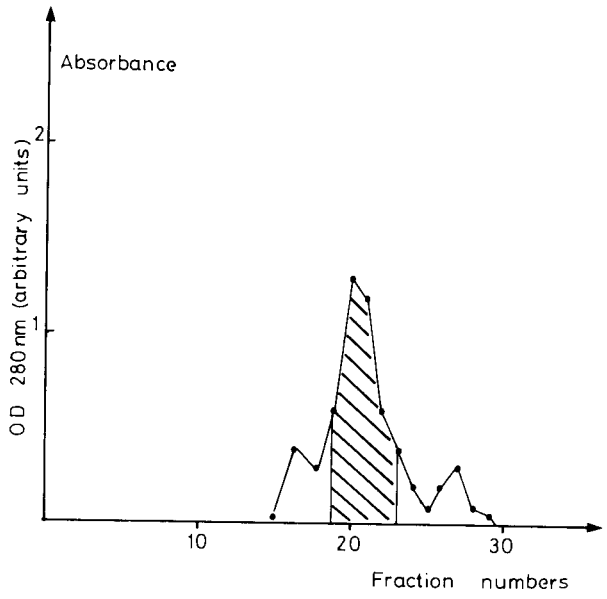


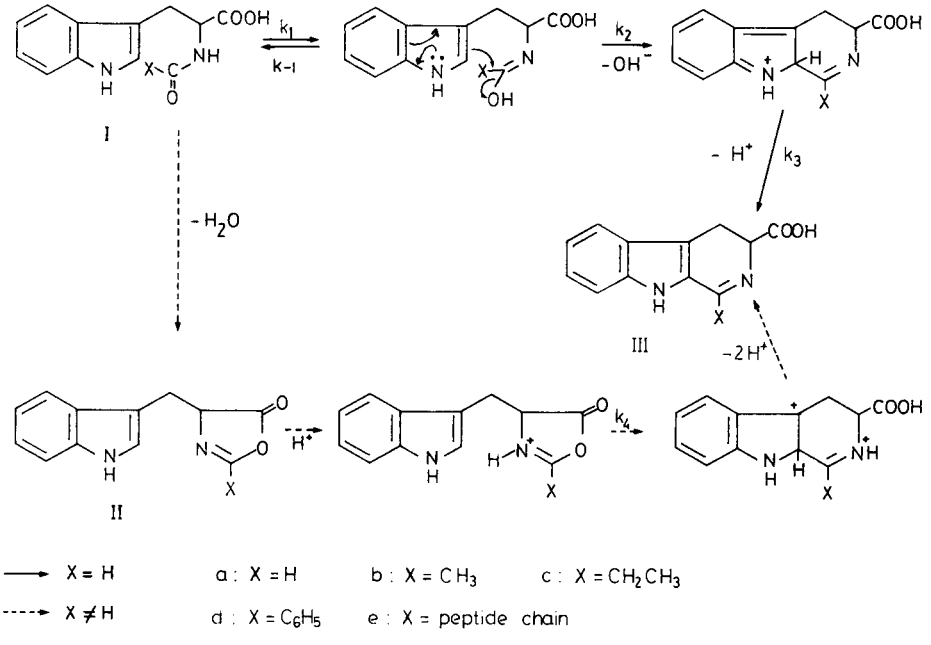
Figure 1. Column chromatography of 1-substituted-3,4-dihydro-β-carboline-3-carboxylic acid structurally related with enkephalin.

Anal. Calcd. for $C_{20}H_{18}N_2O_6S$: C, 57.96; H, 4.38; N, 6.76; S, 7.74. Found: C, 57.84; H, 4.44; N, 6.86; S, 7.50.

A parallel run was performed using the D-enantiomer of the β-carboline derivative and the reaction was followed polarimetrically.

Time (minutes)	0	5	10	20
α (λ = 546)	−575	−589	−588	−584

Scheme 1



After 20 minutes **IIIb** completely disappeared. Isolation of the reaction product was however better performed in the case of racemic **IIIb** as previously described.

Results.

A. Synthesis.

Tryptophan containing peptides **Ie** are potential parents of 3,4-dihydro-β-carboline derivatives **IIIe** as shown in Scheme 1.

A direct cyclisation of **I** into **III** easily occurs only in the case of **Ia** [5,11]. In all the other cases, the reaction does not occur directly or it takes place under drastic conditions which are incompatible with the stability of a peptide chain as well as of a number of organic functional groups. On the contrary, simple N α-acyltryptophan and peptides containing tryptophan at *C*-terminal position can be easily converted into 1-substituted-3,4-dihydro-β-carboline-3-carboxylic acids **III** by acid catalyzed isomerization of their azlactone derivatives **II**.

Figure 2 compares the kinetic of 1-ethyl-3,4-dihydro-β-carboline-3-carboxylic acid (**IIIc**) formation from *N*-α-propionyltryptophan (**Ic**) and its azlactone derivative **IIc**. Propionyltryptophanazlactone is one of the few members of the azlactone family which can be secured in pure crystalline form [12].

Figure 2 shows that the formation of **IIIc** from **IIc** is several times (about 100) faster than from **Ic**, thus on the basis of Scheme 1, k_4 should be greater than k_1.

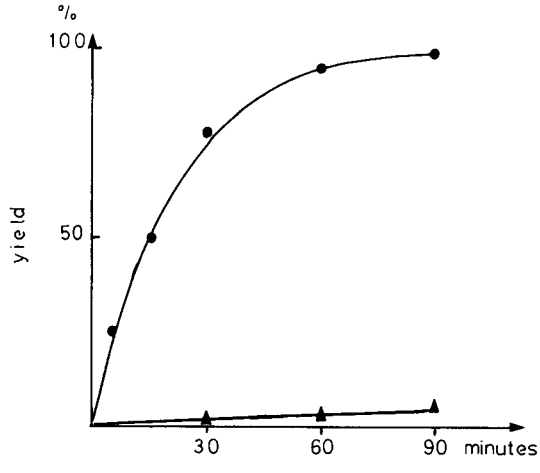


Figure 2. Rate of formation of 1-ethyl-3,4-dihydro-β-carboline-3-carboxylic acid in trifluoroacetic acid at 50° from •-• 10^{-2} *M* propionyltryptophan azlactone, ▲-▲ 10^{-2} *M* propionyltryptophan.

The rates of dihydro-β-carboline formation from other acyltryptophanazlactones are approximately the same as that from **IIc** (Table 1).

Table 1

Yields of 3,4-Dihydro-β-carboline formation from *N*-α-Acyltryptophan Derivatives

3,4-Dihydro-β-carboline-3-carboxylic acid from:	maximum yield %	time mn	T°C
acetyl-Trp	88	60	50
propionyl-Trp	92	90	50
phenyl-acetyl-Trp	60	45	50
Cbz-Gly-Trp	86	10	20
Cbz-Phe-Trp	70	20	20
Cbz-Trp-Trp	67	20	20
Cbz-Ala-Trp	79	10	20
Cbz-Gly-Gly-Trp	88	10	20
Cbz-Gly-Phe-Trp	58	20	20
Cbz-Phe-Gly-Gly-Trp	73	10	20
Boc(OBz)-Tyr-Gly-Gly-Trp	75	10	20
Boc(*O*-*t*-Bu)-Tyr-Gly-Gly-Phe-Leu-Trp	70	20	20

Several *N*-protected peptides containing tryptophan as the *C*-terminal residue (**Ie**) were converted into azlactones **II** by *N,N'*-dicyclohexylcarbodiimide in an inert organic solvent [13] and then isomerized to the corresponding dihydro-β-carboline derivatives **III** by trifluoroacetic acid.

3,4-Dihydro-β-carbolines absorb intensily around 350 nm thus allowing their determination by spectrophotometric analysis [6].

Overall yields of tryptophan containing peptide → β-carboline conversion together with the reaction conditions are reported in Table 1. The rate of formation of **III** from **II** depends on the reactivity of the azlactone ring which is correlated to the nature of the X-substituent (Scheme 1). Reaction conditions to reach maximum yield of **III** from **II** range from few minutes at 20° for peptides to 1.5 hour at 50° for *N*-α-acetyltryptophan.

The 3,4-dihydro-β-carboline structure of compounds listed in Table 1 has been established by comparison with authentic samples in the case of **IIIb** and **IIIc** [6].

D,L-1-(Cbz-aminomethyl)-3,4-dihydro-β-carboline-3-carboxylic acid and D,L-(Cbz-glycylaminomethyl)-3,4-dihydro-β-carboline-3-carboxylic acid were secured in pure crystalline form (see Experimental) and showed the characteristic spectral properties (absorbance [6] and fluorescence [7]) of the 1-substituted 3,4-dihydro-β-carbolines.

When peptides containing optically active amino acids were used, it was assumed that their azlactone derivatives were a mixture of diastereomers in which the *C*-terminal tryptophan residue was fully racemized [14]. The subsequent conversion into 3,4-dihydro-β-carboline derivatives also produced a mixture of diastereomers, thus hindering

their obtention in crystalline form. The conversion **I** → **II** → **III** (Scheme 1) in peptides takes place without cleavage of the peptide chain. An evidence for that is reported by using the peptide enkephalin with a tryptophan added to its *C*-terminal end. According to the described procedure this enkephalin homolog was converted into its 3,4-dihydro-β-carboline derivative and purified by column chromatography (Figure 1). Amino acid analysis of the 3,4-dihydro-β-carboline containing enkephalin yielded the expected amino acids with the exception of tryptophan and its adjacent amino acid, both involved in the β-carboline ring (Scheme 1).

Spectral properties of 3,4-dihydro-β-carboline containing peptides **IIIe** are mainly that of 1-substituted 3,4-dihydro-β-carbolines [6].

In particular they are characterized by a strong fluorescence around 520 nm in the neutral *p*H range [7] which makes them potentially useful substances in physiological studies involving active peptides.

They are also stable in acidic, neutral and slightly basic aqueous solutions and in common organic solvents as well.

When dissolved in water at *p*H values greater than 10, **IIIe** undergoes irreversible modifications which also depend on the nature of the 1-substituent. For example **IIIe** (X = Cbz-1-aminomethyl) rapidly reacts in basic media releasing benzylalcohol (Figure 3). The mechanism of the reaction presumably involves the initial formation of an ambident carbanion and its intramolecular participation in the hydrolysis of the urethan group as suggested in Scheme 2.

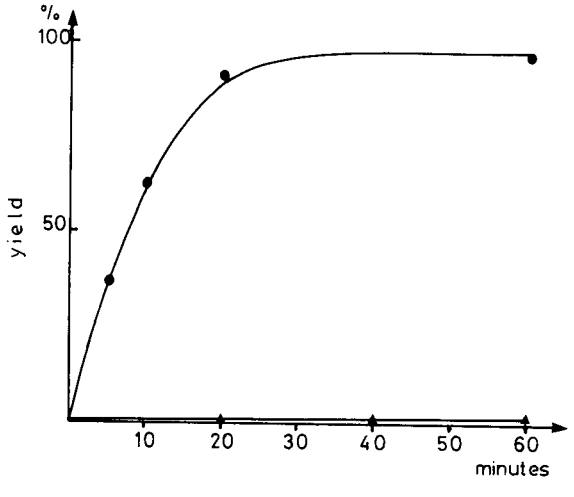


Figure 3. Base catalyzed release of benzyl alcohol from 1-(Cbz-aminomethyl)-3,4-dihydro-β-carboline-3-carboxylic acid (2.5×10^{-2} *M*) in 1 *N* sodium hydroxide (•-•) compared with that of Cbz-glycyltryptophan (▲-▲). Benzyl alcohol was determined by hplc analysis (see Experimental); K′ = 2.15.

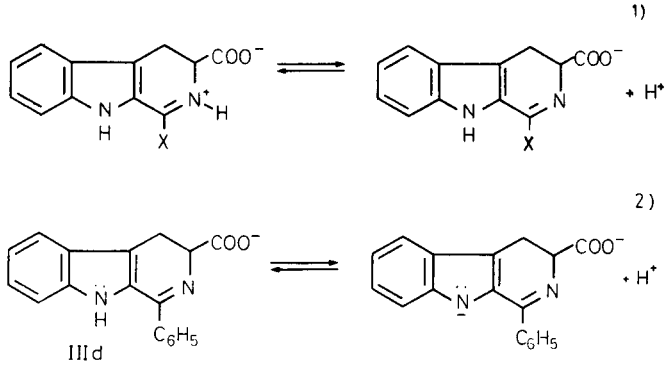
In order to confirm the proposed route, a comparative study of acid base properties was made on 3,4-dihydro-β-carboline-3-carboxylic acid (**III**) and its 1-phenyl, **IIId**, and 1-methyl, **IIIb**, derivatives as model compounds.

In neutral alkaline media **III** (HB) undergoes two successive acid base equilibria:

$$H_2B^+ \rightleftharpoons HB + H^+ \text{ and } HB \rightleftharpoons B^- + H^+$$

The uv spectral characteristics of compounds **IIIa**, **IIId** and **IIIb** in water/methanol (9:1 v/v) buffered solutions are reported in Table 2. For all compounds a first spectral change was observed in the *p*H range 7-11 corresponding to the equilibrium 1 (Scheme 3).

Scheme 3



For example, in the case of **IIIb**, the absorption band at 350 nm decreased with increasing *p*H, while a new band at 310 nm appeared. Spectral changes showed an isosbestic point at 325 nm indicating that a simple acid-base equilibrium was shifted. Analogous spectral changes observed for other model compounds enable us to calculate the p*K*a values listed in Table 3.

Using **IIId** as starting material, a second evolution was evidenced in alkaline media. With increasing *p*H, a decrease of the absorption band at 320 nm occurred, while a new band at 335 nm developed. Spectral changes showed an isosbestic point at 330 nm, indicating that a second acid base equilibrium was shifted [16]. Calculated p*K*a

Scheme 2

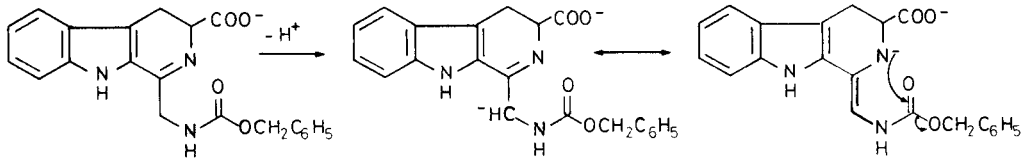


Table 2

Spectral Characteristics of 3,4-Dihydro-β-carboline-3-carboxylic Acids

Compounds	Form	λ nm	ε M^{-1}. cm^{-1}
	H_2B^+	240	8000
		360	13600
IIIa	HB	235	13000
		320	13000
	B^-		[a]
	H_2B^+	255	9600
		300	7400
		370	16000
IIId	HB	245	19000
		320	11000
	B^-	240	20000
		335	10000
	H_2B^+	245	8000
		350	14000
	HB	240 (sh)	10000
IIIb		310	11000
	B^-	245	16400
		325	11200
		400 (sh)	4000
	H_2B^+	250	8000
		355	13600
IIIe	HB	250 (sh)	11000
		315	11600
	B^-		[a]

[a] Non measured because the high instability of the compound (see Figure 3).

values are listed in Table 3. In the case of **IIId**, the p*K*a is obviously related to the acid-base equilibrium 2 (Scheme 3).

Moreover, in the case of **IIIb**, this ionization could involve either the NH indolic or the methyl group according to equilibria 3 and 3′ (Scheme 4).

Actually ionization takes place according to equilibrium 3′ which implies a CH_2-H cleavage and the formation of an ambident carbanion.

Table 3

p*K*a Values of 3,4-Dihydro-β-carboline Derivatives

Compound	Isosbestic point λ nm	wavelength of measurement λ nm	H_2B^+ HB at 20°C ε M^{-1} cm^{-1}		p*K*a₁
IIIa	395	360	13600	3000	8.10
IIIb	328	350	14000	3600	9.10
IIId	340	370	16000	3300	8.00
IIIe	333	355	13600	3000	7.80
			ε HB	ϵB^-	p*K*a₂
IIIb	320	310	11000	8000	14.80
IIId	330	320	11000	8600	15.20

The p*K*a values were determined from the relation: $pH = pKa + \log (A_{H_2B^+} - A) / (A - A_{HB})$ were $A_{H_2B^+}$ and A_{HB} are the respective absorbances of the protonated (immonium) and neutral (imine) species and A the absorbance of their mixture at a fixed *p*H.

The mobility of hydrogen at the 1′ position was confirmed by both hydrogen-deuterium exchange experiments and by reaction of the carbanion with a carbonyl compound.

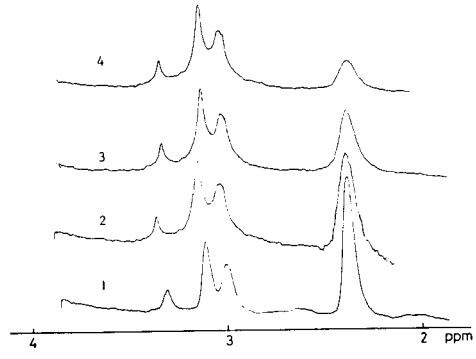


Figure 4. Hydrogen → deuterium exchange of 1-methyl substituent of **IIIb** in 1*N* sodium hydroxide in deuterium oxide; time (minutes): 0 (1), 10 (2), 15 (3), 20 (4).

Scheme 4

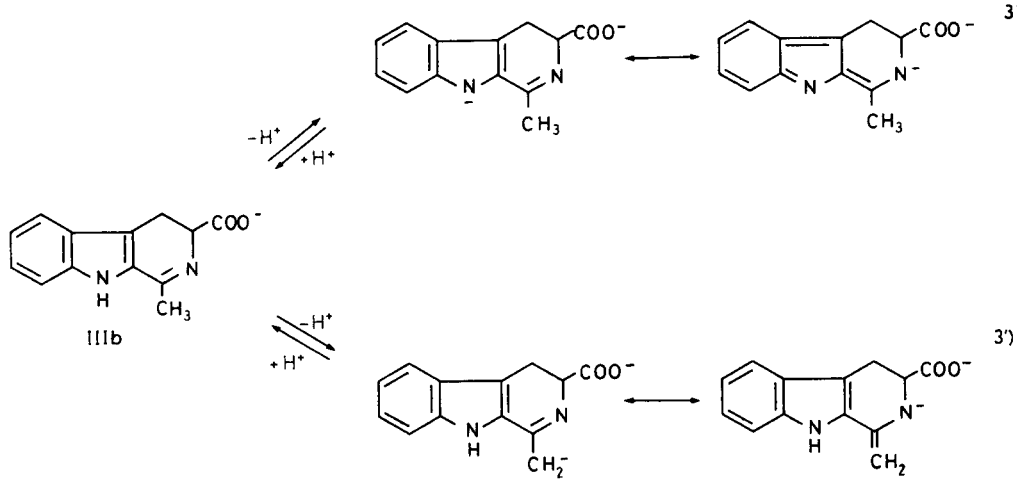


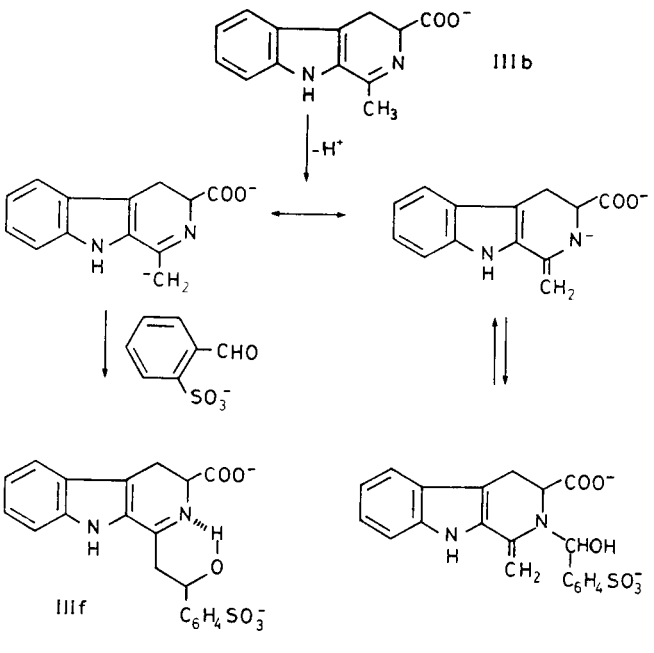
Figure 4 shows the upfield part of the high resolution ^1H NMR spectra of **IIIb** in deuterium oxide solution at *p*D 11, recorded at different times. The incorporation of deuterium into the molecule, which is generally a measure of the rate of carbanion formation concerns only the methyl at the 1 position.

The singlet at 2.35 ppm corresponding to the 1-methyl substituent loses indeed its intensity while the doublet at 3 and 3.12 ppm relative to the methylene at C-4 position remains unchanged. The 1-methyl group has been completely regenerated by a reverse exchange deuterium-hydrogen in water at *p*H value greater than 10. Spectrophotometric and chromatographic controls at the end of the exchange experiments showed no modification of the regenerated **IIIb** molecule when compared with the untreated one.

When an alkaline solution of racemic **IIIb** was treated with 2-formylbenzenesulfonic acid at room temperature, a rapid reaction took place which afforded as main product a yellow crystalline substance **IIIf** separated by acidification. The structure of **IIIf** (see Scheme 5) was proposed on the basis of the following evidence: the product analyzed for $C_{20}H_{18}N_2O_6S$ showed a yellow fluorescence and an absorption spectrum similar to that of 1-substituted 3,4-dihydro-β-carboline [6,7].

When submitted to paper electrophoresis at *p*H 1.8 the product behaved as a sulfonic acid [15].

Scheme 5



Using the D-enantiomer of **IIIb** as starting material (see Experimental) the reaction with 2-formylbenzenesulfonic acid occurred without loss of optical activity, indicating that no participation of the chiral carbon nor base catalyzed oxidation [8] took place. The nmr spectrum of the product showed no presence of the 1-methyl substituent of the parent **IIIb**.

Conclusion.

The results presented show that abnormal peptides belonging to the group of 1-substituted 3,4-dihydro-β-carbolines can be easily obtained from peptides containing tryptophan as the *C*-terminal residue. One of the main properties of these 3,4-dihydro-β-carboline derivatives is the acidity of the C-H proton at the 1′ position. The removal of this proton is probably facilitated by the neighbouring imino group which stabilizes the resulting negative charge. These ambident carbanions undergo addition reactions to carbonyl [17,18] or related unsaturated carbon sites, through both intermolecular and intramolecular mechanism.

Intramolecular reaction of carbanions arising from **IIIe** with neighbouring electrophilic groups on one side accounts for the instability of these abnormal peptides in basic media and on the other hand makes them potential intermediates for synthesizing more complex β-carboline derivatives.

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* To whom all correspondence should be addressed.

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