Reagents for reversible coupling of proteins to the active centres of trypsin-like serine proteinases

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In order to extend the scope for the application of acyl-enzymes as fibrinolytic agents, *p*-amidinophenyl ester enzyme substrates were prepared that gave stabilized acyl-enzymes capable of being coupled to other proteins. Coupling was achieved by reaction of protein thiol functions with a 2-pyridyldithio moiety within the acyl group. Acyl-enzymes derived from such substrates were stable enough to permit isolation of reversible conjugates between proteins and the active centres of plasminogen activators. Hydrolytic release of active enzyme from a conjugate of human immunoglobulin G and urokinase could be demonstrated.

INTRODUCTION

We have described previously the preparation of stabilized acyl-enzymes derived from fibrinolytic serine proteinases and amidinophenyl esters of substituted benzoic acids (Smith *et al.*, 1981). Such derivatives constitute semisynthetic enzyme precursors whose stabilities may be chemically controlled, and they have shown activity as thrombolytic agents in animals (Smith *et al.*, 1982; Dupe *et al.*, 1984) and for the treatment of acute myocardial infarction in man (Been *et al.*, 1985; Marder *et al.*, 1986). The rationale for the use of acyl-enzymes as thrombolytic agents derives in part from the fact that the important endogenous inhibitors of fibrinolytic enzymes in human blood interact directly or indirectly with the active centre of the enzyme (Barrett & Starkey, 1973; Mullertz & Clemmensen, 1976). Temporary protection of the catalytic apparatus of the enzyme by acylation facilitates other protein–protein interactions, notably binding to the fibrin component of a thrombus. In the case of the streptokinase–plasminogen activator complex, blockade of inhibitor interactions also prolongs the plasma residence time of the acyl-enzyme (Gonias *et al.*, 1982; Been *et al.*, 1986). However, two important human plasminogen activators, urokinase (u-PA) and tissue plasminogen activator (t-PA), are known to be removed from the circulation of animals (Korninger *et al.*, 1981; Rijken & Emeis, 1986) and man (Nilsson *et al.*, 1984) by pharmacologically significant processes that do not involve such plasma inhibitors and that are not, therefore, subject to blockade by simple reversible acylation.

We were interested in the possibility of manipulating plasminogen activator (PA) pharmacokinetics by coupling the enzyme through its active centre to a slowly catabolized carrier protein from which the unmodified plasminogen activator might be released by deacylation *in vivo* at a chemically predetermined rate. Such an approach provides the additional advantage of scope for enzyme targeting by exploiting binding specificities in the carrier protein. We report here the first reagents capable of such reversible conjugation. These reagents are heterobifunctional forms of the ‘inverse’ esters previously described as substrates for trypsin-like serine

proteinases (Tanizawa *et al.*, 1977). The preparation of a reversible conjugate between urokinase (u-PA) and non-specific human IgG is also described.

MATERIALS AND METHODS

Materials

High- M_r urokinase was purchased from Serono, Freiburg, W. Germany. Recombinant t-PA was prepared as previously described (Browne *et al.*, 1985). 2-Iminothiolane, 5,5'-dithiobis-(2-nitrobenzoic acid) and human non-specific γ -globulin (electrophoretic purity 99%) were from Sigma Chemical Co., Poole, Dorset, U.K. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was obtained from Pharmacia, Milton Keynes, Bucks., U.K. 4-Fluorobenzoyl chloride, 2-methylpropan-2-ol, 1,2-diaminoethane, 1,6-diaminohexane, *NN'*-dicyclohexylcarbodi-imide and 2-chlorobenzoic acid were supplied by Aldrich Chemical Co. (Gillingham, Dorset, U.K.). 4-Hydrazinobenzoic acid was purchased from Fluka (Buchs, Switzerland). 4-Amidinophenol was prepared as the hydrochloride salt essentially as described by Tanizawa *et al.* (1977).

Determination of deacylation rate constants

Deacylation rate constants were measured in 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl and 0.01% Tween 80 or in 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.14 M-NaCl and 20% (v/v) glycerol (for acyl-plasmins and the acyl-enzyme from t-PA and compound Ic) at 37 °C as previously described (Smith *et al.*, 1985).

Synthesis of coupling reagents

t-Butyl 4-fluorobenzoate (II). 2-Methylpropan-2-ol (6.6 ml) and pyridine (5.6 ml) were dissolved in dry dichloromethane (40 ml), cooled in solid CO₂/acetone to –50 °C, and a solution of 4-fluorobenzoyl chloride (10.0 g) in dry dichloromethane (20 ml) was added slowly under N₂. After warming slowly to room temperature, the mixture was heated under reflux for 3 days. I.r. then indicated that reaction was complete and, after cooling,

Abbreviations used: u-PA, urinary-type plasminogen activator; t-PA, tissue-type plasminogen activator; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)-propionate.

the organic layer was washed successively with 2 M-HCl (40 ml) and satd. Na_2SO_4 (40 ml). The dichloromethane solution was dried, filtered and evaporated to leave an oil (10.6 g), which was a mixture of the desired compound and 4-fluorobenzoic acid anhydride. The components were separated by column chromatography (Merck Kieselgel grade 60 silica) in dichloromethane/light petroleum (b.p. 40–60 °C) (3:7, v/v) to yield the title compound (8.5 g, 69% yield). I.r. (neat): 2970, 1715, 1610, 1510, 1155, 1120, 850 and 770 cm^{-1} . N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}]$ chloroform): 8.05, 7.05 (4H, m) and 1.6 (9H, s).

t-Butyl 4-N-(2-aminoethyl)aminobenzoate (III). Compound (II) (1.19 g) was dissolved in 1,2-diaminoethane (5 ml) and heated at 120 °C under N_2 for 3 h. After cooling and removal of diamine by evaporation, water (25 ml) was added, followed by acidification with conc. HCl. The aqueous layer was extracted with ethyl acetate (25 ml, discarded), then basified with 5 M-NaOH, and the product was extracted with ethyl acetate (2 $\times$ 50 ml). The organic layer was dried, filtered and evaporated to yield the title compound (1.16 g, 81% yield), m.p. 117–119 °C (Found C, 65.94; H, 8.55; N, 11.51; $\text{C}_{13}\text{H}_{20}\text{N}_2\text{O}_2$ requires C, 66.07; H, 8.53; N, 11.85%). I.r. (Nujol): 3360, 3300, 1680, 1610, 1540, 1300, 1160, 1110, 1060, 930, 840, 780 and 620 cm^{-1} . N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}]$ chloroform): 7.8 (2 H, d, $J = 8$ Hz), 6.5 (2 H, d, $J = 8$ Hz), 4.6 (1 H, s), 3.0 (4 H, m), 1.6 (9 H, s) and 1.3 (2 H, s).

t-Butyl 4-N-(2-N-[3-(2-pyridyldithio)propionyl]aminoethyl)aminobenzoate (IV). Compound (III) (74 mg) and SPDP (100 mg) were dissolved in dry pyridine (0.5 ml) and stirred at room temperature overnight. Solvent was removed at reduced pressure, the residue was taken up in ethyl acetate (100 ml), and the organic layer was washed with aq. 10% (w/v) NaHCO_3 (10 ml). The organic phase was dried (over Na_2SO_4), filtered and evaporated to yield the title compound as an oil (131 mg, 96% yield). I.r. (neat): 3350, 3070, 1660, 1610, 1530, 1420, 1300, 1160, 1120, 910, 840, 770 and 735 cm^{-1} . N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}]$ chloroform): 8.25 (1 H, m), 7.4 (6 H, m), 6.5 (2 H, d, $J = 9$ Hz), 4.65 (1 H, br s), 3.4 (4 H, m), 3.05 (2 H, t, $J = 6$ Hz), 2.60 (2 H, t, $J = 6$ Hz) and 1.55 (9 H, s).

4-N-(2-N-[3-(2-Pyridyldithio)propionyl]aminoethyl)aminobenzoic acid (Va). The ester (IV) (66 mg) was dissolved in trifluoroacetic acid (2 ml) and held at room temperature for 90 min. Trifluoroacetic acid was removed at reduced pressure and the bistrifluoroacetate salt of the acid (90 mg) was isolated as an orange oil in quantitative yield. N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}_6]$ acetone/ $[\text{D}_2\text{H}]$ chloroform): 8.6 (1 H, m), 7.75 (5 H, m), 6.65 (2 H, d, $J = 9$ Hz), 3.5 (4 H, br s), 3.05 (2 H, t, $J = 6$ Hz) and 2.65 (2 H, t, $J = 6$ Hz). Mass spectrum: m/z M^+ found 377.0866; $\text{C}_{17}\text{H}_{19}\text{N}_3\text{O}_3\text{S}_2$ requires 377.0868.

4'-Amidinophenyl 4-N-(2-N-[3-(2-pyridyldithio)propionyl]aminoethyl)aminobenzoate hydrochloride (Ia). Compound (Va) (450 mg) was dissolved in dry pyridine (2 ml) and 4-amidinophenol hydrochloride (130 mg), followed by NN' -dicyclohexylcarbodi-imide (160 mg) added. The mixture was stirred for 3 days at room temperature under N_2 , then filtered and evaporated to a dark gum. The latter was triturated successively with chloroform

(3 $\times$ 5 ml), water (1 ml), satd. NaCl (1 ml) and water (1 ml). The gum was dried under high vacuum, then taken up in the minimum quantity of ethanol; the solution was filtered and sufficient diethyl ether was added to precipitate the product. The mixture was held at 0 °C for 48 h, the hardened gum was isolated by decantation and the product was reprecipitated from ethanol/diethyl ether, yielding the title compound as a gummy solid (103 mg, 26% yield). N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}_6]$ dimethyl sulphoxide): 9.25 (4 H, s, $^2\text{H}_2\text{O}$ -exchangeable, amidine NH), 8.45 (1 H, d, $J = 5$ Hz), 8.13 (1 H, s), 7.30 (6 H, m), 7.48 (2 H, d, $J = 8$ Hz), 7.24 (1 H, t, $J = 5$ Hz), 6.81 (1 H, s, $^2\text{H}_2\text{O}$ -exchangeable), 6.69 (2 H, d, $J = 9$ Hz), 3.23 (4 H, s), 3.03 (2 H, t, $J = 7$ Hz) and 2.52 (2 H, t, $J = 7$ Hz). Fast-atom-bombardment mass spectrum (glycerol/methanol): m/z MH^+ free base 496, MH^+ –SS–pyridine 352.

4-(2-N-[3-(2-Pyridyldithio)propionyl]hydrazino)-benzoic acid (Vb). 4-Hydrazinobenzoic acid (25 mg) was dissolved in pyridine (0.5 ml), and SPDP (50 mg) was added. The reaction was stirred at room temperature for 3 h and evaporated to an oil, which was recrystallized from ethanol/water to give a glassy solid. The latter was redissolved in ethanol, filtered and evaporated to a pale-red gum (51 mg, 91% yield). I.r. (Nujol): 2500–3500, 1680, 1610, 1420, 1240, 1175, 1120, 840 and 760 cm^{-1} . N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}_6]$ acetone): 9.45 (1 H, br s), 8.5 (1 H, m), 7.8 (5 H, m), 7.25 (1 H, m), 6.9 (3 H, d, $J = 8$ Hz) and 3.0 (4 H, m).

4'-Amidinophenyl 4-(2-N-[3-(2-pyridyldithio)propionyl]hydrazinobenzoate hydrochloride (Ib). The acid (Vb) (50 mg) was dissolved in pyridine (1 ml), and 4-amidinophenol hydrochloride (100 mg) and NN' -dicyclohexylcarbodi-imide (31 mg) were added. The mixture was stirred under N_2 at room temperature for 10 days, when i.r. showed reaction to be complete. The solution was filtered and evaporated, and the residue was triturated successively with water (5 ml), satd. NaCl (2 ml) and water (1 ml). The solid was dissolved in ethanol (2 ml) and filtered, and diethyl ether was added until a precipitate appeared. After standing for 48 h at 0 °C, the mixture was filtered and yielded the title compound (51 mg, 71% yield). N.m.r. δ (p.p.m.) (in $[\text{D}_2\text{H}_6]$ dimethyl sulphoxide): 10.05 (1 H, s, $^2\text{H}_2\text{O}$ -exchangeable, hydrazide NH), 9.41 (4 H, s, $^2\text{H}_2\text{O}$ -exchangeable, amidine NH), 8.75 (1 H, s, $^2\text{H}_2\text{O}$ -exchangeable, hydrazine NH), 8.48 (1 H, m), 7.85 (6 H, m), 7.50 (2 H, d, $J = 8$ Hz), 7.27 (1 H, m), 6.82 (2 H, d, $J = 9$ Hz), 3.11 (2 H, t, $J = 7$ Hz) and 2.69 (2 H, t, $J = 7$ Hz). Fast-atom-bombardment mass spectrum (3-nitrobenzyl alcohol): m/z MH^+ 468, M^+ – CO_2 424, M^+ –pyridine 390, M^+ –S-pyridine 358.

N-(6-Aminohexyl)anthranilic acid (VI). This compound was prepared following the general procedure of Young & Sweet (1958). 2-Chlorobenzoic acid (31.4 g), 1,6-diaminohexane (55.7 g), anhydrous K_2CO_3 (25.6 g), n-pentanol (68 ml) and copper powder (0.3 g) were refluxed for 5 h. Solvent and 1,6-diaminohexane were distilled off and the residue was poured into cold water (1.5 litres). The mixture was neutralized with conc. HCl and cooled to 4 °C. The precipitated gum was taken up in hot water/ethanol/conc. HCl (8:8:1, by vol.) (850 ml), cooled, filtered and neutralized with 5 M-NaOH. The title

compound was precipitated as a yellow solid at 4 °C, filtered and dried *in vacuo* (22.4 g, 52% yield), m.p. 192 °C (decomp.). N.m.r. δ (p.p.m.) (in $^2\text{HCl}/^3\text{H}_2\text{O}$): 8.20 (1 H, m), 7.65 (3 H, m), 3.50 (2 H, t), 3.0 (2 H, t) and 1.7 (8 H, m).

***N*-6-[3-(2-Pyridyldithio)propionyl]aminoanthranilic acid (Vc).** *N*-6-Aminoanthranilic acid (76 mg) was dissolved in pyridine (1 ml), and SPDP (100 mg) was added. The solution was heated at 60 °C for 48 h and, after cooling, was evaporated. The residue was dissolved in a small quantity of ethanol, and water was added. The solution went cloudy, and after several hours standing at 0 °C a precipitate was obtained. The liquid was removed by decantation to leave a glassy solid. Ethanol was added, and the solution was filtered and evaporated to leave the title compound (29 mg, 21% yield). N.m.r. δ (p.p.m.) (^2H]chloroform): 8.45 (1 H, m), 7.8 (3 H, m), 7.2 (2 H, m), 6.65 (2 H, m), 5.9 (3 H, br s), 3.3 (6 H, m), 2.75 (2 H, s) and 1.5 (8 H, br s).

4'-Amidinophenyl *N*-6-[3-(2-pyridyldithio)propionyl]aminoanthranilate hydrochloride (Ic). The acid (Vc) (29 mg) prepared above was dissolved in pyridine (0.5 ml), and 4-amidinophenol (12 mg) was added, followed by *NN'*-dicyclohexylcarbodi-imide (14 mg). The reaction mixture was stirred at room temperature under N_2 for 48 h, in which time dicyclohexylurea was precipitated. This was removed by filtration, and the solution was allowed to evaporate at reduced pressure. The residual oil was triturated with water (1 ml) and satd. NaCl (1 ml). The residue was taken up in ethanol (2 ml), filtered and precipitated with diethyl ether. The liquid was removed by decantation and the solid dried at reduced pressure to leave the title compound (8 mg, 21% yield). N.m.r. δ (p.p.m.) ($^2\text{H}_6$]dimethyl sulfoxide) 9.25 (4 H, br d), 8.43 (1 H, m), 8.05 (1 H, m), 7.93 (2 H, d, $J = 8$ Hz), 7.75 (2 H, m), 7.50 (3 H, m), 7.24 (1 H, m), 6.85 (1 H, m), 6.66 (1 H, m), 3.01 (4 H, m), 2.75 (2 H, m), 2.60 (2 H, m) and 1.3 (8 H, m). Fast-atom-bombardment mass spectrum (glycerol): m/z MH^+ 552.

Reversible conjugate of u-PA and IgG

Human non-specific γ -globulin (60 mg, 400 nmol) was dissolved in N_2 -purged phosphate buffer (as above) (2 ml) and treated with fresh 2-iminothiolane (1.25 mM) at 25 °C for 20 min and 0 °C for 2 h. The product was gel-filtered (Sephadex G-25, PD10; Pharmacia) into phosphate buffer (3.0 ml) at 4 °C, diluted to 6.0 ml, precipitated with satd. $(\text{NH}_4)_2\text{SO}_4$ (6.0 ml) at 9000 *g* for 20 min at 4 °C and the pellet dissolved in phosphate buffer (2.0 ml). Titration with 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) indicated that approx. 2 mol of thiol/mol of protein was incorporated. Urokinase (63 nmol) was dissolved in phosphate buffer (1.0 ml) and treated with compound (Ia) (20 μl , 10 mM in dry dimethyl sulfoxide) for 30 min at 25 °C.

Amidolytic assay with S-2444 (Claeson *et al.*, 1978) indicated that the enzyme was 96% acylated, and the product was gel-filtered into phosphate buffer as above. Acyl-enzyme (53 nmol, 2.7 ml) was mixed with thiolated IgG (191 nmol of protein, 1.1 ml), held on ice for 30 min and concentrated (Centricon 10; Amicon, Stonehouse, Gloucs., U.K.) to 1.0 ml. The product was chromatographed on a 21.5 mm $\times$ 300 mm TSK G3000 SW gel-permeation column in 20 mM-Tris/HCl buffer, pH 7.4,

Fig. 1. General structure and functional features of *p*-amidinophenyl ester serine proteinase substrates capable of conjugative acylation of trypsin-like serine proteinases

containing 0.5 M-L-arginine, 0.5 M-NaCl and 0.01% Tween 80 at 2 ml/min at 20 °C. Conjugate and unmodified IgG were eluted at about 80 ml whereas acyl-urokinase was eluted at 112 ml. A pool of fractions eluted between 70 and 88 ml was gel-filtered (Sephadex G-25, PD10) at 4 °C into 0.2% D-mannitol in 20 mM-ammonium bicarbonate buffer, pH 7.4, freeze-dried in 1.6 ml portions and stored at -40 °C. Analytical h.p.l.c. was performed on a Dupont GF250 column in the above arginine buffer at 0.5 ml/min at ambient temperature. Fractions were assayed after hydrolysis for 2 h at 37 °C by using a micro-titre-plate modification of the S-2444 amidolytic assay [S-2444, 1.0 mM in 0.1 M-triethanol-amine/HCl buffer, pH 8.0 (100 μl), incubated with sample (50 μl) at 37 °C for 10–30 min and the reaction stopped with 1.0 M-acetic acid (100 μl); plates were read at 410 nm].

RESULTS AND DISCUSSION

Previous studies had suggested (Smith, 1983; Smith *et al.*, 1985) that acyl-enzymes of the required range of stabilities (pseudo-first-order deacylation rate constants under physiological conditions of 10^{-5} to 10^{-3} s^{-1}) could be derived from benzoic acid esters with electron-releasing substituents in the 2- or 4-position of the aromatic ring. We therefore designed a series of compounds (Fig. 1) in which bifunctional amine-containing substituents within such esters were coupled to a functionality capable of reacting with free thiol groups in carrier proteins. The compounds were prepared by treating the appropriate aminoalkylamino- or hydrazino-benzoic acids or their esters with the *N*-oxy-succinimide ester of 3-(2-dithiopyridyl)propionic acid (SPDP) (Carlsson *et al.*, 1978), followed by esterification with 4-amidinophenol hydrochloride with the use of *NN'*-dicyclohexylcarbodi-imide.

The esters (I) could be reduced by dithiothreitol to give quantitative release of pyridine-2-thione. They reversibly inactivated a variety of fibrinolytic enzymes. Deacylation rate constants in buffers are given in Table 1. The most stable acyl-enzymes were those derived from the anthranilic acid analogue (Ic) (which was also the least reactive substrate), and the range of stabilities was within that found previously to be of pharmacological interest (Smith *et al.*, 1982).

The above reagents have been used to couple u-PA and t-PA to carrier proteins by using two general strategies. Firstly, the carrier protein (e.g. human serum

Table 1. Stabilities to hydrolysis of acyl-enzymes derived from heterobifunctional substrates (I)

Values indicated by an asterisk (*) were determined in Tris/glycerol buffer; all other determinations were in phosphate buffer (see the Materials and methods section), pH 7.4 at 37 °C. Values given are means $\pm$ S.E.M. ($n > 4$).

Enzyme	Substrate	$10^4 \times$ Deacylation rate constant (s^{-1})	Half-life (min)
Urokinase	(Ia)	1.62 ± 0.19	71
	(Ib)	2.41 ± 0.15	48
	(Ic)	0.88 ± 0.08	131
Tissue-type plasminogen activator	(Ia)	1.87 ± 0.15	63
	(Ib)	3.18 ± 0.14	36
	(Ic)	$< 0.3^*$	$> 385^*$
Plasmin	(Ia)	$0.25 \pm 0.014^*$	462^*
	(Ib)	$2.40 \pm 0.13^*$	48^*
	(Ic)	$< 0.1^*$	$> 1155^*$

albumin or immunoglobulins) was modified by reaction with 2-iminothiolane (Jue *et al.*, 1978) or SPDP followed by reduction, to give a free thiol content of 1–2 mol/mol of protein. The thiolated protein was either (a) treated with esters (I) to generate a proteinaceous acylating agent (which was then made to react with the enzyme) or (b) directly coupled to the acyl-enzyme derived from (I). Secondly, plasminogen activators themselves were thiolated and made to react with acyl-enzymes derived from other activators or plasmin. The latter method provided a route to combination enzymes.

One conjugate was prepared by the reaction of 2-iminothiolane with human non-specific IgG followed by coupling of the thiolated IgG with the acyl-enzyme derived from compound (Ia) and u-PA.

This material (for which we suggest the designation IgG→u-PA) was purified from excess uncoupled acyl-u-PA by preparative high-performance gel-permeation chromatography (see the Materials and methods section) and was found to regenerate active u-PA with a first-order rate constant of approx. $4.8 \times 10^{-4} s^{-1}$ at pH 7.4 at 37 °C. Fig. 2 shows analytical gel-permeation h.p.l.c. of intact IgG→u-PA and a control of pre-hydrolysed conjugate. Potential u-PA activity in IgG→u-PA was measured by hydrolysing individual fractions and appeared nearly coincident with the main protein peak (IgG and IgG→u-PA). Hydrolysed IgG→u-PA was clearly separated into IgG and active u-PA peaks. SDS/polyacrylamide-gel electrophoresis combined with fibrin overlay autography (Graneli-Piperno & Reich, 1978) confirmed that IgG→u-PA conjugates break down to give fibrinolytically active u-PA (results not shown).

Conjugation of fibrinolytic activity in this way differs in several respects from previous studies in which PAs were linked to proteins (Bode *et al.*, 1985; Sevilla *et al.*, 1985; Maksimenko & Torchilin, 1985). Firstly, the linkage between protein and PA is reversible and generates chemically unmodified PA upon hydrolysis; secondly, it is specific (i.e. located at the active-centre serine residue of the PA); thirdly, the PA is delivered in an inactive pro-drug form that is insensitive to plasma inhibitors of PAs (Kruithof *et al.*, 1986); fourthly, the

Fig. 2. High-performance gel-permeation chromatography of IgG→u-PA

(a) Intact conjugate; (b) conjugate hydrolysed before chromatography. For conditions see the Materials and methods section. ●—●, Urokinase activity (A_{410}); —, A_{280} .

rate at which the activity is released is subject to a degree of chemical control through the design of the linker acyl group. Preliminary results suggest that, in certain cases, the reversible conjugation technique can significantly prolong the plasma residence time of plasminogen activator activity as well as conferring novel binding specificities and kinetic properties (Smith, 1986, and unpublished work). We suggest that this chemical approach, together with recombinant DNA methods for the production of the enzymes themselves, may provide new opportunities for therapeutic application of proteolytic activities.

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