Kinetic and stereochemical studies on novel inactivators of C-terminal amidation

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C-terminal amidation, a required post-translational modification for the bioactivation of many neuropeptides, entails sequential enzymic action by peptidylglycine α -mono-oxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5). Here we introduce novel compounds in which an olefinic functionality is incorporated into peptide analogues as the most potent turnover-dependent inactivators of PAM. Kinetic parameters for PAM inactivation by 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid were obtained by using both the conventional dilution assay method and the more complex progress curve method. The results obtained from the progress curve method establish that these compounds exhibit the kinetic characteristics of pure competitive inactivators (i.e. no ESI complex forms during inactivation). On the basis of k_{inact}/K_i values, 4-oxo-5-

acetamido-6-(2-thienyl)-hex-2-enoic acid is almost two orders of magnitude more potent than benzoylacrylate, a chemically analogous olefinic inactivator that lacks the peptide moiety. Stereochemical studies established that PAM inactivation by 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid is stereospecific with respect to the moiety at the P₂ position, which is consistent with previous results with substrates and reversible inhibitors. In contrast, 2,4-dioxo-5-acetamido-6-phenylhexanoic acid, which is a competitive inhibitor with respect to ascorbate, exhibits a low degree of stereospecificity in binding to the ascorbate sites of both PAM and dopamine- β -hydroxylase.

Key words: olefinic, peptidylglycine α -mono-oxygenase, progress curve.

INTRODUCTION

Neuropeptides, which are critical mediators of intercellular communication, are generated biosynthetically from larger precursors via a variety of post-translational modifications. One such processing event is C-terminal amidation, a very prevalent post-translational modification essential to the bioactivity of many neuropeptides [2,3]. We and others [4–9] have demonstrated that formation of peptide amides from their glycine-extended precursors entails the sequential action of peptidylglycine α -mono-oxygenase (PAM, EC 1.14.17.3) and peptidylamidoglycolate lyase (PGL, EC 4.3.2.5); the mono-oxygenase first catalyses the formation of the α -hydroxyglycinated peptide [4,5,10], which is then converted by the lyase to the amidated peptide product plus glyoxylate [4,9].

We have established that PAM and PGL exhibit tandem reaction stereospecificities in performing the two requisite steps of C-terminal amidation, with PAM producing exclusively α -hydroxyglycine moieties of absolute configuration (*S*) and PGL being reactive only towards (*S*)- α -hydroxyglycines [11]. In contrast, PAM and PGL exhibit markedly different subsite stereospecificities towards the residue at the penultimate position (the P₂ residue) in both substrates and inhibitors [12]; PAM exhibits high S₂-subsite stereospecificity, whereas PGL exhibits very low S₂-subsite stereospecificity. However, there have hitherto been no reports of investigations aimed at elucidating the binding stereospecificity for compounds that bind at the ascorbate site of PAM.

It is well recognized that olefinic substrate analogues can act as turnover-dependent inactivators for mono-oxygenase enzymes; the first mechanistic studies of this process were reported for cytochrome P450 [13] and for dopamine β -mono-oxygenase

(DBM) [14–16]. In both cases it was demonstrated that it is not a product epoxide, but rather an intermediate along the olefin oxygenation pathway, that is responsible for enzyme inactivation. Olefinic turnover-dependent inactivators for PAM were first reported in 1990 both by workers in our laboratory [17] and by Bradbury et al. [18], with inactivation exhibiting mechanistic characteristics similar to those of olefinic inactivators of cytochrome P450 and DBM [15–17]. Several other PAM inactivators were reported subsequently [19–22].

We report here on novel compounds, incorporating an olefin functionality into peptide analogues, as the most potent turnover-dependent inactivators of PAM. Kinetic parameters for PAM inactivation by these compounds were obtained by using both the conventional dilution assay method and the more complex progress curve method. The results obtained from the progress curve method establish that these compounds exhibit the kinetic characteristics of pure competitive inactivators. In addition, we report a series of experiments on the stereospecificities of inactivators and inhibitors of PAM. Our results demonstrate stereospecific inactivation by our olefinic inactivators, which interact solely at the substrate-binding site of PAM, but a low degree of stereospecificity for inhibitors that bind at the ascorbate sites of both PAM and DBM.

EXPERIMENTAL

Enzyme purification and assays

The *Xenopus laevis* PAM and PGL were expressed using the *Spodoptera frugiperda* ('Sf9')/baculovirus expression system

Abbreviations used: DBM, dopamine β -mono-oxygenase; PAM, peptidylglycine α -mono-oxygenase; PGL, peptidylamidoglycolate lyase; TMS, tetramethylsilane; N₃Ph, trinitrophenyl; NAc, *N*-acetyl; OEt, ethyl ester; the nomenclature for amino acid residues (or analogues) of substrates (P₁, P₂, etc.) and the corresponding enzyme subsites (S₁, S₂, etc.) is that originally proposed by Schechter and Berger [1].

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[23]; they were isolated as described previously, with minor modifications [24]. DBM was isolated from bovine chromaffin granules as described previously [15]. PAM and PGL assays were performed in 100 mM Mes/Na, pH 6.5, or 100 mM sodium acetate buffer, pH 5.5, at 37 °C. PAM assays contained 4.5 mM ascorbic acid, 4 μ M CuSO₄ and 1 mg/ml catalase, and various concentrations of N₃ph-D-Tyr-Val-Gly (in which N₃ph stands for trinitrophenyl), as described previously [25]. The product was analysed quantitatively by HPLC by using a standard curve based on peak height [25]. DBM assays were performed as described previously [15]. All steady-state kinetic measurements reported here were performed at atmospheric oxygen concentration; therefore the apparent mono-oxygenase $V_{\max}$ differs from the 'true' $V_{\max}$ by the factor $1 + K_{O_2}/[O_2]$.

Dilution assay

Inactivation reactions containing 4 μ M CuSO₄, 4.5 mM ascorbate, 1 mg/ml catalase, 0.2 % (v/v) Triton X-100, 80 mM KI, purified PAM and various concentrations of inhibitor in a total volume of 0.8 ml of 100 mM Mes/Na buffer, pH 6.5, were incubated at 37 °C; the reaction was initiated by the addition of enzyme. Aliquots of 20 μ l were withdrawn and diluted into 1 ml of assay solution containing 4 μ M CuSO₄, 4.5 mM ascorbate, 1 mg/ml catalase and an excess of N₃ph-D-Tyr-Val-Gly (60 μ M). Aliquots (500 μ l) of assay solutions were quenched with 50 μ l of 3 M HClO₄ and subjected to HPLC analysis.

Progress curve assay

Reaction solutions containing 4 μ M CuSO₄, 4.5 mM ascorbate, 1 mg/ml catalase, purified PAM and various concentrations of olefinic inhibitors and substrates in a total volume of 3 ml of 100 mM Mes/Na buffer, pH 6.5, were incubated at 37 °C. Reactions were initiated by the addition of enzyme. Aliquots of 250 μ l were withdrawn at intervals of 1–3 min from the assay solution and quenched with 25 μ l of 3 M HClO₄. Product concentrations were then analysed by quantitative HPLC.

Chiral separation of the enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoate

The dioxo tautomer of the enantiomers of 2,4-dioxo-5-acetamido-6-phenylhexanoate (more conveniently referred to as dioxo-NAcPhe pyruvate) were separated on a Chiralpak AD HPLC column (Chiral Technologies) connected to a Waters® LC Module I Plus HPLC system, with a mobile phase of acetonitrile containing 0.1 % (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min. Baseline separation of the two dioxo enantiomers was obtained (results not shown). Chromatography on a C₈ column was used to confirm that both enantiomers collected from the chiral column were dioxo-NAcPhe pyruvate. Analysis of the enantiomers by CD spectropolarimetry confirmed that their spectra were mirror images of each other, as expected for enantiomers. Multiple collections of the two enantiomers were then made and their concentrations were determined spectrophotometrically.

Synthesis of 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid

To a solution of NAc-L-Phe (5.0 g, 24.1 mmol) in 120 ml of ethanol was added concentrated HCl (0.5 ml); the reaction mixture was refluxed overnight and then concentrated under reduced pressure. The residual solid was dissolved in ethyl acetate, washed twice with dilute NaHCO₃ and once with water.

The organic phase was dried over MgSO₄, concentrated under reduced pressure and dried under vacuum to give 5.03 g (21.1 mmol) of NAc-L-Phe-OEt as a white solid (yield 89 %), m.p. 82–84 °C. ¹H NMR ([²H]chloroform, tetramethylsilane (TMS) = 0.0 p.p.m.): δ 1.25 (t, 3H), 1.99 (s, 3H), 3.13 (m, 2H), 4.17 (q, 2H), 4.86 (m, 1H), 5.96 (broad, 1H), 7.12 (m, 2H), 7.27 (m, 3H) (NAc is N-acetyl; OEt is ethyl ester).

n-Butyl-lithium (17.0 ml, 42.5 mmol) was slowly added, under N₂, to a solution of dimethyl methylphosphonate (4.6 ml, 42.4 mmol) in 50 ml of anhydrous tetrahydrofuran kept at –78 °C [26]. The reaction mixture was stirred at –78 °C for 15 min, during which a white precipitate formed. NAc-Phe-OEt (5.0 g, 21.2 mmol) was then added all at once. The reaction mixture was slowly brought to room temperature and stirred overnight (the reaction was monitored by TLC; the eluting solvent was ethyl acetate). The reaction was quenched by the addition of water. Ether was then added, the two layers were separated, and the aqueous layer was extracted with ether. The aqueous layer was acidified by the addition of 4 M HCl and extracted with methylene chloride; the extracts were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude NAc-L-Phe- α -oxophosphonate was purified by flash column chromatography [the eluting solvent was from chloroform/methanol (20:1, v/v) to chloroform/methanol (10:1, v/v)], to give a bright yellow oil (5.0 g, 16.1 mmol, 76 %) which was pure by TLC [chloroform:methanol (20:1, v/v)]. ¹H NMR ([²H]-chloroform, TMS = 0.0 p.p.m.): δ 2.02 (s, 3H), 3.00–3.37 (m, 4H), 3.74–3.84 (m, 6H), 4.95 (q, 1H), 6.52 (broad, 1H), 7.19–7.37 (m, 5H). MS (FAB⁺): 314 (*M* + 1).

A mixture of ethyldiethoxyacetate (13.22 g, 75 mmol), glyoxylic acid monohydrate (6.9 g, 74.5 mmol) and *p*-toluenesulphonic acid (catalyst) was stirred overnight at 80 °C. The reaction mixture was then cooled to 0 °C and P₂O₅ (7.8 g) was added slowly. The resulting thick slurry was then stirred at 80 °C for 4 h and distilled under reduced pressure to give a yellow oil of methylglyoxylate that quickly thickened and turned clear. ¹H NMR ([²H]chloroform, TMS = 0.0 p.p.m.): δ 3.82 (broad singlet). MS (CI⁺): 89.1 (*M* + 1).

Potassium carbonate (4.45 g, 32.2 mmol) dissolved in 20 ml of water was added to a mixture of NAcPhe- α -oxophosphonate (5.04 g, 16.1 mmol) and ethylglyoxylate (1.6 g, 15.7 mmol) in 10 ml of water, kept at 5 °C [27]. The reaction mixture was stirred at 5 °C for 15 min. The pale yellow solid formed was collected by suction, washed with cold water and dried under vacuum over P₂O₅. Recrystallization from hot ethyl acetate/hexane yielded 1.3 g (4.72 mmol, 29 %) of ethyl NAc-L-Phe-acrylate as pale yellow crystals. [a]_D: –67.7 °, m.p.: 105–107 °C. ¹H NMR ([²H]chloroform, TMS = 0.0 p.p.m.): δ 2.00 (s, 3H), 3.05–3.25 (o, 2H), 3.82 (s, 3H), 5.12 (q, 1H), 6.10 (bs, 1H), 6.80 (d, 1H, *J* = 15.9 Hz), 7.05 (m, 2H), 7.15 (d, 1H, *J* = 15.9 Hz), 7.27 (m, 3H). MS (FAB⁺): 276 (*M* + 1).

To a solution of ethyl NAc-L-Phe acrylate (0.5 g, 1.81 mmol) in 7 ml of methanol was added 75 ml of 0.1 M phosphate buffer, pH 7.0. The solution turned cloudy. Pig liver esterase (5000 units) was added and the reaction mixture was shaken vigorously at 30 °C for 48 h (the reaction mixture became clear; the progress of the reaction was monitored by TLC; the eluting solvent was ethyl acetate). The reaction mixture was washed twice with ether (an emulsion formed; ether extracts were discarded), acidified by the addition of 4 M HCl (turned cloudy) and saturated with NaCl. The aqueous layer was thoroughly extracted with methylene chloride (emulsion). The combined organic extracts were dried over MgSO₄, concentrated under reduced pressure and dried under vacuum to give 0.255 g (0.98 mmol, 54 %) of NAc-L-Phe-acrylic acid as a white solid. m.p.: decomposes. ¹H NMR

(DMSO- d_6 , DMSO = 2.49 p.p.m.): δ 1.78 (s, 3H), 2.72–3.07 (m, 2H), 4.70 (m, 1H), 6.56 (d, 1H, J = 15.9 Hz), 7.09 (d, 1H, J = 15.9 Hz), 7.22 (m, 5H), 8.42 (d, 1H). MS (FAB $^+$): 262.3 (M + 1). Calculated for $C_{14}H_{15}NO_4 + \frac{1}{4}H_2O$: C, 63.27%; H, 5.88%; N, 5.27%; found: C, 63.26%; H, 5.85%; N, 5.22%.

X-ray structural analysis confirmed that ethyl NAc-L-Phe acrylate crystallized in the chiral space group $P2(1)2(1)2(1)$.

Synthesis of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid

Synthesis of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid followed the same general procedure as that described above; only significant differences are mentioned here. In the first step, a suspension of 3-(2-thienyl)-L-alanine (4 g, 0.23 mmol), ethanol (125 ml) and concentrated HCl (2.5 ml) was heated under reflux overnight. The mixture was decolorized with activated carbon, filtered and concentrated under reduced pressure. A sample of the resulting pale yellow crystalline solid was recrystallized from ethanol to obtain a white crystalline solid [^1H NMR (DMSO) δ 8.4 (m, 1H), 7.40 (s, 1H), 6.95 (s, 1H), 5.65 (s, 1H), 4.15 (m, 2H), 2.45 (s, broad H_2O), 1.15 (t, 3H)]. The unpurified 3-(2-thienyl)-L-alanine ethyl ester from the previous step was dissolved in 15 ml of pyridine and was treated with acetic anhydride (1.2 equiv.). The resulting mixture was stirred overnight under argon. The reaction mixture was then acidified and extracted with methylene chloride. The methylene chloride phase was washed with water (100 ml) and then saturated sodium bicarbonate solution (150 ml), dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The resulting pale yellow crystalline solid was recrystallized from ethyl acetate/light petroleum to obtain white crystalline solid, NAc-3-(2-thienyl)-L-alanine ethyl ester. ^1H NMR ($[^2H]$ chloroform): δ 7.15–7.17 (m, 1H), 6.91–6.94 (m, 1H), 6.76–6.77 (m, 1H), 6.09 (d, broad, 1H), 4.17–4.24 (m, 1H), 4.21 (q, 2H), 3.26–3.52 (m, 2H), 2.03 (s, 3H), 1.29 (t, 3H). MS (CI) m/z 242 (M^+ + 1).

NAc-3-(2-thienyl)-L-alanyl- α -oxophosphonate was synthesized as described above for the phenylalanyl derivative; the product was chromatographed over silica gel with chloroform:methanol (20:1, v.v) to obtain a pure product. ^1H NMR ($[^2H]$ chloroform): δ 7.13–7.15 (m, 1H), 6.89–6.92 (m, 1H), 6.79–6.80 (m, 1H), 6.66–6.69 (d, broad, 1H), 4.83–4.89 (m, 1H), 3.69–3.76 (m, 6H), 3.25–3.43 (m, 3H), 2.97–3.09 (m, 1H).

Reaction of the oxophosphonate prepared in the previous step with methylglyoxylate was then performed as described above for the phenylalanyl derivative; the pale yellow solid was separated by filtration and dried under vacuum over P_2O_5 . The product was recrystallized from ethyl acetate/light petroleum to give an off-white crystalline solid. ^1H NMR ($[^2H]$ chloroform): δ 7.23 (s, 0.5H), 7.14–7.18 (m, 1.5H), 6.89–6.92 (m, 1H), 6.78–6.83 (m, 1H), 6.17–6.21 (d, broad, 1H), 5.05–5.11 (m, 1H), 3.83 (s, 3H), 3.31–3.51 (m, 2H), 2.05 (s, 3H). MS (CI) 282 (M^+ + 1).

A solution of *N*-acetyl-3-(2-thienyl)-L-alanylacrylate methyl ester (200 mg) in methanol (1.0 ml) was added to a solution of acylase (350 mg) in phosphate buffer (10 ml, 0.1 M, pH 7.0). The resulting suspension was shaken at 30 °C for 24 h. The reaction mixture was centrifuged to remove precipitated protein. The supernatant was acidified, saturated with NaCl and extracted with chloroform. The organic phase was separated, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure.

X-ray structural analysis confirmed that methyl NAc-3-(2-thienyl)-L-alanylacrylate crystallizes in the chiral space group $P2(1)$, whereas racemic methyl NAc-3-(2-thienyl)-D,L-alanyl acrylate crystallizes in the $P(-1)$ space group, which contains a centre of inversion.

RESULTS

It has previously been reported that the olefinic substrate analogues 4-phenylbut-3-enoate, *trans*-benzoylacrylate and monoethylfumarate are mechanism-based inactivators of PAM [17,18]. Because mechanism-based inactivators compete with normal substrates at the substrate-binding sites of the enzyme, inactivators that best resemble the substrates would be expected to have higher potencies. Previous studies have shown that peptide substrates with hydrophobic residues at the penultimate position are preferred in the PAM-catalysed reaction [28]. Therefore, by incorporating an appropriately positioned, activated olefinic moiety into a substrate-like structure, we expected that inactivators with enhanced potencies would be obtained. The structures of the olefinic PAM inactivators described here, and their similarities to the peptide PAM substrate, are shown in Figure 1.

Inactivation of PAM by 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid with the dilution method

The dilution assay is the conventional method used to determine kinetic parameters of time-dependent inactivators [29], and it assumes the minimal inactivation pathway shown in eqn (1).

Inactivation experiments performed with 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid (**I**) and with 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid (**II**) using the dilution method showed

Figure 1 Structures of inactivators, inhibitors and substrates of PAM

I, 4-Oxo (= keto)-5-acetamido-6-phenylhex-2-enoic acid; **II**, 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid; **III**, NAc-Phe-Gly; **IV**, *trans*-benzoylacrylate; **V**, NAc-Phe pyruvate; **VI**, ascorbate.

Table 1 Kinetic parameters for the inactivation of *Xenopus laevis* PAM by olefinic compounds as obtained from dilution assaysIn this Table and Table 3, results are means $\pm$ S.D.

Compound	4-Oxo-5-acetamido-6-phenylhex-2-enoic acid (L-enantiomer)	4-Oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid	
		L-Enantiomer	Racemate
K_i (μ M)	95 ± 12	26.1 ± 1.6	60 ± 3
k_{inact} (min^{-1})	0.99 ± 0.03	0.82 ± 0.04	0.57 ± 0.04
$10^{-3} k_{\text{inact}}/K_i$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	10.4	31.4 ± 0.3	9.5 ± 0.1

Scheme 1 Reaction scheme for progress curve method

that these compounds are indeed potent time-dependent inactivators of PAM. Inactivation was strictly first-order and concentration dependent, with plots of log(percentage initial activity) against time remaining linear through a 90 % loss of initial activity. Inactivation is also strictly ascorbate-dependent and is protected against by the inclusion of either PAM substrates or reversible inhibitors in the inactivation solution. The kinetic results obtained for our olefinic inactivators are listed in Table 1. Comparing the results for the L-enantiomers, it is apparent that 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid is more potent than 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid, as evidenced by its greater k_{inact}/K_i value. (Kinetic parameters for the racemate of the thienyl inactivator are discussed in the stereochemistry section below.) On the basis of their K_i and k_{inact} values, the thienyl derivative has a 4-fold higher binding affinity towards PAM and a slightly slower inactivation rate than the phenyl derivative.

Inactivation of PAM by 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid with the progress curve method

The theory of the progress curve method was first developed by Tsou in 1965 [30–32]; the method has been used in inactivation studies by several research groups [33–36]. As shown in Scheme 1 and summarized by Tsou [30], enzyme inactivation could occur at several stages during the reaction. Progress curves for the reaction are obtained by determining the formation of enzymic product over time in the presence of various concentrations of

substrate and inhibitor. For a single substrate system, by using a plotting method introduced by Wang et al. [33], all of the microscopic rate constants and equilibrium constants (except K_m and K'_m , which represent substrate binding to the free enzyme and the E·I complex respectively) can be determined in one experiment.

Three substrates are involved in the PAM-catalysed reaction: the glycine-extended peptide, ascorbate and molecular oxygen. By analogy with DBM, the reduction of PAM by ascorbate is likely to be the first step in the enzymic reaction [37]. Because the inactivation of PAM by olefinic compounds is ascorbate-dependent [17], E in Scheme 1 can be considered to be reduced PAM. In our inactivation studies, the initial ascorbate concentration was 4.5 mM (K_m 1 mM); the highest concentration of the peptide product formed in the reaction was less than 5 μ M. Therefore the maximum consumptions of ascorbate and molecular oxygen (250 μ M in air) were 10 and 5 μ M respectively. This corresponds to less than 2 % change throughout the inactivation reaction in both cases. Thus the PAM reaction system can be treated as a single-substrate reaction for the purpose of this inactivation study.

The relevant equations (eqns 2–6) are summarized in Table 2, which also lists the expressions for the slopes and y-intercepts of Figures 2–6. In all cases, data were least-squares fitted to the theoretical lines; R values of 0.999 or better were obtained. The progress curve method was used to obtain a full set of kinetic parameters for inactivation by the L-enantiomer of 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid and by both the L-enantiomer and the D,L-racemate of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid. For the sake of brevity, only the data for the L-enantiomer of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid are shown in Figures 2–7.

A plot of $[P]_\infty$ (obtained from Figure 2) against the reciprocal of the concentration of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid at various substrate concentrations (Figure 3) passes through the origin. This indicates that when the inactivator concentration is sufficiently high, there is no product formation in the reaction regardless of substrate concentration. Thus the binding of the inactivator to the enzyme prevents substrate turnover, which implies that the inactivator binds at the substrate-binding site. Furthermore, from the expression for the intercept of Figure 3 [eqn (3)], an intercept at the origin for this plot indicates that:

$$\frac{(k'_2/K'_i)[E]_0[S]}{(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)} = 0 \quad (7)$$

$$(k'_2/K'_i)[E]_0[S] = 0 \quad (8)$$

Because $[E]_0$ and $[S]$ are not zero, either $K'_i \rightarrow \infty$ or $k'_2 = 0$, or both. These indicate that either the inactivator does not bind at

Table 2 Equations used in progress curve assay

Abbreviation: n.a., not applicable.

Equation	Plot	Slope	Intercept	Figure no.
$[P] = [P]_{\infty} [1 - \exp(-k_{\text{obs}} t)]$	(2) [P] against time	n.a.	n.a.	2
$[P]_{\infty} = \frac{k_2[E]_0[S]}{[(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)[I]] + \frac{(k'_2/K'_i)[E]_0[S]}{(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)}}$	(3) $[P]_{\infty}$ against $1/[I]$	$\frac{k_2[E]_0[S]}{[(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)]}$	$\frac{(k'_2/K'_i)[E]_0[S]}{(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)}$	3
$1/s = \frac{k_{\text{inact}}K_m}{K(k_2[E]_0[S])} + \frac{k'_{\text{inact}}}{K'_i k_2[E]_0}$	(4) $1/(\text{slope of Figure 3})$ against $1/[S]$	$\frac{k_{\text{inact}}K_m}{K'_i k_2[E]_0}$	$\frac{k'_{\text{inact}}}{K'_i k_2[E]_0}$	4
$\frac{1}{k_{\text{obs}}} = \frac{K_m + [S]}{[(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)[I]] + \frac{K_m/K_i + [S]/K'_i}{(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)}}$	(5) $1/k_{\text{obs}}$ against $1/[I]$	$\frac{K_m + [S]}{[(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)]}$	$\frac{K_m/K_i + [S]/K'_i}{(k_{\text{inact}}K_m/K_i) + (k'_{\text{inact}}[S]/K'_i)}$	5
$\gamma = \frac{\text{intercept in Figure 6}}{\text{slope in Figure 6}} = \frac{1}{K_i} + \left\{ \frac{1}{K'_i} - \frac{1}{K_i} \right\} \frac{[S]}{K_m + [S]}$	(6) γ against $[S]/(K_m + [S])$	$\frac{1}{K'_i} - \frac{1}{K_i}$	$\frac{1}{K_i}$	6

Figure 2 Progress curve of the PAM reaction in the presence of 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid

Shown are time courses of product formation in reaction mixtures containing 0 μM (●), 8 μM (◆), 12 μM (△), 18 μM (◇), 28 μM (▽) and 40 μM (+) fixed concentrations of the inactivator. A constant initial rate was obtained in the absence of the inactivator. The substrate concentration used here was 15.06 μM N₃ph-D-Tyr-Val-Gly. Experimental data were fitted to eqn (2) in Table 2.

all to the ES complex (K'_i) or the formation of the enzymic product from the ESI complex (k'_2) does not occur during the inactivation process, or both. Thus product formation from an ESI complex would not be possible.

As shown in Figure 4, a secondary double-reciprocal plot of (slope in Figure 3) against substrate concentration gives a straight

Figure 3 Plot of $[P]_{\infty}$ against $1/[I]$ in progress curve assay

Plot of $[N_3\text{ph-D-Tyr-Val-}\alpha\text{-OH-Gly}]_{\infty}$ against $1/[I]$ for the inactivation of PAM at 4.52 μM (●), 6.07 μM (▽), 8.45 μM (△), 11.24 μM (◇) and 15.06 μM (◆) fixed concentrations of N₃ph-D-Tyr-Val-Gly. In this and other Figures, results are means and the bars represent S.D.

line that also passes through the origin. Thus, the y-intercept of this line is zero, or:

$$k'_{\text{inact}}/K'_i k_2[E]_0 = 0 \quad (9)$$

Again, either $K'_i \rightarrow \infty$ or $k'_{\text{inact}} \rightarrow 0$, or both. This again implies that either the inactivator does not bind at all to the ES complex

Figure 4 Plot of $1/(\text{slope in Figure 3})$ against $1/[S]$ in the progress curve assay

Secondary plot of $1/(\text{slope in Figure 3})$ against $1/[N_3\text{ph-p-Tyr-Val-Gly}]$. The slope of the line yields $k_{\text{inact}}K_m/(K_1k_2[E]_0)$.

Figure 5 Plot of $1/k_{\text{obs}}$ against $1/[I]$ at various fixed substrate concentrations

Plot of $1/k_{\text{obs}}$ against $1/[I]$ at $6.07 \mu\text{M}$ ($\circ$), $8.45 \mu\text{M}$ (Δ), $11.24 \mu\text{M}$ (∇) and $15.06 \mu\text{M}$ ($\times$) fixed concentrations of $N_3\text{ph-p-Tyr-Val-Gly}$. Weighted linear regressions of the data give straight lines with same value of the y -intercept at all substrate concentrations.

(K'_1) or inactivation does not occur after the formation of an ESI complex (k'_{inact}), or both. Thus the results in Figures 4 and 5 support the conclusion that the olefinic compounds act as pure competitive inactivators.

The slope of the line in Figure 4 is given by:

$$\text{slope} = \frac{k_{\text{inact}}K_m}{K_1k_2[E]_0} \quad (10)$$

As described below, k_{inact} can be obtained once all other constants in the equation have been determined.

Figure 6 Secondary plot of the ratio of intercept to slope in Figure 5 against $[S]/(K_m + [S])$

By assuming that $K'_1 \rightarrow \infty$, linear regression of the data into eqn (6) in Table 2 gives $K_1 = 31.9 \pm 0.2 \mu\text{M}$.

Figure 7 Competitive inactivation of PAM by 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid

Shown is the determination of inactivation type by plotting $1/A$ against $[N_3\text{ph-p-Tyr-Val-Gly}]$ at $8 \mu\text{M}$ ($\blacklozenge$), $12 \mu\text{M}$ (Δ), $18 \mu\text{M}$ ($\diamond$), $28 \mu\text{M}$ (∇) and $40 \mu\text{M}$ ($+$) fixed concentrations of inactivator.

All of the lines in the plot of $1/k_{\text{obs}}$ (obtained from Figure 2) against $1/[I]$ under various substrate concentrations (Figure 5) give the same value for the y -intercept. This value represents the maximum k_{obs} , i.e. the maximum apparent inactivation rate constant when the inactivator concentration reaches infinity. This indicates that saturation of PAM by the inactivator can be reached.

A secondary plot of γ against $[S]/([S] + K_m)$ based on eqn (6) (Table 2) is shown in Figure 6, where γ is the ratio of the y -intercepts of the lines in Figure 5 to their corresponding slopes.

Table 3 Results of progress curve assay

Compound ...	4-Oxo-5-acetamido-6-phenylhex-2-enoic acid (L-enantiomer)	4-Oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid	
		L-Enantiomer	Racemate
K_i (μM)	90 ± 5	31.9 ± 0.2	60.5 ± 0.3
K_i' (μM)	∞	∞	∞
k_{inact} (min^{-1})	1.1 ± 0.2	0.56 ± 0.01	0.40 ± 0.01
k_{inact}' (min^{-1})	0	0	0
k_2 (min^{-1})	0	0	0
$10^{-3} k_{\text{inact}}/K_i$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	12 ± 1	17.6 ± 0.1	6.6 ± 0.2

Table 4 Kinetic criteria for different types of inactivation

Inhibition type	Definition	Kinetic expression	Expression for A	Plot of straight line
Competitive	I does not bind to ES	$K_i' = \infty$	$\frac{k_{\text{inact}}/K_m}{K_m(K_i + [I]) + K_i[S]}$	$1/A$ against $[S]$
Non-competitive	I does not affect ES binding	$K_i = K_i'$	$\frac{k_{\text{inact}}}{K_i + [I]}$	A independent of $[S]$
Uncompetitive	I binds to ES only	$K_i = \infty$	$\frac{K_{\text{inact}}[S]}{K_m K_i' + [I][S]}$	$1/A$ against $1/[S]$

A weighted least-squares linear fit of the data gives a slope of this line of $(1/K_i' - 1/K_i)$ and a y -intercept of $1/K_i$, from which the value of K_i can be obtained ($K_i = 1/y$ -intercept). When a simple linear least-squares fit was applied to the data points in Figure 6, a small negative value for $1/K_i'$ (-0.004 ± 0.002) was obtained, which is obviously not possible. It was suggested above from the intercepts of both Figure 3 [eqn (8)] and Figure 4 [eqn (9)] that K_i' might approach infinity or $1/K_i'$ might approach zero. It is therefore reasonable to presume that the value of $1/K_i'$ in this plot actually approaches zero (a small negative value being impossible) or $K_i' \rightarrow \infty$. Direct support for this assumption comes from the data in Figure 7 discussed below. The line in Figure 6 was then generated from eqn (6) by setting the value of K_i' to infinity; the value of K_i was obtained from the reciprocal of the y -intercept.

Once the K_m and k_2 values have been obtained from a simple substrate kinetic assay, k_{inact} can then be obtained from eqn (10) as mentioned above. Table 3 compares the inactivation kinetic parameters for 4-oxo-5-acetamido-6-phenylhex-2-enoic acid and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid as PAM inactivators obtained by using the progress curve method.

Effect of substrate concentration on k_{obs} for inactivation

It has been suggested that, like reversible inhibitors, irreversible inactivators can be classified as competitive, non-competitive or uncompetitive, on the basis of considerations analogous to those used for reversible inhibitors [30,38]. The type of inactivation can be ascertained [38] by examining the effect of substrate concentration on the apparent second-order rate constant for inactivation (A), with the values of k_{obs} being obtained from Figure 2. The expression for A can be obtained from the expression for k_{obs} [eqn (5) in Table 2], as shown by:

$$A = \frac{k_{\text{obs}}}{[I]} = \frac{k_{\text{inact}} K_m / K_i + k_{\text{inact}}' [S] / K_i'}{K_m (1 + [I]/K_i) + [S] (1 + [I]/K_i')} \quad (11)$$

Table 4 summarizes the theoretical correlation between A and substrate concentration for different types of inactivation. As shown in Figure 7, for PAM inactivation by **I** and **II**, the plot of $1/A$ against $[S]$ gives a linear plot at all inactivator concentrations, indicating that the inactivation is competitive. At the same time, the plot of $1/A$ against $1/[S]$ gives obviously curved lines instead of straight lines, indicating that the inactivation is not uncompetitive. It is obvious that A is not independent of $[S]$, indicating that the inactivation is also not non-competitive. This analysis therefore leads to the conclusion that **I** and **II** are indeed competitive inactivators for PAM with respect to the peptide substrate.

Stereospecificities of amidating enzyme inhibitors

It has been established in our laboratory [11] that PAM and PGL exhibit tandem reaction stereospecificities in performing the two requisite steps of C-terminal amidation, with PAM producing exclusively α -hydroxyglycine moieties of absolute configuration (*S*) and PGL being reactive only towards (*S*)- α -hydroxyglycines. In contrast, we have shown that PAM and PGL exhibit very different S_2 -subsite binding stereospecificities towards their respective substrates and competitive inhibitors [12]. For PAM, the binding of substrates and inhibitors is severely diminished by the presence of a D-amino acid [or a stereotopically equivalent (*R*)-mandelyl] adjacent to the C-terminal glycine moiety, whereas the configuration of the chiral moiety at the P_2 position of PGL substrates has virtually no effect on binding or catalysis [12]. However, there have hitherto been no investigations aimed at elucidating the binding stereospecificity of inhibitors targeted at the ascorbate site of PAM.

Pyruvate-extended amino acids (2,4-dioxo-5-acetamido-alkanoic acid derivatives) are a novel class of amidation inhibitors designed in our laboratory, and these compounds are inhibitors of both PAM and PGL [25]. By virtue of the fact that these compounds possess a dioxocarboxylic acid moiety, they bear sufficient resem-

Figure 8 Elution profile of dioxo-NAcPhe pyruvate from the Chiralpak AD column

The baseline separation is shown of the enantiomers of the dioxo-NAcPhe pyruvate with two Chiralpak AD columns connected in series in polar organic mode. Dioxo-NAcPhe pyruvate was dissolved in methanol followed by dilution 1:1 (v/v) with acetonitrile. The mobile phase was acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 0.8 ml/min; the detecting wavelength was 300 nm. The retention times of the two peaks are 9.4 and 11.2 min respectively. Abbreviation: AU, absorbance unit.

blance to ascorbate to bind competitively to the ascorbate-binding sites of PAM and DBM, both of which are ascorbate-dependent mono-oxygenases [25]. Because our synthetic procedure for these compounds entails a basic hydrolysis step that causes racemization [25], chiral chromatography was employed to separate the enantiomers of dioxo-NAcPhe pyruvate for the purpose of determining the stereospecificity of PAM inhibition by this compound.

After the evaluation of several chiral chromatography supports and elution procedures, a Chiralpak AD column was found to be capable of successfully resolving the two enantiomers of dioxo-NAcPhe pyruvate in their dioxo tautomeric forms (Figure 8). CD spectra of the two peaks exhibited completely opposite spectral features, indicating that they are enantiomers of each other. The Cotton effects in the spectra indicate that the peak at 9.4 min represents the L-enantiomer and the peak at 11.2 min represents the D-enantiomer [39,40]. Inhibition experiments with PAM gave K_i values of 134 ± 10 , 312 ± 18 and $200 \pm 28 \mu\text{M}$ for the L-enantiomer, the D-enantiomer and the racemate of dioxo-NAcPhe pyruvate respectively; in all cases the inhibition was purely competitive with respect to ascorbate. Similar experiments with DBM with the use of the substrate tyramine gave K_i values of 221 ± 35 , 228 ± 24 and $215 \pm 46 \mu\text{M}$ for the L-enantiomer, the D-enantiomer and the racemate of dioxo-NAcPhe pyruvate respectively; inhibition was purely competitive with respect to ascorbate in all cases.

In contrast with the 2,4-dioxo-carboxylate inhibitors, which are competitive with respect to ascorbate, the kinetic results described above confirm that our olefinic inactivators bind competitively at the substrate-binding site of PAM. Our expectation was therefore that inactivation by these compounds would be stereospecific with respect to the amino acid residue adjacent to the C-terminal acrylate moiety. Accordingly, racemic 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid was synthesized; kinetic parameters for PAM inactivation were obtained by using both the dilution and progress curve methods. The K_i values determined for the racemate were approximately

double those of the L-enantiomer [60.0 compared with $26.1 \mu\text{M}$ by the dilution assay (Table 1) and 60.5 compared with $31.9 \mu\text{M}$ by the progress curve assay (Table 3)]. In contrast, the k_{inact} values determined for the racemate and the L-enantiomer were very similar. As has been well documented for a variety of enzyme inhibitors [41,42], this result is consistent with a K_i for L-4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid at least an order of magnitude lower than that of the D-enantiomer.

DISCUSSION

Previous studies from our laboratory have shown that appropriately designed olefinic compounds are mechanism-based, turnover-dependent inactivators for both PAM and DBM; mechanistic studies have provided evidence that an intermediate along the olefinic oxygenation pathway is the likely actual inactivating species [15–17]. On the basis of this previous work, the novel compounds 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid (**I**) and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid (**II**) were synthesized as potential mechanism-based inactivators for PAM. In these compounds, a C-terminal acrylate functionality is linked to a moiety that would be expected to exhibit enhanced secondary binding interactions at the active site of PAM. The results reported here confirm this expectation; both **I** and **II** are indeed highly potent time-dependent inactivators for PAM, with **II** being almost two orders of magnitude more potent (based on k_{inact}/K_i values) than *trans*-benzoylacrylate.

From the results obtained with the progress curve method, we conclude that an ESI complex does not form in the inactivation of PAM by these olefinic inactivators. This conclusion is based on several lines of evidence. First, the plots shown in Figures 3, 4 and 6 indicate that $K'_i \rightarrow \infty$, which means that the inactivator cannot bind to the ES complex. An alternative pathway by which an ESI complex might form is via substrate binding to EI. The dissociation constant for this process (the K'_m value) cannot be determined with the progress curve method. However, even if such an ESI complex had formed via EI plus S, it could neither have reacted to produce EI plus product nor have undergone inactivation to form an inactivated ESI* complex, because the plots of Figure 3 and Figure 4 indicate that $k'_2 \rightarrow 0$ and $k'_{\text{inact}} \rightarrow 0$ respectively. Therefore our data clearly indicate that neither product formation nor inactivation could have arisen from such an ESI complex, as expected for an inactivator that competes for the substrate-binding site.

Strong support for the conclusion that **I** and **II** are competitive inactivators is provided by the plot of $1/A$ against $[S]$ (Figure 7), in which the linear plot is diagnostic of competitive inactivation. Classically, competitive, non-competitive and uncompetitive reversible inhibitors are distinguished by their effects on the apparent Michaelis constants determined in the presence of the inhibitor. Tsou [32] has shown that similar criteria can also be derived to distinguish these three types of irreversible inhibitor on the basis of their effects on the apparent rate constant of enzyme inactivation determined in the presence of both the substrate and the inactivator. Because such an analysis cannot be made on data obtained from the conventional dilution assay, this capability of determining the type of the inactivation is a distinct advantage of the progress curve method. Wang et al. [33] have pointed out that it becomes difficult to obtain accurate parameters with the dilution method when $k_{\text{inact}} \rightarrow 1 \text{ min}^{-1}$. This probably accounts, at least in part, for the small differences in the kinetic parameters reported here with the two assay methods.

Olefinic mechanism-based inactivators for PAM were first reported in 1990 by us [17] and by Bradbury et al. [18]. Both of

these papers described inactivation by 4-phenyl-3-butenic acid; our paper also described inactivation by *trans*-benzoylacrylate and monoethylfumarate. We demonstrated that inactivation is turnover-dependent, protected against by substrate and not reversed by dialysis; moreover, incubation of PAM with authentic epoxide does not lead to inactivation, which is consistent with the notion that a intermediate along the olefin oxygenation pathway is the actual inactivating species. The novel compounds reported here, 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid and 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid, are much more potent inactivators than any of these olefinic compounds described previously. For example, in parallel dilution assays, 309 μM 4-phenyl-3-butenic acid resulted in 73 % inactivation of PAM activity after 25 min of incubation, whereas 62 μM 4-oxo-5-acetamido-6-phenyl-hex-2-enoic acid resulted in 94 % inactivation of PAM activity under the same conditions. The k_{inact} for PAM inactivation by D-Phe-L-Phe-D-vinylglycine was not reported [19], making it difficult to compare its potency with those of **I** and **II**. However, it was reported, that PAM activity was completely abolished by incubation with 250 μM D-Phe-L-Phe-D-vinylglycine for 45 min; in our hands, the incubation of PAM with 100 μM **I** for 30 min resulted in the complete abolition of PAM activity.

Tamburini et al. [43,44] have reported that substrates possessing a sulphur-containing or planar aromatic structure at the P_2 position exhibit a high binding affinity for PAM. The thienyl ring contains both an aromatic moiety and a sulphur atom that can ligate to the catalytic copper (Cu^{B}) [45]; thus an inactivator possessing a thienyl moiety might be expected to exhibit an enhanced binding affinity. Consistent with this view is the fact that the K_i of inactivator **II** is approx. one-quarter that of inactivator **I**. Examination of the published crystal structure of the PAM catalytic core [45] reveals that the ligation of the thienyl sulphur to Cu^{B} would alter the positioning of the acrylate moiety within the active site; this might account for the lower k_{inact} of inactivator **II**. The fact that PAM substrates possessing a sulphur-containing residue at P_2 position have lower V_{max} values than those containing an aromatic side chain at the same position supports this view.

Our results confirm that the inactivation of PAM by 4-oxo-5-acetamido-6-(2-thienyl)-hex-2-enoic acid exhibits the stereospecificity expected on the basis of previous stereochemical studies with PAM substrates and reversible inhibitors. With regard to the ascorbate-binding site of PAM, our results indicate that binding of dioxo-NAcPhe pyruvate to this site exhibits a low degree of stereospecificity, with the L-enantiomer being approximately twice as potent as the D-enantiomer. Comparison of the amino acid sequences of amidating enzymes from various sources and other ascorbate-dependent enzymes reveals a potential ascorbate-binding region at residues 134–152 of rat PAM [46]. It is apparent from the crystal structure of the catalytic core of rat PAM that this region faces the interdomain cleft and seems to be fully accessible to solvent. Moreover, we have previously demonstrated [47] that *N,N*-dimethyl-1,4-phenylenediamine (DMPD) functions as an electron donor for PAM catalysis. The activity of DMPD is kinetically quite similar to that of ascorbate, despite the vast structural differences between these electron donors. Thus the low stereospecificity of binding of dioxo-NAcPhe pyruvate at the ascorbate-binding site is not surprising.

Enzymic inactivation has been studied with the progress curve method in a number of laboratories. In an early example, Tian and Tsou [38] studied the inactivation of α -chymotrypsin by di-isopropyl fluorophosphate, PMSF and L-1-[(*p*-toluenesulphonyl)-amino]-2-phenylethylchloromethane in the presence of the substrate. On the basis of plots of $(1/A)$ against $[S]$, they

were able to show that all three compounds are competitive inactivators of α -chymotrypsin. The plotting method used in this paper was first introduced by Wang et al. [33], who applied it to the study of the inactivation of aminoacylase by 2-chloromercuri-4-nitrophenol (MNP). Their investigations showed that plots of $[P]_{\infty}$ against $1/[MNP]$ gave positive intercepts at all substrate concentrations, in contrast with our results, where the lines pass through the origin. A slow conformation-change model was proposed by Wang et al. to explain this kinetic behaviour. The progress curve method has also been applied to analyses of irreversible modification reactions, with an example being the proteolysis of reduced spinach chloroplast fructose-1,6-bisphosphatase by subtilisin [36]. In this case the kinetic results indicate that proteolysis occurs at an exposed loop well away from the active site, with substrate binding giving rise to a conformational change that protects against proteolytic modification. The progress curve approach has also been recently applied to kinetic analysis of the activation of dihydrofolate reductase by *p*-chloromercuribenzoate [48].

Amidation represents a potentially attractive target point for modulating the production of peptide hormones. We have shown [49] that chronic administration of a PAM inhibitor markedly decreases carrageenan-induced hindpaw swelling in rats. Moreover, we demonstrated that this effect is correlated with the inhibition of serum PAM as well as decreased tissue levels of the amidated peptide Substance P, which is known to have a key role in the inflammatory process. The availability of new potent amidation inhibitors should facilitate further investigations on biochemical, physiological and pharmacological aspects of neuropeptide amidation.

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