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On the Use of Carboxamidomethyl Esters in the Protease-Catalyzed Peptide Synthesis**

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Carboxamidomethyl esters (*CAM* esters) of *Z*- and *Boc*-protected alanine and phenylalanine were prepared in order to investigate their usefulness as substrates for α-chymotrypsin- and papain-catalyzed hydrolysis and peptide synthesis reactions. The easy removal of the *CAM*-C-protecting group under mild conditions and dependent on the enzyme specificity was demonstrated. Examples are given for the protease-catalyzed synthesis of various peptide derivatives using *CAM* esters as C- and N-components in aqueous-organic media. Comparatively short reaction times were observed.

(Keywords: Carboxamidomethyl ester as C-protecting group; Enzymatic deprotection; Peptide synthesis; α-Chymotrypsin- and Papain-catalyzed peptide bond formation)

Zur Verwendung von Carboxamidomethylestern in der proteasekatalysierten Peptidsynthese

Es wurden die Carboxamidomethylester (*CAM*-Ester) von *Z*- und *Boc*-geschütztem Alanin und Phenylalanin hergestellt mit dem Ziel, ihre Eignung als Substrate für α-chymotrypsin- und papainkatalysierte Hydrolyse- sowie Peptidsynthesereaktionen zu untersuchen. Die leichte, unter milden Bedingungen und in Abhängigkeit von der Enzymspezifität erfolgende Abspaltung der *CAM*-C-Schutzgruppe wurde nachgewiesen. An Beispielen wird die proteasekatalysierte Synthese verschiedener Peptidderivate unter Verwendung von *CAM*-Estern als C- und N-Komponenten in wäßrig-organischen Medien belegt. Die für die Umsetzungen benötigten Reaktionszeiten sind vergleichsweise gering.

** *Abbreviations:* IUPAC-IUB rules for peptides are followed, see Eur. J. Biochem. (1972) 27: 201. *Ac* = acetyl, *Bz* = benzoyl, *Boc* = *tert*-butyloxycarbonyl, *Z* = benzyloxycarbonyl, $-OBu^t$ = *tert*-butyl ester, $-CAM$ = carboxamidomethyl ester, $-OEt$ = ethyl ester, $-OMe$ = methyl ester, TLC = thin layer chromatography.

Introduction

Esters of amino acid and peptide derivatives are preferred substrates in the kinetically controlled approach to enzymatic peptide synthesis, which in general offers a maximum of product formation after short reaction times and with low enzyme concentrations [1, 2]. The solubility of the substrate in aqueous media and its effective binding at the active center of the protease are important factors for a successful synthesis. In this respect we suggested that the recently as carboxyl protecting group recommended carboxamidomethyl esters (*CAM* esters) [3, 4] could have some advantages due to their polar "peptide resembling" part in the alcohol moiety. Besides, these compounds structurally represent a shortened form of the glycolyl glycine esters of N-acylamino acids, the applicability of which in enzymatic peptide synthesis was well documented quite recently [5]. In the following we want to report on our results in using *CAM* esters as substrates in the protease-catalyzed peptide bond formation.

Results and Discussion

For the preparation of *CAM* esters of some N-acylamino acids we used a slightly modified procedure according to *Gisin* [6]. The N-protected amino acids were converted to their potassium salts, from which the corresponding *CAM* esters **1–4** could be obtained in sufficient yields by the reaction with chloroacetamide in *DMF*.

$$X\text{NHCH}R\text{COO}^-\text{K}^+ + \text{ClCH}_2\text{CONH}_2 \xrightarrow[-\text{KCl}]{60\,^\circ\text{C}} X\text{NHCH}R\text{COOCH}_2\text{CONH}_2 \qquad \textbf{1–4}$$

The compounds prepared in this way and their physical properties are listed in Table 1. A solubility test of compounds **1** and **3** in mixtures of water/*DMF* revealed that in comparison to the corresponding methyl esters more than twice of substance can be dissolved. Hence, the use of these substrates in serine and cysteine protease-catalyzed peptide synthesis enables one to work with a diminished portion of organic solvent, which in certain cases negatively affects the protease efficiency [8].

An essential prerequisite for the applicability of *CAM* esters in enzyme-catalyzed peptide synthesis is that the ester group can be split off by an esterolytically active protease forming an acyl enzyme which subsequently reacts with an amino component to the desired peptide derivative. First reports concerning the utilization of *CAM* esters as enzyme substrates have been given in the literature some years ago [9, 10].

At the beginning of our work we determined the rates of hydrolysis of compounds **1–4** in the presence of α-chymotrypsin and papain, respectively. For comparison we also investigated the hydrolysis of *Z*-Phe-O*Me*, *Z*-Ala-O*Me* and the standard substrates under the same conditions.

Table 1. *Yields and physical properties of CAM esters of N-acylamino acids*

	Compound	Yield (%)	M.p. (°C) [Lit.]	$[\alpha]_D^{20}$ (Lit.)	(in *DMF*)	R_F-Values			
						A	B	C	D
1	*Z*-Ala-O*CAM*	56	72–74 (74–75)[7]	−15.8 (−16.8)[7]	($c = 2$)	0.84	0.34	0.79	0.70
2	*Boc*-Ala-O*CAM*	49	97–98 (96–98)[3]	−28.1 (−27.0)[3]	($c = 2.1$)	0.81	0.37	0.77	0.69
3	*Z*-Phe-O*CAM*	58	87–88 (88.5–89.5)[7]	−37.4 (−38.5)[7]	($c = 1$)	0.92	0.47	0.81	0.79
4	*Boc*-Phe-O*CAM*	70	111–114 (113–116)[3]	−16.8 (−16.0)[3]	($c = 2.95$)[a]	0.92	0.44	0.82	0.80

[a] In *Et*OH.

Table 2. *α-Chymotrypsin-catalyzed hydrolysis of ester substrates*[a]

Substrate (17 m*M*)	**3**	**4**	*Z*-Phe-O*Me*	*Ac*-Phe-O*Me*
Specific activity (*μ*kat/mg)	4.52	3.37	0.43	0.29

[a] 0.05 *M* carbonate buffer (*pH* 10.5) with 50% *DMF*, 0.5 *μM* α-chymotrypsin.

Table 3. *Papain-catalyzed hydrolysis of ester substrates*[a]

Substrate (17 m*M*)	**1**	**2**	**3**	**4**	*Z*-Ala-O*Me*	*Bz*-Arg-O*Et*
Specific activity (nkat/mg)	333	233	84	73	40	46

[a] 0.05 *M* phosphate buffer (*pH* 8.5) with 33% (*v*/*v*) *DMSO*, 0.05 *M* thioglycolic acid, 0.025 *M EDTA*, 7.25 *μM* papain.

As it clearly can be seen from Tables 2 and 3, the *CAM* esters of *Z*- and *Boc*-protected phenylalanine and alanine are, in general, amenable to protease-catalyzed hydrolysis reactions. According to the substrate specificity, compounds **1**–**4** were accepted from papain, whereas α-chymotrypsin only catalyzed the hydrolysis of **1** and **2**. In all cases the *CAM* esters reacted more rapidly than the corresponding methyl esters. Under the given conditions the complete deprotection of 0.05 mmol **4** giving *Boc*-Phe-OH occurred within 30 minutes. The spontaneous hydrolysis of the *CAM* esters at *pH* 10.5 amounted to 0.1 *μ*mol ester/min (i.e. 5% of the rate of the enzyme-catalyzed reaction).

The enzymatic elimination of the *CAM* ester group from amino acid and peptide derivatives under mild conditions and without any risk of racemization also offers a valuable means in the classical way of peptide synthesis. This may be especially important in cases where alkaline conditions for the deprotection of *CAM* ester peptides should be avoided, because undesired side reactions may occur. Recently, it was reported in the literature [4] that it was not possible to remove selectively the *CAM* ester group of a peptide which contained a *β*-benzyl protected aspartate residue. On the example of *Boc*-Asp(O*Bzl*)-Phe-O*CAM* **5** we were able to demonstrate that the *CAM* ester group can be split off by α-chymotrypsin- or papain-catalyzed hydrolysis at *pH* 7.0 in some minutes. No damage to the *β*-benzyl aspartyl protection was found.

The experimental results of α-chymotrypsin-catalyzed peptide synthesis using *CAM* esters of *Z*- and *Boc*-protected phenylalanine as

substrates in the reaction with different nucleophilic components are summarized in Table 4. The addition of *DMF* as cosolvent was necessary for working in a homogeneous phase. The progress of the reactions was followed by TLC until no more substrate was found. With the exception of **11** the formed peptide products precipitated. As it could be expected from our previous experiments on this line, the use of the amino component in excess resulted in more favorable yields. From a synthetic point of view reactions with an amino acid amide or peptide amide as nucleophile were more satisfying than those with amino acid esters.

For reasons of comparison we were also interested in using *Z*-Phe-O*Me* as substrate in the reaction with Leu-NH_2, but it was not possible to dissolve it completely even in the presence of 60% (*v/v*) *DMF*. We therefore applied a reaction medium consisting of carbonate buffer and ethyl acetate (1 : 1) and worked under the conditions given in Table 4 with a substrate-nucleophile ratio of 1 : 3. While with **3** and *Z*-Phe-O*Me* almost the same yields (83 and 78%, resp.) were obtained, we noticed a remarkable difference in the reaction times, namely 15 min for **3** and 5.5 h for the methyl ester. Presumably the reason for this is to be found in the better solubility of **3** in the protease-containing aqueous phase, from which an increased reaction rate follows.

Examples for papain-catalyzed peptide synthesis reactions with N-blocked amino acid *CAM* esters are given in Table 5. The used reaction conditions and the yield dependence on increased nucleophile concentrations were similar to those for the α-chymotrypsin-catalyzed conversions. With the exception of **14** and **15** the formed dipeptide derivatives precipitated. An increase of the *DMF* content from 15 to 50% (*v/v*) in the synthesis of **13** at a substrate-nucleophile ratio of 1 : 2 had no distinct influence on the outcome of the reaction. The usefulness of a two-phase system in cases where at least one reactant is sparingly soluble in water was demonstrated with the synthesis of the model peptide **15**.

CAM esters of N-blocked peptides are possible intermediates for a further prolongation of a peptide by protease-catalyzed coupling steps. To verify this, compound **10** was used as carboxyl component for a papain-catalyzed reaction with Leu-NH_2 under conditions given in Table 5. The C/N-ratio was 1 : 5. In order to get a homogeneous phase 50% (*v/v*) *DMF* were added. The formed *Z*-Phe-Ala-Leu-NH_2 precipitated. The yield after a reaction time of 10 min amounted to 61%.

Furthermore, the synthesis of the tetrapeptide derivative *Z*-Phe-Phe-Ala-Leu-NH_2 was accomplished using the *CAM* ester substrates **3**, **10** (after deblocking with HBr/CH_3COOH) and **11** and the biocatalysts α-chymotrypsin and papain, respectively (Fig. 1). The last reaction step was carried out in the presence of 80% (*v/v*) 1,3-butanediol/*DMSO* (1 : 1) and with a tenfold amount of Leu-NH_2 giving 66% peptide product after 2.5 h.

Table 4. *α-Chymotrypsin-catalyzed peptide synthesis using CAM esters*[a]

Substrate (0.2 mmol)	Nucleophile	Ratio of reactants	Synthesized peptide		Yield (%)
3	Leu-NH_2	1 : 1	*Z*-Phe-Leu-NH_2	**6**	36
		1 : 2			60
		1 : 3			76
		1 : 5			87
	Leu-Ala-NH_2	1 : 1	*Z*-Phe-Leu-Ala-NH_2	**7**	25
		1 : 2			48
	Ala-O*Me*	1 : 5	*Z*-Phe-Ala-O*Me*	**8**	30
	Ala-O*Bu*^{t}	1 : 5	*Z*-Phe-Ala-O*Bu*^{t}	**9**	45
	Ala-O*CAM*	1 : 5	*Z*-Phe-Ala-O*CAM*	**10**	26
	Phe-Ala-O*CAM*	1 : 1	*Z*-Phe-Phe-Ala-O*CAM*	**11**	27
		1 : 2			48
4	Leu-NH_2	1 : 2	*Boc*-Phe-Leu-NH_2	**12**	62

[a] Reaction volume 3 ml, 0.2 *M* carbonate buffer (*pH* 9.0) with 40% (*v*/*v*) *DMF*, 10 *μM* α-chymotrypsin, reaction time 5 min.

Table 5. *Papain-catalyzed peptide synthesis using CAM esters*[a]

Substrate (0.2 mmol)	Nucleophile	Ratio of reactants	Synthesized peptide		Cosolvent *DMF* (% *v/v*)	Yield (%)
1	Leu-NH_2	1 : 1	*Z*-Ala-Leu-NH_2	**13**	15	66
		1 : 2			15	74
		1 : 4			15	80
		1 : 5			15	83
	Ala-O*Bu*^{t}	1 : 5	*Z*-Ala-Ala-O*Bu*^{t}	**14**	40	70
	Leu-O*Bu*^{t}	1 : 5	*Z*-Ala-Leu-O*Bu*^{t}	**15**	50[b]	78
3	Leu-NH_2	1 : 2	*Z*-Phe-Leu-NH_2	**6**	40	70
	Leu-O*Bu*^{t}	1 : 2	*Z*-Phe-Leu-O*Bu*^{t}	**16**	50	48
4	Leu-NH_2	1 : 2	*Boc*-Phe-Leu-NH_2	**12**	40	67

[a] Reaction volume 3 ml, 0.2 *M* phosphate buffer (*pH* 8.5), 0.15 m*M* papain, 0.2 *M* thioglycolic acid, reaction time 10 min.
[b] Ethyl acetate.

Summing up, it may be said that *CAM* ester derivatives of N-blocked amino acids and peptides are suitable substrates for the α-chymotrypsin- and papain-catalyzed peptide synthesis. Considering the substrate specificity of the protease they can be used both as carboxyl and amino components. In comparison to the corresponding methyl esters they are

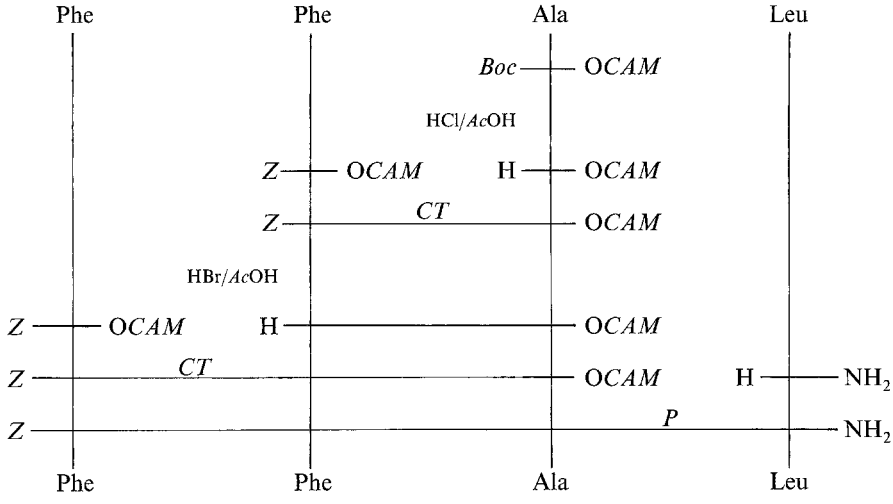

Fig. 1. Protease-catalyzed synthesis of Z-Phe-Phe-Ala-Leu-NH_2 using *CAM* ester substrates, *CT* = α-chymotrypsin, *P* = papain

somewhat better soluble in aqueous media, thus enabling a reduction of the organic solvent content. This may be favourable for enzyme activity. Using *CAM* esters, comparatively short reaction times are observed for peptide syntheses in homogeneous media as well as in two-phase systems. Finally, the protease-catalyzed removal of the *CAM* ester group under neutral conditions is advantageous in cases where treatment with alkali should be avoided due to undesired side chain deprotection.

Experimental

α-Chymotrypsin (EC 3.4.21.1) (3 times crystallized and lyophilized) and papain (EC 3.4.22.2) were commercially available from Lečiva Narodni Podnik (Prague, Czechoslovakia) and Merck (Darmstadt, FRG), respectively. Amino acids were obtained from Reanal (Budapest, Hungary). All other chemicals and reagents were either reagent grade quality or were purified and dried prior to use.

Melting points were determined with a *Boëtius* apparatus and are corrected. Optical rotations (Polamat A of VEB Carl Zeiss Jena, 1 dm cells) are accurate to at least $\pm 0.5°$. Compound **5** was obtained by mixed anhydride coupling of *Boc*-Asp(O*Bzl*)-OH with H-Phe-O*CAM*. Analytical data: m.p. 110–115 °C, $[\alpha]_D^{22} - 24.6$ ($c = 1$, *DMF*). Rates of hydrolysis were determined under *pH*-stat conditions using the autotitrator TTT1d from Radiometer (Copenhagen, Denmark). Amino acid analyses were accomplished with an amino acid analyser Kd-1200 E (ZSNP, Czechoslovakia). TLC was performed on silica-precoated foils (Kavalier, Czechoslovakia) using the following solvent systems: A: $CHCl_3$/*Me*OH/NH_3 (25%) (6:3:1), B: $CHCl_3$/*Me*OH (9:1), C: pyridine/*n*-butanol/H_2O (2:2:1), D: pyridine/*n*-butanol/H_2O/*Ac*OH (40:60:48:12).

Preparation of CAM Esters of N-Acylamino Acids

2 g N-acylamino acid were dissolved in 10–15 ml *Et*OH and diluted with 3–5 ml H_2O. Then the equivalent amount of KOH solution was added. The mixture was concentrated *in vacuo*, and after addition of benzene the water was eliminated by azeotropic distillation. The residue was then dried *in vacuo* over P_4O_{10}. It was dissolved in a minimal volume of *DMF*, to which a 20% excess of chloroacetamide was added. The mixture was stirred on a water bath at 60 °C for 8 h. During the reaction KCl precipitated as a whitish powder. The progress of the reaction was followed by TLC. The solvent was removed *in vacuo* and the residue dissolved in *Et*O*Ac*, washed repeatedly with saturated $NaHCO_3$ solution and then with H_2O. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was recrystallized from *Et*O*Ac*/petroleum ether. The *CAM* esters were obtained as crystalline compounds and characterized as usual (see Tab. 1).

Enzymatic Hydrolysis of **5**

22.4 mg (0.04 mmol) **5** were dissolved in 0.7 ml *DMF*. To this solution 1.8 ml 0.02 *M* phosphate buffer (*pH* 7.0) with 1.2 mg α-chymotrypsin were added. The progress of hydrolysis was followed under *pH*-stat conditions using the autotitrator. After 10 min the reaction came to an end. Examination by TLC (solvent systems A and B) revealed that no more *CAM* ester was present in the reaction mixture and that only one hydrolysis product had been formed. The same result was obtained using 8.7 mg papain as catalyst in the buffer which additionally contained 0.02 *M* thioglycolic acid.

Enzymatic Peptide Syntheses

The carboxyl and the amino component were dissolved in a mixture of buffer and organic solvent as indicated in Tab. 4 and 5. After the addition of the protease from a stock solution the resulting mixture was stirred at room temperature until the ester compound was no more detectable by TLC. In cases where the product precipitated, it was collected on a glass filter, successively washed with H_2O, 1 *M* HCl, saturated $NaHCO_3$ solution, H_2O, and then dried *in vacuo*. If necessary, the peptides were recrystallized from *Me*OH/H_2O, *Et*O*Ac*/petroleum ether or *DMF*/H_2O. Reaction mixtures without product precipitation were extracted with *Et*O*Ac*, and the organic phase was washed as described above for the solid material. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. After recrystallization from *DMF*/H_2O or *Me*OH/H_2O the yield was determined by weight. Physical properties of the products are complied in Tab. 6.

Table 6. *Physical properties of the enzymic reaction products*

Peptide	M.p. (°C)	Optical rotation $[\alpha]_D^{22}$ ($c = 1$, *DMF*)	R_F-System[a]	
			A	B
Z-Ala-Ala-O*Bu*^{t}	67–69	−22.7	0.90	0.75
Z-Ala-Leu-NH_2	187–189	−17.1	0.86	0.30
Z-Ala-Leu-O*Bu*^{t}	86–88	−16.2	0.87	0.74
Boc-Phe-Leu-NH_2	184–187	−22.3	0.92	0.45
Z-Phe-Ala-O*Bu*^{t}	99–100	−17.2	0.96	0.80
Z-Phe-Ala-O*CAM*	117–120	−15.7	0.85	0.41
Z-Phe-Ala-O*Me*	119–121	−20.9[b]	0.86	0.60
Z-Phe-Leu-NH_2	195–196	−19.7	0.93	0.46
Z-Phe-Leu-O*Bu*^{t}	89–91	−20.6	0.91	0.78
Z-Phe-Ala-Leu-NH_2[c]	240–242		0.90	0.34
Z-Phe-Leu-Ala-NH_2	211–213	−9.2	0.93	0.38
Z-Phe-Phe-Ala-O*CAM*	165–167	−28.7	0.92	0.45
Z-Phe-Phe-Ala-Leu-NH_2[c]	248–250		0.89	0.38

[a] See Experimental.
[b] $c = 0.2$, *Me*OH.
[c] Also characterized by amino acid analysis.

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