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Prodrugs of peptides. 14. Bioreversible derivatization of the tyrosine phenol group to effect protection of tyrosyl peptides against α-chymotrypsin

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Summary

Various derivatives of the tyrosine phenol group in *N*-protected tyrosine amides and esters, used as models of tyrosyl peptides, were synthesized to assess their suitability as prodrug forms with the aim of protecting the tyrosyl peptide bond against cleavage by α-chymotrypsin. The derivatives studied included aliphatic and aromatic carboxylic acid esters, a glutarate ester, carbonate esters and carbamates. All derivatives greatly improved the stability of the parent tyrosine compounds toward hydrolysis by α-chymotrypsin but some esters were themselves readily attacked by the enzyme. Thus, the usefulness of this prodrug approach is greatly dependent on the type of derivatization performed. Useful derivatives were found to include aliphatic carboxylic acid and carbonate esters with a short or branched side chain, a glutarate ester and carbamate esters. Most of these esters are readily bioreversible, the conversion to the parent peptide being catalyzed by plasma or liver esterases.

Introduction

A major cause of the poor or lacking oral absorption of peptides is their rapid degradation by enzymes in the gastrointestinal tract including both luminal proteases and mucosal cell enzymes (Wiedhaup, 1981; Humphrey and Ringrose, 1986; Lee and Yamamoto, 1990). In recent years, we have been studying the possibility of overcoming this enzymatic barrier in peptide drug delivery by the prodrug approach. Thus, *N*-α-hydroxyalkylation of the peptide bond has been shown to make the bond involved as well as adjacent peptide bonds completely resistant to cleavage by carboxypeptidase A and at the same time allow regeneration of the parent peptide by spontaneous, pH-dependent hydrolysis (Bundgaard and Rasmussen, 1991). Likewise, various means of bioreversible derivatization of the C-terminal amide group in amino acid and peptide amides have been shown to afford protection against cleavage by α-chymotrypsin, another pancreatic proteolytic enzyme (Kahns and Bundgaard, 1991).

The aim of the present work was to develop prodrug derivatives capable of protecting tyrosine-containing peptides against degradation by α-chymotrypsin. The impetus to this study was our finding of a very rapid α-chymotrypsin-catalyzed degradation of the HIV-inhibiting peptide

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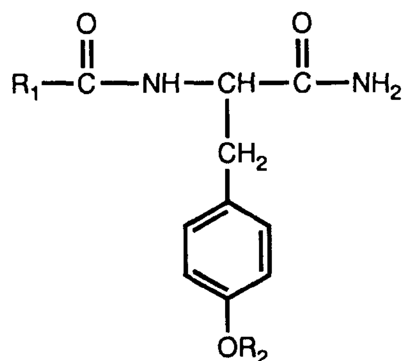
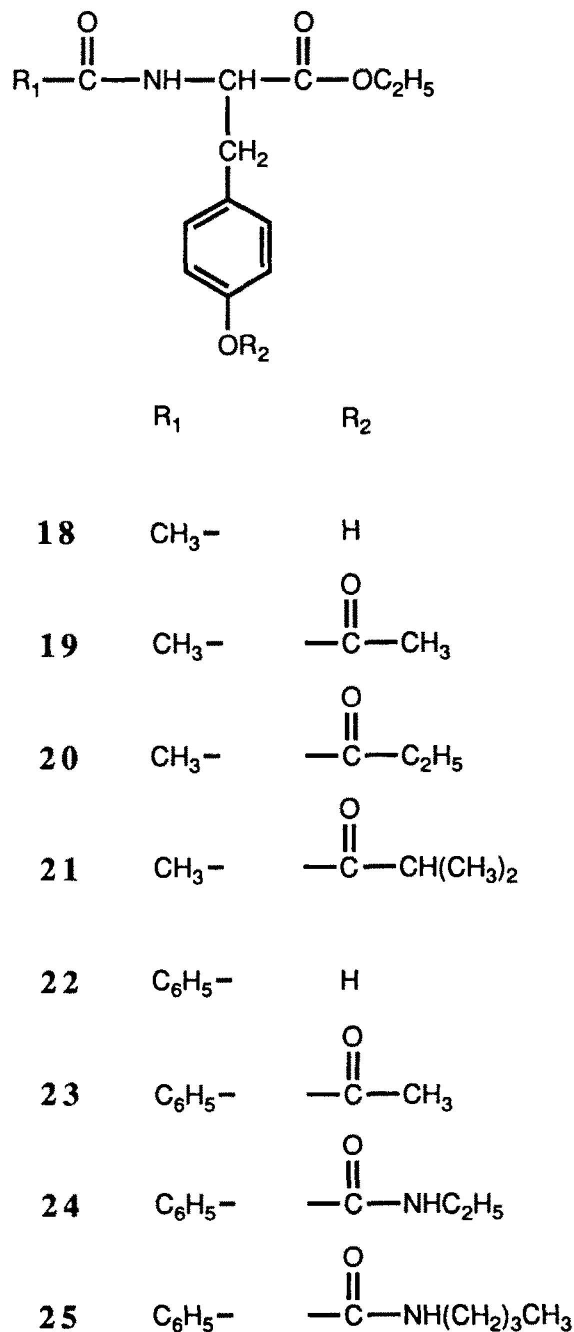


	R_1	R_2
1	CH_3-	H
2	CH_3-	$-C(=O)-CH_3$
3	CH_3-	$-C(=O)-C_2H_5$
4	CH_3-	$-C(=O)-CH(CH_3)_2$
5	CH_3-	$-C(=O)-C(CH_3)_3$
6	CH_3-	$-C(=O)-(CH_2)_3CH_3$
7	CH_3-	$-C(=O)-(CH_2)_4CH_3$
8	CH_3-	$-C(=O)-C_6H_5$
9	CH_3-	$-C(=O)-OCH_3$
10	CH_3-	$-C(=O)-O(CH_2)_3CH_3$
11	CH_3-	$-C(=O)-(CH_2)_3COOH$
12	CH_3-	$-C(=O)-NHC_2H_5$
13	CH_3-	$-C(=O)-NHCH_2COOC_2H_5$
14	C_6H_5-	H
15	C_6H_5-	$-C(=O)-CH_3$
16	C_6H_5-	$-C(=O)-C_2H_5$
17	C_6H_5-	$-C(=O)-OCH_3$

Scheme 1.

[D-Ala[1]]-peptide T amide (D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr amide). As expected from the known specificity of α-chymotrypsin (Hess, 1971), this degradation primarily involved cleavage of the Tyr-Thr bond. Thus, at an α-chymotrypsin concentration as low as 0.01 mg ml^{-1} the half-life for the cleavage of this peptide at pH 7.4 and 37 °C was only 0.6 min. The normal concentration of α-chymotrypsin in the gut including the stool is about 0.5 mg ml^{-1} (Goldberg et al., 1968; Dockter et al., 1986). Therefore, a means to protect [D-Ala[1]]-peptide T amide against cleavage by α-chymotrypsin is mandatory for the development of an orally absorbable preparation of the peptide.



Scheme 2.

Several years ago, Peterson et al. (1963) reported that the facile α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine methyl ester is diminished by several orders of magnitude when the tyrosine phenol group is replaced by a methoxy, ethoxy or isopropoxy group. Later, Kundu et al. (1972) reported that the chymotryptic hydrolysis of *N*-acylated tyrosine esters could also be diminished by esterification of the phenolic group with acetic, propionic or pivalic acid. These findings inspired us to examine the possibility of developing prodrug derivatives of the tyrosine phenol group with the aim of obtaining derivatives which, on the one hand, are stable toward α-chymotrypsin and, on the other, are readily hydrolyzed in the blood, e.g. by plasma enzyme-catalyzed hydrolysis. To this end, we have prepared a number of esters and carbamates of α-*N*-acylated L-tyrosine amide or ethyl ester, used as α-chymotrypsin-reactive model compounds, and studied their reactivity toward the enzyme as well as their chemical stability and esterase-catalyzed hydrolysis. The structures of the compounds investigated are depicted in Schemes 1 and 2.

Materials and Methods

Apparatus

High-performance liquid chromatography (HPLC) was performed with a Shimadzu system consisting of an LC-6A pump, an SPD-6A variable-wavelength UV detector and a Rheodyne 7125 injection valve with a 20 μl loop. Separations were generally performed with a deacti-

vated Supelcosil LC-8-DB reversed-phase column (33 × 4.6 mm, 3-μm particles) in conjunction with a Supelguard precolumn (both from Supelco Inc., U.S.A.). In some cases, a Chrompack Spherisorb ODS-2 column (100 × 3 mm, 5-μm particles) was used. Readings of pH were carried out on a Radiometer Type PHM 83 Autocal instrument. Melting points were taken on a capillary melting point apparatus and are uncorrected. Microanalysis was performed at Leo Pharmaceuticals, Ballerup, Denmark.

Chemicals

N-protected amino acid derivatives (all of the L-configuration) were purchased from Bachem AG, Bubendorf, Switzerland. α-Chymotrypsin (type II; from bovine pancreas) (56 units/mg) was obtained from Sigma Chemical Co., MO, U.S.A. Chemicals and solvents used in the kinetic studies were of reagent grade.

Preparation of tyrosine derivatives

The *O*-acyl derivatives **2–4**, **6**, **7**, **15**, **16**, **19–21** and **23** were prepared by stirring a mixture of 1.5 mmol of the parent *N*-protected tyrosine amide or ethyl ester and 4.5 mmol of the appropriate acid anhydride in 5 ml of pyridine at room temperature for 1 h. The *O*-pivaloyl (**5**) and *O*-benzoyl (**8**) derivatives were prepared by refluxing a mixture of *N*-Ac-Tyr-NH_2 (4 mmol), the appropriate acid chloride (5 mmol) and *N*,*N*-dimethylaminopyridine (0.4 mmol) in 10 ml of pyridine for 2 h. Upon evaporation of pyridine in vacuo, water was added to the residue. For the tyrosine amide derivatives **2–8**, **15** and **16**, the precipitate formed was filtered off, washed with water and recrystallized from ethanol. In the case of the tyrosine ester derivatives **19–21** and **23**, the residue was extracted with ethyl acetate. The extract was washed with 0.1 M hydrochloric acid, 2% aqueous bicarbonate and water, dried over anhydrous sodium sulfate and evaporated in vacuo. The residue obtained was crystallized from ethyl acetate-petroleum ether.

The carbonate esters **9**, **10** and **17** were prepared by adding 6 mmol of the appropriate alkyl chloroformate to a mixture of 4 mmol of the *N*-protected tyrosine amide in 5 ml of pyridine. The mixture was stirred at room temperature overnight and then evaporated under reduced pressure. The residue obtained was taken up in water and ethyl acetate. The organic phase was separated and washed as described above. The compounds obtained upon evaporation of the solvent were recrystallized from ethyl acetate-petroleum ether.

The glutarate ester **11** was prepared by the method of Bischoff and von Hedenström (1902). *N*-Acetyltyrosine amide (1.11 g, 5 mmol) was dissolved in 10 ml of ice-cold 0.5 M sodium hydroxide. Glutaric anhydride (0.57 g, 5 mmol) was added and the mixture was stirred for 5 min and acidified with 4 M hydrochloric acid. The resulting precipitate was filtered, dried and recrystallized from ethanol-water.

The carbamates **12**, **13**, **24** and **25** were prepared by adding 2.5 mmol of the appropriate alkyl isocyanate to a mixture of 2.0 mmol of the *N*-protected tyrosine amide and 2.5 mmol of triethylamine in 10 ml of acetonitrile. The reaction mixture was stirred at room temperature overnight. The precipitate formed was filtered off, washed with water and recrystallized from acetone-*N*,*N*-dimethylformamide.

N,*O*-Diacetyltyrosine was prepared by adding acetic anhydride (0.28 ml, 3.0 mmol) to a solution of *N*-acetyltyrosine (0.45 g, 2.0 mmol) in 5.0 ml of 0.5 M sodium hydroxide. The pH was kept at 9.0 ± 0.2 by addition of 1 M sodium hydroxide. After 5 min the reaction solution was acidified to pH about 2.5 and extracted with ethyl acetate. The extract was dried over anhydrous sodium sulfate and evaporated in vacuo. The residue obtained was recrystallized from ether-ethanol-petroleum ether, m.p. 157–159 °C.

Anal.: Calc. for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 58.51; H, 5.77; N, 5.20.

Physical and analytical data for the derivatives prepared are given in Table 1.

Kinetic measurements

The rates of degradation of the various tyrosine derivatives were followed by using reversed-phase HPLC procedures capable of separating the compounds from their products of degradation. Mobile phase systems of 5–30% (v/v) ace-

TABLE 1

Melting points and analytical data of various derivatives of N-protected tyrosine amides and esters

Compound	M.p. (°C)	Formula		Analysis (%)	
				Calculated	Found
2	225–226	$C_{13}H_{16}N_2O_4$	C	59.08	59.04
			H	6.10	6.08
			N	10.60	10.51
3	196–197	$C_{14}H_{18}N_2O_4$	C	60.42	60.55
			H	5.52	6.31
			N	10.07	10.09
4	215–216	$C_{15}H_{20}N_2O_4$	C	61.63	61.74
			H	6.90	6.89
			N	9.58	9.63
5	159–163	$C_{16}H_{22}N_2O_4$	C	62.73	61.93
			H	7.24	7.21
			N	9.14	9.16
6	200–201	$C_{16}H_{22}N_2O_4$	C	62.73	62.66
			H	7.24	7.30
			N	9.14	9.20
7	184–185	$C_{17}H_{24}N_2O_4$	C	63.73	63.95
			H	7.55	7.53
			N	8.74	8.74
8	257–260	$C_{18}H_{18}N_2O_4$	C	66.25	66.02
			H	5.56	5.57
			N	8.58	8.66
9	190–191	$C_{13}H_{16}N_2O_5$	C	55.71	55.62
			H	5.75	5.80
			N	9.99	9.88
10	195–197	$C_{16}H_{22}N_2O_5$	C	59.62	59.32
			H	6.88	6.95
			N	8.69	8.56
11	172–176	$C_{16}H_{20}N_2O_6$	C	57.14	57.12
			H	5.99	6.05
			N	8.33	8.28
12	206–207	$C_{14}H_{19}N_3O_4$	C	57.33	57.37
			H	6.53	6.76
			N	14.33	14.06
13	186–188	$C_{16}H_{21}N_3O_6$	C	54.70	54.96
			H	6.02	6.10
			N	11.96	11.96
15	216–217	$C_{18}H_{18}N_2O_4$	C	66.25	66.27
			H	5.56	5.53
			N	8.58	8.51
16	231–232	$C_{19}H_{20}N_2O_4$	C	67.05	66.66
			H	5.92	5.91
			N	8.23	8.14
17	213–215	$C_{18}H_{18}N_2O_5$	C	63.15	63.30
			H	5.30	5.51
			N	8.18	8.18
19	87– 88	$C_{15}H_{19}NO_5$	C	61.42	61.50
			H	6.53	6.47
			N	4.78	4.88
20	97– 98	$C_{16}H_{21}NO_5$	C	62.53	62.64
			H	6.89	6.79
			N	4.56	4.52

(continued)

TABLE 1 (continued)

Compound	M.p. (°C)	Formula		Analysis (%)	
				Calculated	Found
21	83– 84	$C_{17}H_{23}NO_5$	C	63.54	63.76
			H	7.21	7.11
			N	4.36	4.37
23	81– 82	$C_{20}H_{21}NO_5$	C	67.59	67.58
			H	5.96	6.02
			N	3.96	3.90
24	146–148	$C_{21}H_{24}N_2O_5$	C	65.61	65.64
			H	6.29	6.40
			N	7.29	7.33
25	128–130	$C_{23}H_{28}N_2O_5$	C	66.97	66.87
			H	6.84	6.88
			N	6.79	6.70

tonitrile in 0.1% (v/v) phosphoric acid were used with triethylamine added at a concentration of 10^{-3} M to improve peak shapes. The concentration of acetonitrile was adjusted for each compound to give an appropriate retention time (2–8 min). The column effluent was monitored at 215 nm and the flow rate was 1.5 ml min^{-1}. Quantitation of the compounds was carried out by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

The degradation reactions in buffer solutions were initiated by adding 100 μl of a stock solution of the compounds in acetonitrile or methanol to 10 ml of preheated buffer solution in screw-capped test tubes, the final concentration of the compounds being 5×10^{-4} M. The buffers used were hydrochloric acid, acetate, borate and carbonate buffers at a total concentration of 0.02 M. A constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The solutions were kept in a water bath at 37 ± 0.2 °C and at appropriate intervals samples were taken and chromatographed immediately. Pseudo-first-order rate constants for the degradation were determined from the slopes of linear plots of the logarithm of residual derivative against time.

Degradation studies in the presence of α-chymotrypsin were performed at 37 °C in a 0.1 M phosphate buffer solution of pH 7.40 containing the enzyme at a concentration of 0.50 mg ml^{-1}. In the case of the more reactive compounds (**18**, **19**, **22** and **23**), a concentration of 5×10^{-5} mg ml^{-1} of α-chymotrypsin was also used. The initial concentration of the compounds was $0.5–1.0 \times 10^{-4}$ M. At appropriate intervals samples of 250 μl were withdrawn and added to 250 μl of a 5% (v/v) aqueous solution of perchloric acid in order to deproteinize the samples and stop the reaction. After mixing and centrifugation for 3 min at 13 000 rpm, 20 μl of the clear supernatant was analyzed by the HPLC procedures described above.

The hydrolysis of some derivatives was also studied in human plasma diluted to 80% with 0.05 M phosphate buffer of pH 7.40 as well as in 10% rat liver homogenate. Samples of 250 μl were withdrawn and deproteinized by the addition of 500 μl of a 2% solution of zinc sulfate in methanol-water (1 : 1 v/v). The supernatant obtained following centrifugation was analyzed by HPLC as described above.

Results and Discussion

Stability toward α-chymotrypsin

α-Chymotrypsin is an endopeptidase (serine protease) which catalyzes the hydrolysis of peptide bonds in which the reactive carbonyl group belongs to the L-amino acids tryptophan, tyrosine, phenylalanine and, to a lesser extent, leucine and methionine (Hess, 1971). It also catalyzes the

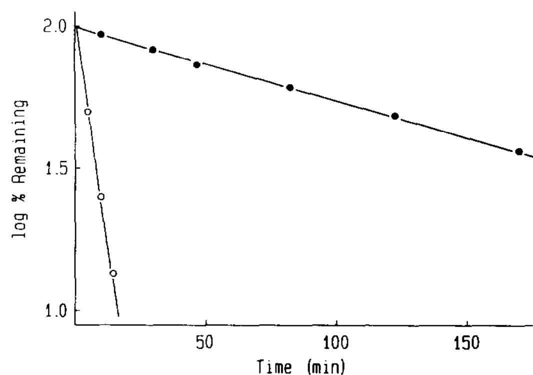


Fig. 1. First-order kinetic plots for the hydrolysis at 37 °C of *N*-Bz-Tyr-NH_2 (**14**) (○) and its *O*-acetyl derivative (**15**) (●) in 0.1 M phosphate buffer solution (pH 7.40) containing 0.50 mg ml^{-1} of α-chymotrypsin.

hydrolysis of various simple esters and amides (Bruice and Benkovic, 1966; Hansch and Coats, 1970; Bender and Killheffer, 1973). In this study, *N*-acetyltyrosine amide (**1**) and *N*-benzoyltyrosine amide (**14**) were used as models for the tyrosyl residues in tyrosine-containing peptides. The corresponding ethyl esters **18** and **22** are known to be particularly readily hydrolyzed by α-chymotrypsin (Bruice and Benkovic, 1966) and were also used as model compounds.

The stability of these substrates and the various derivatives obtained by esterification or carbamoylation of the tyrosine phenolic group was examined at 37 °C in a 0.1 M phosphate buffer solution of pH 7.40 containing 0.50 mg ml^{-1} of α-chymotrypsin, corresponding to approx. 2×10^{-5} M. Under these conditions the compounds were found to degrade according to strict first-order kinetics. Typical first-order plots obtained are shown in Fig. 1. The half-lives of degradation are listed in Tables 2 and 3 along with the half-lives observed in the same buffer solution without α-chymotrypsin. The *N*-acylated tyrosine ethyl esters **18** and **22** are so reactive toward α-chymotrypsin that it was necessary to use a much lower enzyme concentration than 0.50 mg ml^{-1} in order to obtain specific rate data. The data obtained at a concentration of 5×10^{-5} mg ml^{-1} for these compounds and some of their ester derivatives are included in Table 3.

TABLE 2

Half-lives of hydrolysis of various derivatives of N-acetyl- and N-benzoyl-L-tyrosine amide in 0.1 M phosphate buffer solution with and without α-chymotrypsin at 37 °C

Compound	Half-lives	
	pH 7.4 (buffer)	0.50 mg ml^{-1} α-chymotrypsin
1		72 min
2	40 h	4.8 h
3	76 h [a]	64 min
4	137 h [a]	40 min
5	905 h [a]	4.4 h
6	136 h [a]	41 s
7	134 h [a]	25 s
8	215 h [a]	4.9 min
9	50 h	2.7 h
10	76 h	2.3 min
11	2.4 h	2.6 h
12	32 h	36 h
13	10 h	6.3 h
14		5.1 min
15	22 h	1.9 h
16	15 h	36 min
17	20 h	1.6 h

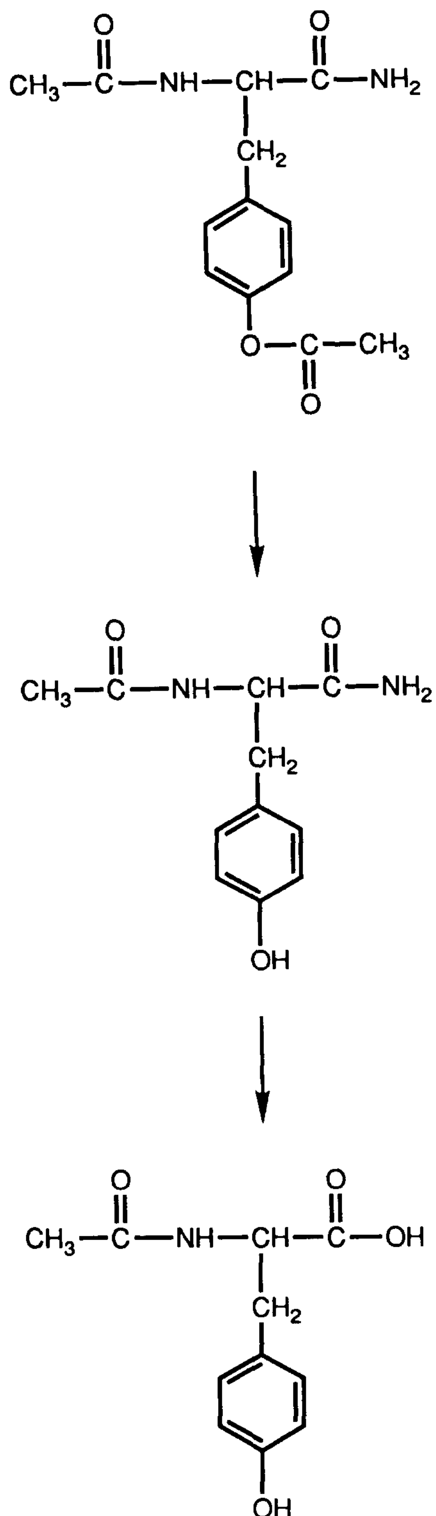
[a] Half-lives calculated from k_{OH} values determined in alkaline solutions.

The reaction of α-chymotrypsin with esters, amides and peptides follows a three-step process. In the first step an enzyme-substrate complex is formed and in the next, the substrate acylates the

TABLE 3

Half-lives of hydrolysis of various derivatives of N-acetyl- and N-benzoyl-L-tyrosine ethyl ester in the presence of α-chymotrypsin and in buffer solution at 37 °C

Compound	Half-lives		
	pH 7.4 (buffer)	α-Chymotrypsin	
		5×10^{-5} mg ml^{-1}	0.50 mg ml^{-1}
18		4 min	< 2 s
19	22 h	9.3 h	18 min
20	29 h		25 min
21	43 h		19 min
22		6 min	< 2 s
23	16 h	17 h	3 min
24	32 h		5.8 h
25	31 h		18.0 h



Scheme 3.

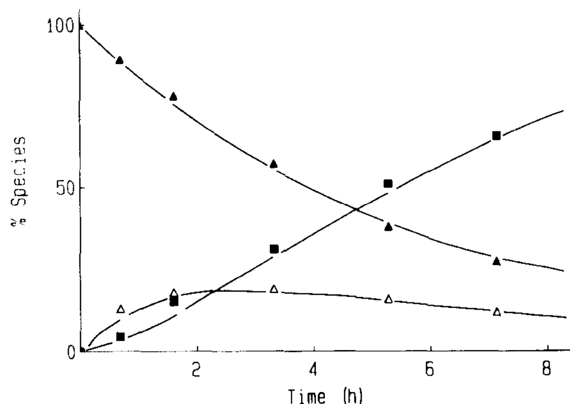


Fig. 2. Plots showing the time courses of degradation of the *O*-acetyl derivative of *N*-Ac-Tyr-NH_2 (**2**) (▲) and formation of *N*-Ac-Tyr-*N*H_2 (**1**) (△) and *N*-Ac-Tyr (■) in 0.1 M phosphate buffer solution (pH 7.40) containing 0.50 mg ml^{-1} of α-chymotrypsin at 37 °C.

active site (a serine hydroxyl group) in the enzyme. Thirdly, the acylated enzyme is solvolyzed to liberate a carboxylic acid and regenerate the enzyme (Bender and Killheffer, 1973). For hydrolysis of the amide or peptide bond the rate-determining step is the acylation of the enzyme whereas that for cleavage of esters is the deacylation reaction (Bender and Killheffer, 1973). It has been shown that the most meaningful kinetic parameter for comparing the specificity of various substrates to α-chymotrypsin is the ratio V_{max}/K_m, also called the specificity constant, where V_{max} is the maximum rate of substrate consumption and K_m is the Michaelis constant (apparent binding constant) (Bender and Kezdy, 1965; Brot and Bender, 1969). When the substrate concentration is lower than K_m the enzymatic reaction is first-order with the rate constant being equal to V_{max}/K_m. Such conditions prevailed during the present study and accordingly, the half-lives given in Tables 2 and 3 refer directly to the specificity constant:

$$t_{1/2} = 0.693/(V_{max}/K_m) \quad (1)$$

Inspection of the rate data in Table 2 reveals a widely different pattern of reactivity of the OH-modified tyrosine peptide amides. It is readily apparent that esterification of the tyrosine phenol group can afford a large degree of protection

against α-chymotrypsin-catalyzed hydrolysis of the C-terminal amide bond in the model compounds *N*-acetyl-L-tyrosine amide (**1**) and *N*-benzoyl-L-tyrosine amide (**14**) but this is quite dependent on the chain length of the ester. The acetate esters of both **1** and **14** (compounds **2** and **15**) are seen to be stabilized by a factor of 4 and 22, respectively. The time courses for the degradation pattern of *N*,*O*-diacetyl-L-tyrosine amide (**2**) as obtained by HPLC analysis of the reaction solution are shown in Fig. 2. Kinetic analysis of the data in Fig. 2 in the usual way for consecutive reactions showed that the α-chymotrypsin-catalyzed degradation of compound **2** proceeds via an initial and quantitative formation of *N*-acetyl-L-tyrosine amide (**1**) which is subsequently hydrolyzed in a more rapid step to *N*-acetyl-L-tyrosine (Scheme 3). In Fig. 2 the curves for the intermediately formed compound **1** and the final product *N*-acetyl-L-tyrosine were constructed on the assumption that compound **2** is hydrolyzed exclusively to yield compound **1** as depicted in Scheme 3. The good agreement observed between the experimental and calculated data demonstrates that the sole or predominant ($>90\%$) reaction of the *O*-acetyl ester **2** is hydrolysis of the phenol ester moiety. It can thus be concluded that acetylation of the tyrosine phenolic group affords a complete protection of the tyrosyl amide bond against α-chymotrypsin. These findings also show that, although the *O*-acetylation (and most likely esterification in general) makes the tyrosyl amide bond resistant to the enzyme, the esterolytic activity of α-chymotrypsin is responsible for the lower stability of the derivatives in the enzyme solutions as compared with pure buffer solutions. As seen from Table 2, the *O*-acetyl ester derivatives **2** and **15** are degraded about 10-times faster in the presence of α-chymotrypsin at a concentration of 0.50 mg ml^{-1} than in the same buffer solution without the enzyme. This cleavage of the protecting ester group becomes more predominant as the alkyl chain of the esters is enlarged. Thus, the ester functions in the valerate (**6**) and hexanoate (**7**) esters are cleaved much more rapidly than the amide group in the parent compound **1** so that the net result is that no protection of the latter is achieved at all.

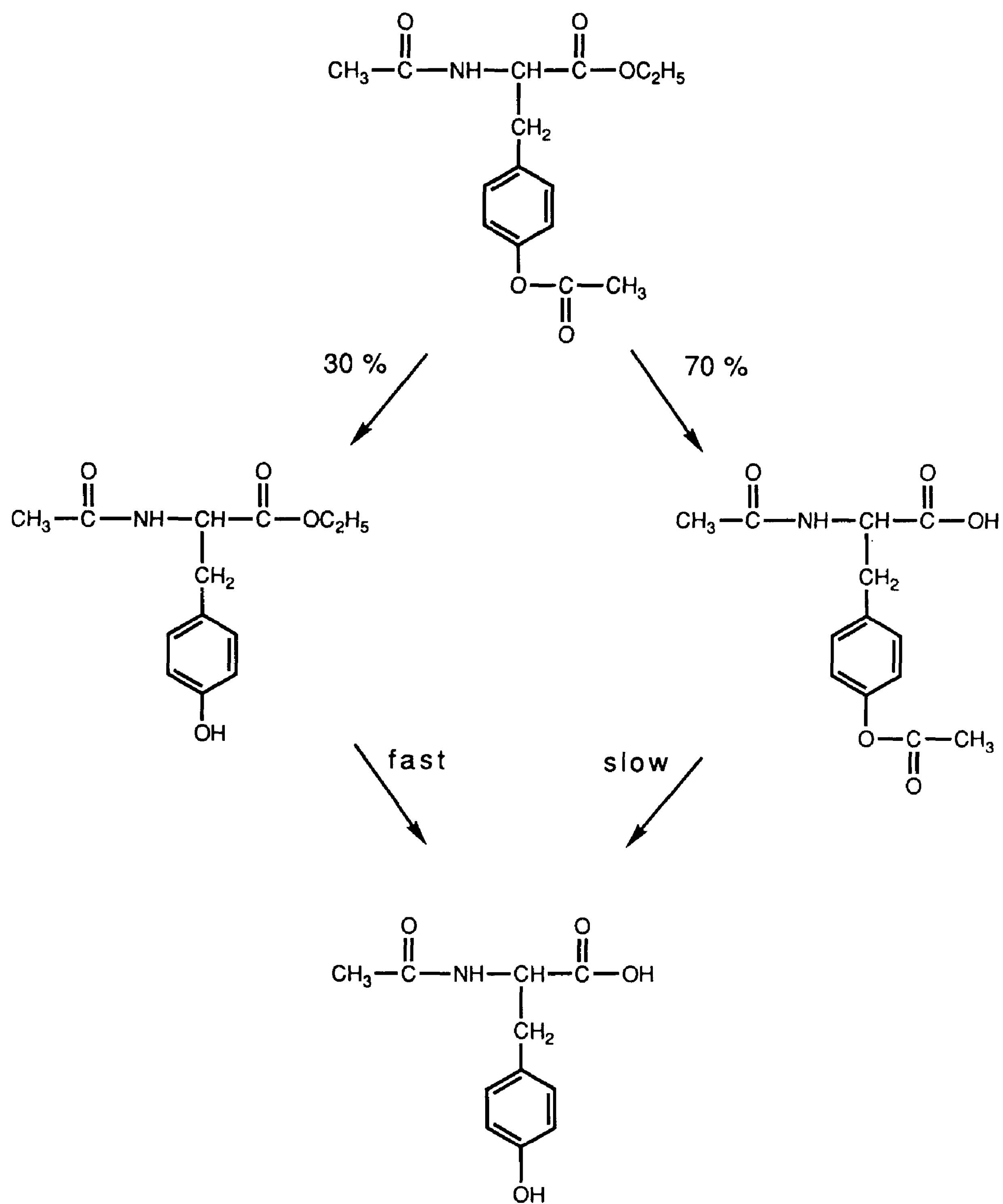
The same picture is seen with the carbonate esters. While the methyl carbonate esters **9** and **17** are more stable than the parent *N*-acylated tyrosine amide the butyl carbonate ester **10** is rapidly hydrolyzed by α-chymotrypsin.

Besides being influenced by the chain length of the acyl group, the α-chymotrypsin-catalyzed hydrolysis of the protecting ester moiety is dependent on steric factors. Thus, the *O*-pivaloyl ester derivative **5** is almost as stable as the *O*-acetyl ester **2**.

Introduction of a negative charge in the ester group as exemplified with the *O*-glutarate ester **11** is seen to have a dramatic influence upon the reactivity toward α-chymotrypsin. In fact, compound **11** shows the same stability in the pH 7.40 buffer solution containing α-chymotrypsin as in the solution without the enzyme. The rather facile hydrolysis of the glutarate ester **11** at pH 7.40 is due to intramolecular nucleophilic attack of the ionized carboxy group on the ester function with the formation of glutaric anhydride as has been reported for glutarate esters of other phenols (Bruice and Pandit, 1960; Gaetjens and Morawetz, 1960).

The conversion of the tyrosine phenol group into a carbamate derivative likewise results in derivatives showing a high resistance toward α-chymotrypsin-catalyzed degradation. Thus, the ethyl carbamate **12** is completely stable toward the enzyme as seen from the data in Table 2 whereas the carbamate **13** derived from glycine ethyl ester is only slightly more unstable in the presence of the enzyme as compared to pure buffer solution. This difference may be due to a slight chymotrypsin-catalyzed hydrolysis of the ethyl ester function in compound **13**.

The same dependence between ester structure and susceptibility to undergo α-chymotrypsin-catalyzed cleavage was observed with the ethyl ester substrates **18** and **22**. As seen from Table 3, the *O*-acetyl derivatives **19** and **23** afford a marked protection of the ethyl ester moiety in the parent compounds **18** and **22**, respectively, the degree of protection amounting to factors of 140 and 170. A noticeable difference from the amide substrates **1** and **14** is, however, that the *O*-propionyl and *O*-isobutyryl esters **20** and **21** are as stable as



Scheme 4.

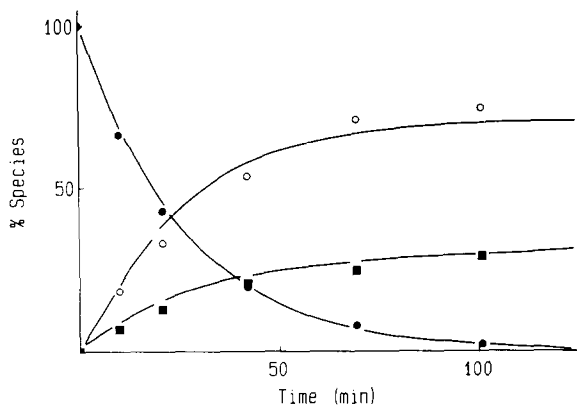


Fig. 3. Plots showing the time courses of degradation of the *O*-acetyl derivative of *N*-Ac-Tyr-OEt (**19**) (●) and formation of *N,O*-diacetyl-Tyr (○) and *N*-Ac-Tyr (■) in 0.1 M phosphate buffer solution (pH 7.40) containing 0.50 mg ml^{-1} of α-chymotrypsin at 37 °C.

the *O*-acetyl ester (**19**) toward α-chymotrypsin. As is the case for the carbamate esters of the *N*-acyl tyrosine amides, the corresponding derivatives (**24** and **25**) of *N*-benzoyl-L-tyrosine ethyl ester are seen to be highly stable in the presence of α-chymotrypsin.

Whereas the kinetic analysis of the degradation of compound **2** revealed a complete protection of the amide group toward α-chymotrypsin by the *O*-acetylation as described above, similar analysis of the degradation pattern of the *O*-acetyl ester of *N*-acetyl-L-tyrosine ethyl ester (**19**) showed a different result. HPLC analysis of the reaction solutions of compound **19** revealed the formation of *N,O*-diacetyl-L-tyrosine in an amount of 70% and *N*-acetyl-L-tyrosine in a yield of 30% (Fig. 3). Since the former compound is rather stable under the assay conditions (the half-life of conversion to *N*-acetyl-L-tyrosine being 15.2 h), the observed 30% formation of *N*-acetyl-L-tyrosine must derive from the intermediate formation of the parent *N*-acetyl-L-tyrosine ethyl ester (Scheme 4). Consequently, the hydrolysis of the ethyl ester function in compound **19** proceeds 2.3-times (i.e., 70 : 30) more rapidly than that of the *O*-acetyl ester moiety. Although this finding implies that the *O*-acetylation of the phenol group in the reactive ethyl ester **18** does not completely protect the latter against α-chymotrypsin, a very marked degree of protection is achieved. This can also be observed from the rate data in Table 3 for the run performed with an α-chymotrypsin concentration of 5×10^{-5} mg ml^{-1}. The derivative **19** is 140-times more stable than compound **18**.

These results are in harmony with the findings of Peterson et al. (1963) on the hydrolysis of *O*-alkyl derivatives of *N*-acetyl-L-tyrosine methyl ester. They found that replacement of the tyrosine phenol group with a methoxy, ethoxy or isopropoxy group afforded protection against α-chymotrypsin by factors of about 500, 100 and 14×10^3, respectively. The *O*-alkylation of the phenolic group greatly diminishes the susceptibility toward α-chymotrypsin but does not completely abolish it. The degree of protection achieved with the ester groups used in the present study is also dependent on the structure of the groups. While *O*-acetylation of compound **22** affords a degree of protection of 170 the butylcarbamate derivative **25** is at least 3×10^4 times more stable than the parent compound **22**.

Kundu et al. (1972) have reported that the *O*-acetyl, *O*-propionyl and *O*-pivaloyl esters of *N*-acetyl-L-tyrosine ethyl ester remained com-

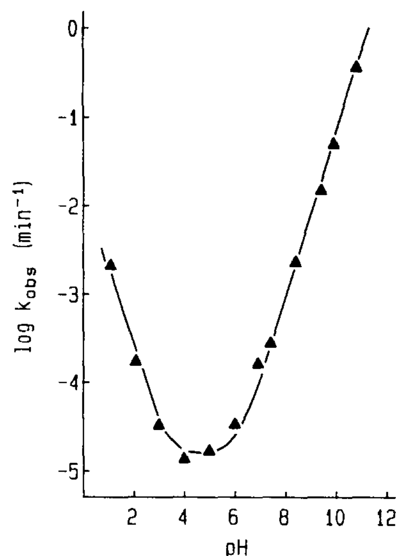


Fig. 4. The pH-rate profile for the decomposition of the *O*-acetyl derivative of *N*-Ac-Tyr-NH_2 (**2**) in aqueous buffer solution ($\mu = 0.5$) at 37 °C.

pletely stable on incubation with α-chymotrypsin at a concentration of 0.05 mg ml^{-1} for 3 h. This led the authors to conclude that acylation of the tyrosine phenol group completely abolishes the esterase activity of the enzyme toward an *N*-acylated tyrosine ester. As described above, this absolute conclusion is not supported by the present findings. Moreover, this study demonstrates that although esterification of the tyrosine phenol group affords a large degree of protection, this is limited by the fact that the ester grouping introduced can itself be hydrolyzed by α-chymotrypsin.

The stabilizing effect achieved by esterification of the tyrosine phenol group may be ascribed to steric factors hindering the interaction of the compounds with the enzyme. Plattner et al. (1988) have recently shown that whereas the aromatic side chains of phenylalanine and tyrosine fit into the hydrophobic binding pocket of α-chymotrypsin, the *O*-methyl group in *O*-methoxytyrosine collides with the wall in the pocket. An ester group may perform similarly.

Chemical stability and bioconversion

The kinetics of degradation of the *O*-acetyl derivative **2** was studied in aqueous solution as a function of pH in order to provide basic information on the stability of a potential *O*-acyl tyrosine peptide prodrug. The influence of pH on the rate of hydrolysis of compound **2** to produce *N*-acetyl-L-tyrosine amide at 37 °C is shown in Fig. 4 in which the logarithm of the observed pseudo-first-order rate constants (k_{obs}) has been plotted against pH. The U-shaped pH-rate profile can be accounted for by the following rate expression:

$$k_{obs} = k_H a_H + k_0 + k_{OH} a_{OH} \quad (2)$$

where a_H and a_{OH} are the hydrogen ion and hydroxide ion activity, respectively, k_H and k_{OH} are second-order rate constants for specific acid- and base-catalyzed hydrolysis, respectively, and k_0 is a first-order rate constant for spontaneous or water-catalyzed hydrolysis. The following values were found for these rate constants at 37 °C: $k_H = 2.5 \times 10^{-2}$ M^{-1} min^{-1}; $k_0 = 1.5 \times 10^{-5}$ min^{-1}; $k_{OH} = 3.5 \times 10^2$ M^{-1} min^{-1}.

TABLE 4

Half-lives of hydrolysis of various derivatives of N-acetyl-L-tyrosine amide in buffer solution, 80% human plasma and in 10% rat liver homogenate at 37 °C

Compound	Half-lives (h)		
	pH 7.4 (buffer)	80% human plasma	10% rat liver homogenate
2	40	1.8	< 10 s
9	50	0.5	< 10 s
11	2.4	4.1	1.0
12	32	20	22
13	10	3.4	1.6

The rate data given in Tables 2 and 3 for the other derivatives studied were either obtained directly in a pH 7.40 phosphate buffer or extrapolated from k_{OH}-values obtained at higher pH values. HPLC analysis of the solutions of the *O*-acetyl derivatives of *N*-acetyl- or *N*-benzoyl-L-tyrosine ethyl ester (**19** and **23**) showed that deacetylation was the predominant hydrolysis reaction as the parent compounds **18** and **22** were produced in stoichiometric amounts.

In order to be useful as prodrug forms for tyrosine-containing peptides, the derivatives should be readily converted to the parent compounds in vivo. Esters are in general readily hydrolyzed by virtue of plasma or liver esterases and, as observed from the data in Table 4, this is also the case for the acetyl ester **2** and the carbonate ester **9**. The hydrolysis of the glutarate ester **11**, which is completely stable against α-chymotrypsin, is, however, only slightly catalyzed by rat liver enzymes and not at all by human plasma. In fact, plasma shows an inhibiting effect on the rate of hydrolysis, probably due to binding of the compound to proteins. The lack of plasma catalysis for compound **11** is similar to the behavior of other negatively charged esters (Nielsen and Bundgaard, 1987). The hydrolysis of the carbamates **12** and **13** is slightly catalyzed in human plasma and rat liver homogenate (Table 4). For the glycine ethyl ester carbamate **13** a large part of this catalytic effect could, however, be ascribed to hydrolysis of the ethyl ester moiety to give a glycine carbamate ester as revealed by HPLC analysis.

Conclusions

The results obtained show that esterification of the tyrosine phenol group in tyrosine-containing peptides may be a useful prodrug approach to protect the peptides against cleavage by α-chymotrypsin. However, since the protecting ester moiety itself may be a substrate for α-chymotrypsin, this prodrug concept is limited to certain types of esters. As demonstrated with the tyrosine amide and ester model compounds, such esters include aliphatic carboxylic or carbonate esters with a short or branched side chain, a glutarate ester and carbamate esters. The concept of replacing a tyrosine hydroxyl group with a methoxy group has previously been used to obtain a peptide with improved stability toward α-chymotrypsin (Plattner et al., 1988), but since such alkylation of the phenolic group is not bioreversible, this approach may have a pronounced effect on the biological activity of the peptide. The presently described prodrug approach may achieve the same aim but with retention of the original peptide. Studies are in progress to apply this prodrug approach to the HIV-inhibiting peptide [D-Ala[1]]-peptide T amide and other tyrosine-containing peptides that are susceptible to α-chymotrypsin-catalyzed degradation.

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