The synthesis and properties of peptidylmethylsulphonium salts with two cationic residues as potential inhibitors of prohormone processing

Albrecht ZUMBRUNN, Stuart STONE and Elliott SHAW

Friedrich Miescher-Institut, CH 4002 Basel, Switzerland

Peptidylmethylsulphonium salts incorporating consecutive basic residues at the C-terminus of the peptidyl portion such as -Arg-Arg-, -Arg-Lys-, -Lys-Lys- and -Lys-Arg- were synthesized and examined as proteinase inhibitors. Serine proteinases with a specificity directed towards hydrolysis at cationic residues were found to be unaffected by these derivatives. On the other hand, cysteine proteinases, cathepsin B and, in particular, clostripain were readily inactivated by affinity labelling. The reagents thus are of promise for the study of prohormone processing promoted by cysteine proteinases.

INTRODUCTION

Peptidylmethylsulphonium salts represent a new class of proteinase affinity-labelling reagents of the general structure $R-C(=O)-CH_2-S^+(CH_3)_2$, in which the ketonic function is derived from the C-terminal carboxy group of the peptide $R-CO_2H$. Like chloromethyl ketones and diazomethyl ketones, these derivatives are of interest in the study of serine and cysteine proteinases. Changes in the chemical nature of the alkylating function influence the relative susceptibilities of serine and cysteine proteinases to each type of reagent. Thus the chloromethyl ketones (or peptidyl-chloromethanes) irreversibly inactivate both classes of proteinases if the amino acids in the R- portion of the structure satisfy the specificity requirements of members of each class (Schoellmann & Shaw, 1962; Shaw *et al.*, 1965; Whitaker & Perez-Villasenor, 1968; Kettner & Shaw, 1981). On the other hand, the diazomethyl ketones alkylate almost exclusively cysteine proteinases (Green & Shaw, 1981). The exploitation of this great specificity for the study of cysteine proteinases presumably involved in prohormone processing with a specificity directed towards hydrolysis after paired cationic residues is not readily achieved, however, since diazomethyl ketones incorporating these residues are very difficult to synthesize (Zumbrunn *et al.*, 1988). On the other hand, peptidylmethylsulphonium salts appeared to fall somewhere between chloromethyl ketones and diazomethyl ketones with respect to relative effectiveness against these proteinase classes, judged from initial results. Cathepsin B was very susceptible to $Cbz-Phe-AlaCH_2S^+(CH_3)_2$, alkylation resulting from transfer of $Cbz-Phe-AlaCH_2-$ to the enzyme (presumably to the essential thiol group) rather than by methyl transfer (Shaw, 1988). With lysine-containing derivatives irreversible inhibition of some trypsin-like serine proteinases was obtained with dimethylsulphonium salts, whereas reversible inhibition without covalent bond formation was observed with a benzylmethylsulphonium salt (Rauber *et al.*, 1988). Nevertheless, the susceptibility

of a cysteine proteinase with a specificity directed towards a cationic amino acid, namely clostripain (Porter *et al.*, 1971), was considerably greater than that of the serine proteinases.

The possible application of peptidylmethylsulphonium salts to the study of prohormone processing was of interest since there is evidence that cysteine proteinases are involved in some cases (Loh *et al.*, 1984). In fact, it has been shown that a yeast hormone, the alpha factor, is liberated from a precursor form by the product of the *KEX2* gene, apparently a cysteine proteinase (Julius *et al.*, 1984) also known as yscF (Achstetter & Wolf, 1985). In earlier work use was made of $Ala-Lys-Arg-CH_2Cl$ (Kettner & Shaw, 1981) to attempt to identify the enzyme(s) involved in the conversion of proinsulin into insulin and resulted in the labelling of cathepsin B (Docherty *et al.*, 1983, 1984). Cathepsin B was not viewed as the converting proteinase but as a possible low- M_r maturation product of the actual converting enzyme. The likelihood that proinsulin processing and analogous prohormone activations are brought about by a cysteine proteinase was also indicated by studies in anglerfish eyelets (Fletcher *et al.*, 1981). However, other views have been put forward (ole-MoiYoi *et al.*, 1979; Virji *et al.*, 1980). Recent studies on the proinsulin conversion in rat insulinoma tissue have indicated the importance of a Ca^{2+} -dependent proteinase, which was inactivated by *p*-hydroxymercuribenzoate; however, other thiol-blocking reagents were without effect (Davidson *et al.*, 1987). The peptidylmethylsulphonium salts, as a class of reagents, seemed quite amenable to synthesis with double cationic residues that appear to be a characteristic feature of processing sites in prohormones (Andrews *et al.*, 1987), and to have some advantage over chloromethyl ketones with respect to class reactivity. We therefore undertook the synthesis of some peptidylmethylsulphonium salts containing the Lys-Lys, Lys-Arg, Arg-Lys, and Arg-Arg sequences as described below along with some preliminary observations of their properties as inhibitors.

Abbreviations used: Cbz-, benzyloxycarbonyl-; Boc-, t-butoxycarbonyl-; Bz-, benzoyl-; Mtr-, 4-methoxy-2,3,6-trimethylbenzenesulphonyl-; -NH-Mec, 4-methylcoumarin-7-ylamide; -NH-Np, 4-nitroanilide; f.a.b., fast atom bombardment.

EXPERIMENTAL

Materials

Bz-Phe was from Senn Chemicals (Dielsdorf, Switzerland). Boc-Ala and Lys(Boc) were from Bachem (Bubendorf, Switzerland) and Arg(Mtr) was from Nova Chemical (Laufelfingen, Switzerland). All other chemicals were from Fluka (Buchs, Switzerland). Peptide 4-nitroanilides used as substrates were purchased from Kabi-Vitrum (Stockholm, Sweden); Cbz-Phe-Arg-NH-Mec was from Bachem. Plasma kallikrein was purchased from Kabi-Vitrum; clostripain and pig pancreatic kallikrein were from Boehringer (Mannheim, Germany); bovine trypsin [tosylphenylalanyl-chloromethane ('TPCK')-treated] was from Worthington (Freehold, NJ, U.S.A.). Cathepsin B was prepared from pig liver as described previously (Evans & Shaw, 1983).

Synthesis of peptidylmethyl-dimethylsulphonium salts

In this work the ϵ -amino group of lysine was protected with the Boc- group, and the arginine side chain with the Mtr- group (Fujino *et al.*, 1981). By using standard methods of peptide synthesis (Wuensch, 1974) Bz-Phe-Arg(Mtr), Boc-Ala-Lys(Boc)-Arg(Mtr), Boc-Ala-Arg(Mtr)-Lys(Boc), Boc-Ala-Lys(Boc)-Lys(Boc) and Boc-Ala-Arg(Mtr)-Arg(Mtr) were prepared. The fully protected peptides were converted into the sulphonium salts by the steps summarized in Scheme 1, involving conversion of the free carboxyl group (I) successively into the diazomethyl ketone (II), chloromethyl ketone (III) and methylmercaptomethyl ketone (IV). Methylation of this provided the fully protected peptidylmethyl-dimethylsulphonium salt (V) (Shaw, 1988), which was deblocked at room temperature with trifluoroacetic acid, leaving the sulphonio group unchanged (VI).

General. N.m.r. spectra were measured at 250 MHz in [$^2\text{H}_6$]dimethyl sulphoxide. Analytical h.p.l.c. was performed on a Whatman RAC II ODS-3 column with a gradient mixture of 0.1% (v/v) trifluoroacetic acid in water and acetonitrile [gradient 1, linear gradient

from 10% (v/v) acetonitrile to 100% acetonitrile within 7 min; gradient 2, linear gradient from 40% (v/v) acetonitrile to 100% acetonitrile within 7 min]. Preparative h.p.l.c. was performed on a Whatman Magnum 9 ODS-3 column.

Benzoyl-phenylalanyl-*N*^w-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-diazomethane [Bz-Phe-Arg(Mtr)CHN₂ (Ib)]. A solution of Bz-Phe-Arg(Mtr) (Ia) 319 mg, 0.5 mmol in tetrahydrofuran (7 ml) was treated at -10°C with *N*-methylmorpholine (0.11 ml, 1 mmol) and isobutyl chloroformate (0.13 ml, 1 mmol) for 5 min. Ethereal diazomethane (15 ml, 5 mmol) was added and the stirred mixture allowed to warm to room temperature during 3 h. Usual work-up and preparative chromatography on silica gel with methanol/dichloromethane (1:20, v/v) gave 221 mg (69% yield) of a yellow oil. It had h.p.l.c. (gradient 2) $t = 5.51$ min (92%) and $t = 5.33$ min (2%, DL-isomer), i.r. (film) 3340, 3240, 2100, 1635 and 1550 cm^{-1} , and n.m.r. δ (p.p.m.) 1.4–1.9 [4H, m, $2\text{CH}_2(\text{Arg})$], 2.13, 2.58, 2.68 [9H, 3s, $3\text{CH}_3(\text{Mtr})$], 3.05–3.35 [4H, m, $\text{CH}_2(\text{Arg}) + \text{CH}_2(\text{Phe})$], 3.85 (3H, s, $\text{OCH}_3(\text{Mtr})$], 4.38 [1H, m, $1\text{H}-\alpha(\text{Arg})$], 5.00 [1H, t $\times$ d, $J = 6$ Hz and 6 Hz, $1\text{H}-\alpha(\text{Phe})$], 5.32 (1H, s, CHN_2), 6.21 (2H, m, 2NH), 6.33 (1H, s, 1NH), 6.53 [1H, s, $1\text{H}(\text{Mtr})$], 7.30 [7H, m, $5\text{H}(\text{Phe}) + 2\text{NH}$] and 7.4–7.6 [5H, m, $5\text{H}(\text{Bz})$].

Benzoyl-phenylalanyl-*N*^w-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-chloromethane [Bz-Phe-Arg(Mtr)-CH₂Cl (IIIa)]. To an ice-cooled stirred solution of Bz-Phe-Arg(Mtr)-CHN₂ (IIa) (193 mg, 0.30 mmol) in ethyl acetate (4 ml) was added 2 M-HCl in ethyl acetate (4 ml) and, after 2 min stirring, water (20 ml). The ethyl acetate layer was washed with water, satd. NaHCO_3 and satd. NaCl, dried over MgSO_4 and concentrated. Preparative chromatography on silica gel with methanol/dichloromethane (1:20, v/v) gave 140 mg (70% yield) of a colourless oil. It had h.p.l.c. (gradient 2) $t = 6.11$ min (96%).

3-(Benzoyl-phenylalanylamido)-6-[3-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-guanidino]-2-oxohexyl methyl sulphide [BzPhe-Arg(Mtr)-CH₂SCH₃ (IVa)]. To a solution of Bz-Phe-Arg(Mtr)-CH₂Cl (IIIa) (140 mg, 0.21 mmol) in methanol (2 ml) was added 1 drop of 1% phenolphthalein solution. Then 1 M-sodium methanethiolate in methanol was added dropwise until the red colour persisted for 10 min. The mixture was poured into water. Usual work-up and preparative chromatography on silica gel with methanol/dichloromethane (1:20, v/v) gave 78 mg (55% yield) of a colourless oil. It had h.p.l.c. (gradient 2) $t = 6.11$ min (97%).

3-(Benzoyl-phenylalanylamido)-6-(3-methoxy-2,3,6-trimethylphenylsulphonyl)-guanidino-2-oxohexyl-dimethylsulphonium tetrafluoroborate [Bz-Phe-Arg(Mtr)-CH₂S⁺(CH₃)₂BF₄⁻ (Va)]. A solution of Bz-Phe-Arg(Mtr)-CH₂SCH₃ (IVa) (285 mg, 0.40 mmol) in nitromethane (7 ml) was treated with methyl iodide (0.09 ml, 1.5 mmol) and AgBF_4 (86 mg, 0.43 mmol) and stirred for 4 h. Acetone (20 ml) was added, and the mixture was filtered through Celite and concentrated. The residue was submitted to preparative chromatography on silica gel with dichloromethane/propan-2-ol (1:1, v/v). The main fraction crystallized upon concentration and was

- (a): $\text{R}' = \text{Bz-Phe-Arg(Mtr)-}$
 $\text{R} = \text{Bz-Phe-Arg-}$ with $\text{CF}_3\text{CO}_2\text{H}$, $\text{X}^- = \text{BF}_4^-$
 (b): $\text{R}' = \text{Boc-Ala-Lys(Boc)-Arg(Mtr)-}$
 $\text{R} = \text{Ala-Lys-Arg-}$ with $3\text{CF}_3\text{CO}_2\text{H}$, $\text{X}^- = \text{CF}_3\text{CO}_2^-$
 (c): $\text{R}' = \text{Boc-Ala-Arg(Mtr)-Lys(Boc)-}$
 $\text{R} = \text{Ala-Arg-Lys-}$ with $3\text{CF}_3\text{CO}_2\text{H}$, $\text{X}^- = \text{CF}_3\text{CO}_2^-$
 (d): $\text{R}' = \text{Boc-Ala-Lys(Boc)-Lys(Boc)-}$
 $\text{R} = \text{Ala-Lys-Lys-}$ with $3\text{CF}_3\text{CO}_2\text{H}$, $\text{X}^- = \text{CF}_3\text{CO}_2^-$
 (e): $\text{R}' = \text{Boc-Ala-Arg(Mtr)-Arg(Mtr)-}$
 $\text{R} = \text{Ala-Arg-Arg-}$ with $3\text{CF}_3\text{CO}_2\text{H}$, $\text{X}^- = \text{CF}_3\text{CO}_2^-$

Scheme 1. Synthesis of peptidylmethyl-dimethylsulphonium salts

recrystallized from acetone/propan-2-ol to give 68 mg (21 % yield) of a very hygroscopic material. It had h.p.l.c. (gradient 1) $t = 4.18$ min (87 %).

3-(Benzoyl-phenylalanyl-amido)-6-guanidino-2-oxohexyl-dimethylsulphonium trifluoroacetate hydrotrifluoroacetate [Bz-Phe-Arg-CH₂S⁺(CH₃)₂CF₃CO₂⁻, CF₃CO₂H (VIa)]. A solution of Bz-Phe-Arg-CH₂SCH₃ (Va) (68 mg, 0.087 mmol) in trifluoroacetic acid (1 ml) was kept at room temperature overnight and evaporated. The residue was submitted to preparative h.p.l.c. [linear gradient mixture of 0.1 % (v/v) trifluoroacetic acid in water and acetonitrile, from 20 % (v/v) acetonitrile to 100 % acetonitrile within 15 min, at a flow rate 3 ml/min]. The main fraction was freeze-dried to give 37 mg (yield 60 %) of a fluffy colourless powder. It had h.p.l.c. (gradient 1) $t = 5.52$ min (93 %), and n.m.r. δ (p.p.m.) 1.3–1.9 (4H, m, 2CH₂(Arg)), 2.80, 2.89 (6H, 2s, 2CH₃-S⁺), 5.1 [4H, m, CH₂(Arg) + CH₂(Phe)], 3.5–4.1 [5H, m, 5NH(guanidino)], 4.3 [1H, m, 1H- α (Arg)], 4.66 [1H, m, 1H- α (Phe)], 4.7–4.9 (2H, m, CH₂-S⁺), 7.2–7.9 (10H, m), 8.80 and 8.95 (2H, 2m, 2NH).

t-Butoxycarbonyl-alanyl-N^t-t-butoxycarbonyl-lysyl-N^w-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-diazomethane [Boc-Ala-Lys(Boc)-Arg(Mtr)-CHN₂ (IIb)]. The same procedure as for compound (IIa) was used to give a yellow oil (76 % yield). It had h.p.l.c. (gradient 2) $t = 5.41$ min (95 %).

t-Butoxycarbonyl-alanyl-N^t-t-butoxycarbonyl-lysyl-N^w-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-chloromethane [Boc-Ala-Lys(Boc)-Arg(Mtr)-CH₂Cl (IIIb)]. The same procedure as for compound (IIIa) was used to give a colourless oil (62 % yield). It had h.p.l.c. (gradient 2) $t = 5.82$ min (86 %).

3-(t-Butoxycarbonyl-alanyl-N^t-t-butoxycarbonyl-lysyl-amido)-6-[3-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-guanidinol]-2-oxohexyl methyl sulphide [Boc-Ala-Lys(Boc)-Arg(Mtr)-CH₂SCH₃ (IVb)]. The same procedure as for compound (IVa) was used to give a colourless oil (43 % yield). It had h.p.l.c. (gradient 2) $t = 5.82$ min (95 %).

3-(t-Butoxycarbonyl-alanyl-N^t-t-butoxycarbonyl-lysyl-amido)-6-[3-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-guanidinol]-2-oxohexyl-dimethylsulphoniumtrifluoroacetate [Boc-Ala-Lys(Boc)-Arg(Mtr)-CH₂S⁺(CH₃)₂CF₃CO₂⁻ (Vb)]. To a solution of Boc-Ala-Lys(Boc)-Arg(Mtr)-CH₂SCH₃ (IVb) (282 mg, 0.34 mmol) in nitromethane (5 ml) was added methyl iodide (0.085 ml, 1.36 mmol) and AgBF₄ (86 mg, 0.44 mmol). The mixture was stirred overnight, diluted with acetone, filtered through Celite and concentrated. Preparative h.p.l.c. [linear gradient mixture of 0.1 % (v/v) trifluoroacetic acid in water and in acetonitrile, from 30 % (v/v) acetonitrile to 100 % acetonitrile within 15 min, at a flow rate of 3 ml/min], isolation of the second of two peaks and freeze-drying yielded 137 mg (44 % yield) of a fluffy colourless powder. It had h.p.l.c. (gradient 2) $t = 4.78$ min (98 %) and n.m.r. δ (p.p.m.) 1.16 [3H, d, $J = 7$ Hz, CH₃(Ala)], 1.31 (18H, s, 3Boc), 1.3–1.8 (10H, m, 5CH₂), 2.04, 2.50, 2.58 [9H, 3s, 3CH₃(Mtr)], 2.80 (3H, s, CH₃-S⁺), 2.86 [5H, s + m, CH₃-S⁺ + CH₂(Lys)], 3.04 (2H, m, CH₂(Arg)], 3.79 [3H, s, OCH₃(Mtr)], 3.98, 4.10, 4.20 (3H, 3m, 3H- α), 4.66, 4.81 (ab system, 2H, $J = 15$ Hz, CH₂-S⁺), 6.5 [2H, br m,

2NH(guanidino)], 6.66 (1H, s, 1H(Mtr)], 6.75 [1H, m, 1HN(Lys)], 7.00, 7.95 and 8.66 (3H, 3d, $J = 6$ Hz, 3NH).

3-(Alanyl-lysyl-amido)-6-guanidino-2-oxohexyl-dimethylsulphonium trifluoroacetate trihydrotrifluoroacetate [Ala-Lys-Arg-CH₂S⁺(CH₃)₂CF₃CO₂⁻, 3CF₃CO₂H (VIb)]. A solution of Boc-Ala-Lys(Boc)-Arg(Mtr)-CH₂S⁺(CH₃)₂ (Vb) (20 mg, 0.021 mmol) in trifluoroacetic acid (0.5 ml) was prepared in a vial. When the gas evolution ceased the vial was stoppered and kept at room temperature overnight. The solution was concentrated by a stream of N₂ and the residue was purified by preparative h.p.l.c. [0.1 % (v/v) trifluoroacetic acid in water for 4 min, then linear gradient mixture of this solvent and acetonitrile, from 0 % acetonitrile to 100 % acetonitrile within 15 min, at a flow rate of 3 ml/min]. Fractions (1.5 ml) were collected; 1 drop of every fraction was added to 0.1 M-Tris/HCl buffer, pH 8.8 (1 ml), and the u.v.-absorption spectrum was measured. All fractions showing a maximum at 253 nm were collected and freeze-dried to give 15.6 mg (84 % yield) of a colourless very hygroscopic solid. It had u.v. (0.05 M-Tris/HCl buffer, pH 8.8) 253 nm ($\epsilon = 10400$ M⁻¹·cm⁻¹), n.m.r. δ (p.p.m.) 1.32 [3H, d, $J = 7$ Hz, CH₃(Ala)], 1.25–1.9 (10H, m, 5CH₂), 2.78 [2H, m, CH₂(Lys)], 2.84, 2.98 (6H, 2s, 2CH₃-S⁺), 3.13 [2H, m, CH₂(Arg)], 3.9, 4.3 (3H, 2m, 3H- α), 4.81 (2H, s, CH₂-S⁺), 7.0–7.4 [5H, m, 5NH(guanidino)], 7.82, 8.21 (6H, 2m, 2NH₃⁺), 8.65, 8.85 and (2H, 2d, $J = 6$ Hz, 2NH), and m.s. (f.a.b.⁺) 660 (M -CF₃CO₂⁻-CF₃CO₂H), 546 (M -CF₃CO₂⁻-2CF₃CO₂H), and 432 (M -CF₃CO₂⁻-3CF₃CO₂H), (f.a.b.⁻) 886 (M -H), 772 (M -H-CF₃CO₂H), 711 [M -H-CF₃CO₂H-(CH₃)₂S], 658 (M -H-2CF₃CO₂H), 597 [M -H-2CF₃CO₂H-(CH₃)₂S], 544 (M -H-3CF₃CO₂H) and 483 [M -H-3CF₃CO₂H-(CH₃)₂S].

3-(Alanyl-arginyl-amido)-7-amino-2-oxoheptyl-dimethylsulphonium trifluoroacetate trihydrotrifluoroacetate [Ala-Arg-Lys-CH₂S⁺(CH₃)₂CF₃CO₂⁻, 3CF₃CO₂H (VIc)]. The same procedures as for compound (VIb) were used to give a colourless hygroscopic material. It had u.v. (0.05 M-Tris/HCl buffer, pH 8.8) 254 nm ($\epsilon = 9700$ M⁻¹·cm⁻¹), and n.m.r. δ (p.p.m.) 1.38 [3H, d, $J = 7$ Hz, CH₃(Ala)], 1.3–1.8 (10H, m, 5CH₂), 2.75 (2H, m, CH₂(Lys)], 2.87, 2.92 (6H, 2s, 2CH₃-S⁺), 3.13 [2H, m, CH₂(Arg)], 3.88, 4.30 (3H, 2m, 3H- α), 4.83 (2H, s, CH₂-S⁺), 7.0–7.3 [5H, br m, 5NH(guanidino)], 7.80, 8.13 (6H, 2m, 2NH₃⁺), 8.67 and 8.77 (2H, 2d, 2NH).

3-(Alanyl-lysyl-amino)-7-amino-2-oxoheptyl-dimethylsulphonium trifluoroacetate trihydrotrifluoroacetate [Ala-Lys-Lys-CH₂S⁺(CH₃)₂CF₃CO₂⁻, 3CF₃CO₂H (VIId)]. Boc-Ala-Lys(Boc)-Lys(Boc)-CH₂S⁺(CH₃)₂ (Vd) (15 mg, 0.018 mmol), prepared by the same procedures as described for compound (VIb), was dissolved in trifluoroacetic acid (1 ml). After 10 min the solvent was evaporated and the residue was submitted to preparative h.p.l.c. as described for compound (VIb) to give a glassy solid (yield 12.3 mg, 80 %). It had u.v. (0.05 M-Tris/HCl buffer, pH 8.8) 254 nm ($\epsilon = 8800$ M⁻¹·cm⁻¹), n.m.r. δ (p.p.m.) 1.33 [3H, d, $J = 7$ Hz, CH₃(Ala)], 1.3–1.8 (12H, m, 12H, 6CH₂), 2.77 [4H, m, 2CH₂(Lys)], 2.85, 2.90 (6H, 2s, 2CH₃-S⁺), 3.92, 4.28 (3H, 2m, 3H- α), 4.84 (2H, s, CH₂-S⁺), 7.89, 8.21 (9H, 2m, 3NH₃⁺), 8.66 and 8.88 (2H, 2d, $J = 6$ Hz, 2NH), and m.s. (f.a.b.⁺) 859 (M), 745

($M - CF_3CO_2H$), 632 ($M - CF_3CO_2H - CF_3CO_2^-$), 518 ($M - 2CF_3CO_2H - CF_3CO_2^-$) and 404 ($M - 3CF_3CO_2H - CF_3CO_2^-$), (f.a.b.⁺) 744 ($M - H - CF_3CO_2H$), 630 ($M - H - 2CF_3CO_2H$), 516 ($M - H - 3CF_3CO_2H$) and 402 ($M - H - 4CF_3CO_2H$).

t-Butoxycarbonyl-alanyl-*N*^ω-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-*N*^ω-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginyl-chloromethane [Boc-Ala-Arg(Mtr)-Arg(Mtr)-CH₂Cl (IIIe)]. A solution of Boc-Ala-Arg(Mtr)-Arg(Mtr)-CHN₂ (IIe) (756 mg, 0.80 mmol), prepared as described for compound (IIb), in dichloromethane (5 ml) was added dropwise to ice-cold 1 M-HCl in ethyl acetate (10 ml) during 3 min. The mixture was stirred for another 2 min. Water (50 ml) and dichloromethane (50 ml) were added. Usual work-up and preparative chromatography on silica gel with methanol/dichloromethane (1:20, v/v) gave 335 mg (44 % yield) of a colourless oil. It had h.p.l.c. (gradient 2) $t = 5.87$ min (82 %).

3-[t-Butoxycarbonyl-alanyl-*N*^ω-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-arginylamido]-6-[3-(4-methoxy-2,3,6-trimethylphenylsulphonyl)-guanidino-2-oxohexyl-dimethylsulphonium trifluoroacetate [Boc-Ala-Arg(Mtr)-Arg(Mtr)-CH₂S⁺(CH₃)₂CF₃CO₂⁻ (Ve)]. Boc-Ala-Arg(Mtr)-Arg(Mtr)-CH₂SCH₃ (IVe) (100 mg, 0.103 mmol), prepared as described for compound (IVb), was mixed with 1 M-methyl iodide in nitromethane (0.11 ml) and 0.47 M-AgBF₄ in nitromethane (0.23 ml; excess reagent must be avoided) and kept overnight in the dark. Work-up as described for compound (Vb) gave 21 mg (21 % yield) of starting material and 41 mg (36 % yield) of a fluffy colourless powder. It had h.p.l.c. (gradient 2) $t = 5.11$ min (90 %).

3-(Alanyl-arginylamido)-6-guanidino-2-oxohexyl-dimethylsulphonium trifluoroacetate trihydrotrifluoroacetate [Ala-Arg-Arg-CH₂S⁺(CH₃)₂CF₃CO₂⁻, 3CF₃CO₂H (Vle)]. The same procedure as described for compound (VIb) was used and gave a colourless hygroscopic solid (yield 58 %). It had u.v. (0.05 M-Tris/HCl buffer, pH 8.8) 254 nm ($\epsilon = 9600 \text{ M}^{-1} \cdot \text{cm}^{-1}$), n.m.r. δ (p.p.m.) 1.31 [3H, d, $J = 7$ Hz, CH₃(Ala)], 1.4–1.9 (8H, m, 8H, 4CH₂), 2.83, 2.88 (6H, 2s, 2CH₃-S⁺), 3.13 [4H, m, 2CH₂(Arg)], 3.90, 4.30 (3H, 2m, 3H- α), 4.79 (2H, s, CH₂-S⁺), 7.0–7.4, 7.8, 8.1 (13H, 3m, 2guanidino + NH₃⁺), 8.67 and 8.84 (2H, 2d, $J = 6$ Hz, 2NH), and m.s. (f.a.b.⁺) 710 ($M + Na - 2CF_3CO_2H$), 688 ($M + H - 2CF_3CO_2H$), 596 ($M + Na - 3CF_3CO_2H$), 574 ($M + H - 3CF_3CO_2H$), 482 ($M + Na - 4CF_3CO_2H$) and 460 ($M + H - 4CF_3CO_2H$), (f.a.b.⁻) 800 ($M - H - CF_3CO_2H$), 686 ($M - H - 2CF_3CO_2H$) and 572 ($M - H - 3CF_3CO_2H$).

Enzyme assays

Plasma kallikrein and tissue kallikrein were assayed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl and 0.1 % poly(ethylene glycol) (M_r 6000). The release of *p*-nitroaniline that resulted from the hydrolysis of Bz-Pro-Phe-Arg-NH-Np (plasma kallikrein) or of D-Val-Leu-Arg-NH-Np (tissue kallikrein) was monitored by measuring the increase in absorbance at 405 nm (Stone & Höfsteenge, 1985). Clostripain was assayed at

37 °C in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.05 M-CaCl₂, 0.1 % poly(ethylene glycol) (M_r 6000) and 5.0 mM-dithiothreitol. Before assay, clostripain was pre-incubated in the above buffer solution for 1 h at 37 °C. The release of *p*-nitroaniline from Bz-Pro-Phe-Arg-NH-Np was monitored as described above. The K_m for this substrate with clostripain was found to be $248 \pm 19 \mu\text{M}$. Cathepsin B was assayed at 37 °C in 0.1 M-EDTA with D-Phe-Pro-Arg-NH-Np as substrate. The reaction was monitored at 405 nm as described above. Kinetic constants for the inactivation of the enzyme in the presence of the substrate were determined as described by Stone & Hofsteenge (1985) and recently applied to these enzymes by Zumbrunn *et al.* (1988). Trypsin was assayed fluorimetrically in 0.05 M-Tris/HCl buffer, pH 7.5, containing 1 mM-Ca²⁺ and 0.1 M-NaCl with 20 μM -Cbz-Phe-Arg-NH-Mec.

RESULTS AND DISCUSSION

The synthesis of peptidylmethylsulphonium salts with consecutive cationic residues was readily achieved with the use of Mtr- and Boc- as the protecting group for arginine and lysine side chains respectively. These methods should be readily extendable to elongated derivatives in which other useful features may be introduced. The approach to the sulphonium function by way of the thiomethyl ether is an alternative to the direct reaction of the chloromethanes with dimethyl sulphide (Shaw, 1988), but has the advantage of making available the thioether, which is of interest as a potential competitive inhibitor of processing enzymes as well as providing a branching point in syntheses leading to alternative sulphonium salts. About 10 % racemization occurred during synthesis, estimated from n.m.r. spectra. Since a ketone is much more susceptible to enolization than an amide, it is probably the modified C-terminal amino acid that racemizes, leading to contamination of the products with their LLD- or LD- isomers. Strongly alkaline conditions had to be avoided in order to prevent complete racemization. The final products were stable for several months at -20 °C. Dissolved in water or buffers of pH 6–8 they decomposed slowly on standing at room temperature. Decomposition was accelerated by the presence of 1 mM-2-mercaptoethanol (alkylation of thiol groups). Under the conditions tested, however, half-lives were at least several hours, indicating sufficient stability for enzyme inhibition studies.

The properties of the new sulphonium salts with respect to enzyme inhibition were surprising. Among the known serine proteinases of trypsin specificity implicated in prohormone processing, the kallikreins have a prominent place in current thinking (Bothwell *et al.*, 1979; ole-MoiYoi *et al.*, 1979; Richards *et al.*, 1982; Mason *et al.*, 1983). Consequently, plasma and tissue kallikreins were examined. In view of the fact that Cbz-Phe-Lys-CH₂S⁺(CH₃)₂ irreversibly inactivated plasma kallikrein, although the affinity was not very great ($K_i = 60 \mu\text{M}$) (Raubert *et al.*, 1988), it was our expectation that Bz-Phe-Arg-CH₂S⁺(CH₃)₂ would be at least as effective, since this enzyme has a characteristically greater affinity for arginine over lysine in P₁; for example, K_i for Ala-Phe-Arg-CH₂Cl is 0.08 μM compared with 4.9 μM for the lysine analogue (Kettner & Shaw, 1978). The inertness of Bz-Phe-Arg-CH₂S⁺(CH₃)₂ at 0.1 M towards both plasma

Table 1. Characteristics of the inactivation of clostripain and cathepsin B by sulphonium salts containing cationic residues, $RCH_2S^+(CH_3)_2$

Clostripain			
R	$10^3 \times k_1$ (s ⁻¹)	K_i (nM)	k_1/K_i ($\mu M^{-1} \cdot s^{-1}$)
Ala-Lys-Lys	31 ± 8	6.0 ± 2.0	5.27 ± 0.32
Ala-Lys-Arg*	36 ± 12	23 ± 11	1.54 ± 0.08
Ala-Arg-Lys*	24 ± 2	32 ± 4	0.77 ± 0.02
Ala-Arg-Arg*	—	—	0.55 ± 0.02†
Bz-Phe-Arg	58 ± 20	45 ± 17	1.30 ± 0.07
Cathepsin B			
R	$10^3 \times k_1$ (s ⁻¹)	K_i (μM)	k_1/K_i (mM ⁻¹ · s ⁻¹)
Ala-Lys-Lys	—	—	0.050 ± 0.002†
Ala-Lys-Arg	—	—	0.069 ± 0.001†
Ala-Arg-Arg	2.42 ± 0.20	96 ± 13	0.025 ± 0.001
Ala-Arg-Lys	4.5 ± 1.0	86 ± 28	0.052 ± 0.006

* Weighted means of two independent determinations.
† Formation of an EI complex was not kinetically observed.

and pancreatic kallikreins was therefore unexpected. Trypsin was also not inhibited by this reagent. In fact, the kallikreins were similarly unaffected by all of the sulphonium salts containing two cationic residues that were examined at 0.1 mM. These inhibitors are therefore not suitable for investigating the possible role of kallikreins in prohormone processing. Conceivably, the sulphonium salts with two cationic residues may have been proteolytically cleaved between the cationic residues by the kallikreins, thus destroying the ability of the reagents to function as affinity labels. Some support for this possibility is found in the observations (Prado *et al.*, 1986) that blocked tetrapeptides containing the Arg-Arg and Lys-Arg sequences may be hydrolysed by the kallikreins between the basic residues. In the case of the -Lys-Arg-derivative examined there was also hydrolysis at the arginine carboxy group in -Lys-Arg-. Enzymic destruction of these sulphonium salts with two cationic residues by the kallikreins has not yet been examined.

The susceptibility of the cysteine proteinases was examined with cathepsin B and clostripain. Clostripain was found to be readily inactivated by all the sulphonium salts studied with affinity for the reagents in the 0.01 M region. There was relatively small variation with reagent structure (Table 1). The contents refer to the standard process:

in which an intermediate complex is reversibly formed followed by covalent bond formation leading to E-I*. Cathepsin B was also inactivated, but at a lower rate (Table 1). In the two cases where complex-formation was detectable, both the affinity of the reagent for the enzyme and the rate of covalent bond formation were found to be less than for clostripain. Clostripain is a bacterial

cysteine proteinase with a trypsin-like specificity (Porter *et al.*, 1971). Cathepsin B, on the other hand, although showing affinity for small-molecule arginine-containing substrates and inhibitors, in the case of proteins appears to be positioned by hydrophobic side chains (Barrett & Kirschke, 1981). The amino acid sequence of clostripain (Gilles *et al.*, 1983) is not homologous with the cathepsin cysteine proteinase family. Because of its greater specificity and the relevance of this specificity, it represents for the current work an available model of a cysteine proteinase functioning in prohormone processing. The reagents described in this paper should be useful for the study of such as yet uncharacterized cysteine proteinases, and in such studies the lack of action on trypsin-like serine proteinases should be an asset. Initial observations (C. J. Rhodes, A. Zumbrunn, H. W. Davidson, E. Shaw & J. C. Hutton, unpublished work) on proinsulin processing suggest that this will be the case.

REFERENCES

- Achstetter, T. & Wolf, D. H. (1985) *EMBO J.* **4**, 173–177
 Andrews, P. C., Brayton, K. & Dixon, J. E. (1987) *Experientia* **43**, 784–790
 Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561
 Bothwell, M. A., Wilson, W. H. & Shooter, E. M. (1979) *J. Biol. Chem.* **254**, 7287–7294
 Davidson, H. W., Peshavaria, M. & Hutton, J. C. (1987) *Biochem. J.* **246**, 279–286
 Docherty, K., Carroll, R. & Steiner, D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3245–3249
 Docherty, K., Hutton, J. C. & Steiner, D. F. (1984) *J. Biol. Chem.* **259**, 6041–6044
 Evans, E. & Shaw, E. (1983) *J. Biol. Chem.* **258**, 10227–10232
 Fletcher, D. J., Quigley, J. P., Bauer, G. E. & Noe, B. D. (1981) *J. Cell Biol.* **90**, 312–322
 Fujino, M., Wakimasu, M. & Kitada, C. (1981) *Chem. Pharm. Bull.* **29**, 2825–2831
 Gilles, A.-M., De Wolf, A. & Keil, B. (1983) *Eur. J. Biochem.* **130**, 473–479
 Green, G. & Shaw, E. (1981) *J. Biol. Chem.* **256**, 1923–1928
 Julius, D., Brake, A., Blair, L., Kunisawa, R. & Thorner, J. (1984) *Cell* **37**, 1075–1088
 Kettner, C. & Shaw, E. (1978) *Biochemistry* **17**, 4778–4784
 Kettner, C. & Shaw, E. (1981) *Methods Enzymol.* **80**, 826–841
 Loh, Y. P., Brownstein, M. J. & Gainer, H. (1984) *Annu. Rev. Neurosci.* **7**, 189–222
 Mason, A. J., Evans, B. A., Cox, D. R., Shine, J. & Richards, R. I. (1983) *Nature (London)* **303**, 300–307
 ole-MoiYoi, O., Seldin, D. C., Spragg, J., Pinkus, G. S. & Austen, K. F. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3612–3616
 Porter, W. H., Cunningham, L. W. & Mitchell, W. M. (1971) *J. Biol. Chem.* **246**, 7675–7682
 Prado, E. S., Araujo-Viel, M. S., Juliano, M. A., Juliano, L., Stella, R. C. R. & Sampaio, C. A. M. (1986) *Hoppe-Seyler's Z. Physiol. Chem.* **367**, 199–205
 Rauber, P., Walker, B., Stone, S. & Shaw, E. (1988) *Biochem. J.* **250**, 871–876
 Richards, R. I., Catanzaro, D. F., Mason, A. J., Morris, B., Baxter, J. D. & Shine, J. (1982) *J. Biol. Chem.* **257**, 2758–2761
 Schoellmann, G. & Shaw, E. (1962) *Biochem. Biophys. Res. Commun.* **7**, 36–40
 Shaw, E. (1988) *J. Biol. Chem.* **263**, 2768–2772
 Shaw, E., Mares-Guia, M. & Cohen, W. (1965) *Biochemistry* **4**, 2219–2224

- Stone, S. & Hofsteenge, J. (1985) **230**, 497–502
- Virji, M. A. G., Vassalli, J.-D., Estensen, R. D. & Reich, E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 875–879
- Whitaker, J. R. & Perez-Villasenor, J. (1968) *Arch. Biochem. Biophys.* **124**, 70–78
- Wuensch, E. (1974) *Methoden der Organischen Chemie (Houben-Weyl)*, vol. 15: *Syntheses von Peptiden*, part 1, pp. 46–305, Georg Thieme Verlag, Stuttgart
- Zumbrunn, A., Stone, S. & Shaw, E. (1988) *Biochem. J.* **250**, 621–623

Received 9 February 1988/11 May 1988; accepted 30 May 1988