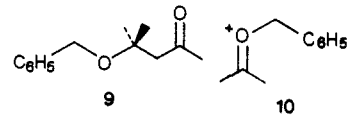
Table I. ^1H NMR Chemical Shifts for the Benzyl CH_2 Group of Labile Reactants and Products

compd	$\delta^{a,b}$
2A	4.91
2B	5.01 (5.13)[c]
2C	5.01
2D	3.26[d]
6A	5.47[e]
6B	5.56
6C	5.56
6D	4.20[d]
$C_6H_5CH_2N^+{\equiv}CCD_3$	5.31 (t, J_{NCH} = 2.5 Hz)
$4\text{-}NO_2C_6H_4CH_2N^+{\equiv}CCD_3$	5.46 (t, J_{NCH} = 2.5 Hz)
2'B	5.19 (5.13)[c] (5.30)[f] (4.76)[g]
6'B	5.47 (5.39)[c] (5.61)[f] (4.78)[g]

[a] Relative to TMS. [b] In $CDCl_3$ unless noted. [c] In CD_3CN. [d] For the CH_3 group. [e] The corresponding value for benzyl tosylate is 5.06 ppm. [f] In acetone-d_6. [g] In benzene-d_6.

to these salts produced the corresponding acetamides (80-90% yields).[16]

In acetone-d_6 in the absence of bases, the major product from the decomposition of **2A** was mesityl oxide [$(CH_3)_2C{=}CHCO\text{-}CH_3$], whereas in the presence of BMP the reaction product was compound **9**; the trapped benzyl cation **10** apparently reacts rapidly with the enol tautomer present in bulk acetone to form compound **9**.



9 **10**

A hierarchy of alkylating agents can thus be prepared conveniently and under mild reaction conditions: the method can produce—in decreasing order of reactivity the following: *carbonium ions*, *nitrilium salts* and other products formed from relatively unreactive solvents, *alkyl sulfonate esters*, and *alkyl derivatives* prepared from the sulfonates (quaternary ammonium salts, e.g.).

^1H NMR chemical shift values for the labile compounds mentioned in this article are listed in Table I.

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(14) Unexpectedly, the decomposition of **2'B** in CD_3CN at 25 °C (~2 days required) yielded only traces of the nitrilium salt; the major products were recovered amide (**1'B**) and *N*-(4-nitrophenyl)acetamide. The runs were assembled on a vacuum line (solvent distilled from P_2O_5), and the NMR spectra showed no trace of the water resonance (2.1 ppm). The formation of amides may stem from the greater base strength of the dinitrobenzene sulfonate ion versus the triflate ion, but the pathway and the sources of the protons and oxygen atoms are unknown.

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(16) Yields not maximized.

Modification of Proteases to Esterases for Peptide Synthesis: Methylchymotrypsin[1]

J. Blair West, Jeff Scholten, N. J. Stolowich, John L. Hogg,* A. I. Scott,* and Chi-Huey Wong*

Departments of Chemistry and of Biochemistry and Biophysics Texas A&M University, College Station, Texas 77843

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Recent investigations[2] have demonstrated the utility of protease-catalyzed synthesis of short peptides and semisynthesis of larger peptides via segment coupling. A serious drawback of this technique, however, is the potential loss of product due to the hydrolysis of sensitive peptide bonds by the protease. In our examinations of possible solutions to this problem, we have found certain target peptides that allow for the irreversible formation of product[3] as well as reaction conditions which selectively inhibit the amidase versus the esterase activities of several serine and cysteine proteases.[4] An alternate approach is the use of esterases which have no amidase activity,[5] although reaction rates are often slow.

A different approach was recently developed by Kaiser,[6] where an amidase-damaged protease, thiolsubtilisin, was used in conjunction with a mildly activated ester as acyl donor for segment coupling.

We report here the use of α-chymotrypsin methylated at $N^{\epsilon 2}$ of histidine 57[7] in peptide synthesis. This derivative, methylchymotrypsin (MeCT), is known to be some 4-5 orders of magnitude less active than α-CT toward ester substrates and inert toward amide substrates yet is known to have virtually identical binding properties to the native enzyme.[8] Although the modified enzyme is slowly active toward methyl esters, the use of cyanomethyl esters[9] increased rates considerably.

Kinetic parameters of the modified enzyme toward the two types of esters are shown in Table I. The lack of a common value for k_{cat} for the different esters indicates that with this enzyme, under these conditions, deacylation is no longer as strongly rate determining[10] so that both steps are kinetically significant. Both re-

Table I. Kinetic Parameters for the Hydrolysis of Z-L-Phe-OCH_2-X by Methylchymotrypsin[a]

X	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
H	1.5	4.5	0.33
CN	5.1	4.5	1.1

[a] All reactions performed in 50 mM KCl/DMSO (1:1), pH 8.8. Initial velocities determined by Radiometer pH-Stat at four or more substrate concentrations. Enzyme concentration 29 μM. Parameters determined by Lineweaver-Burke plots, with correlation coefficients of 0.98 or greater. All velocities corrected for spontaneous hydrolysis of the respective ester at this pH. Enzyme purity assessed by FPLC (phenyl sepharose column, 1.7-0 M linear gradient of NaCl in 20 mM Tris-HCl, pH 7.8, in 35 min) and active site concentrations determined by measuring the burst of nitrophenol from Z-Tyr-ONp (pH 5.0, 5% MeCN) spectrophotometrically.

(1) Abbreviations: Y, Tyr: tyrosine; G, gly: Glycine; F, phe: phenylalanine; L, leu: leucine; Z: benzyloxycarbonyl; CT: α-chymotrypsin; MeCT: *N*-methylchymotrypsin; Np: *p*-nitrophenyl. All amino acids are of the L configuration unless otherwise specified. This work was supported by the NSF (CHE 8318217) to C.H.W. and NIH Grants GM 31960 and 32596 to J.L.H. and A.I.S., respectively.

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(7) MeCT was prepared by reaction of the enzyme with methyl *p*-nitrobenzenesulfonate (Ryan, D. S.; Feeney, R. E. *J. Biol. Chem.* **1975**, *250*, 843) as modified by M. S. Matta (personal communication to J. S.). To further remove possible contamination by native chymotrypsin, the methylated enzyme was again reacted with phenylmethylsulfonyl fluoride and purified by affinity chromatography with lima bean trypsin inhibitor agarose.

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(9) These esters are mildly activated and have the advantages of being readily prepared from chloroacetonitrile and the given carboxylate without prior activation of the acid. They also possess enhanced solubility in aqueous media.

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Table II. Methylchymotrypsin Catalyzed Peptide Synthesis[a]

no.	acyl donor	acyl acceptor	product	enzyme	rxn time (h)	yield (%)
1	Z-L-Phe-OCH_2CN	L-Leu-NH_2	Z-L-Phe-L-Leu-NH_2	MeCT[b]	0.7	88
2	Z-D-Phe-OCH_2CN	L-Leu-NH_2	Z-D-Phe-L-Leu-NH_2	MeCT	0.7	4
3	Z-L-Phe-OCH_2CN	L-Leu-NH_2	Z-L-Phe-L-Leu-NH_2	none	48	5
4	Z-L-Phe-OMe	L-Leu-NH_2	Z-L-Phe-L-Leu-NH_2	MeCT	48	41[c]
5	Z-L-Phe-OCH_2CN	D-Leu-NH_2	Z-L-Phe-D-Leu-NH_2	MeCT	0.5	18
6	Z-L-Phe-OCH_2CN	D-Leu-NH_2	Z-L-Phe-D-Leu-NH_2	α-CT	0.5	21
7	Z-L-Phe-OCH_2CN	L-Leu-OMe	Z-L-Phe-L-Leu-OMe	α-CT	0.5	2
8	Z-L-Phe-OCH_2CN	L-Leu-OMe	Z-L-Phe-L-Leu-OMe	MeCT	0.5	91
9	Z-L-Leu-OCH_2CN	L-Leu-NH_2	Z-L-Leu-L-Leu-NH_2	MeCT	3	77
10	Z-Y-G-G-F-OCH_2CN	L-Leu-NH_2	Z-Y-G-G-F-L-NH_2	α-CT	0.7	56[d]
11	Z-Y-G-G-F-OCH_2CN	L-Leu-NH_2	Z-Y-G-G-F-L-NH_2	MeCT	0.7	99[e]

[a] Reaction conditions unless otherwise indicated: 100 mM acyl donor, 200 mM acyl acceptor, 1–2 mg enzyme in 1 mL Tris HCl/DMSO (1:1), pH 8.8. Reactions monitored by HPLC, peaks identified by coelution with samples authenticated by NMR. Yields from reactions containing Tris were normalized for small amounts of Tris adduct formed. [b] In all reactions, MeCT was pretreated ith phenylmethylsulfonyl fluoride for 30 min to inhibit traces of native CT. [c] 41% of ester unreacted. [d] Reaction buffer: 0.1 M phosphate/dioxane (35%), pH 10. After 11 h, 21% Z-Tyr-OH (secondary hydrolysis product) was detected. Similar results were obtained in 35% acetonitrile, with a 66% yield of peptide and 3% yield of Z-Tyr-OH. [e] No hydrolysis product detected after 24 h.

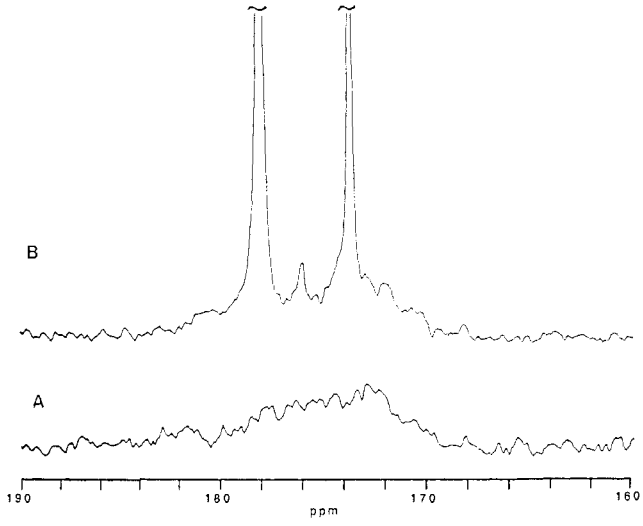

Figure 1. 75 MHz ^{13}C NMR spectrum of 1.6 mM MeCT in 0.1 phosphate/D_2O buffer, 50 mM NaCl, pH 4.4, 25 °C; (A) enzyme alone and (B) 24 h after the addition of 6 mM *N*-acetyl[1-13C]-L-Phe-OEt. Each spectrum represents 50 000 scans (5 h) of accumulation.

actions are still general-base catalyzed, thus accounting for the increase of activities at higher pH.[11]

The catalytic competency of MeCT can be further demonstrated by nuclear magnetic resonance (NMR) spectroscopy. The MeCT catalyzed hydrolysis of *N*-acetyl[1-^{13}C]-L-phenylalanine ethyl ester was monitored by ^{13}C NMR under conditions expected to accumulate acyl–enzyme intermediate (Figure 1). In addition to resonances assignable to substrate ester (δ 173.8) and product acid (δ 178.2) is a third signal at δ 176.2 ppm which can be attributed to the acyl intermediate formed with serine 195 of the enzyme. The result is in agreement with the value of δ 176.5 observed for the acyl intermediate formed between *N*-CBZ-lysine *p*-nitrophenyl ester and trypsin at −21 °C.[12] In this case, the acyl enzyme is observable at *room temperature*. Additionally, based on the relative areas of the three signals, it is estimated that approximately 20% of the enzyme is present as the acyl intermediate.

Table II summarizes our initial investigations. MeCT efficiently catalyzes the coupling of phenylalanine and leucine at rates much higher than the direct chemical coupling of the activated ester and the nucleophile (reaction 1 versus 3). The reaction is stereospecific in that Z-D-Phe-OCH_2CN is not an effective acyl donor. The range of acyl acceptor specificity is very broad, including L- and D-amino acid derivatives. Higher nucleophile concentrations will give correspondingly higher yields. Interestingly, the differing reactivities of the esters allow for the use of the methyl ester as a *protecting* group for hydrophobic, L-nucleophiles, which is not possible with the native enzyme (reactions 8 and 9).

The acyl donor specificity is the same as that of the native enzyme but does not appear to be as broad as that for thiol-subtilisin. Aromatic and hydrophobic amino acids such as tyrosine, phenylalanine, leucine, and methionine are reactive, while less hydrophobic amino acid derivatives such as Z-Gly-OCH_2CN were not. The narrow specificity, however, is often desirable when an enzyme is used in fragment coupling.

As a model of fragment coupling, the synthesis of Z-Leu-enkephalinamide was undertaken. This peptide contains a chymotrypsin sensitive peptide bond (Tyr-Gly). Hydrolysis of this bond will be diagnostic of secondary hydrolysis by the catalyst. Indeed, upon extended incubation of the reaction containing α-CT, free Z-Tyr-OH was detected (4–20%, depending on cosolvent). No Z-Tyr-OH was detected with MeCT, even after 24 h.

In summary, this communication provides a simple chemical procedure for alteration of chymotrypsin to an amidase-free esterase useful for peptide synthesis. Other serine proteases could be modified similarly to have such new enzymatic activities. The histidine residue in the active site of serine proteases may be a target for site-directed mutagenesis in order to develop novel esterases. Application of this esterase as a catalyst for the condensation of large peptide fragments containing usual and unusual amino acids is in progress.

(10) At pH 7.8, the ratio of the first-order rate constants for acylation (k_2) to deacylation (k_3) is smaller for methylchymotrypsin than for the native enzyme (7 versus 27 for Ac-Tyr-OEt) (ref 8). Native chymotrypsin exhibits similar reactivities in high organic solvent and high pH (unpublished data).

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Photoelectrochemical Evolution of Elemental Fluorine at TiO_2 Electrodes in Anhydrous Hydrogen Fluoride Solutions

Chong Mou Wang,[1] Qui-Chee Mir,[2] Simin Maleknia,[1] and Thomas E. Mallouk*,[1]

Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712
Department of Chemistry, Yakima Valley College Yakima, Washington 98907

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Although elemental fluorine has been known for more than 100 years,[3] it is still produced by Moissan's original method, electrolysis of anhydrous HF/alkali fluoride solutions. Only one convenient alternative route for preparing fluorine has been reported to date.[4] Several oxide and oxyfluoride semiconductors have valence band

* To whom correspondence should be addressed.

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