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FLUORESCENT ENKEPHALIN DERIVATIVES WITH BIOLOGICAL ACTIVITY

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SUMMARY :
Fluorescent dansylated derivatives of enkephalins were prepared, with the dansyl group either at the N- or C-terminal part of methionine-enkephalin (Met-E) or its peptidase-resistant analogue D-Ala$_2$-Met-E. An energy transfer, from the Tyr to the dansyl group, was found for all compounds and spectral properties were dependent upon the environment polarity. Met-E-(CH$_2$)$_2$ dansyl II and D-Ala$_2$-Met-E-(CH$_2$)$_2$ dansyl III exhibit biological activities on guinea-pig ileum and binding affinities on striatal membranes, similar to Met-E. III exhibits significant analgesic activity in mice after i.v. administration (about 35 % that of morphine). Fluorescent enkephalins potentially represent useful probes for the exploration of opiate receptors, studies of ligand-receptor interactions and evaluation of enkephalins cleaving peptidases in various tissues.

Fluorescent derivatives of enkephalins potentially represent useful experimental tools for the visualization of opiate receptors and the exploration of ligand-receptor interactions. We report here the synthesis and opiate-like properties of such compounds. The dansyl, 1-(5-dimethylaminonaphtalene) sulfonyl group, has been selected as fluorescent probe in view of i) the marked changes in spectral properties induced by the modifications of its environment, particularly as a consequence of ligand-receptor interaction (1,2) ii) the good energy transfer with tyrosine (or tryptophane) residues it allows (3) iii) its relatively easy covalent attachment to peptides.

The dansyl group has been introduced at either the N- or C-terminal moiety of methionine-enkephalin (Met-E) or of its peptidase-resistant analogue (4), D-Ala$_2$-methionine-enkephalin (D-Ala$_2$-Met-E).

The fluorescence properties of all these compounds were studied in ethanol and aqueous medium. Finaly, the ability of the D-Ala$_2$-Met-E to cross the blood brain barrier was investigated by studies of analgesic activity in mice after *i.v.* administration.

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MATERIAL AND METHOD.

1. Synthesis.

Met-E and D-Ala_2-Met-E were prepared as previously described (5,6) by liquid phase method using terbutyloxycarbonyl (t.boc) and methylesters as protecting groups and dicyclohexylacarbodiimide (DCC) for the coupling reactions. The N-dansyl Met-E I was prepared by direct reaction of one equivalent of dansyl chloride on Met-E in 95 % EtOH at 5°C for 3 hours, in presence of one equivalent of triethylamine. The solid compound obtained by evaporation *in vacuo* was washed by acetone in order to eliminate the triethylammonium hydrochloride ; it migrated as a single spot on silicagel glass plates (Rf=0.89 in BuOH:AcOH:H_2O ; 4:1:1). The position of the dansyl group was checked by ^1H NMR : presence of the phenolic proton at 9.3 ppm and the sulfamidic NH at 8.8 ppm (doublet by coupling with Tyr H_α).

The synthesis of N-1-(2-dansylamino) ethyl-Met-enkephalinamide (Met-E-$(CH_2)_2$ dansyl) II and of N-1-(2-dansylamino) ethyl-D-Ala_2-Met-enkephalinamide (D-Ala_2-Met-E-$(CH_2)_2$ dansyl) III and of N-1-(2-dansylamino pentyl-Met-enkephalinamide (Met-E-$(CH_2)_5$ dansyl) IV were performed by coupling the t.boc protected peptides with the corresponding N-aminoalkyl dansylamides in the presence of DCC, and purified by chromatography on silicagel column using $CHCl_3$:MeOH (9:1) as eluent. N-aminoalkyl dansylamides were obtained according to NILSSON et al. (7). Deprotection was performed using trifluoroacetic acid. The structure of the compounds used as trifluoroacetates, was confirmed by NMR spectroscopy and their purity was checked by thin-layer chromatography on silicagel glass plates using n-BuOH:AcOH:H_2O (4:1:1) as solvent. Met-E-$(CH_2)_2$ dansyl, Rf=0.62 ; D-Ala_2-Met-E-$(CH_2)_2$ dansyl, Rf=0.80 ; Met-E-$(CH_2)_5$ dansyl, Rf=0.71.

2. Fluorescence experiments.

The fluorescence spectra were recorded on a Perkin Elmer MPF 44A equiped with a differential corrected spectra unit. The spectra were performed in ethanol, water or tris-HCl buffer (pH:7.4) at various concentrations.

3. Biological activities.

Opioid peptide activity was measured on the guinea-pig ileum electrically stimulated at 0.15Hz with rectangular pulses of 0.5ms (8). At least 5 different concentrations of each compound (3-6 assays for each) were tested and the specificity of the inhibition of the contractions was established by reversal with naloxone. IC_{50} were determined from regression analysis. Relative potencies represent the ratio expressed in percent of IC_{50} of the peptide/IC_{50} of morphine on the same preparation. Leu-E was equipotent to morphine.

For binding studies, Swiss mice were decapitated and their striatum homogenized in 20 volumes of tris-HCl buffer (0.05M, pH:7.4). After two centrifugations (18Og, 5min and 1000g, 20min) the resulting pellet was preincubated for 15min at 30°C in fractions of 100µl containing about 150µg proteins. The incubations were performed for 15min in the presence of 20nM ^3H-Leu-Enk (41 Ci/mmol, the Radiochemical Center, Amersham), 20µM bacitracin and increasing concentrations of the various peptides. After addition of 3ml cold buffer the membranes were separated by vacuum filtration (Whatman, GF/B) and the radioactivity counted by liquid scintillation spectrometry. The IC_{50} of each compound was estimated from the effects of at least 6 concentrations with triplicate assays and the Ki calculated on the assumption of a competitive inhibition. Means ± S.E.M. form 3 independent experiments.

Analgesic activity of III was measured on the hot plate test (9) after administration of solutions of III (1, 3 or 10 mg/kg) or morphine 0.3, 1, 3 mg/kg) to male mice weighing from 21 to 26g (7 animals a dose).

RESULTS AND DISCUSSION

Spectral properties of all compounds have been studied and those of Met-E-$(CH_2)_2$ dansyl, II, are shown in figure 1. The energy transfer from the Tyr to

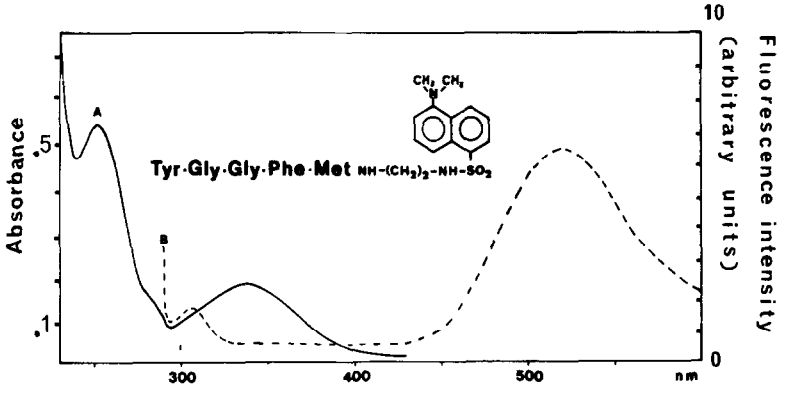

Figure 1. Spectral properties of Met-E-$(CH_2)_2$ dansyl at 2.10^{-5}M in ethanol. A. Absorption spectum. B. Fluorescence spectrum (λexc : 270nm). The absorption spectrum A shows two maxima at 250 and 335nm, corresponding to the dansyl chromophore and a shoulder at 270nm corresponding to the Tyr residue. Upon excitation at 270nm a strong fluorescence transfer from the Tyr (donnor) to the dansyl (acceptor) is indicated by the band at 520nm.

the dansyl group, evidenced by fluorescence emission of the latter upon excitation at one of the absorption maxima of Tyr (λexc.:270nm), was found for all compounds. The occurrence of energy transfer from Tyr to dansyl can also nicely be demonstrated by inspection at 520nm of the dansyl fluorescence excitation spectrum of Met-E-$(CH_2)_2$ dansyl, II, which shows additional excitation in the wavelength range corresponding to the Tyr absorption (not shown).

As could be expected, the spectral properties of the compounds are dependent upon the environment polarity : there is approximately a ten-fold increase in fluorescence intensity accompanied by a 45nm shift towards the blue from water to ethanol (figure 2.).

For biological studies, it was necessary to evaluate the energy transfer in 0.05M tris buffer (pH:7.4) by the percent decrease in emission intensity of Tyr (λ:305nm) in the fluorescent derivatives as compared to Met-E (figure 3.). The following values were found : 94 % for N-dansyl-Met-E, 96 % for Met-E-$(CH_2)_2$ dansyl and 92 % for Met-E-$(CH_2)_5$ dansyl.

The opiate-like activity of the fluorescent derivatives has been evaluated on two different biological systems, *i.e.* the inhibition of electrically-induced contractions of guinea-pig ileum (8) and the inhibition of ^{3}H-Leu-E binding to striatal membranes (10). Both tests gave similar results(Table I) as regard the potency of the various compounds relatively to that of Leu-F (or Met-E which was of the same magnitude). In agreement with previous reports (11), N-substitution of the enkephalins leads to a decreased, although still evaluable, activity. As regard C-substituted compounds, it can be noted that the size of the

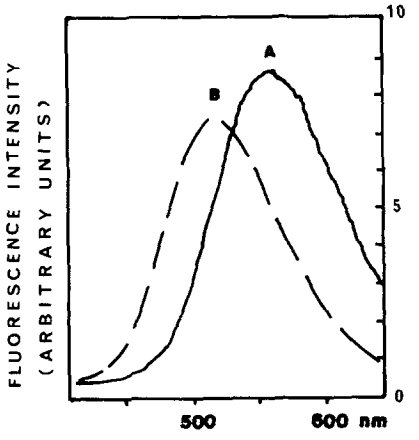

Figure 2. *Changes in fluorescence properties of Met-E-$(CH_2)_2$ dansyl, II, induced by modification of the solvent polarity. A. II (1.3 10^{-5}M) in water (——). B. II (1.3 10^{-6}M) in ethanol (- - - -). Note the shift and the increased fluorescent intensity of the band corresponding to the dansyl group.*

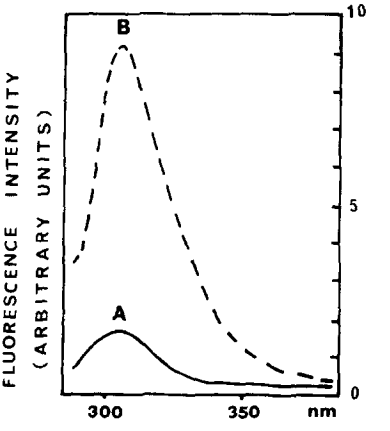

Figure 3. *Emission intensity of Tyr upon excitation at 275nm for Met-E-$(CH_2)_2$ dansyl (A) and Met-E (B) at the same concentration (4.10^{-5}M) in 0.05M Tris-HCl buffer.*

chain is crucial for activity as also shown by BAJUSZ et al. (12). Met-E-$(CH_2)_2$ dansyl, II, exhibits on both tests an activity similar to that of Leu-E. The same is true for the potentially enzyme-resistant compound, D-Ala_2-Met-E-$(CH_2)_2$ dansyl, III, (table I). In contrast, Met-E-$(CH_2)_5$ dansyl, IV, shows a large decrease both in binding properties and activity.

The ability of D-Ala_2-Met-E-$(CH_2)_2$ dansyl, III, to cross the blood-brain barrier is clearly demonstrated by the significant analgesic properties of this compound after *i.v.* administration. So, III at 10mg / kg elicits the same potency as morphine at 1mg / kg (35 % of the activity of morphine on a molar basis) in the mouse hot-plate test, the duration of action being equivalent. It can be observed that few compounds exhibit analgesic properties after *i.v.* injection but all these derivatives, including III, contain a D-aminoacid in position 2 and a modification of the C-terminal Met_5 residue (14,15,16) or its replacement by Pro_5 (13).

Hence it appears that both Met-E-$(CH_2)_2$ dansyl and its D-Ala_2 analogue meet the criteria of a fluorescent prove, *i.e.* adequate spectral properties and strong biological activity.

Different applications of these probes are in progess. These compounds, and specially the D-Ala_2 derivative, could be utilized for the visualisation of opiate receptors by an histofluorescence technique similar to that already developed

Table I. *Inhibitory potencies of Leu-enkephalin and fluorescent peptides on the contractions of guinea-pig ileum and on the binding of ^{3}H-Leu-enkephalin by membranes from mouse striatum.*

Compound	Guinea-pig ileum		^{3}H-Leu-enkephalin binding
	IC_{50} (nM)	Relative Potency	Ki (nM)
Leu-E	67 ± 12	100	5 ± 1
Met-E-$(CH_2)_2$ dansyl	50 ± 11	86	10 ± 3
D-Ala_2-Met-E-$(CH_2)_2$ dansyl	27 ± 4	147	5 ± 2
Met-E-$(CH_2)_5$ dansyl	288 ± 15	13	21 ± 5
N-dansyl-Met-E	1,306 ± 328	4	50 ± 25

for β-adrenergic receptors (17). The fine analysis of energy transfer within such molecules in solution can provide interesting data regarding their preferred conformation (18) and could be compared to those provided by other approaches (19,20, 21). In addition, the energy transfer between the Tyr and the dansyl groups of the molecule provides an indirect index of the integrity of the molecule : since the biological inactivation consists primarily in the hydrolysis of the Tyr-Gly bond (22), Met-E-$(CH_2)_2$ dansyl can be used as a substrate for the peptidases involved in this cleavage and, monitoring the *in vitro* changes in fluorescence, provides a convenient mean to evaluate the activity of these enzymes and the actions of inhibitors.

Finally, and perhaps more interestingly, the analysis of energy transfer between tryptophane residues of the receptor molecule and the dansyl group of the probe (8,23) or between Tyr and dansyl chromophores within the latter, could provide interesting data regarding the conformational changes possibly occuring during attachment of the peptide to its recognition sites. Such an approach can be extended to the different endorphins which contain only one Tyr residue at the N-terminal part of the peptide.

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