Partially Modified Retro-Inverso-Enkephalinamides: Topochemical Long-Acting Analogs in vitro and in vivo

Abstract. *The synthesis of four enkephalinamide analogs is described in which the peptide bond between residues 4 and 5 is reversed with or without simultaneous reversal of the carboxyl-terminal amide bond. These so-called partially modified retro-inverso-isomers are new, potent, topochemical analogs of the enkephalins. Tests, both in vitro and in vivo, have shown that these analogs are considerably longer acting than any previously studied enkephalins. Thus, partial reversal of the peptide bonds of the backbone can result in peptides with enhanced activity compared to a parent compound, provided that the structural complementarity of both the side chains and end groups are conserved.*

The discovery by Hughes *et al.* (*1*) of methionine and leucine enkephalins which exhibit morphinomimetic activity was followed by the observation of their physiological interaction with opiate receptors (*2*). These findings prove the existence of endogeneous substances (*3*) that can interact specifically with opiate receptors (*4*) and competitively with an opiate antagonist such as naloxone.

Two bioassays in vitro are widely used to quantify the relative agonist potencies of various opiates and opioid peptides; the mouse vas deferens (*5*) and the guinea pig ileum-myenteric plexus (*6*). In both, the ability to inhibit electrically induced concentration of the muscles is measured. In the in vivo assays the compounds are injected directly either into the ventricular spaces of the brain or into the peripheral veins and monitored by observation of induced behavioral effects such as tranquilization, analgesia, wet-dog shake, immobilization, and catatonia (*7*).

Minute amounts of an enkephalin induce intense morphinomimetic activity as demonstrated in the in vitro tests. In the in vivo tests, large doses of the enkephalins are required to elicit a weak analgesia, which dissipates entirely after several minutes (*2, 8*). The large discrepancies in opioid activity between the in vitro and the in vivo assays of the enkephalins can be explained by the difference in peptide stability in both assays. The major biodegradation pathway in vivo was postulated (*9*) and subsequently proven (*10*) to be facile cleavage of the Tyr-Gly (*11*) bond by an aminopeptidase. This is followed by cleavage of the COOH-terminal amino acid by carboxypeptidase and subsequent slower breakdown of the Gly-Gly fragment by a specific dipeptidase (*12*).

The weak analgesia and transient behavioral effects of the enkephalins in vivo has led to the synthesis of potent analgesic analogs that are not susceptible to proteolytic degradation (*13-16*). The compounds [D-Ala²]-Met-enkephalinamide (*13*) and [D-Ala²]-Met-enkephalin (*14*), showed only intracerebroventricular activity; [D-Ala², Met⁵-ol]-enkephalin was active after being administered subcutaneously, intracerebroventricularly, or intraveneously (*15*); [D-Ala², Met(O)⁵-ol] and [D-Ala², *N*-MePhe⁴-, Met(O)⁵-ol]-enkephalin produced significant analgesic activity even when given orally. Recently it was demonstrated that the enhanced in vivo activity of some of these analogs can be explained by their greater resistance to biodegradation by enzymes extracted from mouse brain homogenates (*17*).

We undertook the synthesis of a novel class of enkephalin analogs in which one or more of the peptide bonds in the sequence is reversed. We expected that these retro-inverso-isomers would exhibit enhanced stability toward enzymatic degradation. Figure 1 shows the structural relationships between a parent peptide and two partially modified retro-inverso-isomers (*18*). Both modified structures contain *gem*-diaminoalkyl and 2-substituted malonyl residues. In structure (**A**) the modifications occur on adjacent residues, while in structure (**B**) an amino acid residue is interposed. This interposed amino acid residue must be of opposite configuration to that of the parent peptide in order to result in an absolutely complementary topochemical analog. Such an approach does not result in the reversal of end groups, which we consider as a major disadvantage of the

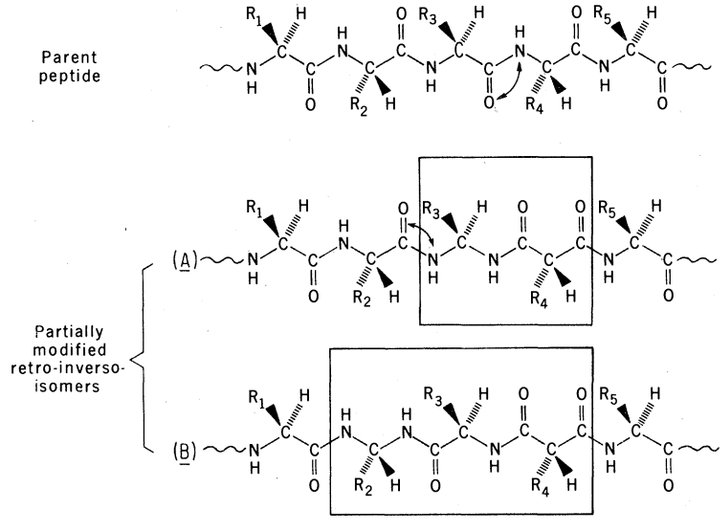
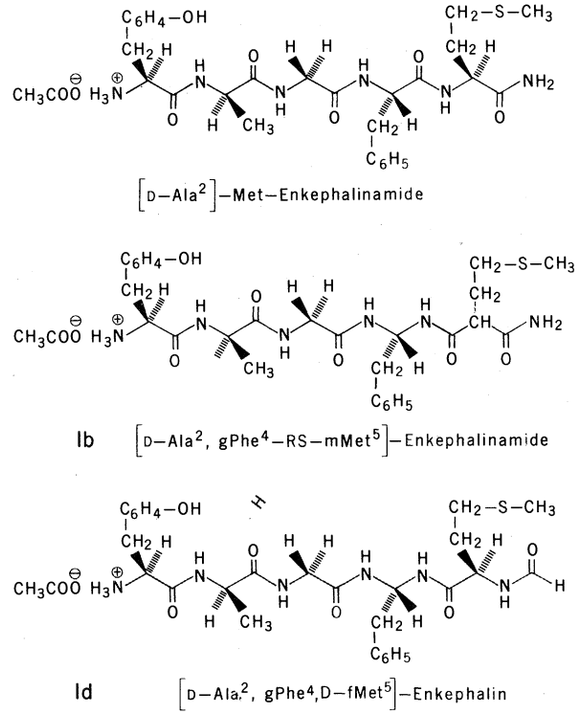


Fig. 1 (left). Schematic representation of partially retro-inverso modifications of a parent peptide. The modification (*A*) is performed on two adjacent amino acid residues containing side chains R_3 and R_4. In (*B*) the modified amino acid residues (containing side chains R_2 and R_4) are moved apart, and D-amino acid residue carrying side chain R_3 is inserted in between. Fig. 2 (right). Schematic representation of [D-Ala²]-enkephalinamide, the parent compound, and the two types of partially modified retro-inverso-analogs **Ib** and **Id**.



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old retro-peptide modification (*18*). In previous work we have applied this topochemical approach to dipeptide sweeteners (*19*) and the luteinizing hormone-releasing hormone (*20*).

On the basis of our previous studies (*19–21*), we have synthesized partially modified retro-inverso-isomers of [D-Ala^2]-enkephalinamides in which the L-phenylalanine residue in position 4 is replaced by a *gem*-diaminoalkyl residue and the methionine or leucine in position 5 is also suitably modified. Figure 2 shows the specific analogs we synthesized and their structural relationship to the parent enkephalines. Analogs **Ia** and **Ib** follow the type (**A**) modification in which a single peptide bond is reversed resulting in adjacent *gem*-diamino and malonyl residues (Figs. 1 and 2). Analogs **Ic** and **Id** follow the type (**B**) modification, in which two consecutive peptide bonds are reversed requiring adjacent *gem*-diamino and D-amino acid residues. In compounds **Ic** and **Id**, D-Leu and D-Met, respectively, are used. Since the second peptide bond that is reversed involves the COOH-terminal carboxamide group, its reversal dictates use of the formyl group as an end group.

Our synthetic approach was based on the synthesis of a fully protected tetrapeptide by standard stepwise coupling procedures. Figure 3 indicates how the tetrapeptide was modified and converted into the partially modified retro-inverso-analogs. The key step involved a Curtius rearrangement of an acylazide to an isocyanate, which is trapped as the protected *gem*-diamino compound, *N*-benzyloxycarbonyl-*N'*-[*N*-*tert*-butyloxycarbonyl-*O*-benzyl-L-tyrosyl-D-alanyl-glycyl]-1-diamino-2-phenylethane (**2**). We recently reported the synthetic methodology for such reactions (*21*). Deprotection followed by immediate coupling with an appropriate COOH-terminal fragment resulted in the fully protected partially modified retro-inverso-analogs. Treatment of these compounds with trifluoroacetic acid liberated the desired analogs shown in Figs. 2 and 3.

Purification of the final products was accomplished by partition chromatography with a mixture of *n*-butanol, acetic acid, and water (4:1:5) on Sephadex G-25. The purity of the compounds was assessed by thin-layer chromatography (TLC) and amino acid analysis. The structures were confirmed by high-resolution nuclear-magnetic-resonance (NMR) spectroscopy with homonuclear decoupling techniques to obtain peak assignments.

In two of the analogs (**Ia** and **Ib**) the COOH-terminal residues were malonamic acid derivatives (where Y represents $-CONH_2$) to complement the corresponding side chains of leucine and methionine (Fig. 3). In the case of analogs **Ic** and **Id**, the retromodifications were extended to include the COOH-terminal blocking amide function. Thus, we incorporated *N*-formyl-D-leucine and *N*-formyl-D-methionine, respectively, to achieve the desired structures (Fig. 3). The electronic and steric characteristics of the formyl amide function are closely related to that of a carboxamide. Compounds **Ia** and **Ib** are diastereoisomeric

Table 1. Equimolar potencies of synthetic analogs (relative to [Met^5]enkephalin) were assayed by their ability to inhibit the electrically induced contractions of the guinea pig ileum–myenteric plexus. Also displayed are closely related enkephalinamide analogs and their potencies relative to [Met^5]enkephalin (Met^5-Enk-OH). To apply the three-letter notation for amino acid residue in our nonclassical modified analogs, we suggest the following prefixes: g- for a *gem*-diamino alkyl corresponding to the amino acid residue denoted and m- for a malonic acid residue corresponding to the amino acid residue denoted.

	Analogs	Relative potency	95 percent confidence limits
	Standard		
	Met^5-Enk-OH	100	
	Data presented here		
Ia	[D-Ala^2, gPhe^4, *RS*-mLeu^5]-Enk-NH_2	206	(174 to 240)
Ib	[D-Ala^2, gPhe^4, *RS*-mMet^5]-Enk-NH_2	175	(150 to 205)
Ic	[D-Ala^2, gPhe^4, D-For-Leu^5]-Enk	1464	(1255 to 1668)
Id	[D-Ala^2, gPhe^4, D-For-Met^5]-Enk	1499	(1314 to 1710)
	Data from (22)		
e	[D-Ala^2, L-Leu^5]-Enk-NH_2	528	(491 to 570)
f	[D-Ala^2, L-Met^5]-Enk-NH_2	528	(492 to 569)
	[D-Ala^2, D-Leu^5]-Enk-NH_2	406	(341 to 484)
	[D-Ala^2, D-Met^5]-Enk-NH_2	246	(207 to 297)
	Projection		
g	[D-Ala^2, DL-Leu^5]-Enk-NH_2	467*	
h	[D-Ala^2, DL-Met^5]-Enk-NH_2	387*	

*These relative potencies were derived from an arithmetic average of the potencies of [D-Ala^2, L-Leu^5]- and [D-Ala^2, D-Leu^5]-Enk-NH_2 and that of [D-Ala^2, L-Met^5]- and [D-Ala^2, D-Met^5]-Enk-NH_2.

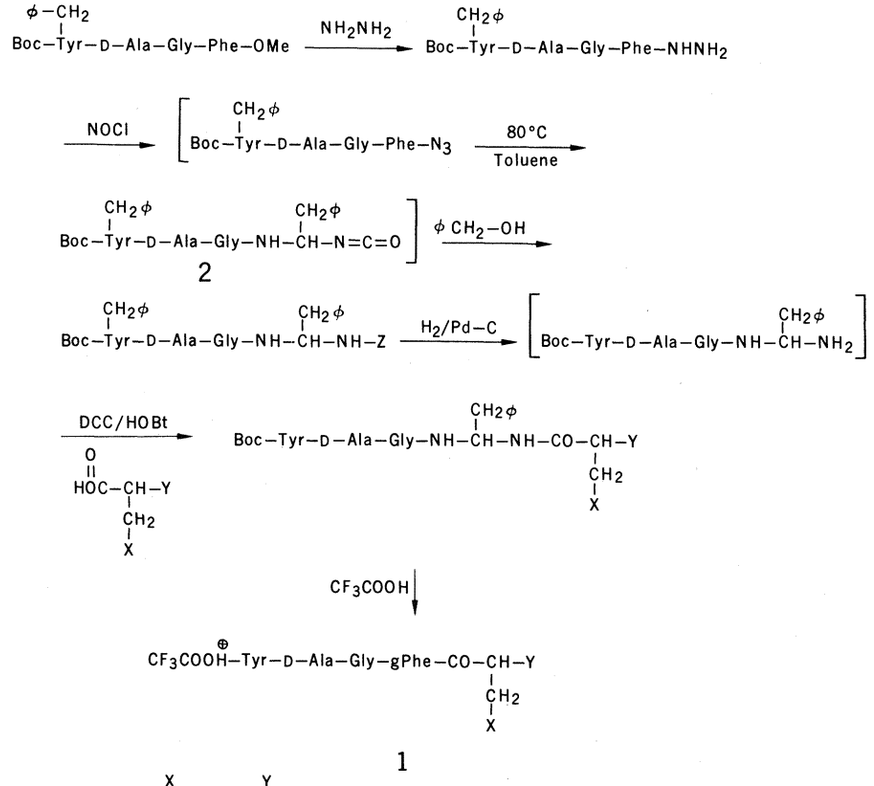

Fig. 3. The synthetic manipulation for the preparation of the partially modified retro-inverso analogs **1a** to **1d**; gPhe represents *gem*-diamino methane substituted with a side chain corresponding to side chain of phenylalanine.

mixtures because of the incorporation of the racemic malonamic acid derivatives; compounds **Ic** and **Id** are single isomers since all the incorporated residues possess a single defined configuration. The in vitro biological activity of these enkephalin analogs is summarized in Table 1. Potency of analogs **Ia** and **Ib** is half that of the closest related analogs with a nonmodified backbone, that is, analogs **g** and **h**, respectively. Analogs **Ic** and **Id** are three times as potent as their corresponding analogs with a nonmodified backbone, that is, analogs **e** and **f**, respectively (see Table 1).

The four analogs were also tested in vivo by injecting them into the cisterna magna of the brains of rats. On a weight basis, **Ic** and **Id** appear to be as potent as β-endorphin in producing catatonia (*7*). The animals remain rigid, displaying general analgesia and a loss of the righting reflex for about 3 hours after an intracisternal injection of 10 μg of **1c** or **1d**. Analogs **Ia** and **Ib** produce a similar syndrome but with effects lasting only 30 to 45 minutes. This state is instantaneously reversed by an injection of naloxone, indicating an opiate receptor mediated effect. In both in vitro and in vivo tests, all the partially modified retro-inverso-isomers tested are considerably longer acting than any of the enkephalin analogs studied previously.

Our findings indicate that this novel approach to structural modification results in analogs with enhanced biological activity. The application of topochemical complementarity can yield spatially equivalent structures when viewed in an extended conformation as shown in Fig. 2. Structure **1** has the same spatial distribution of side chains when the end groups are matched together. Conceptually, the reversal of part of the peptide bonds should not alter biological activity. However, if the modification is carried out properly, compounds with new and enhanced activities result. Thus, the incorporation of *gem*-diamino-, malonyl-, and D-amino acid residues introduces additional possibilities for the synthesis of a new variety of biologically active peptide analogs.

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11. Abbreviations for the amino acid residues are Tyr, tyrosine; Gly, glycine; Met, methionine; Met-ol, methionineol; Met(O)-ol, methionineol sulfoxide; Ala, alanine; Phe, phenylalanine; *N*-MePhe, *N*methyl phenylalanine; Leu, leucine.
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Diffusion-Like Processes Can Account for Protein Secretion by the Pancreas

Abstract. *When fluid secretion by the pancreas was mechanically blocked, amylase secretion into the duct ceased. When flow was reduced in a graded fashion by the application of a back pressure, amylase output was reduced proportionately and amylase concentration in secretion was maintained constant. Thus, the secretion of digestive enzyme from the cell into the duct appears to be dependent upon the concentration of enzyme in the duct system. This behavior is most simply explained by diffusion-like (concentration dependent, bidirectional) fluxes of digestive enzyme across the plasma membrane. A unidirectional process, such as exocytosis, whose rate should be unaffected by fluid flow, cannot readily explain these results.*

Our concept of how proteins are secreted by cells has been greatly influenced by experimental studies on the secretion of digestive enzyme by the exocrine pancreas. During the past 25 years, a rather specific and complex model has been developed which has become the accepted paradigm for this process and for protein secretion in general (*1*). This hypothesis proposes that secretory proteins are "segregated" from the cytoplasm (and other newly synthesized proteins) in special membrane-bound compartments, and subsequently released from one of these intracellular compartments, the secretion granule, into the extracellular environment by a process called exocytosis. In exocytosis, the transfer of secretory product out of the cell is thought to be accomplished by a specialized fusion of the membrane of the secretion granule and the cell membrane, which produces a direct connection between the granule's contents and the extracellular environment.

A variety of experimental evidence from work on the pancreas has made us question this paradigm in general and the final step in the process (exocytosis) in particular (*2*). We have suggested that protein may leave the cell from a free cytoplasmic pool instead of or in addition to exocytosis. In this view, enzyme moves across specialized membranes, particularly granule and cell membranes, by a diffusion-like process in which fluxes are concentration-dependent and bidirectional. We have called this the "equilibrium" hypothesis.

The output or amount of protein that would be secreted (the net secretory flux) in a given state by the two mechanisms that have been proposed—exocytosis and equilibrium-dependent mem-

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