Pergamon

Tetrahedron Letters 39 (1998) 997–1000

TETRAHEDRON LETTERS

Remarkable Effects of Donor Esters on the α-Chymotrypsin-catalyzed Couplings of Inherently Poor Amino Acid Substrates

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Received 21 October 1997; revised 2 December 1997; accepted 5 December 1997

Abstract: The extremely low efficiency during the α-chymotrypsin-catalyzed coupling of an inherently poor amino acid substrate, *e.g.*, alanine, using the methyl ester as an acyl donor was significantly improved using esters such as the 2,2,2-trifluoroethyl or carbamoylmethyl ester. The ameliorating effect of the latter ester was especially significant.

The use of proteases for peptide synthesis has attracted much attention in recent years.[1] The enzymatic methods have several advantages over the chemical couplings: enzyme specificity suppresses side reactions and ensures the production of chemically and chirally pure peptides. However, a narrow substrate specificity shown by the proteases is often regarded as a major drawback from a synthetic standpoint: only a limited number of amino acid residues can be incorporated into peptides. Subsequently, broadening the applicability of the protease-catalyzed peptide synthesis remains a challenging task. Recently, we investigated the α-chymotrypsin-catalyzed peptide synthesis using esters of *N*-protected amino acids or peptides as acyl donors and found that the use of the 2,2,2-trifluoroethyl ester instead of the conventional methyl ester is advantageous for the preparation of peptides containing non-protein amino acids such as halophenylalanines.[2] In the present work, we examined the applicability of several activated esters to the couplings of its inherently poor amino acid substrates such as alanine which lacks a large hydrophobic side chain.[3]

A series of *N*-protected L-alanine esters (Z-L-Ala-OR)[4] were allowed to react with L-leucine amide in acetonitrile containing 4% Tris buffer (pH 7.8). The yields of the desired peptide and the hydrolysis product of the donor ester after 48 h of incubation are compiled in Table 1 together with the relative initial rate (v_{rel}) of consumption of the substrate ester.[5,6] As expected, the yield of the desired peptide, as well as that of the hydrolyzed donor ester, was extremely low when the methyl ester was used as the acyl donor. This was also the case with other alkyl esters carrying a longer chain. In contrast, when halogenated alkyl esters such as the 2,2,2-trifluoroethyl ester were employed, the coupling efficiency was profoundly improved, while the competing hydrolysis of the donor ester was little accelerated.[7] The use of the benzyl ester also considerably improved the peptide yield, and its *p*-substituted esters showed a distinct substituent effect on the coupling efficiency: the *p*-nitrobenzyl ester gave a result similar to that obtained with the trifluoroethyl ester. In addition, a substantial decrease in the peptide yield caused by the insertion of a methylene group also suggests the inductive (electron-withdrawing) effect of the phenyl group. Alkyl esters bearing other electron-withdrawing groups, *e.g.*, the cyanomethyl,[8] methoxymethyl or acetylmethyl ester, considerably improved the coupling efficiency. Finally, it was gratifying to find that the carbamoylmethyl ester[9] was superior even to the halogenated alkyl esters. The advantage of this ester can be seen from its reaction profile (Fig. 1). Even after 2 h of incubation, the peptide yield reached 77%.

PII: S0040-4039(97)10798-5

Table 1. α-Chymotrypsin-catalyzed couplings of Z-L-Ala-OR with L-Leu-NH_2[a]

R	v_{rel}	σ*[b]	Yield/%[c]	
			Peptide	Z-L-Ala-OH
CH_3	1[d]	0	6.7	1.0
CH_2CH_3	0.54	-0.100	4.2	0.7
$(CH_2)_2CH_3$	0.39	-0.115	2.5	0.5
$(CH_2)_3CH_3$	0.53	-0.130	3.4	0.6
$CH_2CH(CH_3)_2$	0.76	-0.125	10.2	1.0
$CH_2C_6H_{11}$-*c*	0.68	-0.06	4.9	0.7
CH_2CF_3	17.5	+0.92	82.4	8.6[f]
$CH_2CF_2CF_3$	8.8		63.2	6.8[f]
CH_2CH_2Cl	3.4	+0.385	30.5	3.8[f]
CH_2CCl_3	14.1		87.8	6.9[f]
CH_2Ph	4.2	+0.215	28.6	4.2
$CH_2Ph(4NO_2)$	18.7		79.3	9.2
$CH_2Ph(4CN)$	11.4		72.7	9.6
$CH_2Ph(4Cl)$	5.3		33.6	4.0
$CH_2Ph(4OMe)$	2.8		19.2	2.2
$(CH_2)_2Ph$	0.61	+0.080	9.1	0.7
$(CH_2)_3Ph$	0.42	+0.02	6.8	0.5
CH_2CN	29.6	+1.300	88.3	6.4[f]
CH_2OCH_3	6.7	+0.64[e]	45.6	5.6[f]
CH_2COCH_3	6.9	+0.60	43.0	7.0[f]
CH_2CONH_2	133		88.4	10.9[f]
$CH_2CONHCH_3$	110		89.1	10.8[f]
$CH_2CON(CH_3)_2$	4.8		34.4	5.2

[a] A mixture of 0.05 mmol of Z-L-Ala-OR, 0.2 mmol of L-Leu-NH_2·HCl, 0.2 mmol of TEA, and 150 mg of the immobilized enzyme on Celite (corresponding to 4.7 mg of α-chymotrypsin) was incubated with shaking in a solvent composed of 2 ml of acetonitrile and 80 μl of Tris buffer (pH 7.8) at 30 °C. [b] Ref. 10. [c] After 48 h of incubation. [d] 2.79 $mM \cdot h^{-1} \cdot mg^{-1}$. [e] P. Ballinger and F. A. Long, *J. Am. Chem. Soc.*, 1960, **82**, 795. [f] Corrected for non-enzymatic hydrolysis (see Ref. 6).

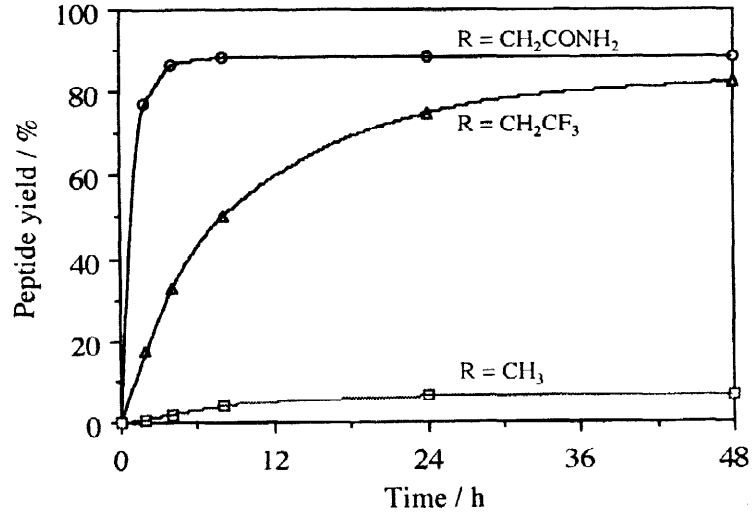

Fig. 1. Reaction profiles in the α-chymotrypsin-catalyzed couplings of Z-L-Ala-OR with L-Leu-NH_2.

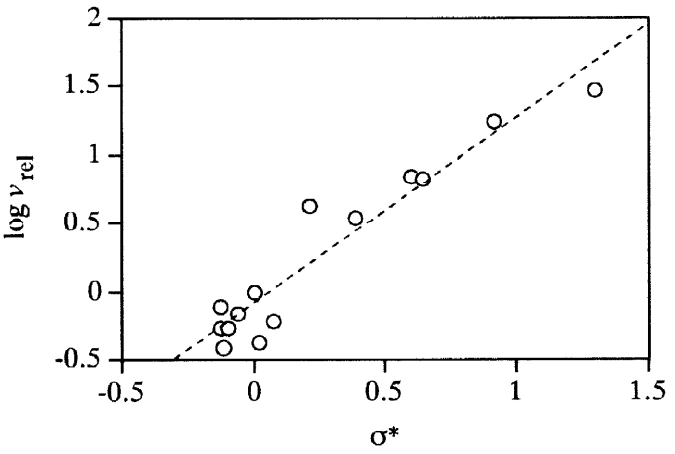

Fig. 2. Plot of log v_{rel} against σ* of R for the α-chymotrypsin-catalyzed coupling of Z-L-Ala-OR with L-Leu-NH_2.

As shown in Fig. 2, there is a rough proportionality between the log v_{rel} value for each ester substrate (Z-L-Ala-OR) in Table 1 and the polar substituent constant, σ*,[10] of R which is a measure of its polar or inductive effect: the higher the electron-withdrawing ability of the R group, the higher the v_{rel} value. The log v_{rel} value corresponds to the relative activation free energy which is dependent both on the stability of the enzyme-substrate (ES) complex and on the rate of acyl-enzyme formation. The correlation between log v_{rel} and σ* is indicative of the predominance of the effect of the R group on the acyl-enzyme formation over the binding of the substrate ester onto the enzyme. In the case of the carbamoylmethyl ester, however, its effect on the binding stage must also be relatively important, because the electron-withdrawing ability of this group should not be very large compared with other groups, *e.g.*, the acetylmethyl group. As shown in the bottom row of Table 1, the *N,N*-dimethylated carbamoylmethyl ester considerably diminished the peptide yield, probably suggesting the necessity of an amide proton of the carbamoylmethyl ester for the stabilization through the hydrogen bond of the ES complex.

As shown in Table 2, a marked enhancement of peptide yield using the trifluoroethyl ester or the carbamoylmethyl ester was also observed during the coupling of glycine or serine as the carboxyl component. The superiority of the carbamoylmethyl ester over other activated esters, *e.g.*, the trifluoroethyl ester, was also demonstrated by the coupling of the bulky valine as a carboxyl component. The coupling yield of 2-chloro- or 2-bromo-DL-phenylalanine bearing a bulky halogen atom at the *o*-position as a carboxyl component was significantly improved using the carbamoylmethyl ester. Furthermore, the use of this ester even allowed the formation of a peptide of D-alanine in moderate yield.

Acknowledgments. This work was supported in part by a grant from the Hirao Taro Foundation of the Konan University Association for Academic Research.

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1. a) C.-H. Wong and G. M. Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon, Oxford, 1994, p. 46; b) H.-D. Jakubke, *Enzyme Catalysis in Organic Synthesis*, eds. K. Drauz and H. Waldmann, VCH, Weinheim, 1995, B.2.5 (p. 431).
2. T. Miyazawa, S. Nakajo, M. Nishikawa, K. Imagawa, R. Yanagihara and T. Yamada, *J. Chem. Soc.*,

Table 2. α-Chymotrypsin-catalyzed couplings of Z-Xaa-OR with L-Leu-NH_2[a]

Xaa	R	Time/h	Yield/%	
			Peptide	Z-Xaa-OH
Gly	CH_3	48	0.4	0.6
	CH_2CF_3	48	74.3	10.9
	CH_2CONH_2	48	90.9	8.1
L-Ser	CH_3	48	8.7	1.2
	CH_2CF_3	48	87.9	10.7
	CH_2CONH_2	48	91.3	8.5
L-Val	CH_3	48	0	0.3
	CH_2CF_3	48	0.9	0.8
	CH_2CONH_2	48	13.6	6.8
DL-Phe(2Cl)	CH_2CF_3	1	19.2	3.2
	CH_2CONH_2	1	39.4	6.7
DL-Phe(2Br)	CH_2CF_3	1	13.9	3.4
	CH_2CONH_2	1	24.7	7.2
D-Ala	CH_3	48	0	0.8
	CH_2CONH_2	48	40.6	7.0

[a] The coupling conditions are the same as described in Table 1, using 0.05 mmol of acyl donor (0.1 mmol in the case of DL-amino acid derivatives).

Perkin Trans. 1, 1996, 1214.

3. W. Kullmann, *Enzymatic Peptide Synthesis*, CRC Press, Boca Raton, 1987, p. 41.
4. The abbreviations given by the IUPAC-IUB Commission are used throughout. Additional abbreviations: Z, benzyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; TEA, triethylamine; Phe(2Cl), 2-chlorophenylalanine; Phe(2Br), 2-bromophenylalanine; Boc, *t*-butoxycarbonyl.
5. The amounts of the peptide product and the hydrolyzed donor ester were quantified on an ODS column using 30 - 50% MeOH aq. containing 0.01 M H_3PO_4 as the eluent.
6. Non-enzymatic peptide synthesis was not detected even when the activated esters were employed, while a small amount of the donor ester was hydrolyzed non-enzymatically in some cases: R, % yield of Z-L-Ala-OH after 48 h; CH_2CF_3, 1.9; $CH_2CF_2CF_3$, 2.3; CH_2CH_2Cl, 0.2; CH_2CCl_3, 2.0; CH_2CN, 5.3; CH_2OCH_3, 2.9; CH_2COCH_3, 1.6; CH_2CONH_2, 0.7; $CH_2CONHCH_3$, 0.1.
7. In the kinetically controlled approach of protease-catalyzed peptide synthesis, the acyl-enzyme intermediate is partitioned between aminolysis and hydrolysis, affording the peptide product and the hydrolysis product of the donor ester. Once the acyl-enzyme intermediate is formed, its competitive partitioning is supposed to be unaffected by the ester moiety of the acyl donor, according to the simplified reaction scheme. It may have some effect in the case when the leaving alkoxy group (OR) is not completely detached from the acyl-enzyme intermediate before the deacylation by the nucleophiles occurs.
8. This ester was proposed as an active ester for the conventional peptide synthesis a long time ago: R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.
9. The use of carbamoylmethyl esters as enzyme substrates was reported some decades ago: R. J. Kerr and C. Niemann, *J. Org. Chem.*, 1958, **23**, 304. The carbamoylmethyl ester was also examined as a donor ester in the α-chymotrypsin-catalyzed coupling of Z- or Boc-phenylalanine in aqueous organic media, mainly taking advantage of its better solubility in aqueous phase: P. Kuhl, U. Zacharias, H. Burckhardt and H.-D. Jakubke, *Monatsh. Chem.*, 1986, **117**, 1195.
10. R. W. Taft, Jr., *Steric Effects in Organic Chemistry*, ed. M. S. Newman, John Wiely, New York, 1956, Chap. 13.