



AN ACTIVE-SITE TITRANT FOR CHYMOTRYPSIN, AND EVIDENCE THAT AZAPEPTIDE ESTERS ARE LESS SUSCEPTIBLE TO NUCLEOPHILIC ATTACK THAN ORDINARY ESTERS

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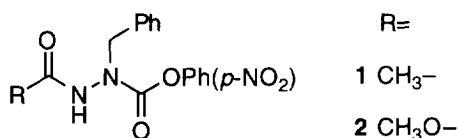
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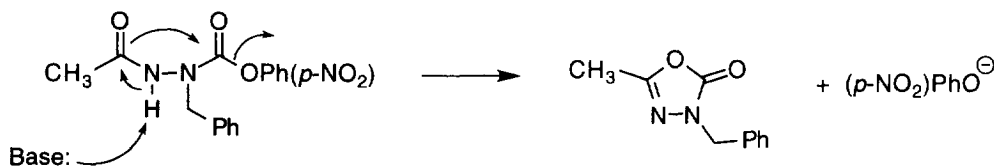
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Abstract: The *p*-nitrophenyl ester of *N*-methoxycarbonylazaphenylalanine reacts with chymotrypsin in 1:1 stoichiometry, and the enzyme concentration can be quantified from the absorbance of the *p*-nitrophenolate produced in the reaction. This azapeptide does not decompose at pH 7 in the presence or absence of 1 mM hydroxylamine. Copyright © 1996 Elsevier Science Ltd

A common problem in enzymology is determining the amount of active enzyme present in a protein sample. It has been reported¹ that the *p*-nitrophenyl ester of *N*-acetylazaphenylalanine² (**1**) forms a stable, acyl enzyme with chymotrypsin, and that the enzyme concentration can be quantified from the absorbance of the *p*-nitrophenolate produced in the reaction.³ However, **1** spontaneously decomposes (also producing *p*-nitrophenolate) at an appreciable rate at pH 7; and this background reaction must be subtracted from the reaction with enzyme.



The spontaneous release of *p*-nitrophenol observed for **1** is due to a cyclization reaction⁴ (Scheme 1); as is also found to be the case for normal peptides possessing good leaving groups.⁵ This reaction can be suppressed by replacing the acetyl function with a less electron-withdrawing carbonyl moiety, as in **2**, thereby decreasing the acidity of the N-H proton.^{6,7}



Scheme 1. Cyclization of an Azapeptide.

The *p*-nitrophenyl ester of *N*-methoxycarbonylazaphenylalanine (**2**) also reacts stoichiometrically with chymotrypsin, releasing *p*-nitrophenol(ate). As expected,^{6,7} **2** does not decompose spontaneously at pH 7; making it a more convenient active-site titrant.

Preparation of **2**: *N*¹-Benzyl-*N*²-methoxycarbonylhydrazine⁸ (180 mg, 1 mmol) and triethylamine⁹ (101 mg, 1 mmol) were dissolved in 3 mL dry CH₂Cl₂ and cooled to -5° in an ice-acetone bath. To this solution was added *p*-nitrophenyl chloroformate (201 mg, 1 mmol) dissolved in CH₂Cl₂. The solution was stirred and allowed to warm to room temperature. After 2 h, 20 mL EtOAc was added, and the triethylammonium hydrochloride was removed by filtration. The solution was extracted with 0.1 N HCl, saturated bicarbonate, dried with K₂CO₃, and the solvent was removed in vacuo. The crystalline product was isolated by flash chromatography (*R*_f 0.26, 2% EtOAc in CH₂Cl₂), and recrystallized from ether/hexanes (113 mg, 34%, m.p. 114.3–115.8).¹⁰ Starting from the corresponding hydrazide,⁸ **1** was prepared similarly (m.p. 122.7–123.9, lit. 116°).

Enzyme assay: Titrant stock solutions were made by dissolving 3.3 mg **1** (or 3.5 mg **2**) in 1 mL DMSO (titrant concentrations = 10 mM). The absorbance of 4 μL of the stock solution of **2** in 996 μL pH 7 buffer (100 mM, potassium phosphate) at 404 nm was determined (1.4 mA units). Enzyme stock was made from lyophilized α-chymotrypsin, approximately 10 mg of enzyme in 1 mL of 1 mM HCl. Into a cuvette, thermostatted at 25°, was pipetted 100 μL enzyme stock and 896 μL pH 7 buffer. After the temperature equilibrated, the absorbance at 404 nm was zeroed, and 4 μL of the stock solution of **2** was added. The new absorbance was read immediately or any time within 2 min,¹¹ the corrected absorbance (ΔA_{corr}) was obtained by subtracting the absorbance of the titrant (alone) in the buffer. Under our conditions ε_{*p*-nitrophenolate} = 1.0 × 10⁴; thus, the concentration of chymotrypsin (μM) was equal to ΔA_{corr} × 100.

The assay was validated by obtaining identical results with **1** and **2** (use of **1** requires simultaneous injection of titrant in the sample and reference cuvettes). Chymotrypsin concentrations determined as described above were reproducible +/- 4% over the range of 0.9 to 1.6 mg enzyme in the assay. Trypsin also reacts with **2**, but the reaction takes at least 5 minutes at pH 7; **1** behaves similarly.¹

Chymotrypsin that has reacted with **2** is inactive (it does not release *p*-nitroaniline from Succ-Ala-Ala-Pro-Phe-*p*-nitroanilide).¹² Also, chymotrypsin that has been inactivated by TPCK (which is known to alkylate the active site)¹³ does not release *p*-nitrophenol from **2**. Taken together with the fact that *p*-nitrophenol is released in the reaction of **2** with the enzyme, these results indicate that, as for **1**, **2** forms a stable acyl enzyme with chymotrypsin.

One would expect azapeptide esters to be less reactive with nucleophiles, compared to true esters; and this effect would contribute to the stability of the acyl enzyme. Some representative esters were treated with hydroxylamine (a nonbasic nucleophile)¹⁴ in order to address the question of whether azapeptide esters and normal esters are similarly reactive with nucleophiles (Table 1). The azapeptide esters (**1** and **2**) are unaffected by addition of hydroxylamine, whereas ordinary esters decompose more rapidly when hydroxylamine is added. If hydroxylamine were accelerating decomposition of the *p*-nitrophenyl ester of CBZ-Gly via base-promoted

cyclization (Scheme 1), then it should also increase the rate of decomposition of **1**; which it does not do. Thus, we feel that hydroxylamine is behaving as a nucleophile and attacking the carbonyl moieties of the normal esters. The lack of reactivity of **2**, and constant reactivity of **1**, in the absence and presence of hydroxylamine suggests that they are less susceptible to nucleophilic attack at the carbonyl function than normal peptide esters.

Table 1. Release of *p*-nitrophenol from various esters in the absence, and presence, of hydroxylamine; as determined by ΔA at 404 nm.

Ester ^a	pH 7 $\Delta A \text{ s}^{-1}$	1 mM H_2NOH , pH 7 $\Delta A \text{ s}^{-1}$
1	4.1×10^{-4}	4.1×10^{-4}
2	0 ^b	0 ^b
<i>p</i> -Nitrophenyl acetate	0 ^b	$1.9 \times 10^{-2} \text{ c}$
CBZ-Gly-OPNP ^d	1.0×10^{-4}	$1.0 \times 10^{-2} \text{ c}$

(a) All esters at 100 μM concentration in 100 mM phosphate buffer, temperature = 25°C.

(b) 0 means $\Delta A \leq 0.004$ units over 20 min. (c) First minute.

(d) The *p*-nitrophenyl ester of CBZ-Gly.

In conclusion, **2** is a stable, convenient active-site titrant for chymotrypsin. Additionally, we present the first evidence that azapeptide esters may be less reactive with nucleophiles than regular esters and suggest that this factor contributes to the stability of azapeptides and stable acyl-enzymes derived therefrom. We became aware of a similar titrant (N^α -(*N,N*-dimethylcarbamoyl)-azalysine *p*-nitrophenyl ester, selective for trypsin) while preparing this manuscript.¹⁵

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9. Triethylamine, silica gel 60, *p*-nitrophenol, and *p*-nitrophenyl chloroformate were purchased from Aldrich Chemical Co. α -Chymotrypsin (3 \times crystallized, type 1S, from bovine pancreas), TPCK, Succ-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from Sigma Biochemical Co. Solvents and buffer salts were purchased from Fisher Scientific Co.
10. Analytical data for **2**: $^1\text{H-NMR}$ 8.28 (d, 2H, aryl, $J=9$ Hz), 7.37 (m, 7H, aryl), 6.64 (br-s, 1H, NH), 4.87 & 4.79 (br, 2H, benzyl, split by the presence of two, slowly interconverting conformers), 3.79 (s, 3H, OCH_3). $^{13}\text{C-NMR}$ 155.7, 153.5, 145.2, 135.1, 128.8, 128.3, 125.1, 122.3, (55.0 and 53.8, the benzyl peak is doubled because of the amide rotation barrier), 53.2.
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