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Anisylazoformylarginine: A Superior Assay Substrate for Carboxypeptidase B Type Enzymes

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Abstract—Anisylazoformylarginine ($CH_3OC_6H_4$–N=N–CO–Arg–OH) is rapidly hydrolyzed at the acyl-arginine linkage by the zinc-enzyme porcine carboxypeptidase B. The catalytic reaction is readily monitored spectrophotometrically by disappearance of the intense absorption (349 nm, ε 19,100) of the azo chromophore, which chemically fragments after substrate cleavage.

Assay for catalytic activity of metalloproteases is much simplified by employment of substrates incorporating the anisylazoformyl residue (Fig. 1). As previously exemplified for thermolysin[1] and carboxypeptidases A[2] and B,[3] enzymic removal of the $CH_3OC_6H_4$–N=N–CO– moiety (acronym: Aaf) from a substrate is followed by a spontaneous fragmentation of the released acyl segment. The intense light absorption of the Aaf chromophore (λ_{max} ~350 nm, ε ~19,000 M^{-1} cm^{-1}) is thereby abolished, allowing a convenient and sensitive spectrophotometric detection of enzymic catalysis.

This assay tool is especially valuable for exopeptidases that remove cationic amino acids (Lys–OH or Arg–OH) from the C-terminus of oligopeptides, as during metabolism of a number of hormones,[4–6] as well as in the attenuation of fibrinolysis.[7,8] An internal-quench fluorescent-type of substrate for an enzyme having this specificity may be difficult to secure, due to absence of an excitation acceptor or donor in the P′ half of the substrate. A preparation of substrate Aaf-Lys–OH was previously reported.[3] However, that procedure was laborious in that it entailed phthaloyl-group protection of the lysine side chain, and a two-step deprotection. We now report an expedient synthesis of Aaf-Arg–OH that avoids this difficulty. Catalytic activity of the new substrate with carboxypeptidase B is superior to that of Aaf-Lys–OH.

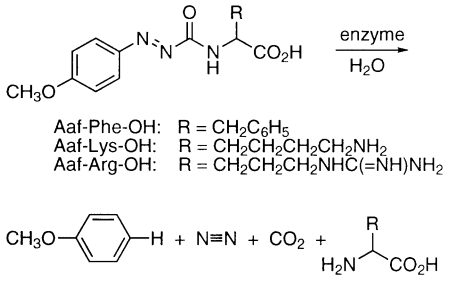

Figure 1. Structure of substrates and chemical transformation catalyzed by zinc peptidases (Aaf-Phe–OH cleaved by carboxypeptidase A; Aaf-Lys–OH and Aaf-Arg–OH cleaved by carboxypeptidase B).

Due to the greater basicity of the guanidine functionality, it may be protected by protonation, while the α-amino group in arginine is acylated during substrate preparation (Fig. 2). The proto-chromophore reactant $CH_3OC_6H_4NHNHCOOPh$ is secured from commercially available anisylhydrazine and diphenyl carbonate.[1,2] The initial hydrazo acylation product is oxidized directly to the azo substrate, which forms a crystalline hydrochloride suitable for storage.

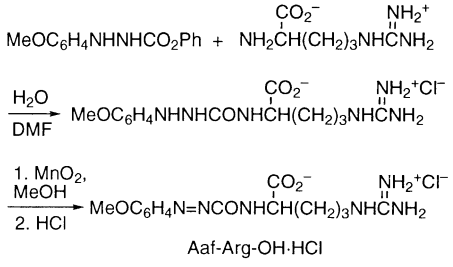

Figure 2. Preparation of Aaf-Arg–OH·HCl.

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The new substrate was directly compared with Aaf-Lys–OH with regard to cleavage by porcine carboxypeptidase B. Michaelis–Menten kinetics was observed as previously in a continuous spectrophotometric analysis, by following a decrease in substrate absorption at 350 nm:[3] For Aaf-Arg–OH a value for the specificity constant k_{cat}/K_m of 1.0×10^6 s^{-1} M^{-1} was obtained at 25 °C, pH 7.9 (0.05 M Tris buffer), which exceeds that for Aaf-Lys–OH by a factor of 9.9-fold. The kinetic difference between the two substrates largely resides in the value for K_m (44 ± 2 μM for Aaf-Arg–OH; k_{cat} 44 ± 1 s^{-1}).

Preparation

Nitrogen-purged dimethylformamide (4 mL) was added to a stirred solution of L-arginine (1.32 g, 7.62 mmol) in nitrogen-purged water (6 mL) maintained at 70 °C. Phenyl *p*-anisylhydrazoformate[1,2] (2.93 g, 11.3 mmol) in nitrogen-purged dimethylformamide (6 mL) was added. The brown homogeneous solution was stirred under a nitrogen atmosphere for 1.5 h at 60 °C. After cooling, the solution was diluted with water (50 mL) and then was extracted with ethyl acetate (3×50 mL). The aqueous layer was submitted to rotary evaporation under reduced pressure, and the residue was redissolved in methanol and again concentrated, yielding 3.9 g of a yellow residue of crude *p*-anisylhydrazoformyl-L-arginine. This material was immediately taken up in methanol (50 mL) and was stirred with excess manganese dioxide (3.31 g, 38.1 mmol) at room temperature for 2 h. The mixture was then passed through a pad of diatomaceous filter agent to remove the Mn remainder. The resulting clear orange solution was concentrated by rotary evaporation under reduced pressure. The residue was taken up in methanol (20 mL) and was diluted with ethanol (20 mL), and the homogeneous solution was again evaporated to dryness, yielding 2.24 g (87% from L-arginine) of an orange, glassy powder. A portion of this (0.424 g, >90% by NMR, ~1.13 mmol) was dissolved in 5.0 mL of methanol and was titrated with 0.8 mL of 1.23 N methanolic hydrochloric acid solution, to a pH of ~3 by pH paper, yielding a dark brown solution near the end of the titration. Solvent was removed by rotary evaporation under reduced pressure. The residue was redissolved in methanol and again rotary evaporated (2×) to remove any excess hydrochloric acid. The residue was taken up in a minimum amount of hot methanol and was diluted to the cloud point with ethyl ether. Orange rosettes formed; the material was recrystallized by repeat precipitation from methanol by ether, with drying by evacuation. This yielded 0.222 g (53% from powder) of orange needles of *p*-anisylazoformyl-L-arginine hydrochloride: mp 121–123 °C (dec); IR (KBr) 1653, 1708 cm^{-1}; UV–vis (H_2O) 349 nm, ε 19,100 M^{-1} cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ 1.73–1.80 (m, 2H), 1.87–1.97 (m, 1H), 2.07–2.16 (m, 1H), 3.24–3.28 (m, 2H), 3.92 (s, 3H), 4.54–4.58 (m, 1H), 7.11 (d, 2H, $J=9$ Hz), 7.98 (d, 2H, $J=9$ Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 26.6, 30.0, 42.1, 54.6, 56.6, 115.9, 127.5, 147.2, 158.8, 164.3, 166.4, 174.5. Anal. calcd for $C_{14}H_{21}N_6O_4Cl$ (372.8): C, 45.10; H, 5.68; N, 22.54. Found: C, 44.96; H, 5.49; N, 22.22.

References and Notes

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