A quenched fluorescent substrate for thimet peptidase containing a new fluorescent amino acid, DL-2-amino-3-(7-methoxy-4-coumaryl)propionic acid

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DL-2-Amino-3-(7-methoxy-4-coumaryl)propionic acid, a new fluorescent amino acid (abbreviated to Amp), has been synthesized to provide an alternative to tryptophan in quenched fluorescent peptide substrates for peptidases. The model compound Ac-DL-Amp-NH₂ was intensely fluorescent with an excitation maximum at 328 nm and an emission maximum at 392 nm. Fmoc (fluoren-9-ylmethoxycarbonyl)-DL-Amp was made to allow the solid-phase synthesis of Amp-containing peptides by the Fmoc-polyamide method. The peptide derivative Dnp (2,4-dinitrophenyl)-Pro-Leu-Gly-Pro-DL-Amp-D-Lys was cleaved by thimet peptidase at the Leu-Gly bond, with a 20-fold enhancement of fluorescence. The value of k_{cat}/K_m for thimet peptidase was $6.7 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, compared with the value of $2.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the tryptophan-containing analogue, Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys.

INTRODUCTION

Quenched fluorescent peptides, in which a short sequence of amino acids separates a fluorescent moiety from a group that acts as an internal quencher, are valuable substrates for the assay of peptidases specific for the cleavage of bonds between amino acid residues (Yaron *et al.*, 1979). Commonly, these substrates have contained a fluorescent 2-aminobenzoyl group at the *N*-terminus and a quenching 4-nitrophenyl group (Carmel & Yaron, 1978; Nishino & Powers, 1980; Castillo *et al.*, 1983). For these groups the quenching mechanism is collisional (Yaron *et al.*, 1979), and the efficiency of quenching decreases markedly when they are separated by more than three amino acids (Nishino & Powers, 1980). If quenching is to be efficient over longer distances it is necessary to employ a fluorophore, the emission spectrum of which is overlapped by the absorption spectrum of another group, so that quenching occurs by resonance energy transfer (Stryer, 1978). A number of substrates employing this principle have been described recently, in which the fluorophore is tryptophan and the quencher a Dnp (Barrett *et al.*, 1989; Stack & Gray, 1989) or dansyl group (Ng & Auld, 1989).

Tryptophan-containing substrates are unsuitable for the assay of crude enzymes, which contribute a high background fluorescence because tryptophan is abundant in proteins. To overcome this problem, we prepared a peptide containing the highly fluorescent Mcc group, which is also quenched by Dnp (Tislar *et al.*, 1990). On cleavage of Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) at the Leu-Gly bond there was a 27-fold increase in fluorescence, and the assay was unaffected by BSA in concentrations up to 4 mg/ml.

A limitation of the Mcc/Dnp fluorophore-quencher pair is that each group must be coupled to the peptide at either the *N*-terminus or on the *N*⁶-amino group of a lysine residue, where they produce an un-naturally bulky side chain. The present paper describes the synthesis of a new fluorescent amino acid, DL-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (abbreviated to Amp), in which the indole ring of tryptophan is replaced by a coumarin. Amp allows a more flexible approach to the design of coumarin-labelled peptides. This is illustrated here by a

new quenched fluorescent substrate for thimet peptidase (EC 3.4.24.25), an enzyme previously known as Pz-peptidase, soluble metallo-endopeptidase and endo-oligopeptidase A (Barrett, 1990).

EXPERIMENTAL

Materials

Diethyl acetamidomalonate, 4-bromomethyl-7-methoxycoumarin and fluoren-9-ylmethylsuccinimidyl carbonate were from Fluka. Sodium ethoxide was from Aldrich. Pepsyn KA resin was from MilliGen. Fmoc-amino acid reagents and benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate were from NovaBiochem. Dnp-Pro and Dnp-Pro-Leu were made as described by Porter & Sanger (1948). Vydac 218TPB1520 was from Technicol. Thimet peptidase, prepared from chicken liver (Barrett & Brown, 1990), was provided by Mrs. M. A. Brown of this Department.

Syntheses

DL-2-Amino-3-(7-methoxy-4-coumaryl)propionic acid. Diethyl acetamidomalonate (4.34 g, 20 mmol), sodium ethoxide (1.36 g, 20 mmol) and 4-bromomethyl-7-methoxycoumarin (5.38 g, 20 mmol) in dry ethanol (20 ml) were boiled under reflux for 24 h. NaBr was removed by filtration and the intermediate adduct was allowed to crystallize on cooling. The product (5.7 g, 70% yield) was suspended in 6 M-HCl (50 ml) and boiled under reflux for 18 h. The solution was filtered and DL-Amp precipitated by neutralization with 35% (w/v) NH₃ in water. The product was filtered, washed with water to remove NH₄Cl and dried *in vacuo* (3.31 g, 63% yield). Crude DL-Amp (1.66 g, 6.4 mmol) was recrystallized by dissolving it in a hot mixture of ethanol (30 ml) and 1 M-NaOH (12 ml) and acidifying with acetic acid (5 ml). The precipitate was collected, washed with water and dried *in vacuo* (1.52 g, 91% yield). DL-Amp (*M*_r 263.25) had m.p. 252–253 °C (Found: C, 59.1%; H, 5.0%; N, 5.3%; C₁₃H₁₃NO₅ requires C, 59.3%; H, 5.0%; N, 5.3%).

Abbreviations used: Dnp, 2,4-dinitrophenyl; Fmoc, fluoren-9-ylmethoxycarbonyl; Mcc, 7-methoxycoumarin-3-carboxyl; Boc, t-butoxycarbonyl; Amp, DL-2-amino-3-(7-methoxy-4-coumaryl)propionic acid.

Ac-DL-Amp-NH₂. A solution of DL-Amp (132 mg, 0.5 mmol) and toluene-*p*-sulphonic acid monohydrate (190 mg, 1.0 mmol) in ethanol (15 ml) was boiled under reflux for 16 h. DL-Amp-OEt-toluene-*p*-sulphonate crystallized on cooling and was collected and dried *in vacuo* (183 mg, 79 % yield). The ester was dissolved in dry pyridine (2 ml) and treated with acetic anhydride (0.2 ml, 2.1 mmol) and 4-dimethylaminopyridine (10 mg, 0.08 mmol) for 75 min at 21 °C. After treatment with methanol (0.5 ml), ethyl acetate (50 ml) was added and the mixture extracted in turn with 10 % (w/v) citric acid (50 ml), saturated NaHCO₃ (50 ml), water (50 ml) and saturated NaCl (50 ml). The solution was dried over anhydrous CaSO₄ and evaporated. The residue of Ac-DL-Amp-OEt (homogeneous by h.p.l.c., retention time 16.8 min) was dissolved in methanol (15 ml), treated with NH₃ for 1.5 h at 0 °C and left for 16 h in a sealed tube at 21 °C. The methanol was removed *in vacuo* and the solid remaining was recrystallized from boiling water (65 mg, 42 % yield). Ac-DL-Amp-NH₂ (*M_r* 304.3) had m.p. 229–230 °C (Found: C, 59.2 %; H, 5.3 %; N, 9.2 %; C₁₅H₁₆N₂O₅ requires C, 59.2 %; H, 5.3 %; N, 9.2 %).

Fmoc-DL-Amp. DL-Amp (263 mg, 1 mmol) was suspended by stirring in acetonitrile/water (1:1, v/v, 10 ml). Di-isopropylethylamine (0.35 ml, 2 mmol) was added, followed by a solution of fluoren-9-ylmethylsuccinimidyl carbonate (337 mg, 1 mmol) in acetonitrile (2 ml). After 30 min, acetonitrile was removed at 40 °C *in vacuo* and the solution remaining was added to 1 M-HCl (10 ml) at 0 °C. The precipitate of Fmoc-DL-Amp was filtered and dried *in vacuo*. This product was dissolved in chloroform (5 ml), and an equal volume of light petroleum (b.p. 80–100 °C) was added. The precipitate was filtered, washed with light petroleum and dried *in vacuo* (442 mg, 91 % yield). Fmoc-DL-Amp (*M_r* 485.5) had m.p. 198–199 °C (Found: C, 68.9 %; H, 5.0 %; N, 2.9 %; C₂₈H₂₃NO₇ requires C, 69.3 %; H, 4.8 %; N, 2.9 %).

Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys. This was synthesized stepwise from the C-terminus by the Fmoc-polyamide method by standard procedures (Atherton & Sheppard, 1989) on a manually operated continuous-flow synthesizer (cf. Dryland & Sheppard, 1986). Pepsyn KA resin (1.0 g, 0.2 mmol) was treated with the pentafluorophenyl ester (1.0 mmol) of Fmoc-D-Lys(Boc) in the presence of 4-dimethylaminopyridine (0.2 mmol). Fmoc-DL-Amp (0.6 mmol) was coupled with benzotriazol-1-yloxytris (pyrrolidino)phosphonium hexafluorophosphate (Coste *et al.*, 1990) in the presence of di-isopropylethylamine (0.6 mmol of each). Subsequent amino acids were coupled as their pentafluorophenyl esters (1.0 mmol) in the presence of 1-hydroxybenzotriazole (1.0 mmol). Dnp-Pro (0.6 mmol) was coupled with PyBOP and di-isopropylethylamine (0.6 mmol of each). At the completion of the synthesis the resin was washed and dried (Atherton & Sheppard, 1989), and the peptide was released by treatment with a mixture of trifluoroacetic acid (38 ml) and water (2 ml) for 2 h. Trifluoroacetic acid was removed *in vacuo*, and the residue was taken up in water (100 ml) and applied to a column (15 mm × 420 mm) of Vydac 218TPB1520. Elution with a gradient of 5–50 % acetonitrile in water containing 0.1 % trifluoroacetic acid yielded the product (86 mg, 47 % yield). Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys (*M_r* 921.85) was homogeneous by h.p.l.c., but displayed a partially resolved double peak, with retention times of 17.2 min (57 %) and 17.3 min (43 %).

H.p.l.c.

This was done on a Varian LC5000 instrument equipped with the Vista 402 data-processing system and a Techopak 10C18 column (4.6 mm × 250 mm) (HPLC Technology). The following

solvents were used: A, 0.1 % trifluoroacetic acid in water; B, 0.1 % trifluoroacetic acid in acetonitrile. A linear gradient of 5–100 % B was run over 25 min at a flow rate of 1.0 ml/min. The eluate was monitored at 220 nm and 360 nm.

Fluorimetric analyses

These were made in a Perkin-Elmer LS-3 spectrofluorimeter controlled by an IBM-compatible computer running the FLUSYS software of Rawlings & Barrett (1990). The reaction mixtures were stirred and maintained at 30 °C in quartz cuvettes during the experiments. The instrument was set to zero with Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys in assay buffer (50 mM-Tris/HCl, pH 7.8, containing 5 mM-2-mercaptoethanol and 0.05 % Brij 35), and then calibrated (at 30 °C) to read 1000 units of fluorescence with 1 μM-Ac-DL-Amp-NH₂ with λ_{ex} 328 nm and λ_{em} 392 nm. One fluorescence unit then corresponded to the formation of 1 nM-product in 2.5 ml by hydrolysis of the substrate.

Continuous-rate assays were done in a constant volume of 2.5 ml. An appropriate volume of assay buffer prewarmed to 30 °C was pipetted into the cuvette, followed by 50 μl of enzyme sample. After 3 min, the reaction was started by addition of up to 50 μl of substrate stock solution in dimethyl sulphoxide and the fluorescence was recorded every 1 s. The rate of increase in fluorescence over a suitable time interval (5–15 min) was calculated by linear regression analysis.

RESULTS AND DISCUSSION

DL-Amp (Fig. 1) was made by a standard method for the synthesis of unnatural amino acids, as described, for example, by Masaoka *et al.* (1982). On treatment with sodium ethoxide, diethyl acetamidomalonate forms a carbanion which can be C-alkylated by reaction with an alkyl halide. On acid hydrolysis, the amino and carboxyl groups are liberated from the intermediate adduct and decarboxylation occurs to give the racemic product. DL-Amp was obtained in satisfactory yield and was recrystallized by a method described for DL-tryptophan (Furniss *et al.*, 1989).

The spectral properties of Amp were characterized with the model compound Ac-DL-Amp-NH₂, which was made by established procedures (Bodanszky & Bodanszky, 1984). The molar absorptivity of Ac-DL-Amp-NH₂ at 325 nm was 13900. The fluorescence excitation spectrum had a maximum at 328 nm and the emission was centred around 392 nm (Fig. 2). There was considerable overlap between the emission spectrum of Ac-DL-Amp-NH₂ and the absorption spectrum of Dnp-Pro-Leu (Fig. 2), which suggested that Amp fluorescence should be efficiently quenched by an adjacent Dnp group.

The peptide derivative Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys was made by the Fmoc-polyamide method using benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate as the coupling agent for Fmoc-DL-Amp. The coumarin side chain of Amp is less reactive towards carbonium ions than the indole ring

Fig. 1. Structure of DL-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (DL-Amp)

Fig. 2. Spectral overlap of Dnp-Pro-Leu and Ac-DL-Amp-NH₂

The absorption spectrum of 48 μM -Dnp-Pro-Leu (—) and the fluorescence excitation (.....) and emission (----) spectra of 1 μM -Ac-DL-Amp-NH₂ were recorded in 50 mM-Tris/HCl, pH 7.8.

Fig. 3. Quenching of Ac-DL-Amp-NH₂ fluorescence by Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys

The fluorimeter was calibrated to read 1000 with 1 μM -Ac-DL-Amp-NH₂ in assay buffer. The residual fluorescence of each substrate solution was set to zero and the increase in fluorescence on adding Ac-DL-Amp-NH₂ to a concentration of 1 μM was recorded.

of tryptophan (Atherton *et al.*, 1988), so that side-chain deprotection and cleavage from the resin occurred cleanly in trifluoroacetic acid/water (19:1, v/v). This constitutes a major advantage over the synthesis of the tryptophan-containing analogue (Barrett *et al.*, 1989), which required synthesis on the base-labile Pepsyn KB resin and cleavage of the Boc protecting group on D-Lys with trifluoroacetic acid/anisole/ethanedithiol, before release of the peptide with 1 M-NaOH.

The absorption spectrum of Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys in aqueous solution had peaks at 332 nm and 377 nm, with molar absorptivity values of 16400 and 14000 respectively. Thus the fluorescence of Amp in solutions of Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys is significantly quenched by internal absorption at substrate concentrations above 1 μM (Fig. 3). In experiments

Fig. 4. Self-quenching of Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys fluorescence

The fluorimeter was calibrated as in the legend to Fig. 3 and the fluorescence of increasing concentrations of substrate were recorded.

Fig. 5. H.p.l.c. analysis of Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys hydrolysis by chicken thimet peptidase

The chromatograph was run as described in the Experimental section, and the effluent was monitored at 220 nm. The part of the chromatograph is shown (9–21 min) which shows the unhydrolysed peptide (elution time 16.7 min) and the two fragments (A: Gly-Pro-Amp-D-Lys, elution time 10.4 min; B: Dnp-Pro-Leu, elution time 19.1 min) produced by complete hydrolysis by thimet peptidase.

to determine k_{cat} and K_m for the cleavage of the substrate by thimet peptidase, it was necessary to correct for this quenching by standardizing the fluorimeter with Ac-DL-Amp-NH₂ at each substrate concentration. If this was not done, spuriously low values of the kinetic constants were obtained. Absorptive quenching also leads to a marked non-linearity when the residual fluorescence of the substrate is plotted against concentration (Fig. 4). These results show, however, that at a concentration of 1 μM , the fluorescence of the substrate is only 3% that of Ac-DL-Amp-NH₂.

When Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys (20 μM in assay buffer) was incubated with chicken liver thimet peptidase (1.6 $\mu\text{g}/\text{ml}$) at 30 °C, two new peaks (A and B) were observed by h.p.l.c. (Fig. 5). Both peaks were visible in separate runs monitored at 220 and 360 nm, and peak B had the same retention time as the standard Dnp-Pro-Leu. After incubation for 5 h, a peak of unhydrolysed peptide remained, but the Amp fluorescence was identical with that measured at 1 h and 2 h, and the h.p.l.c. traces also were unchanged. It was assumed that the peptide remaining was Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys. During this period the fluorescence of the solution (diluted 25-

Fig. 6. Standard curve for the continuous assay of thimet peptidase with Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys

Rates of increase in product concentration were measured with various amounts of enzyme/assay, as described in the Experimental section.

fold for measurement) increased 20-fold, although, as more than 50 % of the peptide remained unhydrolysed, the true degree of quenching must be close to the value of 97 % reported above (cf. Figs. 3 and 4).

Continuous-rate assays of thimet peptidase activity were done initially with 10 μM -substrate and with up to 0.2 μg of the enzyme from chicken liver, pre-activated with 5 mM-2-mercaptoethanol (Fig. 6). The assays were comparable in sensitivity with those previously made with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys and the rabbit muscle enzyme (Barrett *et al.*, 1989). The rates of hydrolysis at substrate concentrations in the range 0.2–8 μM were fitted to the Michaelis–Menten equation by non-linear regression and gave values of k_{cat} and K_m of 0.82 s^{-1} and 1.23 μM respectively ($k_{\text{cat}}/K_m = 6.7 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). The values of both k_{cat} and K_m are lower than those of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys, which had $k_{\text{cat}} = 1.79 \text{ s}^{-1}$ and $K_m = 7.52 \mu\text{M}$ ($k_{\text{cat}}/K_m = 2.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). The higher value of k_{cat}/K_m for the Amp-containing substrate confirms that thimet peptidase interacts favourably with peptides having large aromatic residues at the P_3' position (cf. Orlowski *et al.*, 1988), provided that these are in the appropriate stereochemical configuration. This selectivity may be contrasted with that of clostridial collagenase, which completely hydrolyses both isomers of Dnp-Pro-Leu-Gly-Pro-DL-Amp-D-Lys (M. A. Brown & A. J. Barrett, unpublished work).

The results reported here show that the replacement of tryptophan by the coumarin amino acid Amp in quenched fluorescent peptides has a number of advantages. Amp fluorescence is excited and emitted at wavelengths at which tryptophan makes a negligible contribution, so that background signals are expected to be low in assays made at high protein concentrations or with tryptophan-containing inhibitors. Thus,

Amp has the spectral properties of the Mcc group (Tisljar *et al.*, 1990), and the additional benefit of flexibility of placement in the peptide chain. Also, the synthesis of Amp-containing peptides is more straightforward, as the coumarin side chain is far less susceptible to modification reactions.

A limitation of the present study is the racemic nature of the synthetic product. We have attempted a stereospecific synthesis of L-Amp through the imidazolidinone reagents of Fitz & Seebach (1988), but yields have been low. A more promising approach may be to resolve the racemate by chiral chromatography or by enzyme digestion.

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