Mannose–pepstatin conjugates as targeted inhibitors of antigen processing†

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The molecular details of antigen processing, including the identity of the enzymes involved, their intracellular location and their substrate specificity, are still incompletely understood. Selective inhibition of proteolytic antigen processing enzymes such as cathepsins D and E, using small molecular inhibitors such as pepstatin, has proven to be a valuable tool in investigating these pathways. However, pepstatin is poorly soluble in water and has limited access to the antigen processing compartment in antigen presenting cells. We have synthesised mannose–pepstatin conjugates, and neomannosylated BSA–pepstatin conjugates, as tools for the *in vivo* study of the antigen processing pathway. Conjugation to mannose and to neomannosylated BSA substantially improved the solubility of the conjugates relative to pepstatin. The mannose–pepstatin conjugates showed no reduction in inhibition of cathepsin E, whereas the neomannosylated BSA–pepstatin conjugates showed some loss of inhibition, probably due to steric factors. However, a neomannosylated BSA–pepstatin conjugate incorporating a cleavable disulfide linkage between the pepstatin and the BSA showed the best uptake to dendritic cells and the best inhibition of antigen processing.

Introduction

Uptake, proteolytic degradation, and display of foreign antigens by professional antigen-presenting cells such as dendritic cells are essential steps in the immune response to antigens. Understanding how foreign antigens, once internalised, are proteolysed is therefore key to understanding both the normal function of the immune system[1] and the abnormal antigen processing which occurs in autoimmune diseases.[2] One approach to the study of the role of proteolysis in antigen processing and presentation is to use proteolytic inhibitors, such as pepstatin, to block the processing enzymes, such as cathepsins D and E.[3] However, there are several major problems with this approach. Aspartic protease inhibitors such as pepstatin are only sparingly soluble in water, and are inefficiently transported across membranes. Moreover, in the absence of any tissue or cell targeting specificity, it is also difficult to carry out reliable *in vivo* studies. We therefore sought to develop a system for the solubilisation, cell-specific delivery and receptor-mediated uptake of pepstatin.

Antigen uptake by dendritic cells and macrophages is mediated by the mannose receptor, a membrane-associated protein bearing eight carbohydrate recognition domains. The mannose receptor binds to mannosylated polysaccharides and glycoproteins and internalises such ligands;[4] mannosylation of peptide and protein antigens has been shown to result in a significant increase in T-cell response.[5] High affinity synthetic ligands for the mannose receptor have recently been developed, based on multivalent cluster mannosides which can simultaneously bind to the multiple carbohydrate recognition domains. In particular, lysine-based cluster mannosides bearing up to six terminal mannose groups, connected to the lysine residues by flexible spacers, have been studied and shown to have subnanomolar affinity for the mannose receptor.[6] These and similar cluster mannosides have been used to block mannose receptors[7] and for the efficient, cell-specific delivery of antigens,[8] fluorophores[9] and PNA.[10] Mannose-targeted liposomes have also been synthesised for gene[11,12,13] and antigen[14,15] delivery to dendritic cells, and other scaffolds, such as mannosylated cyclodextrins[16] and mannosylated bovine serum albumin[17] have also been used to target the mannose receptor and deliver therapeutic agents to macrophages. In spite of the advantages of such an approach, targeted delivery of pepstatin has seldom been explored,[18,19] and receptor-mediated uptake to macrophages and dendritic cells *via* the mannose receptor has not previously been disclosed.

In this paper we report the design, synthesis and biological characterisation of four mannose–pepstatin bioconjugates, designed for receptor-mediated uptake of pepstatin to dendritic cells. We have studied the enzyme inhibition properties of these bioconjugates and their uptake into dendritic cells, and report their utility as tools for studying the antigen processing pathway.

Results and discussion

Design of mannose–pepstatin conjugates

We considered several factors for the design of the mannose–pepstatin conjugates. Although many studies have indicated the

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† Electronic supplementary information (ESI) available: NOESY spectrum of **7**; ^{1}H and ^{13}C assignments for the acetylated compound, **13**; MALDI-TOF mass spectra of BSA, mannose–BSA conjugate **17**, and **4**; details of the analysis of the number of sugars, and of free amino groups, on mannose–BSA conjugate **17**; ^{1}H NMR spectra of **7–9**, **11**, **13**, **1**, **14–16**, **2**, *N*-(*t*-butyloxycarbonyl), *N'*-(pepstatinyl)cystamine, *N*-(pepstatinyl)cystamine, and **19**. See DOI: 10.1039/b600060f

importance of polyvalent mannose clusters for successful receptor binding and uptake,[20] it is clear that for some receptors a single glycosidic unit is sufficient,[18] and we therefore sought to compare the delivery of pepstatin bearing a single mannose unit to pepstatin conjugated to a polymannose cluster. The optimal spacer length between individual mannoses in mannose clusters, and between the cluster mannoside and the antigen or inhibitor being delivered, has not been determined for all systems: however, studies of the interaction of mannosylated liposomes with human phagocytes indicated that longer spacer lengths such as PEG 6, PEG 8 led to good uptake by the mannose receptor.[11] It had previously been reported that the conjugation of pepstatin to PEG, whilst increasing the solubility, seriously decreased the aspartic protease inhibition, probably due to steric hinderance between the inhibitor and the conjugated PEG.[21] In order to reduce the possibility of steric hinderance between the polymannose cluster and the proteolytic enzymes under study, we also designed probes where the pepstatin and mannose units were conjugated *via* a disulfide bond. Recent studies have shown that specific redox enzymes, as well as reducing agents, are prevalent in endosomes and lysosomes, and that therefore targeted drug delivery strategies can be enhanced by the use of cleavable disulfide linkages between the receptor targeting moiety and the drug[22] allowing the drug to be released from the targeting moiety by reductive cleavage in the endosome. We hypothesised that mannose–pepstatin conjugates with a disulfide bond in the linker could also be internalised *via* receptor-mediated endocytosis and would subsequently be cleaved in the endosome to release the pepstatin.

We therefore designed mannose–pepstatin conjugates **1–4** (Fig. 1). Conjugates **1** and **2** bear a single mannose unit, in contrast to **3** and **4**, in which the pepstatin is attached to neomannosylated bovine serum albumin (BSA). In addition, conjugates **2** and **4** have a cleavable disulfide bond between the pepstatin and the targeting moiety, whereas **1** and **3** are not cleavable. We envisaged that these four conjugates would enable us to explore the effects of polyvalent and cleavable mannose targeting moieties on pepstatin delivery.

Synthesis of mannose–pepstatin conjugates 1 and 2

A convergent route to the monomannosylated conjugates **1** and **2** was used that would allow incorporation of either a cleavable or a non-cleavable linker at a late stage. The mannose was attached to the linker *via* a flexible spacer group using standard methods, as follows. Mannose was converted to the known 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl trichloroimidate **5**[23] and was reacted with benzyl 4-hydroxybutanoate **6**[24] to afford **7** (Scheme 1). The benzyl ester was then deprotected to give **8** and converted to the *N*-hydroxysuccinimide **9**.

For the synthesis of mannose–pepstatin conjugate **1**, **9** was then reacted with the non-cleavable linker *N*-(*t*-butyloxycarbonyl) propane-1,3-diamine[18] **10** to give **11** (Scheme 2). Deprotection with TFA was followed by coupling to pepstatin-*N*-hydroxysuccinimide

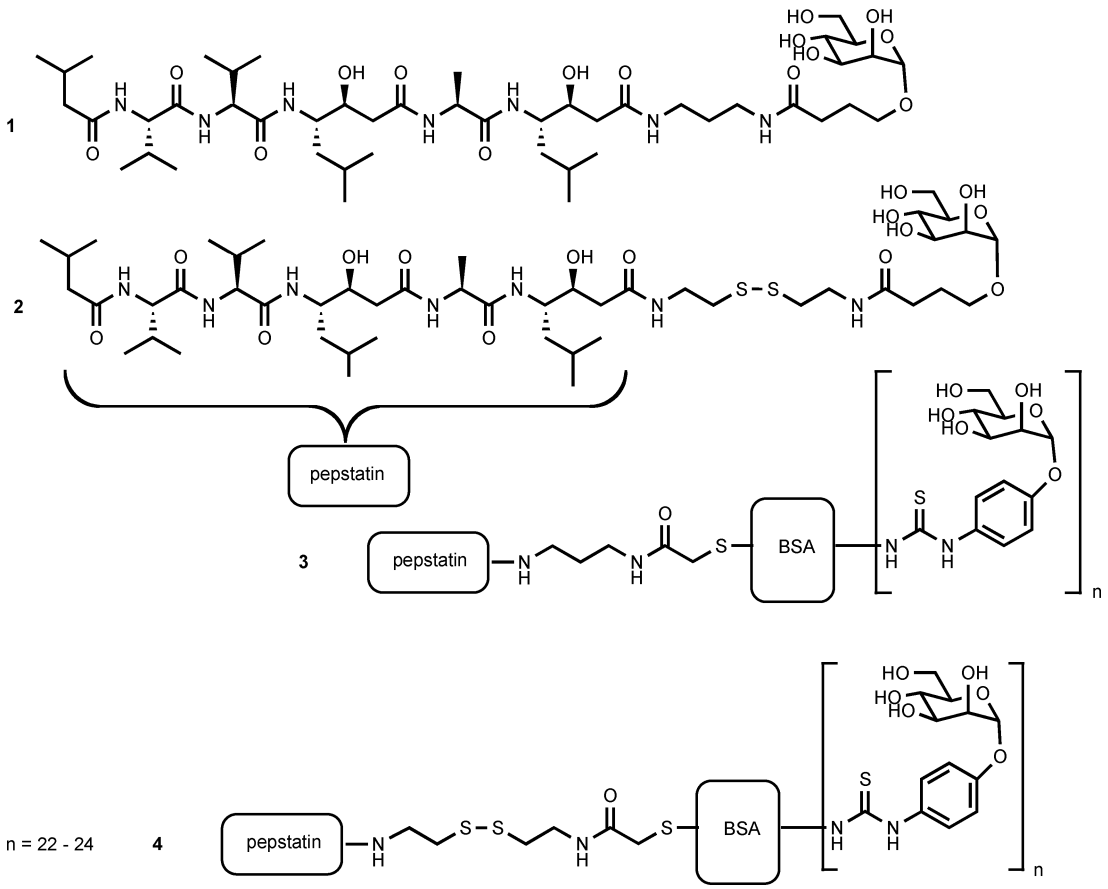
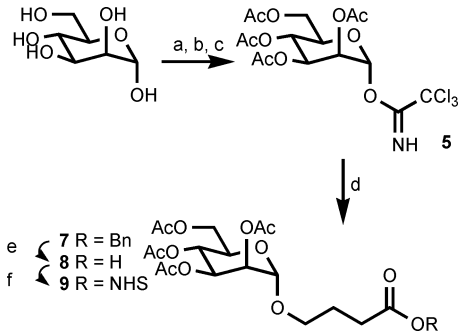


Fig. 1 Structure of the mannose–pepstatin conjugates **1** (non-cleavable) and **2** (cleavable) and of the neomannosylated BSA–pepstatin conjugates **3** (non-cleavable) and **4** (cleavable).



Scheme 1 Synthesis of mannose-linker **9**: a) Ac_2O, Et_3N (99%); b) H_2NNH_2.AcOH, DMF (77%); c) K_2CO_3, Cl_3CCN, CH_2Cl_2 (96%); d) **6**, BF_3OEt_2 (55%); e) H_2, Pd/C (85%); f) EDCI, NHS (quant.).

12 to give **13**. Removal of the acetate groups then afforded **1**. Similarly, **9** was reacted with the cleavable linker *N*-(*t*-butoxycarbonyl) cystamine **14** to give **15**; deprotection with TFA and coupling to pepstatin-*N*-hydroxysuccinimide **12** gave **16**, which was likewise deprotected to afford **2**.

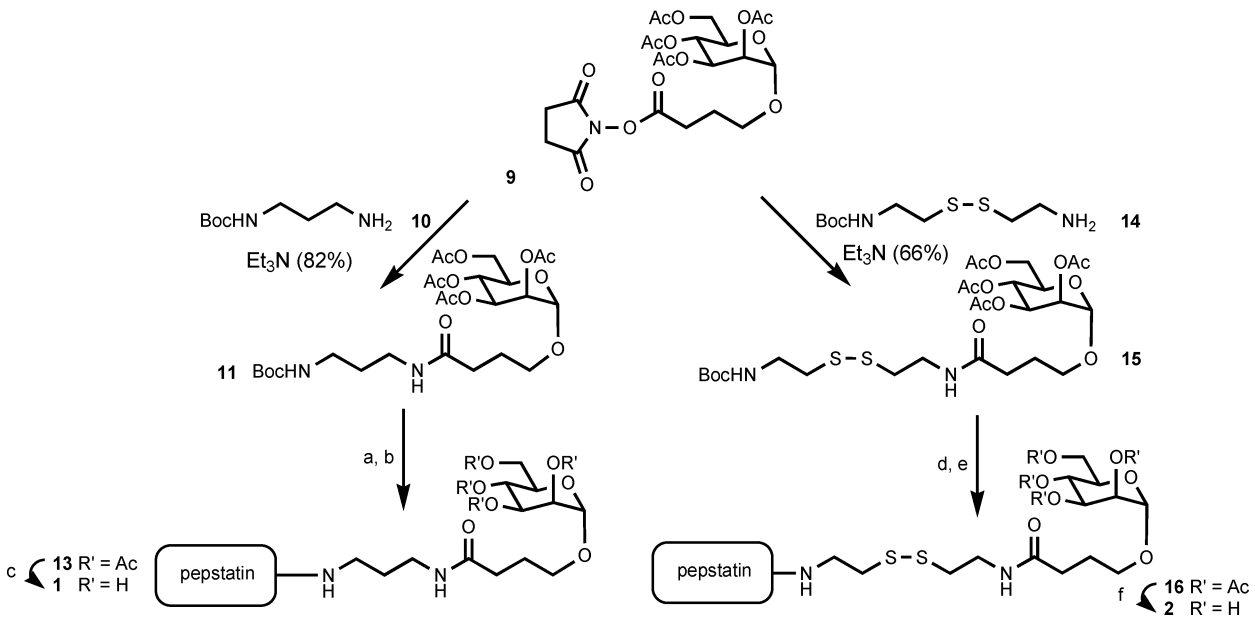
Synthesis of neomannosylated BSA–pepstatin conjugates 3 and 4

BSA is an inexpensive and readily available protein, soluble in many different aqueous buffers,[25] with a large number of Lys residues and a single Cys residue that is not involved in a disulfide bridge. We planned to use this inherent orthogonality of functional groups to form the desired conjugates **3** and **4**. It was envisaged that the mannose moieties could be selectively conjugated to the Lys-NH_2 groups, leaving the Cys-SH available for chemoselective coupling to a suitable linker–pepstatin moiety. As we wished to avoid a final deacetylation step in the synthesis of the neomannosylated BSA–pepstatin conjugates, it was also necessary to alter the strategy for mannosylation of BSA. Conjugation of oligovalent amines with unprotected NCS-functionalised mannose derivatives to give thiourea-bridged glycodendrimers has previously been reported,[26] and we therefore elected to use this approach in our synthesis. Thus, 4-isothiocyanatophenyl α-D-mannopyranose was reacted with BSA in 0.15 M NaCl[27] to give mannose–BSA conjugates **17** (Scheme 3). Three methods were used to determine the average amount of conjugation of isothiocyanomannosyl sugar derivatives: mass spectroscopy (MALDI-TOF); biochemical analysis for total mannose content;[28] and analysis of free amino groups.[29] These methods indicated that the mannose–BSA conjugates **17** had, on average, between 22 and 24 mannosyl units attached.

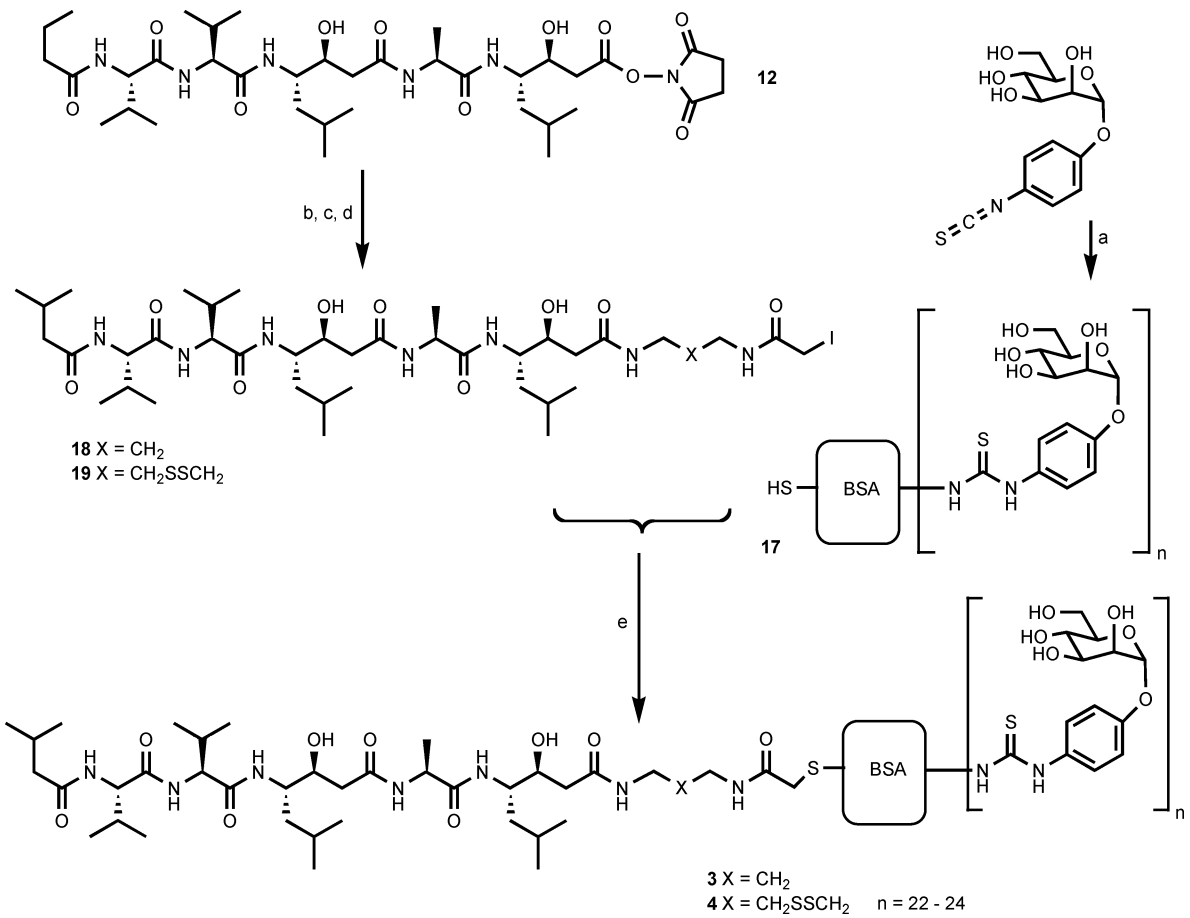
In order to chemoselectively attach a pepstatin–non-cleavable linker moiety, pepstatin-*N*-hydroxysuccinimide **12** was reacted with *N*-(*t*-butyloxycarbonyl) propane-1,3-diamine **10**, the Boc group removed and the resulting amine conjugated to iodoacetic anhydride to give the iodoacetamide **18**. Similarly, pepstatin-*N*-hydroxysuccinimide **12** was reacted with *N*-(*t*-butyloxycarbonyl) cystamine **14**, the Boc group removed and the resulting amine conjugated to iodoacetic anhydride to give the iodoacetamide **19**. The inherent insolubility of pepstatin in many solvents ensured that **18** and **19**, and the intermediates in their synthesis, could be readily purified prior to coupling with the mannose–BSA conjugate. Thus, reaction of **18** with **17** gave the non-cleavable neomannosylated BSA–pepstatin conjugate **3** directly, and reaction of **19** with **17** afforded the cleavable neomannosylated BSA–pepstatin conjugate **4**.

Biochemical evaluation of the mannose–pepstatin and neomannosylated BSA–pepstatin conjugates

It was evident during synthesis that both monomannosylated conjugates **1** and **2** and the neomannosylated BSA–pepstatin conjugates **3** and **4** had much improved solubility compared to pepstatin A alone. The monomannosylated conjugates **1** and **2** had excellent solubility in organic solvents, especially methanol, and



Scheme 2 Synthesis of mannose–pepstatin conjugates **1** and **2**; a) TFA; b) pepstatin *N*-hydroxysuccinimide **12**, Et_3N (45% over 2 steps); c) LiOH, MeOH (70%); d) TFA; e) pepstatin *N*-hydroxysuccinimide **12**, Et_3N (55% over 2 steps); f) LiOH, MeOH (66%).



Scheme 3 Synthesis of neomannosylated BSA–pepstatin conjugates **3** and **4**; a) BSA, 0.15 M NaCl (74%); b) **10** or **14**, DMF; c) TFA, Et_3SiH, thioanisole, phenol; d) iodoacetic anhydride (**18**: 40% over 3 steps: **19**: 42% over 3 steps); e) PBS, DMF (**3**: 73%: **4**: 70%).

their solubility in water was much higher than that of pepstatin. At high concentrations in water (>0.5 mM) a slight cloudiness was visible, but at the lower concentrations used in biological assays (<54 μM, see below) the compounds appeared to be completely water soluble. The neomannosylated BSA–pepstatin conjugates were highly soluble in water (greater than 1 mM).

The inhibitory activity of the conjugates was tested against cathepsin E using a fluorogenic assay.[30] Fig. 2 shows representative plots of this data for monomannosylated conjugate **2** and neomannosylated BSA–pepstatin conjugate **4**. Similar data were obtained for conjugates **1** and **3** (not shown). The best fit curve for the data allowed estimates for IC_{50} values. IC_{50} for monomannosylated conjugates **1** and **2** are in the picomolar range (conjugate **1** IC_{50} = 133 ± 23 pM, $n = 3$; conjugate **2** IC_{50} = 86 ± 47 pM, $n = 4$) similar to that of pepstatin (not shown). IC_{50} for neomannosylated BSA–pepstatin conjugate **3** and **4** are in the low nanomolar range (conjugate **3** $IC_{50} = 1.3 \pm 0.4$ nM, $n = 3$; conjugate **4** $IC_{50} = 3.5 \pm 0.4$ nM, $n = 3$. In contrast, the neomannosylated BSA conjugate **17** without the addition of pepstatin had little inhibitory activity ($IC_{50} > 1$ μM).

Attachment of a small linker and sugar unit does not therefore cause any significant reduction in the inhibitory effects of pepstatin A, while attachment of the larger neomannosylated BSA causes a reduction in IC_{50} of approximately 10 fold, perhaps because the large carrier reduces the accessibility of the pepstatin.

Effect of the mannose–pepstatin and neomannosylated BSA–pepstatin conjugates on antigen processing in A20 cells and dendritic cells

In order to test the activity of the pepstatin conjugates on antigen processing, we made use of a well-established assay[31,32] in which ovalbumin is processed and presented to an antigen-specific T cell hybridoma, DO11-10, which recognizes ovalbumin amino acids 323–339. Co-culture of antigen presenting cells, antigen and the T cell hybridoma DO11-10 induces the release of the cytokine interleukin 2 (IL-2). We explored the activity of the inhibitors using two types of antigen presenting cell types, A20 a B cell lymphoma which does not express the mannose receptor[33] but is known to use the aspartic protease cathepsin E for processing, or mouse bone marrow-derived dendritic cells which do express mannose receptor(s).[33,34]

Pepstatin inhibited processing of ovalbumin by A20 cells as described previously,[31] and did not inhibit presentation of the "preprocessed" ovalbumin peptide 323–339 (Fig. 3, lower panels), demonstrating that inhibition was at the level of processing and not presentation, or T cell activation. High concentrations of inhibitor were required (around 10^5 higher than the IC_{50} measured in enzymatic assays), and some toxicity (reflected in the inhibition of ovalbumin peptide presentation (lower right panel) was observed at the highest concentration.

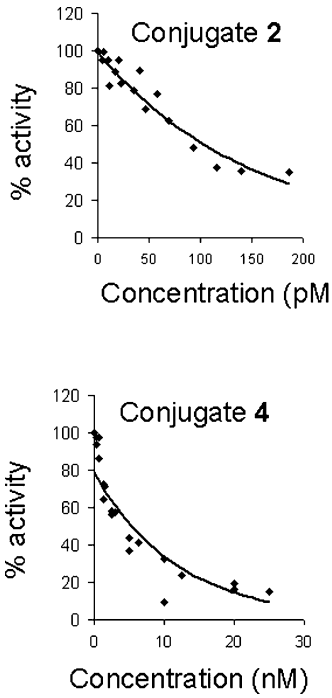


Fig. 2 Inhibitory activity of mannose–pepstatin and neomannosylated BSA–pepstatin conjugates against cathepsin E. Inhibitors were incubated at the concentrations shown in the presence of fluorogenic peptide substrate (5 μM) and purified rat cathepsin E (50 ng ml^{-1}) as described in Experimental procedures. Results for each inhibitor are representative of at least three experiments and expressed as % activity relative to the activity in the absence of inhibitor.

Preliminary experiments showed that although the monomannosylated conjugates were soluble in water at the μM concentrations used, they precipitated out of solution in the cell culture media required for these assays. Addition of DMSO to the stock solution (to a final concentration of $\frac{1}{3}$ that required for pepstatin A) restored solubility. The monomannosylated conjugates **1** and **2** also inhibited processing of ovalbumin, showing a dose response rather similar to pepstatin. No toxicity or inhibition of peptide presentation was observed at any concentration tested, perhaps because the solutions contained only $\frac{1}{3}$ the concentration of DMSO as solvent. These conjugates therefore both retain the inhibitory activity of pepstatin, albeit with improved solubility properties.

In contrast, neither neomannosylated conjugates **3** or **4** showed consistent inhibition of ovalbumin processing/presentation by A20 cells (Fig. 4). Although some slight inhibition was observed with conjugate **4** this did not exceed that shown by the mannose–BSA conjugate **17** which did not carry pepstatin, and was presumably due to non-specific effects. For reasons which remained

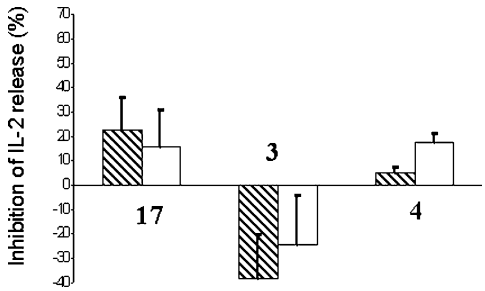


Fig. 4 Antigen processing by A20 cells in the presence of neomannosylated BSA–pepstatin conjugates 3 and 4, or the mannose–BSA conjugate 17. The results are expressed as % inhibition of the IL-2 responses in the presence of 54 μM inhibitor. The figure shows the average of four independent experiments. Other experimental details as for Fig 3.

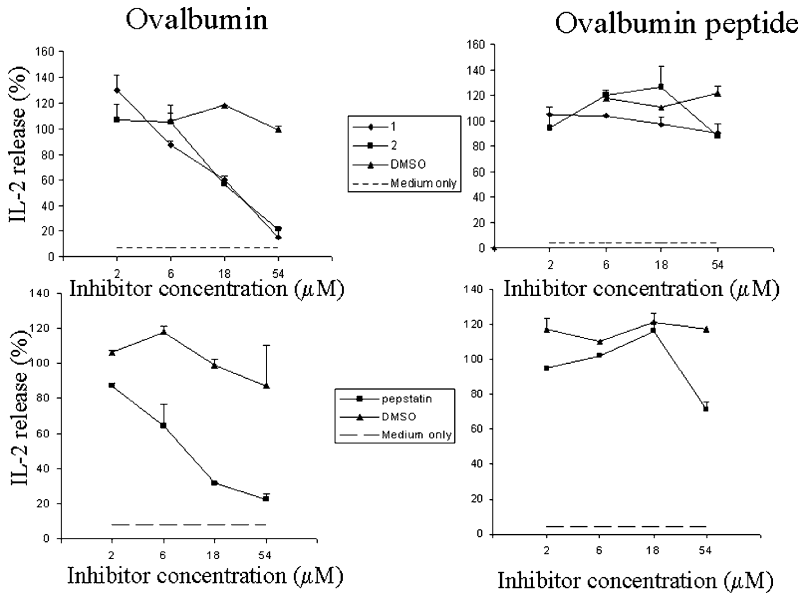


Fig. 3 Antigen processing by A20 cells in the presence of mannose–pepstatin conjugates 1 and 2, or pepstatin. A20 cells and DO11-10 T cells were co-cultured in the presence of ovalbumin (left panels, 3 mg ml^{-1}) or ovalbumin peptide 323–339 (right panels, 0.1 μg ml^{-1}) and inhibitor or an equivalent concentration of DMSO in culture medium. IL-2 was collected and measured by ELISA as described in Experimental procedures and results are expressed as % of IL-2 in the presence of antigen but no inhibitor (corresponds to approximately 1 ng ml^{-1} IL-2). Dotted line shows IL-2 levels in absence of antigen or inhibitor.

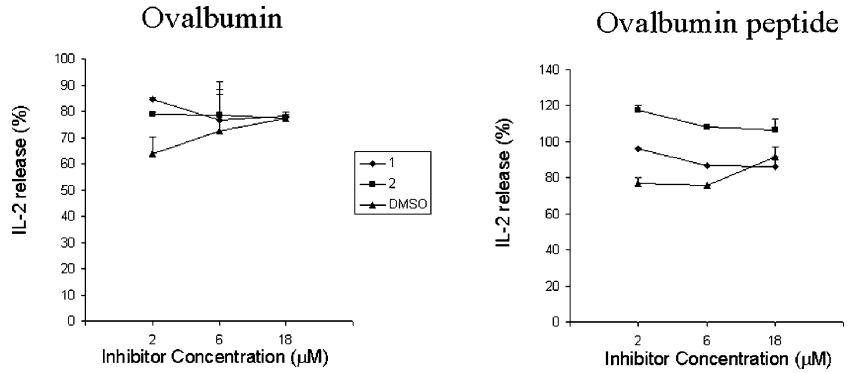


Fig. 5 Antigen processing by dendritic cells in the presence of mannose–pepstatin conjugates 1 and 2. Dendritic cells and DO11-10 T cells were co-cultured in the presence of ovalbumin (left panel, 3 mg ml^{-1}) or ovalbumin peptide 323–339 (right panel, 0.3 μg ml^{-1}) and inhibitor or an equivalent concentration of DMSO in culture medium. IL-2 was collected and measured by CTLL bioassay as described in Experimental procedures and results are expressed as % of IL-2 in the presence of antigen but no inhibitor (corresponds to approximately 1 ng ml^{-1} IL-2). IL-2 levels in absence of antigen were less than 10%. The result shows one representative of four experiments.

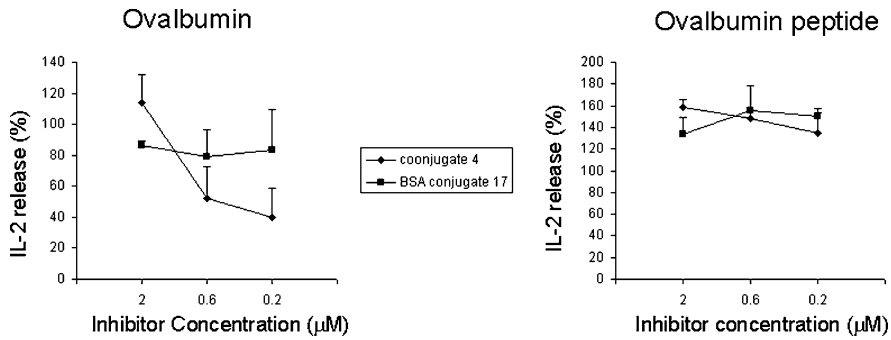


Fig. 6 Antigen processing by dendritic cells in the presence of neomannosylated BSA–pepstatin conjugates 4, or the mannose–BSA conjugate 17. Dendritic cells and DO11-10 T cells were co-cultured in the presence of ovalbumin (left panel, 0.5 mg ml^{-1}) or ovalbumin peptide 323–339 (right panel, 0.05 μg ml^{-1}) and inhibitor in culture medium. IL-2 was collected and measured by CTLL bioassay as described in Experimental procedures, and results are expressed as % of IL-2 in the presence of antigen but no inhibitor (corresponds to approximately 1 ng ml^{-1} IL-2). IL-2 levels in absence of antigen were less than 10%. The result shows one representative of eight experiments.

unclear, conjugate **3** actually appeared to enhance IL-2 release, but this phenomenon was not explored further. The lack of inhibition by the mannosylated BSA–pepstatin conjugates may reflect either the increased IC_{50} of these compounds demonstrated in Fig 2, and/or a decreased ability of these much larger molecules to enter the A20 cells, and reach the target enzyme.

A20 cells, although efficient at processing *via* fluid phase uptake,[35] do not carry the mannose receptor, and cannot therefore take up the mannosylated conjugates *via* receptor mediated uptake. We therefore used dendritic cells, which show extremely efficient receptor mediated uptake *via* the mannose receptor to present ovalbumin to the DO11-10 T cells. Neither pepstatin (not shown) nor the monomannosylated conjugates **1** and **2** inhibited ovalbumin presentation above the level of the DMSO solvent, up to concentrations of 18 μM (Fig. 5). Higher concentrations were toxic to the dendritic cells.

Initial experiments using the neomannosylated-BSA conjugates were carried out at the same concentration ranges. Although conjugates **3** and **4** inhibited processing of ovalbumin at these concentrations, there was no difference between those conjugates carrying pepstatin (*i.e.* **3** and **4**) and the mannose–BSA conjugate **17**. Inhibition was presumed to be due to non-specific effects of the high concentrations of mannose BSA conjugates, and these experiments were therefore not pursued further. However, conjugate **4** showed consistent inhibition of ovalbumin, but not ovalbumin peptide presentation, at concentrations of around 1×10^{-5} μM. A representative experiment is shown in Fig. 6, and an average inhibition of 71% ± 8 was obtained at 2 μM in 8 different dendritic cell preparations. Preliminary experiments suggested that the neomannosylated conjugate **3** (without the cleavable disulfide bond linker) was much less active, suggesting the presence of a cleavable disulfide bond within the linker was an essential feature of the design.

Conclusion

Our objective in synthesising the mannose derivatives of pepstatin was to increase solubility, and at the same time target this inhibitor to the endocytic pathway of antigen presenting cells thus enhancing bio-availability. All the conjugates synthesised showed the expected increase in solubility in aqueous solution, although for the monomannosylated derivatives, this increase was limited, and the compounds still required significant concentrations of DMSO to remain in solution in physiological media, which contain salts and serum proteins. Nevertheless, synthesis of these compounds was useful, since it demonstrated that the synthetic strategy of linking a pepstatin to the mannose *via* a linker did not

interfere with the inhibitory activity of the pepstatin, measured either enzymatically or in the A20 antigen processing bioassay.

The monomannosylated derivatives and pepstatin itself were not able to inhibit processing by dendritic cells. Although the molecular reasons for this are not known, it is likely to reflect the greatly enhanced efficiency of processing/presentation by dendritic cells, which provides a much more stringent test of inhibitors of processing enzymes. Indeed it is notable that few previous studies have published data showing inhibition of antigen processing in dendritic cells by small molecular weight protease inhibitors.[36]

However, inhibition of processing by dendritic cells could be achieved by the use of the neomannosylated BSA–pepstatin conjugate **4**. Since the inhibitory activity of this conjugate against purified enzyme was actually less than free pepstatin (see Fig. 2), this increased bioactivity presumably reflects the much higher avidity of binding and uptake *via* the mannose receptor by the conjugate carrying multiple mannose residues, over that of a monomannosylate.[6,17,20] Once inside the cell, the intracellular environment is likely to favour reduction of the disulfide linker, releasing pepstatin to interact efficiently with its target.

In summary, we have demonstrated that conjugation of pepstatin to single, or multiple, mannose units constitutes a powerful method for solubilising such small molecule inhibitors, for targeting them to the desired cells and facilitating their uptake. Such conjugates constitute important new tools for investigating the antigen processing pathway, *in vitro* and *in vivo.* Inhibition of processing by pepstatin conjugates clearly implicates aspartic proteinases in the antigen processing pathway of dendritic cells. At least two aspartic proteinases, cathepsins D and E, are known to be expressed in the immune system, and we have extended this work, using neomannosylated conjugate **4** in conjunction with genetic approaches, to further dissect which enzyme is actually functionally the most important.[37] Further studies, using fluoresceinated derivatives of the conjugate to track uptake and intracellular distribution, are planned.

Experimental

General experimental procedures

Unless specified, all reagents were purchased from commercial suppliers and were used without further purification. Pepstatin was obtained from Calbiochem and Bachem. BSA was purchased from Sigma-Aldrich. Isothiocyanato-phenyl α-D-mannopyranose is available from Sigma-Aldrich, or was synthesised from 4-nitrophenyl-α-D-mannopyranose (Acros) *via* hydrogenation of the nitro group followed by reaction with $CSCl_2$.[27] ^1H and ^{13}C spectra were recorded on Bruker AC 300, 400 and 500 instruments. The chemical shift data for each signal is given in units of δ relative to tetramethylsilane (TMS) where δ (TMS) = 0. Coupling constants (J) are quoted in Hz. CI and FAB mass spectra were recorded using a VG ZAB SE mass spectrometer. APCI and ES mass spectra were recorded on a Micromass Quattro mass spectrometer. Optical rotation was recorded using an AA-10 automatic polarimeter (Optical Activity Ltd, UK) and a 5 cm length, 1.5 ml cell ($d = 0.5$). Melting points were measured using a Gallenkamp melting point apparatus and are uncorrected. Infra-red (IR) spectroscopy was carried out using a Shimadzu FTIR-8700 Fourier transform infrared spectrometer and analysed with Shimadzu FT IRS software.

Analytical HPLC (flow rate 1 ml min^{-1}) was carried out using a Millipore Waters 600E system controller and pump system, Pye Unicam PU4025 UV detector, Shimadzu C-R6A chromatopac and a Vydac 218TP54 protein & peptide C18 column, 4.6 mm × 25 cm. Preparative HPLC (flow rate 15 ml min^{-1}) was carried out using a Millipore Waters 600E system controller and pump system, Gilson Holochrome UV detector, Millipore Waters 745B data module and a Vydac 218TP1022 protein & peptide C18 column, 21.4 mm × 25 cm. tlc was carried out on pre-coated 0.25 mm thick Merck 60 F_{254} silica plates. Reverse phase silica plates used were Merck RP-8 $F_{254}S$. Silica for chromatography was obtained from BDH.

Dialysis was performed using standard 12 000 molecular weight cut-off (MWCO) dialysis tubing. Before use, the tubing was prepared by first boiling for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0), then rinsing the tubing thoroughly in 1 mM EDTA (pH 8.0), before boiling again for 10 minutes in 1 mM EDTA (pH 8.0). The tubing was allowed to cool before use, and was stored in solution (water or buffer) at 4 °C. Centrifugal filtration devices used were either Millipore centriplus YM-30 (Millipore (UK) Ltd) with a maximum 15 ml capacity and used within a Beckman J2-21 swing bucket centrifuge, or Millipore ultrafree-4 filtration unit (5 ml maximum capacity) using a fixed 35° angle centrifuge (Jouan MR 1812, Jouan S. A., France).

Tetrahydrofuran (THF) was dried by distillation from a suspension of THF with sodium and benzophenone. Dimethyl formamide (DMF) was dried by distillation from a suspension of DMF and calcium hydride. CH_2Cl_2, Et_3N and EtOH were dried over CaH powder and stored over molecular sieves when needed. With all reactions that used dry solvents, an inert atmosphere of N_2 or Ar was used.

(3'-Benzyloxycarbonylpropyl)-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside 7

Benzyl 4-hydroxybutanoate **6**[24] (2.68 g, 5.4 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl trichloroacetimidate **5**[23] (2.11 g, 10.8 mmol, 2 eq.) were added to a flask. Water was azeotropically removed from the mixture with anhydrous toluene (4 × 10 ml). Anhydrous CH_2Cl_2 (30 ml) was added and the mixture stirred vigorously and cooled to −40 °C using an MeCN–$CO_{2\,(S)}$ bath. $BF_3{\cdot}Et_2O$ (1.4 ml, 10.9 mmol, 2 eq.) was added slowly to this reaction mixture over 10 min during which time the reaction mixture turned yellow. The disappearance of the acetimidate **5** was monitored by tlc with maximum conversion to **7** by 4 h. The reaction mixture was washed with ice-cold 50% sodium bicarbonate solution (3 × 60 ml) and then with ice-cold 50% brine solution (3 × 60 ml). The organic layer was dried over $MgSO_4$ and filtered. Removal of the CH_2Cl_2 *in vacuo* gave a yellow oil, which was purified by reverse phase chromatography (gradient: 100% water to 80% MeOH–water: R_f 0.35 (3 : 1 MeOH–water; reverse-phase tlc plates)). The column fractions containing **7** were pooled and the MeOH removed *in vacuo.* The remaining aqueous solution was extracted with CH_2Cl_2 (50 ml). The CH_2Cl_2 layer was dried ($MgSO_4$) and the solvent removed *in vacuo* to give the titled product as a yellow wax (1.57 g, 3.0 mmol, 55%); $[\alpha]_D^{21}$ + 38.0°

(*c* 1, $CHCl_3$); ν_{max} ($CHCl_3$) 3024–3014 (w), 1751–1740 (s), 1454–1429 (w), 1369 (m), 1240 (s) cm^{-1}; ^1H NMR δ_H (500 MHz, $CDCl_3$) 1.94 (2H, m, $CH_2CH_2CH_2O$), 1.96 (3H, s), 2.01 (3H, s), 2.07 (3H, s), 2.13 (3H, s), 2.45 (2H, t, *J* 8.5, $CH_2CH_2CH_2O$), 3.37 (1H, ddd, *J* 5.7, 6.6 and 9.8, $CH_2CH_2CH_2O$), 3.72 (1H, ddd, *J* 5.7, 6.9 and 9.8, $CH_2CH_2CH_2O$), 4.05 (1H, ddd, *J* 2.4, 5.3 and 9.7, H-5), 4.08 (1H, dd, *J* 2.4 and 12.3, H-6), 4.25 (1H, dd, *J* 5.3 and 12.3, H-6), 4.76 (1H, d, *J* 1.8, H-1), 5.12 (2H, s, $PhCH_2$), 5.21 (1H, dd, *J* 1.8, and 3.4, H-2), 5.24 (1H, t, *J* 9.7, H-4), 5.29 (1H, dd, *J* 3.4 and 9.7, H-3), 7.34 (5H, s, Ph); ^{13}C NMR δ_H (100 MHz, $CDCl_3$) 20.58, 20.59, 20.62, 20.8, 24.5, 30.8, 62.3, 66.0, 66.3, 67.1, 68.4, 69.0, 69.4, 97.3, 128.2, 128.5, 135.8, 169.6, 169.8, 169.9, 170.5, 172.7; *m/z* (FAB) 547.1796 ($M + Na^+$. $C_{25}H_{32}O_{12}$ requires 547.1792), *m/z* ($APCI^+$) 169 (100%), 331 (29%) and 547 (16%). NOESY experiments were used to determine which anomer had been produced during the coupling reaction: NOE correlations were observed between H-3 and H-5, but not H-1, implying that H-1 is equatorial and that the α-anomer had been produced. The relevant portion of the spectrum is shown in the ESI.†

(3'-Oxycarbonylpropyl)-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside 8

To a flask containing 3-benzyloxycarbonylpropyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **7** (1.16 g, 2.2 mmol) was added anhydrous EtOH (30 ml) and 10% Pd/C (0.1 g). This was hydrogenated at room temperature and 1 atm with continuous stirring in the presence of an excess of H_2 over 4 h, monitoring by tlc. Filtration through Celite and removal of the ethanol *in vacuo* yielded the title compound as an opaque viscous yellow oil (0.82 g, 1.89 mmol, 85%); R_f 0.6 (1 : 4 ethanol–CH_2Cl_2); $[\alpha]_D^{21}$ + 36.0° (*c* 1, $CHCl_3$); ν_{max} ($CHCl_3$) 3024–3014 (w), 1757–1730 (s), 1429 (w), 1369 (m), 1252 (s) cm^{-1}; ^1H NMR δ_H (300 MHz, $CDCl_3$) 1.94–2.02 (2H, m, $CH_2CH_2CH_2O$) 1.98 (3H, s), 2.03 (3H, s), 2.08 (3H, s), 2.13 (3H, s), 2.44 (2H, t, *J* 6.9, $CH_2CH_2CH_2O$), 3.49 (1H, dt, *J* 5.9 and 9.6, $CH_2CH_2CH_2O$), 3.68 (1H, dt, *J* 5.9 and 9.6, $CH_2CH_2CH_2O$), 3.93–3.99 (1H, m, H-5), 4.07 (1H, dd, *J* 2.3 and 12.3, H-6), 4.25 (1H, dd, *J* 5.3 and 12.3, H-6), 4.79 (1H, d, *J* 1.6, H-1), 5.21 (1H, dd, *J* 1.6 and 3.0, H-2), 5.25–5.31 (2H, m, H-3 and H-4), 7.98 (1H, br s, –COOH); ^{13}C NMR δ_H (100 MHz, $CDCl_3$) 20.6, 20.7, 20.8, 24.4, 30.7, 62.4, 66.0, 67.0, 67.2, 68.5, 69.1, 69.4, 97.5, 169.8, 170.1, 170.8, 176.7, 177.9; *m/z* (FAB) 557.1316 ($M + Na^+$. $C_{18}H_{26}O_{12}$ requires 557.1322), *m/z* (ES^+) 169 (8%), 331 (5%) and 457 (100%)

3-Succinimidoxycarbonylpropyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside 9

3-Oxycarbonylpropyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **8** (1.4 g, 2.7 mmol) was dissolved in anhydrous CH_2Cl_2 (30 ml), and the flask cooled in an ice–water bath. To the reaction was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 1 g, 5.4 mmol, 2 eq.) and *N*-hydroxysuccinimide (NHS) (0.62 g, 5.4 mmol, 2 eq.). The reaction was stirred for 18 h and allowed to reach room temperature. The reaction mixture was washed with 20% brine (3 × 50 ml), dried over Na_2SO_4, filtered and the solvent removed *in vacuo* to obtain **9** as a pale yellow oil (1.55 g, 2.9 mmol) which was used without further purification; ν_{max} ($CHCl_3$) 3024–3014 (w), 1741 (s), 1431 (w), 1369 (w), 1229 (s) and 1203 (s) cm^{-1}; ^1H NMR δ_H (300 MHz, $CDCl_3$) 2.01–2.12 (2H, m, $CH_2CH_2CH_2O$) 1.97 (3H, s), 2.02 (3H, s), 2.08 (3H, s), 2.13 (3H, s), 2.73 (2H, t, *J* 7.0, $CH_2CH_2CH_2O$), 2.80–2.82 (4H, m, NHS), 3.52 (1H, dt, *J* 5.9 and 10.0, $CH_2CH_2CH_2O$), 3.81 (1H, dt, *J* 6.1 and 10.0, $CH_2CH_2CH_2O$), 3.92–3.96 (1H, m, H-5), 4.07 (1H, dd, *J* 2.4 and 12.3, H-6), 4.27 (1H, dd, *J* 5.1 and 12.3, H-6), 4.81 (1H, d, *J* 1.4, H-1), 5.22–5.31 (3H, m, H-2, H-3 and H-4); ^{13}C NMR δ_H (100 MHz, $CDCl_3$) 13.9, 13.94, 20.7, 20.9, 24.5, 25.4, 30.8, 62.5, 66.0, 67.3, 68.6, 69.3, 69.4, 97.5, 169.8, 170.2, 170.3, 170.9, 172.2, 177.1; *m/z* (ES^+) 331 (8%) and 554 (100%).

N-(t-Butyloxycarbonyl)propane-1,3-diamine 10

Anhydrous THF (20 ml) was added to a flask containing diaminopropane (4 ml, 47.4 mmol, 4 eq.) and stirred vigorously. Di-*t*-butyl dicarbonate (2.56 g, 11.8 mmol) was dissolved in anhydrous THF (2 ml) and slowly added to the flask. The mixture was stirred vigorously for 3 h, by which time the starting di-*t*-butyl dicarbonate had completely reacted (tlc 20% MeOH–$CHCl_3$ + 1% Et_3N, di-*t*-butyl dicarbonate R_f 0.9, 10 R_f 0.2). The THF was removed *in vacuo* and the crude product redissolved in 100 ml ether. The ethereal layer was washed with water (1 × 100 ml), water–1% brine–1% 0.2 M sodium acetate buffer (2 × 100 ml) and then with brine (1 × 100 ml). The aqueous layers were combined, and brine (40 ml) and saturated sodium bicarbonate (40 ml) added. This was then extracted with $CHCl_3$ (200 ml, then 3 × 50 ml). The combined $CHCl_3$ layers were dried over $MgSO_4$ and the solvent removed *in vacuo* to give **10** (1.175 g, 6.73 mmol, 57%) as a yellow oil identical by NMR to the literature.[18]

N-(3-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyloxy)-propylcarbonyl)-N'-(t-butyloxycarbonyl)propane-1,3-diamine 11

3-Succinimidoxycarbonylpropyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **9** (1 g, 1.9 mmol, 1.2 eq.) was dissolved in anhydrous CH_2Cl_2 (10 ml) in a round-bottomed flask. *N*-(*t*-Butyloxycarbonyl)propane-1,3-diamine **10** (279 mg, 1.6 mmol) was dissolved in anhydrous CH_2Cl_2 (2 ml) and added to the reaction at room temperature with stirring. A further 8 ml of anhydrous CH_2Cl_2 was added to the reaction along with three drops of anhydrous Et_3N. The reaction was stirred overnight, then washed with 20% brine (3 × 50 ml) and the organic layer dried over $MgSO_4$. Removal of the solvent *in vacuo* gave an oil, which was purified by flash chromatography (EtOAc: R_f 0.35 (1 : 19 EtOH–CH_2Cl_2)) to give **11** as an oil (0.78 g, 1.31 mmol, 82%) that partially crystallised overnight at 4 °C; $[\alpha]_D^{21}$ + 23.2° (*c* 2.5, $CHCl_3$); ν_{max} ($CHCl_3$) 3024–3014 (w), 1745 (s), 1700, 1662 (m), 1512 (m), 1440–1433 (br w), 1367 (w), 1230 (s) cm^{-1}; ^1H NMR δ_H (300 MHz, $CDCl_3$) 1.33 (9H, s), 1.52 (2H, quin, *J* 5.9, $NCH_2CH_2CH_2N$), 1.79–1.92 (2H, m, $CH_2CH_2CH_2O$), 1.89 (3H, s), 1.94 (3H, s), 2.0 (3H, s), 2.05 (3H, s), 2.20 (2H, t, *J* 7.3, $CH_2CH_2CH_2O$), 3.05 (2H, q, *J* 6.1 Hz, $NCH_2CH_2CH_2N$ or $NCH_2CH_2CH_2N$), 3.18 (2H, m, $NCH_2CH_2CH_2N$ or $NCH_2CH_2CH_2N$), 3.39 (1H, dt, *J* 6.2 and 9.8, $CH_2CH_2CH_2O$), 3.66 (1H, dt, *J* 6.0 and 9.8, $CH_2CH_2CH_2O$), 3.86–3.91 (1H, m, H-5), 3.97–4.02 (2H, m, H-6 and NH), 4.18 (1H, dd, *J* 5.2 and 12.2, H-6), 4.70 (1H, d, *J* 1.4, H-1), 5.11–5.22 (3H, m, H-2, H-3 and H-4), 6.44 (1H, m, NH); ^{13}C NMR δ_H (100 MHz, $CDCl_3$) 20.7, 20.71, 20.8, 25.1, 28.3, 30.1, 32.8, 62.4, 66.1, 67.5,

68.5, 69.1, 69.5, 97.5, 169.7, 170.0, 170.1, 170.7, 172.5; *m/z* (ES^+) 331 (24%), 629 (100%, $[M + Na]^+$).

Pepstatin N-hydroxysuccinimide 12

Pepstatin A (176 mg, 260 μmol), EDCI (493 mg, 2.6 mmol, 10 eq.) and NHS (296 mg, 2.6 mmol, 10 eq.) were placed in a flask. To this was added anhydrous DMF (5 ml) and the reaction stirred overnight. The DMF was removed *in high vacuo.* The solid was loosened from the vessel walls, washed with water (20 ml) and collected by filtration. The solid was further washed with water (180 ml) and then with diethyl ether (50 ml). The wet solid was then dried for 24 h under vacuum over anhydrous P_2O_5. This gave 187 mg (239 μmol, 93%) of the titled product as a white solid; mp 227–229 °C; ν_{max} (KBr) 3500–3200 (s), 2959–2871 (m), 1818 (w), 1785 (w), 1740 (s), 1650–1617 (s), 1534 (s) cm^{-1}; ^1H NMR δ_H (d_6-DMSO, 300 MHz) 0.50–0.70 (30H, m, CH_3), 0.98 (3H, d, *J* 6.81), 0.95–1.25 (4H, m), 1.25–1.40 (2H, m), 1.70–1.85 (3H, m), 1.85–1.95 (2H, m), 2.25–2.33 (4H, m), 2.60 (4H, s), 3.55–3.77 (4H, m), 3.90–4.10 (3H, m), 4.58 (1H, d, *J* 4.4, OH), 5.04 (1H, d, *J* 9.6, OH), 7.42 (2H, m, NH), 7.73 (1H, d, *J* 9.6, NH), 7.78 (1H, d, *J* 9.1, NH), 7.87 (1H, d, *J* 6.7, NH); ^{13}C NMR δ_H (d_6-DMSO, 75.4 MHz) 18.1, 18.2, 18.3, 18.38, 18.4, 18.41, 19.28, 19.33, 21.6, 21.8, 22.3, 23.3, 23.5, 24.2, 24.8, 25.5, 25.7, 30.1, 30.2, 30.3, 44.4, 48.3, 50.5, 57.8, 169.2, 169.3, 169.7, 170.2, 170.5, 170.6, 171.6, 172.4; *m/z* (ES) 805.4194 ($M + Na^+$. $C_{38}H_{66}N_6O_{11}$ requires 805.4687), *m/z* ($APCI^+$) 215.4 (83%), 283.4 (33%), 511.6 (67%), 668.7 (100%), 783.7 (33%).

N-(3-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyloxy)-propylcarbonyl)-N′-(pepstatinyl)propane-1,3-diamine 13

N-(3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl)-*N′*-(*t*-butyloxycarbonyl)propane-1,3-diamine **11** (650 mg, 1.1 mmol) was added to a flask and dissolved in anhydrous CH_2Cl_2 (20 ml). TFA (2 ml) was added slowly to the flask while stirring. After 30 min the reaction was stopped and 10 ml of CH_2Cl_2 was added. The reaction mixture turned greenish. The solvent and TFA were removed *in vacuo* during which time the colour gradually disappeared to obtain *N*-(3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl)propane-1,3-diamine trifluoroacetate as a viscous oil (0.7 g) which was used without further purification.

Pepstatin *N*-hydroxysuccinimide **12** (30 mg, 38 μmol) and *N*-(3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl)propane-1,3-diamine trifluoroacetate (83 mg, 137 μmol, 5.6 eq.) were both dissolved in anhydrous DMF (5 ml) in a flask. Anhydrous Et_3N (1 ml) was added with stirring and the reaction was stirred at room temperature for 18 h. The DMF and Et_3N were removed *in vacuo*, and the crude product was dissolved in MeOH and filtered through cotton wool to remove pepstatin dimethylamine (insoluble in MeOH). Purification by preparative HPLC (gradient, 60% MeOH–water to 95% MeOH–water over 25 min), and removal of the MeOH *in vacuo* followed by lyophilisation, gave the titled product (RT 11 min, R_f 0.2 (1 : 9 MeOH–CH_2Cl_2)) (20 mg, 17.3 μmol, 45%) as a white powder, mp 180 °C (dec.) ν_{max} ($CHCl_3$) 3400–3200 (w, amide), 3024–2855 (m), 2401 (w), 1747 (s), 1647 (s), 1537 (m), 1466–1435 (br m), 1371 (w), 1227–1213 (s) cm^{-1}; ^1H NMR δ_H (CD_3OD, 500 MHz) 0.84–0.98 (30H, m, CH_3), 1.30–1.36 (2H, m), 1.37 (3H, d, *J* 7.2), 1.54–1.65 (4H, m), 1.69 (2H, quin, *J* 6.8), 1.93–1.95 (2H, m), 1.95 (3H, s), 2.03 (3H, s), 2.05 (3H, s), 2.13 (3H, s), 2.02–2.10 (3H, m), 2.11–2.14 (2H, m), 2.27 (2H, d, *J* 6.7), 2.31 (2H, t, *J* 7.4), 2.34–2.36 (2H, m), 3.17–3.26 (4H, m), 3.51 (1H, ddd, *J* 5.9, 6.4, 9.8), 3.75 (1H, ddd, *J* 6.0, 6.6, 9.8), 3.90–3.96 (2H, m), 3.98–4.03 (3H, m), 4.09 (1H, dd, *J* 2.5, 12.2), 4.13 (1H, d, *J* 7.8 Hz), 4.15 (1H, d, *J* 8.1), 4.23–4.28 (2H, m), 4.82 (1H, d, *J* 1.4), 4.88 (1H, s, OH), 5.20–5.24 (3H, m); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) 18.1 (C-16′), 18.9, 19.1, 19.9, 20.0, 20.6, 20.64, 20.7, 22.39, 22.4, 22.77, 22.8, 23.7, 23.8, 25.8 & 25.9, 26.6, 27.5, 30.1, 31.4 & 31.5, 33.7, 37.8 (2C), 41.3, 41.4, 41.6 & 42.0 (4C), 46.0, 51.4, 52.3 & 52.8, 60.7 & 60.9, 63.6, 67.8, 68.6, 69.8, 70.7, 70.8, 71.1, 71.5, 98.9, 171.5, 171.52, 171.6, 172.4, 173.8, 173.9, 174.1, 174.2, 175.4, 175.5, 174.8; *m/z* (ES^+) 1180.6886 ($M + Na^+$. $C_{55}H_{95}N_7O_{19}$ requires 1180.6580), *m/z* ($APCI^+$) 169.0 (100%), 331.2 (14%), 810.7 (5%, $[M -$ mannopyranose$]^+$), 1158.9 (7% $[M + H]^+$). Full ^1H and ^{13}C NMR analysis was carried out on **13** (COSY, TOCSY) and the chemical shifts fully assigned: these assignments were then used to evaluate other pepstatin conjugates. Details of the ^1H and ^{13}C assignments are given in the ESI.†

N-(3-(α-D-Mannopyranosyloxy)propylcarbonyl)-N′-(pepstatinyl)propane-1,3-diamine 1

N-(3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl)-*N′*-(pepstatinyl)propane-1,3-diamine **13** (20 mg, 17.3 μmol), was dissolved in MeOH (5 ml) in a 25 ml round-bottomed flask. LiOH (1 M, aq) solution was added dropwise with stirring until pH 11 was reached. Stirring was continued for 6 h before removal of the solvents *in vacuo.* The crude product was purified by preparative HPLC (gradient, 20% MeCN–water to 60% MeCN–water over 25 min, RT = 13 min). Removal of the MeCN *in vacuo* and lyophilisation gave the titled product as a white powder (12 mg, 70%, 12 μmol); mp 180 °C (dec.); ν_{max} (KBr) 3400–3200 (br s), 2960–2880 (s), 2500–2380 (w), 1685–1632 (s), 1560–1542 (m), 1468–1437 (m), 1206–1187 (s), 1138 (s) cm^{-1}; ^1H NMR δ_H (CD_3OD, 400 MHz) 0.86–0.99 (30H, m, CH_3), 1.28–1.34 (2H, m), 1.38 (3H, d, *J* 7.2), 1.53–1.64 (4H, m), 1.68 (2H, m), 1.85–1.92 (2H, m), 2.02–2.10 (3H, m), 2.12 (2H, d, *J* 6.5), 2.26–2.36 (6H, m), 3.20–3.23 (4H, m), 3.42–3.52 (2H, m), 3.59 (1H, t, *J* 9.5), 3.66–3.75 (3H, m), 3.78 (1H, dd, *J* 1.7 and 3.3), 3.82 (1H, dd, *J* 2.2 and 11.7), 3.93–4.03 (4H, m), 4.11–4.15 (2H, m), 4.26 (1H, q, *J* 7.1), 4.72 (1H, d, *J* 1.5), 4.85 (1H, s, OH), 7.47 (1H, d, *J* 9.4 NH), 7.70 (1H, d, *J* 9.3, NH), 7.99 (2H, m, NH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) 18.1, 18.9, 19.2, 20.0, 20.1, 22.4, 22.5, 22.8, 22.9, 23.8, 23.9, 25.9, 26.0, 26.9, 27.5, 30.1, 31.5, 31.6, 34.0, 37.9 (2C), 41.3, 41.5, 41.7, 42.0, 46.0, 51.4, 52.4, 52.8, 60.8, 60.9, 63.0, 67.8, 68.7, 71.2, 71.5, 72.2, 72.7, 74.7, 101.7, 173.8, 174.0, 174.1, 174.2, 175.5, 175.7, 175.8; *m/z* (ES^+) 1012.6140 ($M + Na^+$. $C_{47}H_{87}N_7O_{15}Na$ requires 1012.6157), *m/z* (ES^+) 828.6 (32%, $[M -$ mannopyranose$]^+$), 990.7 (100%, $[M + H]^+$), 1012.8 (44%, $[M + Na]^+$).

N-(t-Butyloxycarbonyl)cystamine 14

To a flask containing water (5 ml) and dioxane (4 ml) were added cystamine dihydrochloride (1.29 g, 5.7 mmol, 4 eq.) and sodium bicarbonate (0.48 g, 5.7 mmol, 4 eq.) with vigorous stirring.

Di-*t*-butyl dicarbonate (313 mg, 1.43 mmol) was dissolved in dioxane (1 ml) and slowly added to the flask. The mixture was stirred vigorously. By 2 h a residue had formed which was re-dissolved by the addition of MeCN (5 ml). After 4 h the organic solvents were removed *in vacuo* to give an aqueous suspension. A few drops of acetate buffer (0.2 M) were added, resulting in the precipitation of a solid; this was filtered and washed several times with 10% brine and 10% bicarbonate solution (50 ml total). The filtrate was extracted with CH_2Cl_2 (4 × 50 ml) and the combined organic layers dried over $MgSO_4$. Removal of the solvent *in vacuo* gave **11** as a viscous yellow oil (154 mg, 0.61 mmol, 43%); ν_{max} ($CHCl_3$) 3454 (w), 3000–2900 (w), 1720–1680 (s), 1506 (s) cm^{-1}; ^1H NMR δ_H ($CDCl_3$, 400 MHz) 1.39 (9H, s, *t*-Bu), 1.78 (2H, s, NH_2), 2.72 (2H, t, *J* 6.2, NCH_2CH_2S), 2.73 (2H, t, *J* 6.3, NCH_2CH_2S), 2.96 (2H, t, *J* 6.1, $BocNHCH_2CH_2S$), 3.38–3.44 (2H, m, $SCH_2CH_2NH_2$), 5.03 (1H, br s, NH); ^{13}C NMR δ_H ($CDCl_3$, 100.6 MHz) 28.3, 38.4, 39.3, 40.4, 42.5, 155.7; *m/z* (FAB) 253.1010 (M + H^+. $C_9H_{20}N_2O_2S_2$ requires 253.1044), *m/z* ($APCI^+$) 252.9 (56%).

*N-(3-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyloxy)-propylcarbonyl-N′-(*tert*-butyloxycarbonyl)cystamine 15*

3-Succinimidoxycarbonylpropyl - 2,3,4,6 - tetra - *O* - acetyl - α - D-mannopyranoside **9** (500 mg, 0.94 mmol, 1.7 eq.) was dissolved in anhydrous CH_2Cl_2 (10 ml) in a round-bottomed flask. *N*-(*t*-Butyloxycarbonyl)cystamine **14** (140 mg, 0.55 mmol) was dissolved in anhydrous CH_2Cl_2 (2 ml) and added to the reaction while stirring at room temperature. A further 8 ml of anhydrous CH_2Cl_2 was added to the reaction along with three drops of anhydrous Et_3N. The reaction was stirred overnight, then washed with 20% brine (3 × 50 ml) and the organic layer dried over $MgSO_4$. Removal of the solvent *in vacuo* gave an oil, which was purified by flash chromatography (4 cm, gradient, 50% hexane–ethyl acetate to 10% hexane–ethyl acetate, change in 10% hexane every 50 ml; R_f 0.3 (1 : 9 hexane–ethyl acetate)) to give **15** as an oil (244 mg, 363 μmol, 66%); $[\alpha]_D^{21}$ + 22.4° (*c* 2.5, $CHCl_3$); ν_{max} (film) 3400–3100 (s), 2980–2900 (s), 1760–1650 (s), 1550–1504 (m), 1435 (w), 1367 (w), 1280–1220 (m) cm^{-1}; ^1H NMR δ_H (400 MHz, $CDCl_3$) 1.38 (9H, s), 1.88–1.94 (2H, m, $CH_2CH_2CH_2O$) 1.94 (3H, s), 2.0 (3H, s), 2.05 (3H, s), 2.10 (3H, s), 2.27 (2H, t, *J* 7.2, $CH_2CH_2CH_2O$), 2.73 (2H, t, *J* 6.7, $NHCH_2CH_2S$), 2.79 (2H, t, *J* 5.9, $NHCH_2CH_2S$), 3.38 (2H, q, *J* 6.4, $NHCH_2CH_2S$), 3.41–3.47 (1H, m, $CH_2CH_2CH_2O$), 3.51 (2H, q, *J* 6.0, $NHCH_2CH_2S$), 3.70 (1H, dt, *J* 5.9 and 9.8, $CH_2CH_2CH_2O$), 3.90–3.95 (1H, m, H-5), 4.02–4.07 (1H, m, H-6), 4.27 (1H, dd, *J* 5.2 and 12.2, H-6), 4.75 (1H, d, *J* 1.5, H-1), 5.11 (1H, br d, NH), 5.16–5.27 (3H, m, H-2, H-3 and H-4), 6.53 (1H, br d, NH); ^{13}C NMR δ_H (100 MHz, $CDCl_3$) 20.6, 20.7, 20.8, 25.0, 28.3, 32.5, 37.7, 38.1, 38.2, 39.4, 62.4, 66.0, 67.4, 68.4, 69.0, 69.4, 97.4, 155.9, 169.6, 169.9, 170.1, 170.6, 172.4; *m/z* (FAB) 669.2371 (M^+. $C_{27}H_{44}NO_{13}S_2$ requires 669.2363), *m/z* (FAB^+) 169 (80%), 331 (100%), 669 (50%, [M + H]$^+$).

N-(3-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyloxy)-propylcarbonyl)-N′-(pepstatinyl)cystamine 16

N-(3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl-*N′*-(*tert*-butyloxycarbonyl)cystamine **15** (183 mg, 270 μmol) was dissolved in anhydrous CH_2Cl_2 (5 ml) in a round-bottomed flask. TFA (0.5 ml) was added slowly to the flask while stirring. The reaction was monitored by tlc. After 4 h the solvent was removed *in vacuo* and purified by flash chromatography (4 cm, isocratic, 10% EtOH–CH_2Cl_2) to obtain *N*-(3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonylcystamine trifluoroacetate as a yellow/orange oil (141 mg, 202 μmol, 75%; R_f 0.3 (1 : 9 EtOH–CH_2Cl_2)).

Pepstatin *N*-hydroxysuccinimide **12** (36 mg, 46 μmol) and *N*-(3-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonylcystamine trifluoroacetate (33 mg, 48 μmol, > 1 eq.) were dissolved in anhydrous DMF (4 ml) in a round-bottomed flask. Anhydrous Et_3N (1 ml) was added with stirring and the reaction was stirred for 18 h at room temperature. The DMF and Et_3N were removed *in vacuo*, and the crude product dissolved into MeOH and filtered through cotton wool to remove pepstatin dimethylamine (insoluble in MeOH). Purification by preparative HPLC (gradient, 60% MeOH–water to 95% MeOH–water over 25 min) gave the titled product (RT 11 min, R_f 0.2 (1 : 9 MeOH–CH_2Cl_2)). Removal of the MeOH *in vacuo*, and removal of the water by lyophilisation gave the titled product as a white powder (28.5 mg, 23 μmol, 55%); mp 180 °C (dec.); ν_{max} ($CHCl_3$) 3400–3200 (m), 2962–2850 (m), 1746 (s), 1652 (s), 1540 (m), 1466–1438 (br m), 1370 (w), 1231–1225 (s) cm^{-1}; ^1H NMR δ_H (CD_3OD, 500 MHz) 0.87–0.98 (30H, m, CH_3), 1.28–1.35 (2H, m), 1.38 (3H, d, J 7.2), 1.54–1.64 (4H, m), 1.92–1.97 (2H, m), 1.95 (3H, s), 2.03 (3H, s), 2.06 (3H, s), 2.13 (3H, s), 2.02–2.12 (3H, m), 2.12–2.14 (2H, m), 2.30–2.37 (6H, m), 2.82 (2H, t, *J* 6.8), 2.83 (2H, t, *J* 6.6), 3.40–3.55 (5H, m), 3.73–3.78 (1H, m), 3.90–4.03 (5H, m), 4.10 (1H, dd, *J* 2.6 and 12.3), 4.12–4.16 (2H, m), 4.23–4.29 (2H, m), 4.82 (1H, d, *J* 1.0), 4.89 (1H, s, OH), 5.20–5.26 (3H, m), 7.45 (1H, d, *J* 9.4, NH), 7.67 (1H, d, *J* 9.1, NH), 7.96 (1H, d, *J* 8.4, NH), 8.01 (1H, d, *J* 7.9, NH), 8.06 (1H, t, *J* 5.6, NH), 8.17 (1H, t, *J* 5.6, NH), 8.20 (1H, d, *J* 5.7, NH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) 18.1, 18.9, 19.1, 20.0, 20.1, 20.6, 20.64, 20.66, 20.7, 22.4 (2 C), 22.7, 22.8, 23.7, 23.8, 25.85, 25.9, 26.5, 27.5, 31.4, 31.5, 33.6, 38.3, 38.6, 39.6, 39.7, 41.3, 41.4, 41.6, 41.9, 46.0, 51.4, 52.3, 52.7, 60.8, 60.9, 63.6, 67.3, 68.5, 69.8, 70.7, 70.8, 71.1, 71.5, 98.9, 171.5, 171.55, 171.6, 172.4, 173.7, 173.9, 174.1, 174.2, 175.4, 175.6, 175.8; *m/z* (ES^+) 1258.6237 (M + Na^+. $C_{56}H_{97}N_7O_{19}S_2Na$ requires 1258.6178), *m/z* (ES^+) 1236.6 (30%, [M + H]$^+$), 1258.6 (100%, [M − H + Na]$^+$).

N-(3-(α-D-Mannopyranosyloxy)propylcarbonyl)-N′-(pepstatinyl)cystamine 2

N-(3-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyloxy)propylcarbonyl)-*N′*-(pepstatinyl)cystamine **16** (25 mg, 20.2 μmol), was dissolved in MeOH (5 ml) in a 25 ml round-bottomed flask and stirred. LiOH (1 M, aq) solution was added dropwise with stirring until pH 11 was reached. Stirring was continued for 6 h before removal of the solvents *in vacuo.* The crude product was purified by preparative HPLC (gradient, 20% MeCN–water to 60% MeCN–water over 25 min, RT = 12.8 min). Removal of the MeCN *in vacuo* and lyophilisation gave **2** as a white powder (14.3 mg, 13.4 μmol, 66%); mp 180 °C (dec.); ν_{max} (KBr) 3600–3200 (br s), 2965–2930 (w), 1690–1630 (s), 1565–1534 (m), 1455–1430 (m), 1208 (s), 1146 (s) cm^{-1}; ^1H NMR δ_H (CD_3OD, 500 MHz) 0.87–0.99 (30H, m, CH_3), 1.28–1.36 (2H, m), 1.39 (3H, d, *J* 8.0), 1.58–1.63 (4H, m), 1.85–1.92 (2H, m), 2.04–2.11 (3H, m), 2.14 (2H, d, *J* 6.7), 2.29–2.38 (6H, m), 2.83 (4H, t,

J 6.7), 3.47–3.53 (6H, m), 3.61 (1H, t, *J* 9.6), 3.64–3.72 (3H, m), 3.79 (1H, dd, *J* 1.7 and 3.3), 3.88 (1H, dd, *J* 2.5 and 11.5), 3.94–4.02 (4H, m), 4.12–4.17 (2H, m), 4.27 (1H, m), 4.74 (1H, d, *J* 1.7), 4.92 (1H, s, OH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) 18.1, 19.0, 19.2, 20.0, 20.1, 22.4, 22.5, 22.8, 22.9, 23.8, 23.9, 25.9, 26.0, 26.9, 27.5, 31.5, 31.6, 33.9, 38.4, 38.6, 39.7, 39.74, 41.3, 41.4, 41.6, 42.0, 46.0, 51.4, 52.4, 52.8, 60.9, 61.0, 63.0, 67.8, 68.7, 71.1, 71.5, 72.2, 72.7, 74.7, 101.6, 173.9, 174.0, 174.1, 174.2, 175.5, 175.9, 180.9; *m/z* (ES$^+$) 1090.5787 (M + Na$^+$. $C_{48}H_{89}N_7O_{15}S_2Na$ requires 1090.5755), *m/z* (ES$^+$) 283.2 (38%), 906.5 (22%, [M − mannopyranose]$^+$), 1068.4 (20%, [M + H]$^+$).

Mannosylated BSA 17

4-Isothiocyanatophenyl-*α*-D-mannopyranose (43 mg, 0.14 mmol, approx. 60 eq.) and bovine serum albumin (153 mg, ~2.3 μmol) were dissolved in NaCl solution (0.15 M, 10 ml) in a 50 ml round-bottomed flask and stirred vigorously. The pH was adjusted to pH 9 by the dropwise addition of 0.1 M NaOH, and the reaction was stirred continuously for 6 h, maintaining the same pH. At the end of this time the reaction mixture was stored overnight at 4 °C in the fridge. The pH was then adjusted to pH 7 and the reaction mixture dialysed with 0.15 M NaCl solution (1L) for three days, changing the solution twice a day and stirring the dialysis solution continuously within a fridge. The dialysis tubing contents were further purified and de-salted using centrifugal filtration devices (Millipore) spun within a fixed 35° angle centrifuge at a relative centrifugal force (rcf) of 3000 for 90 min, 21 °C (maximum 4 ml solution per device) before re-suspending the gelatinous product at the bottom of the device with water (4 ml). This was repeated three times to maximise purification. Lyophilisation gave **17** as a fine white powder (126 mg, 74%); mp dec. above 260 °C; MS (MALDI-TOF): MS peak range = 67,000–79,000; top of peak for BSA ~66,430, **17** ~74,032, difference = 7602, implying approximately 24.3 sugar units conjugated to each BSA molecule. Biochemical analysis: Analysis of free TNBS reactive amino groups[28] showed an approximate conjugation of 23 sugar units. Analysis of attached sugar units[27] showed an approximate conjugation of 22.5 sugar units. The mass spectra, and details of the biochemical analysis, are shown in the ESI.†

N-(*t*-Butyloxycarbonyl)-*N′*-(pepstatinyl)propane-1,3-diamine[18]

N-(*t*-Butyloxycarbonyl)propane-1,3-diamine **10** (111 mg, 640 μmol, 10 eq.), pepstatin *N*-hydroxysuccinimide **12** (50 mg, 64 μmol) and anhydrous DMF (6 ml) were added to a round-bottomed flask and stirred continuously for 2 days. The solvent was removed *in vacuo* and the crude product purified by flash chromatography (1 cm^3, isocratic, 10% MeOH–CH_2Cl_2: R_f 0.5 (1 : 9 MeOH–$CHCl_3$)). The solvent was removed *in vacuo* to give an off-white solid. This was re-crystallised from $CHCl_3$–cyclohexane to obtain the title compound as a white powder (48 mg, 57 μmol, 89%); mp 219–221 °C (lit[18] 215–217 °C); ν_{max} (KBr) 3400–3200 (s, amide), 2960–2872 (s), 2360–2331 (m), 1652–1634 (s), 1533 (m), 1465–1437 (m), 1171 (m) cm^{-1}; ^{1}H NMR δ_H (DMSO, 500 MHz) 0.77–0.85 (30H, m, CH_3), 1.18 (3H, d, *J* 7.1), 1.21–1.26 (2H, m), 1.33–1.37 (2H, m), 1.36 (9H, s), 1.46–1.52 (2H, m), 1.92–1.98 (3H, m), 2.00–2.05 (4H, m), 2.11 (2H, d, *J* 6.2), 2.90 (2H, q, *J* 6.4), 2.97–3.05 (2H, m), 3.27–3.34 (2H, m), 3.75–3.83 (4H, m), 4.11 (1H, dd, *J* 7.2 and 8.9), 4.17 (1H, dd, *J* 7.4 and 8.7), 4.24 (1H, quin, *J* 7.2), 4.82 (1H, d, *J* 4.8, OH), 4.83 (1H, d, *J* 4.9, OH), 7.30 (1H, d, *J* 9.2, NH), 7.43 (1H, d, *J* 8.8, NH), 7.75 (1H, d, *J* 8.9, NH), 7.79 (1H, d, *J* 8.8, NH), 7.89 (1H, d, *J* 7.4, NH); ^{13}C NMR δ_H (DMSO, 125.7 MHz) 18.1, 18.2, 19.2, 19.3, 21.6, 21.9, 22.2, 23.2, 23.4, 24.1, 25.6, 28.2, 30.0, 30.3, 36.1, 37.5, 38.6, 39.6, 39.8, 39.9, 40.1, 40.2, 44.4, 48.3, 50.4, 50.7, 54.8, 57.8, 58.0, 69.0, 69.1, 77.4, 155.5, 170.6, 170.7, 170.78, 170.8, 171.1, 171.5, 172.1; *m/z* (ES$^+$) 864.5785 (M + Na$^+$. $C_{42}H_{79}N_7O_{10}Na$ requires 864.5786), *m/z* (ES$^+$) 842.6 (100%, [M + H]$^+$), 864.6 (27%, [M + Na]$^+$).

N-(Pepstatinyl)propane-1,3-diamine[18]

To a flask containing *N*-(*t*-butyloxycarbonyl)-*N′*-(pepstatinyl)-propane-1,3-diamine (75 mg, 89 μmol) was added TFA (4 ml), water (125 μl, 6.9 μmol), triethylsilane (125 μl, 0.77 μmol), thioanisole (250 μl, 2.1 μmol) and phenol (250 μl, 2.8 μmol). The reaction was stirred vigorously at room temperature for 4 h. The TFA was removed *in vacuo*, and the resulting solid purified by preparative HPLC (50% MeOH–water to 100% MeOH over 25 min, RT = 14–15 min; R_f 0.2 (1 : 9 MeOH–$CHCl_3$)) to obtain the title compound as a white powder (60 mg, 81 μmol, 91%); mp 214–216 °C (lit[18] 207–210 °C); ν_{max} (KBr) 3500–3200 (s), 3079 (w), 2960–2872 (s), 2416 (w), 1700–1600 (s), 1537 (s), 1467–1417 (m), 1204–1138 (m) cm^{-1}; ^{1}H NMR δ_H (CD_3OD, 500 MHz) 0.86–0.98 (30H, m, CH_3), 1.27–1.35 (2H, m), 1.39 (3H, d, *J* 7.3), 1.55–1.64 (4H, m), 1.84 (2H, quin, *J* 7.0), 2.02–2.10 (3H, m), 2.14 (2H, d, *J* 6.8), 2.30–2.38 (4H, m), 2.98 (2H, t, *J* 7.1), 3.20–3.25 & 3.32–3.36 (2H, m), 3.92–3.97 (2H, m), 3.99–4.03 (2H, m), 4.10–4.14 (2H, m), 4.21 (1H, q, *J* 7.2), 4.97 (1H, s, OH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) 18.0, 18.9, 19.2, 19.9, 20.0, 22.3, 22.4, 22.7, 22.8, 23.7 & 23.8 (10 C), 25.8, 25.9, 27.4, 28.7, 31.3, 31.4, 36.8, 38.2, 41.2, 41.3, 41.6 (2C), 46.0, 51.6, 51.9, 52.7, 61.0 (2 C), 71.0, 71.3, 173.9, 174.1, 174.3, 174.5, 175.5, 175.9; *m/z* (ES$^+$) 742.5440 (M + H$^+$. $C_{37}H_{71}N_7O_8$ requires 742.5442), *m/z* (ES$^+$) 742.6 (100%, [M + H]$^+$).

N-(Iodoacetyl)-N′-(pepstatinyl)propane-1,3-diamine 18

N-(Pepstatinyl)propane-1,3-diamine (10 mg, 13.5 μmol) was placed in a flask and any water present removed by azeotroping with anhydrous MeCN (3 × 1 ml). The amine was dissolved in anhydrous DMF (3 ml) and iodoacetic anhydride (5.3 mg, 15 μmol, 1.1 eq.) was added dropwise with stirring. The flask was covered with aluminium foil to exclude all light and the reaction stirred continuously for 2 h. The DMF was removed *in vacuo* and the crude product stored overnight in a fridge. The flask contents were then washed with water containing 1% v/v saturated $NaHCO_3$ solution and 10% v/v saturated KI solution. The product precipitated and was filtered through cotton wool. The solid product was washed again with the aqueous KI–$NaHCO_3$ solution (40 ml) and once with water. The precipitate was then dissolved in EtOH and transferred to a round-bottomed flask. Removal of the EtOH *in vacuo* gave **18** as a white powder (6 mg, 6.6 μmol, 49%), which was stored in a fridge under Ar and used in the next reaction as soon as possible; mp 189–191 °C; ν_{max} (KBr) 3500–3150 (s), 3079 (w), 2959–2871 (m), 1700–1635 (s), 1550–1507 (s) cm^{-1}; ^{1}H NMR δ_H (CD_3OD, 500 MHz) 0.86–0.98 (30H, m, CH_3), 1.32–1.39 (2H, m), 1.38 (3H, d, *J* 7.2), 1.54–1.65

(4H, m), 1.70 (2H, quin, *J* 6.8), 2.02–2.10 (3H, m), 2.13 (2H, d, *J* 6.7), 2.27 (2H, d, *J* 6.7), 2.34–2.37 (2H, m), 3.20–3.34 (4H, m), 3.69 (2H, s), 3.93–4.01 (4H, m), 4.13 (1H, d, *J* 7.9), 4.15 (1H, d, *J* 8.1), 4.26 (1H, q, *J* 7.3), 4.89 (1H, s, OH), 7.32 (1H, d, *J* 9.3, NH), 7.45 (1H, s, NH), 7.68 (1H, d, *J* 9.0, NH), 7.89 (1H, s, NH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) −2.0 (CH_2-I), 18.1, 18.9, 19.1, 20.0, 20.1, 22.4 (2H), 22.7, 22.8, 23.7, 23.8, 25.8, 25.9, 27.5, 29.8, 31.4, 31.5, 37.8, 38.4, 41.3, 41.4, 41.7, 42.0, 46.0, 51.4, 52.3, 52.8, 60.7, 60.9, 71.1, 71.4, 171.4, 173.8, 173.9, 174.1, 174.2, 175.4, 175.8; *m/z* (ES^+) 932.4311 (M + Na^+. $C_{39}H_{72}IN_7O_9Na$ requires 932.4334), *m/z* (ES^+) 932.3 (83%, $[M + Na]^+$).

Non-cleavable neomannosylated BSA–pepstatin conjugate 3

Mannosylated BSA **17** (30 mg, ~3.8×10^{-7} moles) was placed in a 25 ml round-bottomed flask and dissolved in 2 ml of phosphate buffer saline (PBS, 1 M, pH 8.0) with stirring. To this was added *N*-(iodoacetyl)-*N′*-(pepstatinyl)propane-1,3-diamine **18** (6 mg ml^{-1} stock in anhydrous DMF, 350 μg, 3.8×10^{-7} moles) and the reaction was stirred overnight at room temperature. The reaction mixture was dialysed once against 1 M PBS solution (1L, pH 7.4) for one day, changing the solution once and stirring the dialysis solution continuously at 4 °C. Further purification and de-salting was achieved by using Millipore ultrafree-4 centrifugal filtration devices spun within a fixed 35° angle centrifuge at a relative centrifugal force (rcf) of 3000 for 90 min at 21 °C (crude product made up to 4 ml with water and total volume of 10% DMSO). The gelatinous product at the bottom of the device was re-suspended in water (4 ml) and re-centrifuged in a new filtration device (3000 rcf, 90 min) and repeated within the same device by re-suspending in water (4 ml). Lyophilisation gave **3** as a fine white powder (22 mg, 73%); mp dec. above 260 °C; MS (MALDI-TOF): MS peak range = 67 500–80 000.

N-(t-Butyloxycarbonyl)-N′-(pepstatinyl)cystamine

N-(*t*-Butyloxycarbonyl)cystamine **14** (161 mg, 640 μmol, 10 eq.), pepstatin *N*-hydroxysuccinimide **12** (50 mg, 64 μmol) and anhydrous DMF (6 ml) were added to a round-bottomed flask and stirred continuously for 2 days at room temperature. The solvent was then removed *in vacuo* and the mixture purified by flash chromatography (1 cm^3, isocratic, 10% MeOH–CH_2Cl_2, R_f 0.55). This gave a crude white powder, which was re-crystallised from hot EtOH to obtain the title compound as a white powder (47 mg, 64 μmol, 80%); mp 244–246 °C; ν_{max} (KBr) 3600–3100 (s), 2959–2871 (s), 1635 (s), 1534 (s), 1466–1437 (m), 1171 (m) cm^{-1}; ^{1}H NMR δ_H (DMSO, 500 MHz) 0.77–0.85 (30H, m, CH_3), 1.18 (3H, d, *J* 7.1), 1.20–1.26 (2H, m), 1.33–1.38 (2H, m), 1.36 (9H, s), 1.48–1.52 (2H, m), 1.90–1.99 (3H, m), 2.00–2.08 (4H, m), 2.11 (2H, d, *J* 6.2), 2.72 (2H, t, *J* 6.5), 2.73 (2H, t, *J* 6.5), 3.16–3.20 (2H, m), 3.25–3.31 (2H, m), 3.74–3.81 (4H, m), 4.11 (1H, d, *J* 7.2), 4.11 (1H, d, *J* 7.3), 4.23 (1H, q, *J* 7.0), 4.82 (1H, d, *J* 5.3, OH), 4.83 (1H, d, *J* 5.2, OH), 7.32 (1H, d, *J* 9.2, NH), 7.45 (1H, d, *J* 8.8, NH), 7.77 (1H, d, *J* 9.0, NH), 7.81 (1H, d, *J* 8.8, NH), 7.91 (1H, d, *J* 7.4, NH); ^{13}C NMR δ_H (DMSO, 125.7 MHz) 18.2, 18.3, 19.2, 19.3, 21.6, 21.9, 22.2, 23.3, 23.5, 24.2, 25.7, 28.2, 30.1, 30.3, 37.1, 37.6, 37.9, 38.0, 38.6, 39.7, 39.8, 40.0, 40.1, 40.2, 44.3, 48.3, 50.4, 50.7, 57.7, 57.9, 69.0, 69.1, 77.8, 155.5, 170.7, 170.8, 170.9, 171.0, 171.02, 171.6, 172.2; *m/z* (ES^+) 942.5378 (M + Na^+. $C_{43}H_{81}N_7O_{10}S_2Na$ requires 942.5384), *m/z* (ES^+) 920.6 (100%, $[M + H]^+$), 942.6 (53%, $[M - H + Na]^+$).

N-(Pepstatinyl)cystamine

To a flask containing *N*-(*t*-butyloxycarbonyl)-*N′*-(pepstatinyl)-cystamine (65 mg, 70 μmol) was added TFA (4 ml), water (125 μl, 6.9 μmol), triethylsilane (125 μl, 0.77 μmol), thioanisole (250 μl, 2.1 μmol) and phenol (250 μl, 2.8 μmol). The reaction was stirred vigorously for 4 h. The TFA was removed *in vacuo*, and the mixture purified by preparative HPLC (40% MeCN–water to 80% MeCN over 30 min; RT = 5 min; R_f 0.2 (1 : 9 MeOH–$CHCl_3$)) to obtain the title compound as a white powder (37 mg, 45 μmol, 64%); mp 219–221 °C; ν_{max} (KBr) 3500–3200 (s), 2960–2872 (m), 1700–1600 (s), 1540 (s), 1470–1419 (w), 1203–1138 (m) cm^{-1}; ^{1}H NMR δ_H (CD_3OD, 400 MHz) 0.86–0.98 (30H, m, CH_3), 1.27–1.35 (2H, m), 1.38 (3H, d, *J* 7.3), 1.55–1.63 (4H, m), 2.02–2.10 (3H, m), 2.13 (2H, d, *J* 6.5), 2.28–2.38 (4H, m), 2.87 (2H, t, *J* 6.7), 2.97 (2H, t, *J* 6.8), 3.24–3.32 (2H, m), 3.42–3.62 (2H, m), 3.91–4.04 (4H, m), 4.10–4.17 (2H, m), 4.23 (1H, q, *J* 7.3), 4.93 (1H, s, OH), 7.47 (1H, d, *J* 9.4, NH), 7.63 (1H, d, *J* 9.7, NH), 7.67 (1H, d, *J* 9.1, NH), 7.97 (1H, d, *J* 8.3, NH), 8.05 (1H, d, *J* 7.63, NH), 7.63 (1H, s, NH); ^{13}C NMR δ_H (CD_3OD, 100.6 MHz) 18.0, 18.9, 19.2, 20.0, 20.1, 22.4 (2C), 22.7, 22.8, 23.7, 23.8, 25.8, 25.9, 27.5, 31.3, 31.5, 35.6, 38.3, 39.4, 39.5, 41.2, 41.3, 41.6, 41.9, 46.0, 51.5, 52.2, 52.7, 60.9 (2C), 71.0, 71.5, 173.8, 173.9, 174.2, 174.3, 175.5, 175.9; *m/z* (ES^+) 820.5039 (M + H^+. $C_{38}H_{73}N_7O_8S_2$ requires 820.5040), *m/z* (ES^+) 820.5 (100%, $[M + H]^+$).

N-(Iodoacetyl)-N′-(pepstatinyl)cystamine 19

N-(Pepstatinyl)cystamine (7 mg, 8.3 μmol) was placed in a round-bottomed flask and any water present removed by azeotroping with anhydrous MeCN (3 × 1 ml). The amine was then dissolved in anhydrous DMF (3 ml) and iodoacetic anhydride (3.3 mg, 9.4 μmol, 1.1 eq.) added dropwise with stirring. The flask was covered with aluminium foil to exclude all light and the reaction was stirred continuously for 2 h. The DMF was removed *in vacuo* and the crude product stored overnight at 4 °C. The next day the flask contents were washed with water containing 1% v/v saturated bicarbonate solution and 10% v/v saturated potassium iodide (KI) solution. The product precipitated and was filtered through cotton wool. The solid product was washed again with the aqueous KI/bicarbonate solution (40 ml) and once with water. The precipitate was then dissolved in EtOH and transferred to a round-bottomed flask. Removal of the EtOH *in vacuo* gave the title compound as a white powder (6 mg, 6.1 μmol, 83%), which was stored in a fridge under Ar and used in the next reaction as soon as possible; mp 198–201 °C; ν_{max} (KBr) 3600–3100 (m), 2959–2871 (m), 1700–1617 (m), 1540 (w), 1067 (s) cm^{-1}; ^{1}H NMR δ_H (CD_3OD, 500 MHz) 0.87–0.99 (30H, m, CH_3), 1.28–1.36 (2H, m), 1.38 (3H, d, *J* 7.3), 1.57–1.62 (4H, m), 2.03–2.11 (3H, m), 2.13 (2H, d, *J* 6.7), 2.28 (2H, d, *J* 6.7), 2.34–2.37 (2H, m), 2.83 (4H, t, *J* 6.6), 3.43–3.61 (4H, m), 3.71 (2H, s), 3.91–4.04 (4H, m), 4.12 (1H, d, *J* 7.8), 4.15 (1H, d, *J* 8.0), 4.26 (1H, q, *J* 9.3), 4.89 (1H, s, OH), 7.43 (1H, s, NH), 7.45 (1H, s, NH), 7.68 (1H, d, *J* 9.0, NH); ^{13}C NMR δ_H (CD_3OD, 125.7 MHz) −2.1 (CH_2-I), 18.1, 18.9, 19.1, 20.0, 20.1, 22.4 (2C), 22.7, 22.8, 23.7, 23.8, 25.8, 25.9, 27.5, 31.4, 31.6, 38.2, 38.3, 39.7, 40.1, 41.3, 41.4, 41.7, 42.0, 46.0, 51.4, 52.3, 52.8, 60.8,

60.9, 71.1, 71.4, 173.8, 173.9, 174.1, 174.2, 175.4, 175.5, 175.8; *m/z* (ES^+) 988.2 (56%, $[M + H]^+$), 1010.3 (100%, $[M - H + Na]^+$).

Cleavable neomannosylated BSA–pepstatin conjugate 4

Mannosylated BSA **17** (30 mg, $\sim 3.8 \times 10^{-7}$ moles) was placed in a 25 ml round-bottomed flask and dissolved in 2 ml of phosphate buffer saline (PBS, 1 M, pH 8.0) with stirring. To this was added *N*-(iodoacetyl)-*N′*-(pepstatinyl)cystamine **19** (taken from 6 mg ml^{-1} stock in anhydrous DMF, 380 μg, 3.8×10^{-7} moles) and the reaction was continually stirred overnight. The reaction mixture was dialysed once against 1 M PBS solution (1L, pH 7.4) for one day, changing the solution once and stirring the dialysis solution continuously within a fridge. Further purification and de-salting was achieved by using Millipore ultrafree-4 centrifugal filtration devices spun within a fixed 35° angle centrifuge at a relative centrifugal force (rcf) of 3000 for 90 min, 21 °C (crude product made up to 4 ml with water and total volume of 10% DMSO). The gelatinous product at the bottom of the device was re-suspended in water (4 ml) and re-centrifuged in a new filtration device (3000 rcf, 90 min) and repeated within the same device by re-suspending in water (4 ml). Lyophilisation gave **4** as a fine white powder (21 mg, 70%); dec. above 260 °C; MS (MALDI-TOF): MS peak range = 69 000–81 500. In order to remove any last traces of solvent or other small molecular weight impurities, 5 mg material was dissolved in 0.5 ml water and passed over a prepacked column of Sephadex G10 chromatography medium (Pharmacia), preequilibrated in tissue culture medium containing 10% foetal calf serum. The conjugate was eluted following the manufacturers instructions, and stored in aliquots at −20 °C until required.

Enzymatic studies of inhibitors

Rat Cathepsin E was prepared as described.[30] Stocks were stored in 0.05 M sodium acetate buffer, 50% glycerol, 0.25 M NaCl, at −20 °C.

The fluorogenic peptide substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)γ-NH_2 (5 μl of 100 μM in H_2O; BAChem, Meyerside, UK) known to be cleaved by cathepsin D or E[30] was added to 80 μl 0.1 M sodium acetate buffer (pH 4.0). To this was added 5 μl of inhibitor solution at a series of two-fold dilutions from various concentrations (dependent on the inhibitor used). Samples were incubated at 37 °C for several minutes in a water bath. 10 μl of enzyme solution (0.01 M sodium acetate buffer, pH 5.5) at 0.5 μg ml^{-1} was added to each sample, and the reaction was allowed to continue at 37 °C for 30 min before adding 200 μl of 5% trichloroacetic acid to precipitate the enzymes. Negative/positive controls were included as a sample without enzyme, or contained no inhibitor, respectively. Samples were centrifuged at 13 000 rpm for a few seconds before reading OD (excitation 328 nm, emission 393 nm) using a Hitachi fluorescence spectrophotometer (Hitachi, Japan).

Data were analysed by the Dixon method[38] plotted as fluorescence (converted to % activation) *vs.* inhibitor concentration to obtain the IC_{50} value for the appropriate inhibitor.

Assays for antigen processing/presentation

A20 cells (B lymphoma) and DO11-10 cells (T cell hybridoma specific for ovalbumin) were cultured in Dulbecco's modified Eagles medium (Cancer Research UK) containing 10% foetal calf serum (FCS; PAA Laboratories, Linz, Austria); 100 U ml^{-1} Penicillin, 100 μg ml^{-1} Streptomycin (Cancer Research UK) and 50 μM 2-mercaptoethanol (2-ME; Gibco, Paisley, UK). Cells were maintained at a density of between 2×10^5 cells/ml and 5×10^5cells/ml. RPMI 1640 with L-Glutamine (Gibco). CTLL-2 (a kind gift from Dr B. Stockinger, National Institute for Medical Research, London, UK) is an IL-2-dependent human T-cell line. Cells were cultured in complete RPMI 1640 medium containing 10% FCS.

Dendritic cells were obtained from bone marrow of Balb/*c* mice as described elsewhere.[37]

The assay for antigen processing/presentation has been described previously.[31] Briefly DO11-10 cells (5×10^4) and either A20 cells (5×10^4) or dendritic cells (2.5×10^4) were co-cultured in wells of a tissue culture plate (Nunc) in a total volume of 200 μl. Cells were stimulated with ovalbumin (Sigma Aldrich, Poole, Dorset), or a synthetic peptide (Cancer Research UK) coding for the ovalbumin sequence 323–339 which is the epitope recognised by the DO11-10 T cells. The cells are cultured for 24 hours at 37 °C, 5% CO_2, and supernatants collected and stored at −20 °C until assayed for IL-2. IL-2 levels were measured either by ELISA (eBioscience, London, UK) according to manufacturer's instructions (Fig. 3), or by bioassay using the IL-2 indicator cell line CTLL, and measuring incorporation of tritiated thymidine (Figs 4–6) as described.[31] Results are expressed as % IL-2, relative to IL-2 levels in the presence of ovalbumin or ovalbumin synthetic peptide, but no inhibitor. 100% levels correspond to 1–10 ng ml^{-1} IL-2 in the culture supernatants.

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