Chemical-enzymatic incorporation of D-amino acids into peptides: synthesis of diastereomeric (D-Ala^2, D-Leu^5)enkephalinamides

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Received 11 February 1985

(D-Ala^2,D-Leu^5)enkephalinamide and its (Ala^2), (Leu^5) and (Ala^2,Leu^5) diastereomers have been prepared by direct coupling of enzymatic and azide methods of peptide bond formation. The low nucleophile reactivity of D-amino acid derivatives has been overcome by an iterative enzymatic synthesis in a nucleophile pool. A striking difference in the biological activity of the chirally pure diastereomeric enkephalinamides has been observed.

Enzymatic synthesis *Opioid peptide* *Enkephalin* *NMR*

1. INTRODUCTION

The chiral specificity of proteases decreases but does not prevent the enzymatic hydrolysis of peptides containing D-amino acids [1,2]. This is relevant for the design and preparation of stable pharmacologically active peptides which, however, are not resistant to enzymatic hydrolysis.

The discovery of the endogenous opioid pentapeptides (Leu^5)- and (Met^5)enkephalins [3] coincided in time with the revival of interest in enzymatic peptide synthesis [4]. This accounts for the reported synthesis of these peptides by enzymatic fragment condensation [5] and by enzyme synthesis of the 4 peptide bonds [6]. Natural enkephalins can also be prepared by recombinant DNA technology [7], but they are not stable against degradation by tissue enkephalinases [8]. Here we report the incorporation of D-amino acids into (Leu^5)enkephalinamide by direct coupling of the enzymatic and azide methods for the formation of peptide bonds.

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2. MATERIALS AND METHODS

The enzymes α-chymotrypsin and thermolysin were purchased from Boehringer Mannheim.

Z-amino acids and amino acid methyl esters were prepared by the classical methods of peptide chemistry [9]; amino acid hydrazides (free base) were obtained by direct hydrozinolysis of the amino acid esters [10].

Enzymatic peptide bond synthesis was carried out by an iterative procedure in a nucleophile pool described previously [11]. A quantity of an amine component, calculated on the basis of the known nucleophile specificity of the enzymes used [12,13], was dissolved in 0.2 M carbonate/bicarbonate buffer, pH 9.3 (chymotrypsin), or 0.2 M maleate buffer, pH 6.8 (thermolysin). Enzyme (10 mg) was then added, followed by the acyl component. After the conversion of the latter the product was filtered and the synthesis was repeated several times by an iterative addition of equivalent amounts of acyl and amine components to the filtrate. The products were pooled and recrystallized from MeOH/H_2O.

The Honzl-Rudinger [14] procedure was used

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00145793/85/$3.30

for azide fragment condensation. The crude products were recrystallized from EtOH/petroleum ether.

Deblocking of Z-peptides was done by hydrogenolysis in the presence of palladized charcoal. Following deprotection, the resulting peptides were obtained as their hydrochlorides from MeOH/Et_2O.

The homogeneity of the intermediate and final products was assessed by ascending thin-layer chromatography (Merck silica plates), amino acid analysis (AAA 881 analyzer) and elemental analysis; their structures were confirmed by ^{1}H-NMR spectroscopy (Bruker WH 250 MHz) in DMSO-d_6.

The biological activity of the synthesized enkephalin analogs was determined by a naloxone-reversible inhibition of electrically-induced contractions of segments from guinea pig distal ileum [15].

3. RESULTS AND DISCUSSION

A synthetic scheme for (D-Ala^2,D-Leu^5)enkephalinamide is shown in fig.1, the strategy for preparation of (D-Ala^2,Leu^5)-, (Ala^2,D-Leu^5)- and (Ala^2,Leu^5)enkephalinamides being strictly identical. The chemical-enzymatic synthesis involves a kinetically controlled enzyme aminolysis of Z-Tyr-OMe by H-D-Ala-$NHNH_2$, followed by azide coupling of the resulting Z-Tyr-D-Ala-$NHNH_2$ with the tripeptide H-Gly-Phe-D-Leu-NH_2. The latter was obtained by thermodynamically controlled enzyme condensation of Z-Gly-OH with H-Phe-OMe, followed by a kinetically controlled enzyme aminolysis of the resulting Z-Gly-Phe-OMe by H-D-Leu-NH_2.

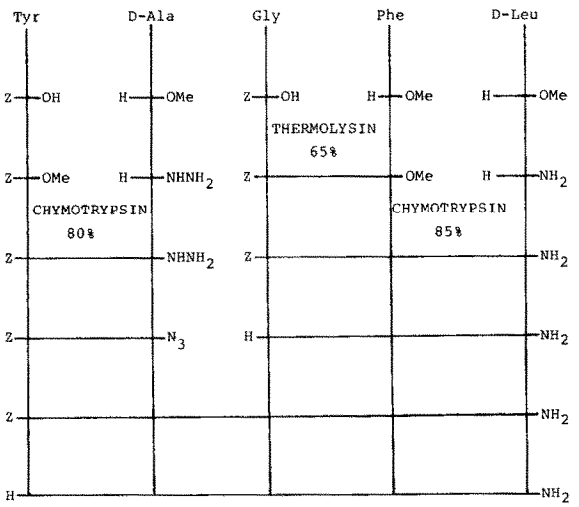

Fig.1.

Due to the radical change of the enzyme-nucleophile interactions, H-D-Ala-$NHNH_2$ and D-H-Leu-NH_2 react much more slowly than the corresponding L-enantiomers. The ratio of the aminolysis rate constants (table 1),

$$(k_n^L/k_w)/(k_n^D/k_w) = k_n^L/k_n^D,$$

suggests that, to favour synthesis, D-enantiomer should be used in a several times higher concentration than L-enantiomer for the same preparative yield.

In the case of such low nucleophile specificity, effective enzyme synthesis is easily achieved by an iterative addition of equivalent amounts of acyl and amine components to a solution (nucleophile pool) containing the enzyme and calculated for the highest possible yield quantity of the nucleophile [11]. Thus, Z-Tyr-D-Ala-$NHNH_2$ and Z-Gly-Phe-D-Leu-NH_2 were obtained with an 80% yield by a chymotrypsin-catalyzed synthesis (fig.1). The same method, applied in the thermolysin synthesis of Z-Gly-Phe-OMe, resulted in a 65% yield (fig.1).

Since proteases exhibit no epimerase activity, the chiral integrity of the final products is controlled by the azide fragment condensation (fig.1). ^{1}H-NMR chemical shifts of the diastereomeric enkephalinamides are reported in table 2. As table 2 shows, the separation between the signals of the diastereomeric pairs is up to 42.5 Hz at 250 MHz;

Table 1

Nucleophile reactivity in chymotrypsin synthesis of diastereomeric fragments of enkephalinamides (fig.1)

Nucleophile	k_n/k_w[a]	k_n^L/k_n^D
H-L-Ala-$NHNH_2$	1100 ± 50	
H-D-Ala-$NHNH_2$	270 ± 20	4.0
H-L-Leu-NH_2	4280 ± 250	
H-D-Leu-NH_2	500 ± 40	8.5

[a] Ratio of the rate constants for aminolysis (k_n) and hydrolysis (k_w) of acylchymotrypsin [11]

hence, NMR is excellent for diastereomeric mixture analysis. No signals of contaminating epimers have been observed, indicating the chiral purity of the prepared enkephalin analogs. This is supported by the fact that they display the highest inhibitory potency (lowest IC_{50} values) reported in the literature (table 3). The striking difference in the IC_{50} values of the diastereomeric enkephalinamides suggests that any diastereomeric impurities would greatly affect their biological activity. The present study would therefore indicate that enzyme incorporation of D-amino acids into peptides ensues preparation of chirally pure, biologically active peptides.

ACKNOWLEDGEMENTS

We are grateful to Dr R. Radomirov and Mrs K. Venkova from the Institute of Physiology, Sofia, for assaying the enkephalin analogs.

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Table 2

^{1}H-NMR chemical shifts of (Ala^2,Leu^5)enkephalinamides[a], epimeric at Ala and Leu α-carbon

Amino acid residue	Proton	Diastereomer		δ (ppm)
		(D-Ala^2,Leu^5)	(Ala^2,D-Leu^5)	
Ala^2	NH	8.69(1)	8.82(1)	0.13
	C$^\alpha$H	1.05(3)	1.22(3)	0.17
Leu^5	NH	7.99(1)	0.05(1)	0.06
	C$^\delta$H	0.86 0.81(6)	0.78 0.71(6)	0.08 0.10

[a] 250 MHz, 0.01 M in DMSO-d_6

Table 3

In vitro biological activity of diastereomeric (Ala^2,Leu^5)enkephalinamides

Diastereomers	IC_{50} (nM)		Relative potency
H-Tyr-L-Ala-Gly-Phe-L-Leu-NH_2	3150.00	200	1
H-Tyr-L-Ala-Gly-Phe-D-Leu-NH_2	8450.00	550	0.34
H-Tyr-D-Ala-Gly-Phe-L-Leu-NH_2	5.80	0.6	540
	11.10	3.2[a]	
	18.00	1.0[b]	
H-Tyr-D-Ala-Gly-Phe-D-Leu-NH_2	0.17	0.02	19000

[a] [16]
[b] [17]

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