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Tetrahydroisoquinoline derivatives of enkephalins: synthesis and properties

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Abstract

Tetrahydroisoquinolines (TIQs) are endogenous alkaloid compounds deriving from the non-enzymatic Pictet–Spengler condensation of catecholamines with aldehydes. These compounds are able to unsettle catecholamines uptake and release from synaptosomes and have been detected in urine and in post-mortem Parkinsonian brains. We have obtained *in vitro*, by the reaction of dopa-enkephalin (dopa-Gly–Gly–Phe–Leu) with acetaldehyde in the presence of rameic ions, a TIQ derivative of Leu-enkephalin. The isolation and the recovery of the peptide was obtained by HPLC. The acid hydrolysis and the subsequent analysis of the peptide lysate by the Amino acid analyser clearly revealed the absence of dopa, while the electrospray ionisation mass spectrometry showed that the sequence of the enkephalin derivative was the following: 3-carboxy-salsolinol-Gly–Gly–Phe–Leu (TIQ-enkephalin). This compound was not a good substrate for microsomal aminopeptidase and pronase with respect to Leu-enkephalin. Tested in the binding assay, the TIQ-enkephalin exhibited a very poor affinity toward the enkephalin receptors. When the TIQ-enkephalin was incubated with tyrosinase or peroxidase/H_2O_2, the formation of TIQ-opio-melanins occurred.

Keywords: Enkephalins; Tetrahydroisoquinolines; Melanin; Opioid receptors; Parkinson's disease; Alcoholism

1. Introduction

In the past recent years, our group was engaged in studying enkephalin metabolism by the investigation of new routes different from those involving Enk cleavage and complete inactivation by peptidases. In particular Enks have been demonstrated—in the presence of hydrogen peroxide—as substrates of myeloperoxidase (E.C. 1.11.1.7) and cytochrome *c* [1,2], with the ensuing formation of *Enk dimers*. These dimeric Enks have been found to suppress the respiratory burst in polymorphonuclear leukocytes [3]. Enks and opioid peptides—exhibiting as a common chemical feature the presence of a tyrosine residue at the amino terminus—are also substrates *in vitro* of mushroom and sepia tyrosinase (catechol:oxidase E.C. 1.14.18.1) giving rise in a first instance to *dopaenkephalins* and *dopaopiopeptides* [4,5]. These compounds in the further steps of the reaction are converted into soluble melanins retaining the peptide moiety (*opiomelanins*) [6–9]. In the presence of cysteine the reaction between tyrosinase and Enks produces *cysteinyl-dopaenkephalins* which are able to yield—by POD (EC. 1.11.1.7) action—the corresponding *pheoopiomelanins* [10]. Both Enks and cysteinyl-dopaenkephalins act as efficient scavengers forming melanin pigments also by ROS action [11,12]. The overall studies about Enk oxidative routes have been recently reviewed [13].

TIQs are endogenous alkaloid compounds deriving from the non-enzymatic Pictet–Spengler condensation of catecholamines with aldehydes [14]. The reaction between dopamine and acetaldehyde entails the generation of salsolinol, whereas the interaction between dopamine and its derived aldehyde, 3,4-dihydroxy phenyl acetaldehyde, leads to tetrahydropapaveroline production. TIQs and TIQs derivatives function as neurotransmitters or neuromodulators at the β-adrenergic receptors [15] and can be involved in biogenic amines regulation by the inhibition of the enzymes engaged in monoamine biosynthesis [16–20].

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Abbreviations: TIQs, tetrahydroisoquinolines; Enk, enkephalin; POD, peroxidase; ROS, reactive oxygen species; LOX, lipoxygenase; PD, Parkinson's disease; TIQ-enk, TIQ-enkephalin; Leu-enk, leucine-enkephalin; MAP, microsomal leucine aminopeptidase; AP, aminopeptidase; DPDPE, [D-Pen^{2,5}]-enkephalin; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; ESI, electrospray ion; CID, collision-induced dissociation; EAU, enzymatic arbitrary unit.

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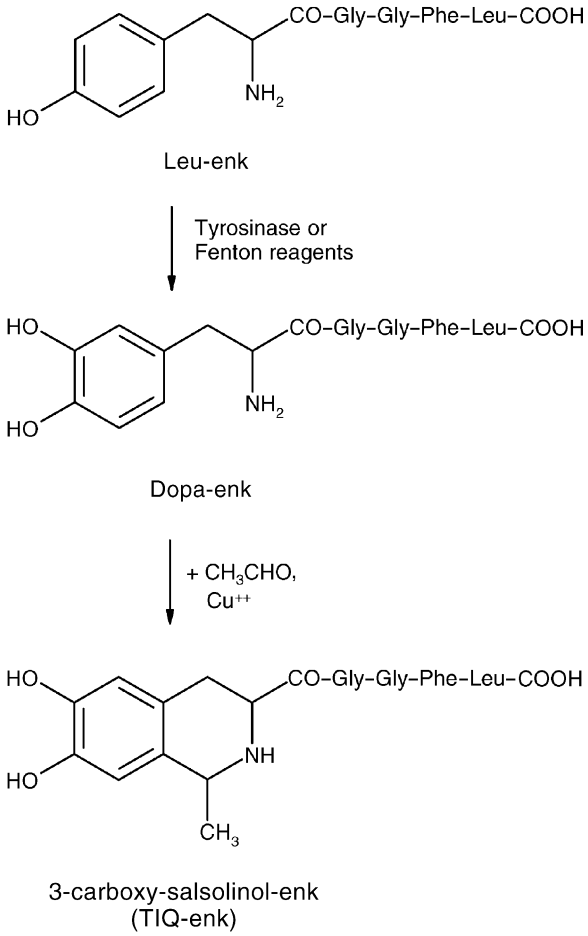
In the last years, the attention of a number of researchers focused on the study of both biochemical and pharmacological properties of these compounds. In particular, it has been reported that TIQs bearing a catecholic moiety in their structure, have the ability—like catecholamines [21–23]—to produce toxic quinones and melanin pigments (*TIQ-melanins*) by either autooxidation [24] or reactions catalysed by tyrosinase or LOX (E.C. 1.13.11.12) [24,25]. The growing interest in TIQs has been hastened by their possible participation in some pathophysiological aspects of both alcoholism [15,26] and PD [27].

As acetaldehyde represents the major by-product of ethanol metabolism, has been hypothesised [28] that TIQs formation could be induced *in vivo* by ethanol consumption and could mediate some ethanol's effects. It has been also reported [29] that the urinary concentration of dopamine-related TIQs significantly increases in humans during long-term alcohol consumption.

Recent data indicate that the biosynthesis, metabolic conversion and cellular levels of TIQs are selectively enhanced in *substantia nigra* dopaminergic neurons in comparison with other brain regions [30,31]. Some of these substances have not only been detected in the urine and in the post-mortem PD brain [32,33] but they were found also to produce parkinsonism in primates [34]. The alkaloids seem to trigger oxidative stress by a mechanism involving $^{\bullet}$OH generation that could be the event leading to mitochondrial impairment and cell death [35].

In the present paper, we demonstrate that TIQ-peptides can derive from the reaction of dopa-enk with aldehydes (Scheme 1). The resulting TIQ-enk differ from Enk by the substitution of amino terminal tyrosine with 3-carboxy-salsolinol or another TIQ. The characterisation, isolation, binding to opiate receptors of this new class of Enk-derivatives and their enzymatic conversion to the corresponding melanins (TIQ-enk-melanins) are described.



Scheme 1. Model for TIQ-enk synthesis: 3-carboxy-salsolinol-enk generation by Leu-enk condensation with acetaldehyde.

2. Materials and methods

2.1. Materials

Leu-enk, mushroom tyrosinase (3000 U/mg), POD from horseradish (1100 U/mg), LOX from soybean type V (1,000,000 U/mg), cytochrome *c* from bovine heart, MAP from porcine kidney microsomes type IV (29 U/mg), AP from *Aeromonas proteolytica* (106 U/mg), protease from *Streptomyces griseus* type XIV (6 U/mg), [D-Pen^{2,5}]-enkephalin (DPDPE) and [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO) were purchased from Sigma Chemical Co. [^{3}H]DPDPE and [^{3}H]DAGO were obtained from DuPont NEN. All other reagents were analytical grade products from Fluka.

Dopa-enkephalin (dopa-enk) was synthesised using Leu-enk as substrate either of mushroom tyrosinase, according to Larsimont *et al*. [5], or of a Fenton system, following Fontana *et al*. [11]. The method is well standardised and the structure of dopa-enk was already assessed by ESI mass spectrometry [5].

For TIQ-enk synthesis, 0.5 mM dopa-enk, 20 mM acetaldehyde and 0.5 mM $CuSO_4$ in 0.2 M potassium phosphate buffer, pH 7.4, were incubated at 37°. After 3 hr, aliquots of incubation mixture were filtered and injected into the HPLC system. For the isolation of TIQ-enk, the eluates corresponding to the product peak were collected and pooled.

To determine dopa- and TIQ-enk concentrations, aliquots of concentrated solutions were submitted to acid hydrolysis and the amino acid content determined by the Amino acid analyser. From the absorption spectra, the extinction coefficients of both dopa- and TIQ-enk have been calculated: $\varepsilon = 3.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 280 nm for dopa-enk; $\varepsilon = 2.1 \times 10^3\ M^{-1}\ cm^{-1}$ at 283 nm for TIQ-enk.

2.2. HPLC analysis

HPLC analysis was performed by a Waters Millipore apparatus (Milford, MA, USA). Samples were applied on a reverse-phase column (Novapak C18, 4 μm 15 cm × 0.39 cm). The chromatography was carried out in isocratic

conditions at 30° in a thermostated apparatus, the eluent being a solution containing 78% 0.05 M citrate/phosphate buffer, pH 5.0, and 22% acetonitrile, at flow rate of 1.0 mL/min. The absorbance of the effluents was monitored at 280 nm. In the above conditions, the retention time of Leu-enk was 6.1 min.

2.3. Mass spectrometry

ESI mass spectra were obtained on a Finnigan LCQ ion trap mass spectrometer, in positive ion mode. The sample was dissolved in 50:50 (v/v) methanol–water solution containing 1% acetic acid to a concentration of 10 pmol/μL and infused into the electrospray needle at flow rate of 5 μL/min. ESI source conditions were as follows: sheath gas (nitrogen) flow rate, 90 (arbitrary units); electrospray needle voltage, 50 kV; capillary voltage and temperature, 12 V and 260°, respectively; electron multiplier and conversion dynode, 800 V and 15 kV, respectively. Full scan mass spectra were obtained in the peak continuum mode over the mass range of 50–1000 every 3 s and then summing each spectrum. CID was achieved on selected trapped ions. A CID energy of 50% (arbitrary unit) was selected.

2.4. TIQ-enk oxidation

The oxidation of TIQ-enk by various enzymatic systems was followed spectrophotometrically through the TIQ-chrome formation [24]. The absorbance at 300 nm was monitored continuously using a Kontron Uvikon 930 spectrophotometer in 1 cm light path thermostated cuvettes at 25°. The standard incubation mixture contained 0.1 mM TIQ-enk, 1 mM H_2O_2 and the indicated EAU of enzyme in 1 mL of 0.1 M potassium phosphate buffer, pH 7.4. One EAU was defined as the amount of enzyme catalysing the production of 1 μmol of dopachrome/min at 25° using an incubation mixture containing 1 mM dopa and 0.1 mM H_2O_2 in 1 mL of 0.1 mM potassium phosphate buffer, pH 7.4. The reaction was started by enzyme addition. As controls, parallel experiments without enzyme or with heat-inactivated enzyme were carried out. The steady-state rate was defined as the slope of the linear zone of the product accumulation curve.

2.5. Membrane preparation and binding assay

Bovine brain was obtained from the local slaughterhouse. The cortex was rapidly removed and membranes were prepared as described by Wood [36]. For the binding assay, brain membranes (0.3 mg protein) were incubated at room temperature for 1 hr in 1 mL of 50 mM Tris–HCl buffer, pH 7.7, containing 4 nM [^{3}H]DPDPE or [^{3}H]DAGO as tracer, in the presence or absence of 10 μM unlabeled ligand or Leu- or TIQ-enk at the indicated concentrations. The radioactivity was determined in a Beckman LS6800 Scintillation Counter.

2.6. Acid or enzymatic hydrolysis

Acid hydrolysis was performed with 0.1 mM TIQ-enk in sealed tubes in 6 N HCl at 110° for 20 hr. Enzymatic hydrolysis was carried out by incubating 0.1 mM substrate in the presence of MAP (0.5 U), protease (0.5 U) or AP (5 U) in 100 mM potassium phosphate buffer, pH 7.4, at 37°. At various times, samples were appropriately diluted and analysed by a 3A30 Carlo Erba Amino acid analyser, using the analysis conditions already described [10].

3. Results

3.1. TIQ-enk synthesis and isolation

TIQ-enk was synthesised by the condensation of dopa-enk with acetaldehyde (Scheme 1) as described in Section 2. Chromatographic profiles of the reaction mixture at zero time and after 3 hr are shown in Fig. 1. In chromatogram (A), the peak with retention time of 6.0 min (a) represents

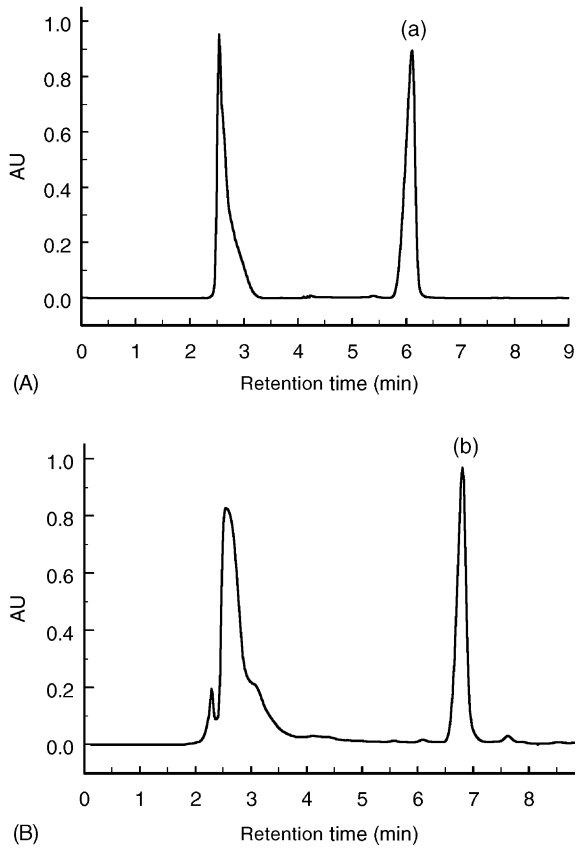


Fig. 1. HPLC profile of dopa-enk reaction with acetaldehyde at zero time (A) and after 3 hr (B). Incubation mixture contained 0.5 mM dopa-enk, 20 mM acetaldehyde and 0.5 mM $CuSO_4$ in 0.2 M potassium phosphate buffer, pH 7.4. The chromatography was carried out in isocratic condition at 30° in thermostated apparatus, utilising as eluent a mixture containing 78% of 0.05 M sodium citrate/phosphate buffer, pH 5, and 22% acetonitrile at a flow rate of 1.0 mL/min. Absorbance was read at 280 nm.

dopa-enk; in chromatogram (B) the same peak significantly falls, whereas a peak with retention time of 6.8 min (b) is clearly evidenced. The spectrophotometric analysis of the eluate corresponding to this latter peak showed an absorption maximum at 283 nm, analogously to a standard sample of salsolinol. The analysis of amino acid composition of eluate (b) subjected to acid hydrolysis unequivocally evidenced the absence of dopa; the calculation of Leu, Gly and Phe amounts allowed to determine the concentration of TIQ-enk solution.

3.2. ESI mass spectrometry

In Fig. 2, the ESI and CID mass spectra of concentrated eluate (b) are shown. Full scan spray ion spectrum, acquired in single MS positive ion mode by infusion injection analysis, shows the predominant ions at $m/z = 598$ and 636, assigned to $[M + H]^+$ and $[M + K]^+$, respectively. These values, compared to those of dopa-enk (not shown), accounted for a 26 Da covalent adduct, in full agreement with $CH–CH_3$ bound. From the CID mass spectrum several important sequence ions (m/z 178, 206, 263, 320, 439, 467) can be assigned, directly derived from the scheme shown at the bottom of the figure. Ion series nomenclature is according to Roepstorff and Fohlman [37]. All the m/z values obtained were 26 Da higher than the fragments obtained from dopa-enk. These data indicated that the sequence of the Enk-derivative was the following: 3-carboxy-salsolinol-Gly–Gly–Phe–Leu-COOH (Scheme 1). This compound was that expected from the condensation of dopa-enk with acetaldehyde. In the following—for clarity—it will be named TIQ-enkephalin (TIQ-enk).

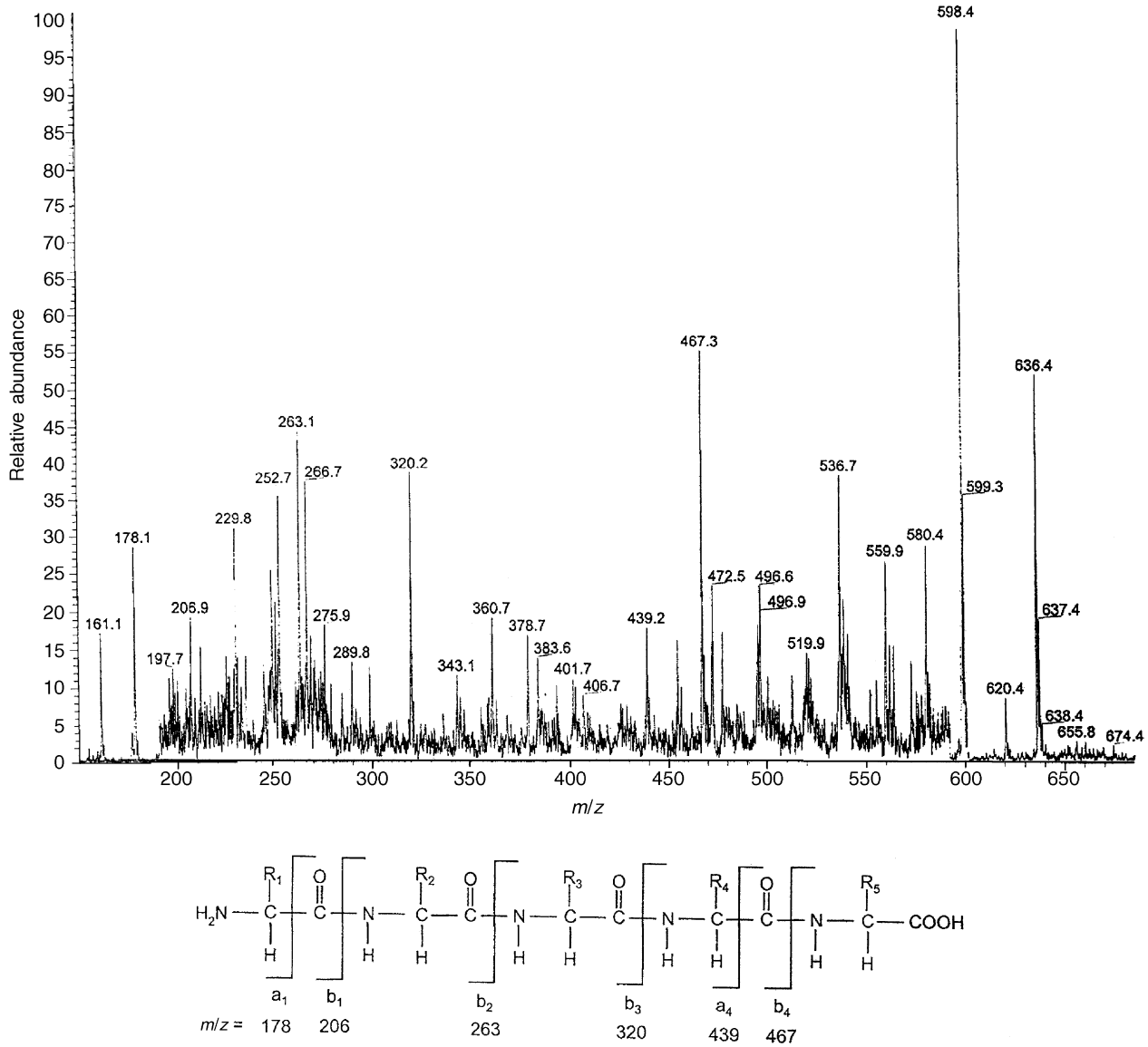


Fig. 2. ESI mass spectrum and CID mass spectrum of pseudomolecular ion at m/z 598.4. The sample was dissolved in 50:50 (v/v) methanol–water solution containing 1% acetic acid to a concentration of 10 pmol/μL. A CID energy of 50% (arbitrary unit) was selected for MS/MS experiments. At 598.4 Da it can be recognised the parental ion TIQ-enk H^+.

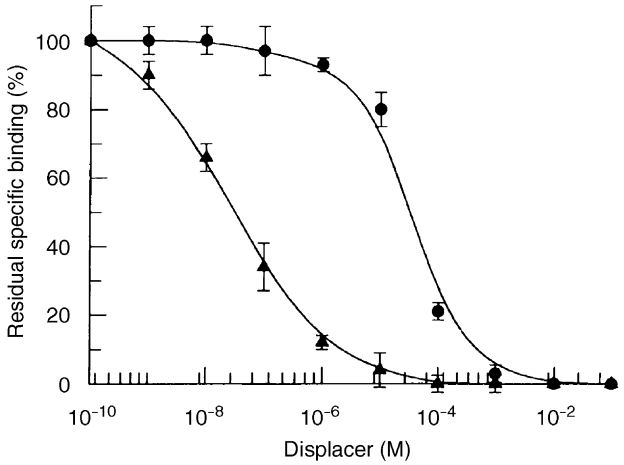


Fig. 3. Displacement of [^{3}H]DPDPE binding in bovine brain membranes by TIQ-enk (●) and Leu-enk (▲). Bovine brain membranes (0.3 mg protein) were incubated at room temperature for 1 hr in 1 mL of 50 mM Tris–HCl buffer, pH 7.7, containing 4 nM [^{3}H]DPDPE in the presence or absence of 10 μM unlabeled ligand and with enkephalins at the specified concentrations. Data are expressed as percentage of the maximum specific binding and are means ± SD for six experiments with assays in triplicate.

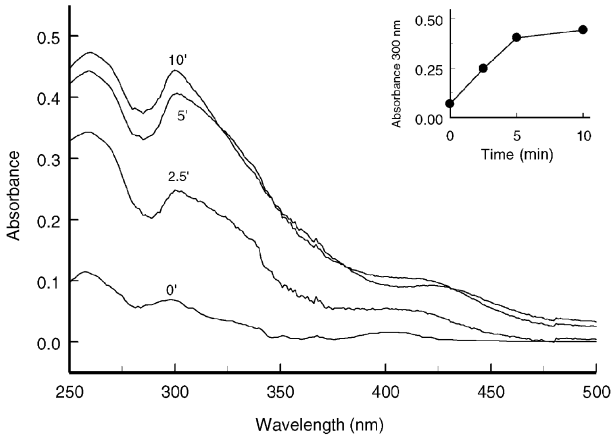


Fig. 4. Spectral modification of TIQ-enk oxidised by the POD/H_2O_2 system. The incubation mixture contained 0.1 mM TIQ-enk, 0.1 mM H_2O_2 and 0.25 EAU of POD in 1 mL of 0.1 M phosphate buffer, pH 7.4. Blank cuvette contained all the reagents except POD. The reaction was started by enzyme addition. Inset: absorbance increase at 300 nm as a function of incubation time.

3.3. Receptor binding affinity

The capacity of TIQ-enk to bind to opiate receptor was tested. The binding to δ and μ receptors in membrane preparations from bovine cerebral cortex was studied using [^{3}H]DPDPE or [^{3}H]DAGO as tracers. Displacement curves of the TIQ-enk in comparison with Leu-enk using [^{3}H]DPDPE are illustrated in Fig. 3. These curves are monophasic, so envisaging the binding to a single site. The required TIQ-enk concentrations for inhibiting 50% (IC_{50}) of the specific binding of 4 nM [^{3}H]DPDPE or [^{3}H]DAGO were about 50 and 530 μM, respectively. The comparison of these values with those of Leu-enk (IC_{50} = 0.05 and 0.48 μM, for δ and μ receptors, respectively) clearly indicates that the presence of the TIQ moiety causes an almost 1000-fold decrease in binding affinity to both opioid receptors.

3.4. TIQ-enk enzymatic oxidation

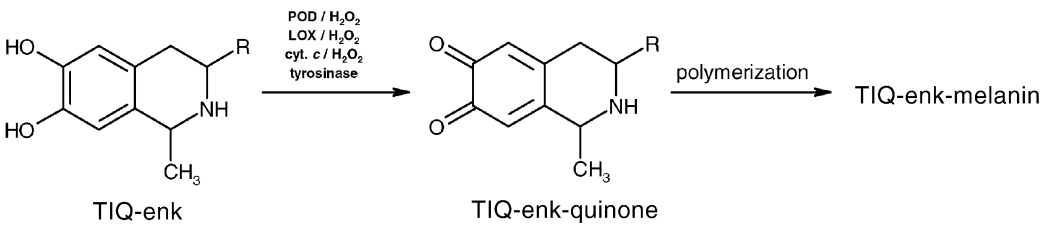
When TIQ-enk was incubated with POD in the presence of hydrogen peroxide, the rapid appearance of two absorption maxima at 260 and 300 nm was evidenced (Fig. 4), this feature being already obtained from the tyrosinase- and POD-catalysed oxidation of salsolinol [24,25]. In the inset, the time course of the reaction—showing a completeness after 10 min—is shown. After 12 hr incubation, a melanin-like pigment was evidenced by the absorption spectrum, indicating TIQ-enk-melanin generation.

TIQ-enk was oxidised also by tyrosinase, LOX/H_2O_2 and cytochrome *c*/H_2O_2 systems (Scheme 2). In Table 1, the TIQ-enk oxidation rates by these enzymatic systems are shown; the reported values indicate that POD was the

Table 1
TIQ-enk oxidation rate by various enzymatic systems

Enzyme	Rate (ΔAbs/min at 300 nm)
POD	0.390 ± 0.052
Tyrosinase	0.039 ± 0.010
LOX	0.070 ± 0.015
Cytochrome *c*	0.063 ± 0.008

Incubation mixture contained 0.1 mM TIQ-enk, 1 mM H_2O_2 (omitted for tyrosinase activity determination) and enzyme in 1 mL of 50 mM phosphate buffer, pH 7.4. The reported values correspond to the increase of absorbance/min at 300 nm, normalised to 1 EAU for each enzyme.



Scheme 2. TIQ-enk-melanin production by TIQ-enk enzymatic oxidation.

Table 2
Chemical and enzymatic hydrolysis of Leu- and TIQ-enk

Amino acid	Acid hydrolysis	Protease[a]	MAP	AP
Leu-enk				
Tyr	10.2 ± 0.1	9.2 ± 0.8	10.0 ± 0.4	9.8 ± 0.5
Gly (or Gly–Gly)[a]	20.1 ± 0.3	10.0 ± 0.7	19.6 ± 1.2	17.4 ± 1.5
Phe	9.8 ± 0.4	10.5 ± 1.1	10.3 ± 0.6	8.6 ± 0.4
Leu	10.0 ± 1.0	10.8 ± 0.9	9.9 ± 0.8	7.5 ± 0.4
TIQ-enk				
Gly (or Gly–Gly)[a]	19.8 ± 1.0	3.9 ± 0.3	3.9 ± 0.2	0
Phe	10.2 ± 0.6	6.1 ± 0.5	3.6 ± 0.3	0
Leu	9.9 ± 0.5	7.4 ± 0.4	4.0 ± 0.2	0

Chemical hydrolysis was performed with 0.1 mM Leu- or TIQ-enk in 1 mL of 6 N HCl in sealed tubes under vacuum for 20 hr at 110°. Enzymatic hydrolysis was carried out incubating 0.1 mM of the same substrates in the presence of MAP (0.5 U) or protease (0.5 U) or AP (5 U) at 37° in 1 mL of 100 mM potassium phosphate buffer, pH 7.4. The amino acid content was determined as described in Section 2. The reported values represent the nmoles of each amino acid in 0.1 mL recovered after 2 hr and are the means ± SD of three different determinations.

[a] The content of glycine alone cannot be reported because the Gly–Gly bond is resistant to cleavage by protease; the reported values are those corresponding to Gly–Gly dipeptide content, obtained by a comparison with an authentic Gly–Gly sample.

most active enzyme while the catalytic efficiencies of the other systems were quite similar one another but lower than that exerted by POD.

Preliminary investigations on TIQ-enk-melanins indicate for these pigments—in sharp contrast with dopa-melanin—chemical and spectroscopic properties similar to those showed by opiomelanins. Indeed they show—alike opiomelanins—an high solubility in hydrophylic solvents, this behaviour being attributed to the elevated number of terminal carboxylate units that both opiomelanins [38] and TIQ-enk-melanins display. Also the UV–VIS spectrum (not shown) exhibits a pattern more similar to that of opiomelanins than to those of dopa- and TIQ-melanins, i.e. a monotonic increase from 700 to 280 nm with a shoulder around 330 nm ascribed up to now to the bond between the melaninic portion and the peptide chain [9].

3.5. TIQ-enk enzymatic hydrolysis

In Table 2, the results of the enzymatic hydrolysis of TIQ-enk compared with those of Leu-enk are reported. While Leu-enk was completely hydrolysed after 2 hr incubation in the presence of either MAP or protease, TIQ-enk with the same amount of hydrolytic enzyme was cleaved only 40% by MAP and 70% by protease. Two hours incubation of Leu-enk in the presence of AP led to almost a complete peptidic bond cleavage, while TIQ-enk in the same conditions was not hydrolysed at all. These results indicate that the presence of the tetrahydroisoquinolinic moiety makes TIQ-enk resistant to aminopeptidase hydrolytic action. The amount of leucine release from the two enkephalins, as a function of incubation time, with the three proteolytic enzymes is illustrated in Fig. 5.

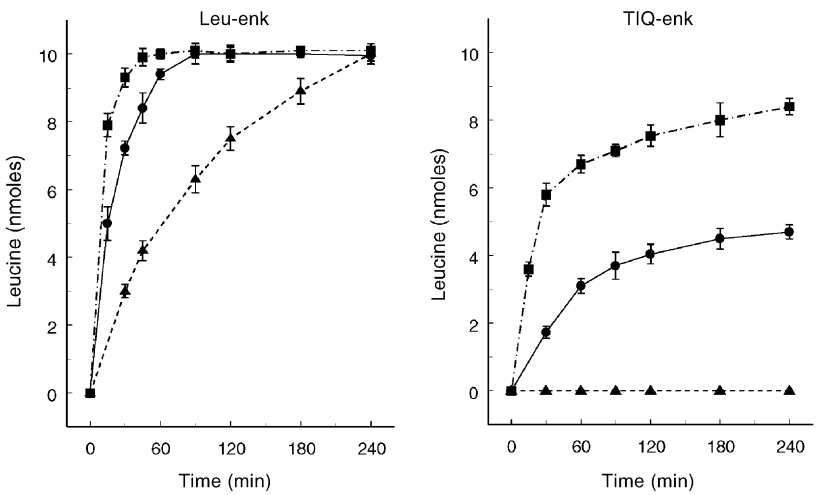


Fig. 5. Time-course release of leucine from TIQ- and Leu-enk by protease (■) or MAP (●) or AP (▲) action. Enzymatic hydrolysis was carried out incubating 0.1 mM substrate in the presence of MAP (0.5 U) or protease (0.5 U) or AP (5 U) at 37° in 1 mL of 100 mM potassium phosphate buffer, pH 7.4. The amino acid content was determined as described in Section 2. The reported values are expressed as nmoles of leucine in 0.1 mL recovered at the indicated time and represent the means ± SD of three different determinations.

4. Discussion

In this paper, we demonstrate that dopa-enk—formed either by the reaction of Leu-enk with tyrosinase or by the Fenton system—can form *in vitro* cyclic adducts with aldehydes (see Scheme 1). The resulting TIQ-enk peptides differ from Enks for the substitution of the amino terminal tyrosine with 3-carboxy-salsolinol or another TIQ linked to the peptide chain.

HPLC analysis clearly shows the production of a single peak with a retention time higher than dopa-enk, indicating the formation of a less hydrophylic compound. The reaction, in accordance with literature data on the condensation rate of amines with aldehydes [27], is rather slow, with about 30% yield. Spectrophotometric analysis of this peak exhibits an absorption spectrum corresponding to that of a synthetic sample of salsolinol, indicating the conversion of dopa into the TIQ-compound. However, the evidence of the 3-carboxy-salsolinol formation at the amino terminus of the Enk has been unequivocally achieved by ESI mass spectrometry.

A lot of synthetic Enk analogues with the insertion of methyl, hydroxyl or acetyl groups have been synthesised and tested at the opioid receptor to find a modification enhancing opioid peptides effect [39]. In this case, we give evidence that the conversion of amino terminal tyrosine into a TIQ compound entails the formation of a TIQ-enk showing a total decline of the ability to bind to the enkephalinergic receptor. This fact indicates that this conversion is able to hamper heavily the enkephalinergic system.

At the same time, the experiments demonstrate that TIQ-enks, in contrast to Enks, are not easily hydrolysed by aminopeptidases signifying that TIQ-peptides once formed, are not easily removed. Also this experiment envisages a possible detrimental effect of TIQ-enks at the enkephalinergic sites.

The data reported clearly demonstrate that a new class of Enks, i.e. TIQ-enks can be easily formed, at least *in vitro*, starting from native Enks by simple reactions. It can be also argued that this new class of peptides is virtually very wide, in fact dopa-enk can react with any type of aldehydes, giving rise—as a consequence of the substitution of the amino terminal tyrosine with salsolinol or another TIQ—to a particular TIQ-enk depending on the reacting aldehyde. On the other hand, if one considers that Enks and opioid peptides represent a large class of tyrosine amino terminal peptides with a diverse number of potential amino acids in the chain, it can be realised that the list of TIQ-enks or TIQ-opiopeptides can be very high.

In our experiments, we have exhibited that TIQ-enk, since presents a cathecolic moiety in its structure, is a fair substrate of tyrosinase/O_2, LOX/H_2O_2, POD/H_2O_2 and cytochrome *c*/H_2O_2, bringing to the light a new class of melanin compounds retaining the peptide moiety (TIQ-enk-melanins/TIQ-opio-melanins). In these pigments, the tetrahydroisoquinolinic moiety has been converted into the melaninic portion of the melanopeptide and the peptidic portion is constituted by the native Enk deprived of tyrosine or dopa. For the above reasons, it is also conceivable that the TIQ-enk-melanins or the TIQ-opio-melanins, can actually constitute a very long array of pigments, considering all the possible combinations that may occur.

As opioid peptides and Enks are involved in both ethanol metabolism [40] and in Parkinson-derived pathological transformations [41], brain synthesis of TIQ-enks and of parent melanins cannot be ruled out being potentially a matter of significance in the course of these pathologies.

For a possible TIQ-enks *in vivo* synthesis, ROS action and aldehydes availability at the synaptosomal level have to be invoked. As ROS are formed in ethanol bioconversion [42] as well as in PD [43], an oxidative stress underlying both these pathophysiological conditions has been advanced [44,45].

As regards aldehyde availability, it has been recognised that these highly reactive substances not only are rapidly formed by ethanol conversion but are produced in neurodegenerative brain illness including PD, as a consequence of membrane lipid oxidation during oxidative stress injuries [46,47]. By the above mentioned argumentation, it is conceivable that oxidative stress conditions, occurring during age or in degenerative disease or in the course of chronic alcoholism, could actually induce the *in vivo* generation of dopa-enk by $^{\bullet}OH$ oxidative action which is able to convert easily tyrosine into dopa.

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