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Synthesis of Chromophoric Dipeptides as Substrates for Papain

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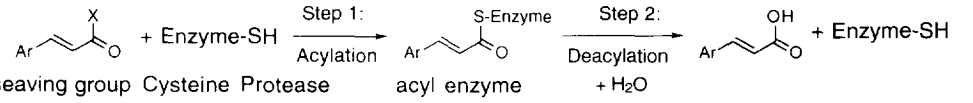
Abstract: Chromophoric dipeptides (**2**) and (**3**) are found to be better substrates for papain in comparison to simpler substrates **1a** or **1b**. A synthetic strategy for obtaining α-NH(COOEt)-5-MTA-Et (**1b**), Ala-5-MTA-Et (**2**), Phe-5-MTA-Et (**3**) has been described.

Chromophoric α,β-unsaturated substrates are useful probes for cysteine protease mechanism. Part of their utility lies in the fact that, since acylation is more rapid than deacylation (**Scheme 1**), they can be accumulated at low pH and analyzed by a number of biophysical techniques.

Scheme 1

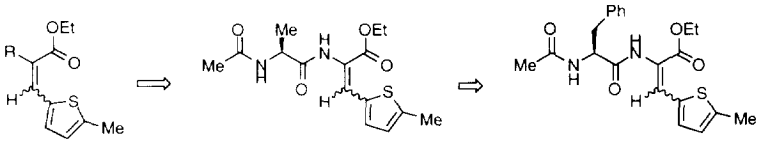


We have been carrying out extensive resonance Raman[3] and more recently Raman difference, spectroscopic studies of acyl-serine and acyl-cysteine proteases, where the acyl group is typically a thienylacryolyl or furylacrylolyl derivative, Ar = thienyl or furyl in Scheme 1. For a series of acyl-serine intermediates it was possible to identify the acyl carbonyl stretching vibration in the Raman spectra and to derive a precise relationship between the carbonyl bond length and the deacylation rate constant.[4] When we started to investigate the papain cysteine protease family to see if similar relationships can be derived, we found that for acyl enzymes based on "simple" aryl acryloyl substrates the range of deacylation rate constants is small. In order to extend the range we are synthesizing more complex "specific" substrates for papain, which have a hydrophobic residue at P_2 and thus take the advantage of papain's known propensity for a hydrophobic group at this position.[5,6]

Smolarsky[7,8] has demonstrated that chromophoric competitive inhibitors and specific substrates of papain can be formed by attaching phenylalanine to the α-carbon of cinnamic acid derivatives. We have elaborated on his approach to incorporate the 5-methylthienylacryloyl- chromophore (**1a** in **Scheme 2**) into specific substrates. This chromophore was selected because of the extensive pool of spectroscopic knowledge in our

laboratory which will facilitate interpretation of the Raman data for the acyl-cysteine protease intermediates. Here we report a novel synthetic strategy to incorporate NH-COOEt **(1b)**, NH-L-alanine **(2)**, NH-L-phenylalanine **(3)** derivatives at the α-position of 5-methylthienylacryloyl ethyl ester. In addition, preliminary kinetic data are reported.

Scheme 2

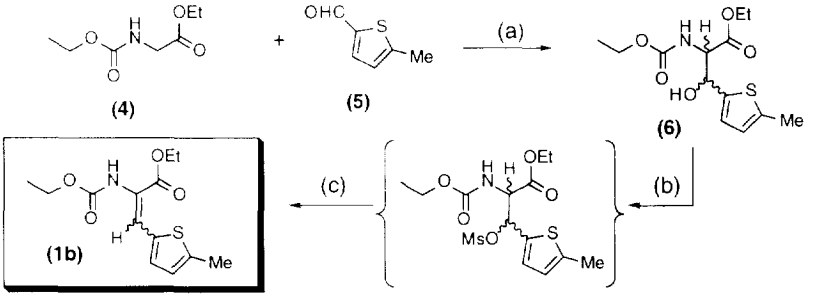


5-MTA-Et
R: H (1a), NHCOOEt (1b)

Ala-5-MTA-Et (2)

Phe-5-MTA-Et (3)

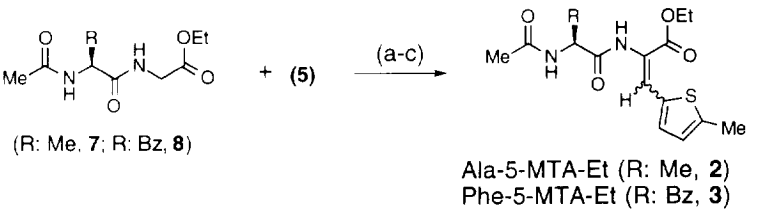
Synthesis of α-NH(COOEt)-5-MTA-Et (1b): The enolate of N(ethoxycarbonyl)-glycine ethyl ester **(4)** was generated by LDA/THF at -60 °C under argon and was reacted with the thiophene aldehyde derivative **(5)** to give the corresponding alcohol **6** as a mixture of diastereomers in 72% yield after purification over silica gel (EtOAc:Hexane, 1:5; EtOAc:Hexane, 1:3). The structure of **6** was assigned on the basis of MS, ^{1}H-NMR and ^{13}C-NMR.[9] The derivatization of the secondary alcohol to its mesityl derivative (MsCl, Et3N, rt) followed by treatment with the excess of base gave the conjugated derivative (5MTA-Et, N:NHCOOEt, **1b**) in 85% yield after purification (EtOAc:Hexane, 1:5).[9] **(Scheme 3)**



Scheme 3: (a) LDA (1.5 eq.), THF, -60 °C (15 min), **5** (1.2 eq., -60 °C to -20 °C); (b) MsCl (1.5 eq), Et3N (1.5 eq), THF, rt, 3h; (c) 4 eq of Et3N, THF, 2h, rt.

Synthesis of Ala-5-MTA-Et (2) and Phe-5-MTA-Et (3): Protected dipeptide **7** was prepared from the coupling of glycine ethyl ester and N(acetyl)-L-alanine or by the standard DCC activation procedure and was purified over silica gel by flash column chromatography. The enolate of N(acetyl)-L-Ala-glycine ethyl ester **(7)** was generated by LDA/THF at -78 °C

under argon and was reacted with the thiophene aldehyde **5** to give the corresponding alcohol derivative which was directly subjected to the mesitylation conditions (MsCl, Et_3N, rt) followed by elimination with the excess base to give Ala-5-MTA-Et **(2)** as a mixture of diastereomers in 58 % yield after purification over silica gel.[10] A similar procedure for the synthesis of Phe-5-MTA-Et **(3)** was utilized in which N(acetyl)-L-Phe-glycine ethyl ester **(8)** was used as a starting material.[10] **(Scheme 4)**



Scheme 4: (a) LDA (1.2 eq.), THF, -78 °C (15 min), 1.2 eq **5**, (-78 °C to rt); (b) MsCl (1.5 eq), Et_3N (1.5 eq), THF, rt, 3h; (c) 4 eq of Et_3N, THF, rt.

Results: Table shown below contains the k_{cat} and K_m parameters obtained from steady state assays for Phe5MTA- and Ala5MTA- papain. It was not possible to ascertain these parameters via steady state assays using the 5MTA or NHCOOEt5MTA ethyl ester substrates because of solubility limitations and their relatively poor reactivity. It was however possible to evaluate k_{cat} independently for these substrates by monitoring the deacylation of acylation complexes prepared from the more reactive imidazolide (Im) derivatives.

Substrate	k_{cat} (min^{-1})	K_m ($M \times 10^{-3}$)	k_{cat} (min^{-1}) / K_m ($M \times 10^{-3}$)
5-MTA-Im	0.07	-	-
NHCOOEt-5-MTA-Im	0.08	-	-
Ala-5-MTA-Et (2)	0.2	3.9	0.05
Phe-5-MTA-Et (3)	0.2	0.062	3.2

Procedure: Papain was purified and activated as described previously.[11] Steady state kinetic assays were performed in 50 mM bicine, 0.2 M NaCl, 1mM EDTA buffer at pH 8.5 containing 20% v/v acetonitrile at 25 °C. Initial velocities were determined at a constant papain concentration of 6.8 μM while the concentrations of either Ala or Phe-5-MTA ethyl ester were varied. k_{cat} and K_m parameters were determined from the initial velocities using Hanes-

Woolfe plots.[12] 5-MTA (**1a**) and NHCOO (**1b**) ethyl esters were converted to their imidazolide derivatives, and purified as described previously.[13] These acyl papain complexes were then diluted into the aforementioned buffer at pH 8.5 and the deacylation kinetics were monitored spectrophotometrically at 390 nm.

The greater reactivity of the new substrates (**2**) and (**3**) containing the dipeptide moieties is qualitatively apparent from the fact that they, but not simpler substrates **1a** or **1b**, could be assayed using steady state kinetics. Consistent with the known specificity of papain's S_2 subsite for phenylalanine[5,6] the k_{cat} / K_m ratio for **3**, with the phenylalanine side chain, is 64 fold greater than for **2** which contains alanine at the P_2 position. The k_{cat} for these two substrates are the same, consonant with the view that specificity for papain is expressed in the acylation step.[14] The dipeptide substrates have kcat 3 times greater than those for the smaller substrates **1a** or **1b**. While this is not a large change it does appreciably extend the range of deacylation values available to explore vibrational spectra-reactivity profiles.

References and Notes:

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9. All the new compounds were well characterized by MS, ^{1}H-NMR, 2D-COSY, ^{13}C-NMR analysis. **6**: LC-MS (thermospray): 302 $(M+H)^+$; ^{1}H-NMR: 6.7.3 (d, 1H, J 2Hz), 6.61 (d, 1H, J 2Hz), 5.6 (bs, 1H), 5.4 (bs, 1H), 4.2 (dq, 2H each, J 7Hz), 2.46 (s, 3H) and 1.27 (dt, 3H each, J 7Hz); ^{13}C-NMR: 170.3, 169.3, 157.0, 140.7, 139.8, 139.6, 124.7, 124.5, 124.4, 71.2, 70.1, 61.6, 61.5, 61.1, 60.2, 59.6, 15.0, 14.3 and 13.8. **1b**: 284 $(M+H)^+$; ^{1}H-NMR: 7.62 (s, 1H), 7.11 (d, 1H, J 2Hz), 6.7 (d, 1H, J 2Hz), 4.24 (q, 4H, J 7Hz), 4.18 (bs, 1H), 2.47 (s, 3H) and 1.29 (t, 3H, J 7Hz); ^{13}C-NMR: 165.2, 154.8, 146.4, 134.4, 133.6, 129.7, 125.6, 120.2, 61.7, 61.3, 15.6, 14.5 and 14.2
10. Dipeptide N(acetyl)Ala-Gly ethyl ester (**7**) and N(acetyl)Phe-Gly ethyl ester (**8**) were purified over silica gel by flash column chromatography and their structures were confirmed by GC-MS, LC-MS and ^{1}H-NMR spectra. **2**: MS (FAB): 325 $(M+H)^+$; ^{1}H-NMR: 1.32 (t, 3H, J 7Hz), 1.52 (d, 3H, J 6.5 Hz), 2.07 (s, 3H), 2.50 (s, 3H), 4.24 (q, 2H, 7Hz), 4.90 (q, 1H, 6.5Hz), 6.33 (bs, 1H), 6.7 (d, 1H, 3.3Hz), 7.14 (d, 1H 3.3Hz), 7.47 (bs, 1H), 7.71 (s, 1H). **3**: MS (FAB): 401 $(M+H)^+$; ^{1}H-NMR: 1.34 (t, 3H, J 7Hz), 2.04 (s, 3H), 2.64 (s, 3H), 3.24 (ddd, 2H, J 6.7, 7.4, 14 Hz), 4.26 (q, 2H, 7Hz), 4.94 (dd, 1H, 6.7, 7.4Hz), 6.12 (bs, 1H), 6.32 (d, 1H, 3.3Hz), 7.10 (d, 1H 3.3Hz), 7.32 (m, 5H), 7.68 (s, 1H); ^{13}C-NMR: 170.8, 170.5, 164.5, 146.5, 136.6, 136.6, 134.0, 133.9, 130.3, 129.6, 129.5, 128.6, 128.3, 126.9, 125.8, 119.7, 61.2, 54.0, 37.2, 23.1, 17.8, 17.0.
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