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Synthesis and properties of D-glucosamine *N*-peptidyl derivatives as substrate analog inhibitors of papain and cathepsin B

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Summary — *N*-peptidyl derivatives of D-glucosamine **7c–7n** were synthesized and tested as reversible, substrate analog inhibitors of cysteine and serine-proteases. D-Glucosamine itself showed fair inhibiting properties against cysteine-proteases. Derivatives **7c–7i**, designed to improve binding at papain active site, displayed reversible inhibition with K_i ranging from 67–860 μM for papain and from 111–2400 μM for cathepsin B. Representative serine proteases were unaffected. No inhibitory activity against human leukocyte elastase was observed for derivatives **7m** and **7n** bearing very effective peptidyl recognizing units for this enzyme.

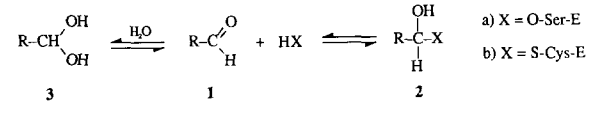
Résumé — Synthèse et propriétés de dérivés *N*-peptidiques de la D-glucosamine comme inhibiteurs analogues du substrat de la papaïne et de la cathepsine B. *Des dérivés* N-*peptidiques de la D-glucosamine* ***7c–7n*** *ont été synthétisés et testés comme inhibiteurs réversibles, analogues du substrat de protéases à cystéine et à sérine. La D-glucosamine, elle-même, présente une certaine activité en tant qu'inhibiteur de protéases à cystéine. Les dérivés* ***7c–7i****, que nous avons préparés en vue d'obtenir une interaction spécifique avec le site actif de la papaïne, ont montré une inhibition réversible avec un* K_i *compris entre 67 et 860 μM pour la papaïne et entre 111 et 2400 μM pour la cathepsine B. Ces substances ont été inefficaces vis-à-vis des protéases à sérine les plus représentatives. Les dérivés* ***7m*** *et* ***7n*** *qui possèdent un groupe peptidique similaire au site actif de l'élastase de leucocytes humains, n'ont aucune activité inhibitrice vis-à-vis de cette enzyme.*

enzyme inhibiting activity / cysteine-protease / serine-protease / D-glucosamine

Introduction

Proteolytic enzymes are ubiquitously distributed in biological tissues and fluids and take part in important and different physiological and pathological processes. Development of efficient inhibitors aims at the control of undesirably high levels of proteolytic activity within biological systems, but is also concerned with the detection and evaluation of the role of targetted enzymes in the living cells and investigations of enzyme chemistry and mechanism [1].

Serine and cysteine proteases make use of the alkoxide (thiolate) of the functional serine (cysteine) unit as an effective nucleophile to attack the carbonyl group of the cleavable peptide bond of the substrate. Several natural and synthetic peptidyl-2-amino-aldehyde derivatives (**1**) behave as potent reversible inhibitors of these families of proteases (E-Ser-OH, E-Cys-SH) by forming stable hemiacetals (**2a**) or hemi-



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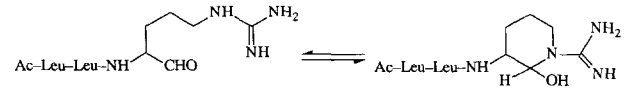
thioacetals (**2b**) which mimic the structure of the tetrahedral reaction intermediate of the enzymes [2, 3]. It is clear that only the unhydrated form of the aldehyde (**1**), which is in equilibrium with the unreactive hydrate form (**3**), is the active form of the inhibitor. For peptidyl argininals such as leupeptin, a natural inhibitor from culture filtrates of actinomycetes [4, 5], the situation is more complex since, in addition to the free aldehyde form **4** (2%) and the inactive hydrate form (42%), it exists as the cyclized form **5** (56%) which is inactive as well [6].



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In spite of the low equilibrium concentration of the active aldehyde form, leupeptin is an effective inhibitor for cysteine proteases and trypsin-like proteases [5].

D-Glucosamine is a 2-aminoaldehyde biosynthesized by living organisms and largely distributed as the *N*-acetyl derivative in macromolecules like glycoproteins, mucopolysaccharides, proteoglycans and peptidoglycans, playing different and important biological roles [7]. While the aldehyde group of *N*-acetyl-D-glucosamine is engaged in very stable β-glucoside bonds used to bind the glycidic units together or to the amino acid units of the macromolecule, simple *N*-acyl derivatives are stable pyranose hemiacetals in equilibrium with other minor cyclic forms and with the open form bearing a free 2-acylamino aldehyde group. Similar derivatives, provided that appropriate peptidyl recognizing units are included, are expected to behave, like leupeptin, as substrate analog inhibitors of serine and cysteine proteases.

Literature references about D-glucosamine and other amino sugars are reported in a review [8]; several *N*-aminoacyl and *N*-peptidyl derivatives have been known since 1932 [9]; others were prepared later [10–13] and were named 'glucopeptides'. A few simple *N*-acyl derivatives have been synthesized and tested as inhibitors of brain hexokinase [14]. More recently, D-glucosamine has been coupled with 4-hydroxybutyric acid, a modulator of dopaminergic activity, in order to obtain derivatives with enhanced biological activity [15]. No references have been found about the activity of 'glucopeptides' as protease inhibitors, while several derivatives of leupeptin have been prepared with the aim of enhancing activity and improve selectivity against different proteases [16].

In view of the widespread diffusion of glucosamine in living organisms, the present work was undertaken to determine to what extent appropriate *N*-peptidyl derivatives, containing the cyclic hemiacetal group of D-glucosamine in place of the cyclic aminoacetal group of argininal, could behave as substrate analog inhibitors of serine and cysteine proteases.

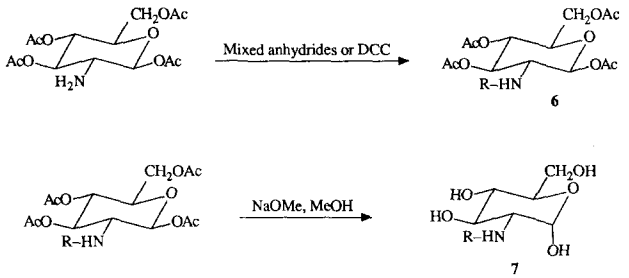
Preliminary experiments with *N*-*Z* and *N*-Ac-D-glucosamine showed no inhibition of papain at 25 and 10 mM concentrations respectively. To our surprise, however, it was found that D-glucosamine hydrochloride itself displayed a fair, reversible inhibiting activity against papain and cathepsin B (K_i 291 and 1136 μM respectively). Representative serine proteases chymotrypsin, trypsin and PPE, on the contrary, were not inhibited at 5.3 mM concentration. This finding seems particularly interesting since treatments with D-glucosamine sulfate are currently used for therapeutic purposes for certain particular disorders *eg* osteoarthritis, due to degenerative processes affecting the cartilage and a study of pharmaco-kinetics of glucosamine in dog and in man has recently been reported [17]. Causes of the degenerative process in osteoarthritis are not known, but other pathological processes, *eg* pulmonary emphysema, muscle dystrophy, malignancy and rheumatoid arthritis have been connected with tissue damage caused by proteolytic enzymes. The lysosomal cysteine proteases cathepsin B, H and L intervene in the breakdown of extracellular-matrix proteins, including collagen and elastin [18, 19] and are probably released at the site of cartilage degradation together with the lysosomal carboxyl protease cathepsin D in rheumatoid arthritis [20]. Supply of exogenous D-glucosamine has been considered important in the treatment of osteoarthritic processes in order to compensate for defects in biosynthesis and turnover of the amino sugar constituents of the cartilage. In the light of our finding, a potential inhibiting activity against proteases responsible for tissue damage could be considered in addition.

Independently of the precise mode of interaction of the free D-glucosamine with papain and cathepsin B, we tried to improve binding at the active site by coupling the amino group with peptidyl units used successfully in known reversible [21] and irreversible [22] inhibitors of cysteine proteases. The peptidyl derivatives **7c–7i** were therefore prepared as substrate analogues of papain and cathepsin B, while **7l–7n** contain amino acid sequences used in trifluoromethyl-ketones [23] which are among the most powerful reversible inhibitors of HLE.

Chemistry

Application of conventional methodologies for direct *N*-acylation of D-glucosamine is hampered owing to the insolubility of the free amino sugar in common aprotic solvents. Different techniques [24–26] have been applied to overcome this problem, but their use in the preparation of peptidyl derivatives has resulted in low yields and complex reaction mixtures. The *N*-peptidyl-D-glucosamines **7c–7n** were therefore prepared according to Lieflænder [11] by acylation of 2-amino-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucose [27] with dicyclohexylcarbodiimide or mixed anhydride methods, followed by deacylation of the peptidyl-tetracetyl derivatives **6c–6n** with sodium methoxide in dry methanol (scheme 1).

The proposed structures of all new compounds are in accordance with the spectroscopic data (IR, ^1H and ^{13}C NMR) and with elemental micro-analyses. Chemical shifts (5.78–5.91 ppm) and coupling constants $J_{1\text{-}2}$ (9 Hz) of the H-1 anomeric proton of the tetracetates **6c–6n** are in accordance with the assigned β-glucoside forms [28–30]. NMR spectra of the corresponding deacetylated products **7c–7n**, obtained



a) R = Ac
b) R = Z
c) R = Ac-Phe
d) R = Z-Phe
e) R = Z-Leu
f) R = Ac-Leu-Leu
g) R = Z-Leu-Leu
h) R = Ac-Gly-Phe
i) R = Z-Gly-Phe
j) R = Z-Pro
m) R = Z-Val-Pro
n) R = Z_2-Lys-Val-Pro
o) R = Ac-D-Phe

Scheme 1.

as crystalline solids, were taken in dimethylsulfoxide, where the equilibration between α and β anomeric forms is prevented. H-1 chemical shifts (4.90–4.97 ppm) are in accordance with the assigned α anomeric forms [15], as well as very small coupling constants $J_{1\text{-}2}$, since unresolved doublets are generally observed. The observed ^{13}C resonances of C-1 (90.34–90.85 ppm) for *N*-peptidylglucosamines **7c–7n** are also characteristic of the α-glucosidic form. These derivatives are thus isolated as crystalline solids in the form of pure α anomers, while equilibria between α and β forms should be readily established in aqueous solution, according to NMR data for D_2O solutions in the case of similar *N*-acyl derivatives of D-glucosamine [15] and ^{13}C NMR spectrum of **7i** in D_2O solution (α/β ratio = 73/27). The diastereoisomer of **7c** containing D-Phe was also prepared, for comparison purposes, by following the same synthetic procedure. All the ^{13}C resonances appear to be clearly differentiated in the 2 diastereoisomers, while the α-glucosidic form is preserved. This result indicates that no epimerization of the amino acid chiral centre intervenes during deacetylation of the tetracetyl derivatives **6** with sodium methoxide under well-controlled conditions.

No spectral signals assignable to the open isomers **7c–7n** with C-1 in the aldehyde form could be detected. This finding is in accordance with the strong preference of the hexoses for the cyclic pyranosic forms. Precise data are not available for our peptidyl derivatives **7c–7n**. Their behaviour, however, is expected to be in accordance with that of D-glucose. For aqueous solutions at pH 6.9, the fractional concentration of the reducible aldehyde form of this sugar has been determined by electrochemical methods and corresponds to 26 ppm [31].

Results

Compounds **7a–7i** were tested as inhibitors of papain and cathepsin B catalyzed hydrolysis of *N*-carbobenzyloxy-glycine-*p*-nitrophenyl ester (Z-Gly-ONp). Reaction progress curves show that inhibition of papain and cathepsin B is time-dependent and the final steady-state velocity is dependent on inhibitor concentration (fig 1). These results indicate that the observed decease in activity was not due to irreversible inhibition of the enzyme or to substrate depletion but to a slow onset of activity loss relative to the rate of diffusion [32–34], *ie* apparent slow-binding inhibition. This type of inhibition may be kinetically described if the substrate concentration is not depleted during the reaction and if the inhibitor concentration is not changed upon formation of the enzyme inhibitor complex [35, 36]. In our case, for competitive inhibitors, the situation may be described by scheme 2, and equations (1) and (2) are valid.

According to this mechanism, the observed rate constant for the approach to steady-state, k_{obs}, is given by equation (2) and a plot of k_{obs} *vs* [I] $K_m/(K_m + [S])$ will yield a straight line with slope and intercept of k_{on} and k_{off}, respectively. Figure 2 shows this plot for cathepsin B inhibition by compound **7c**.

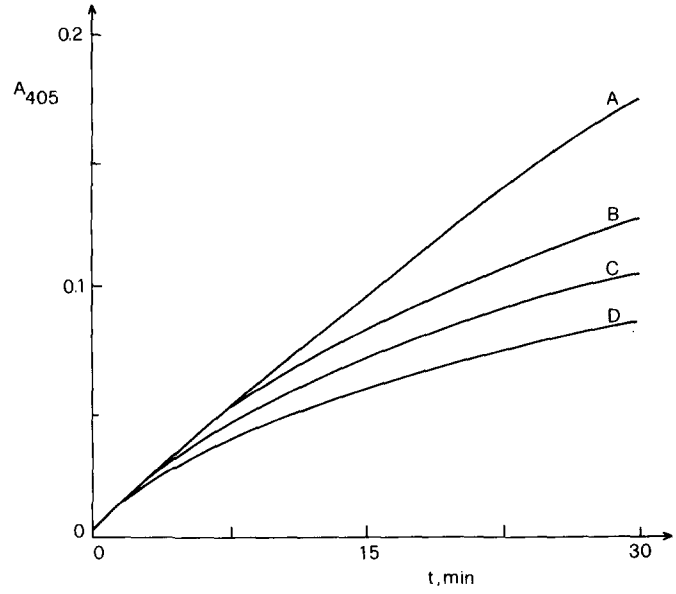
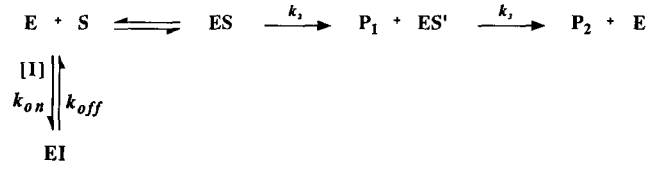


Fig 1. Progress curves for inhibition of cathepsin B by N-Ac-Phe derivative **7c** of D-glucosamine. Reaction conditions were as described in experimental protocols. Inhibitor concentrations are 0, 0.55, 1.25 and 2.50 mM for curves A, B, C and D respectively. Reactions were started by addition of the enzyme.



Scheme 2.

$$K_i = k_{off}/k_{on} \quad (1)$$
$$k_{obs} = k_{on}[I]/(1 + [S]/K_m) + k_{off} \quad (2)$$

All the other inhibitors tested followed the mechanism described. It is worth noting that we always had a linear dependence of k_{obs} from [I] $K_m/(K_m + [S])$ and initial velocities were all identical and equal the initial velocity in the absence of inhibitor (fig 1).

Under our experimental conditions, if an intermediate EI' complex was one of the steps prior to the formation of a stable EI, k_{obs} would be a hyperbolic function of [I] and the initial velocities in figure 1 would decrase [33] with increasing [I]. Our data, of course, are not conclusively against the formation of a preassociation complex EI' prior to the step of k_{on}, since such a complex may be not detectable under the conditions used.

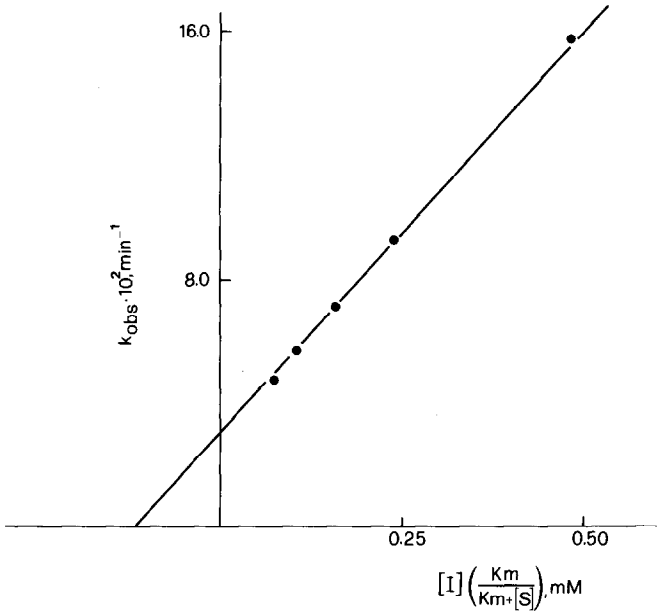
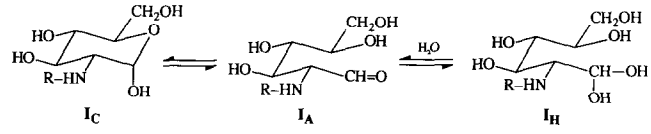


Fig 2. Kinetics of cathepsin B inhibition by N-Ac-Phe derivative **7c** of D-glucosamine plotted according to eqn 2 (see text). First order rate constants for the approach to the steady-state are a linear function of [I]. Determination of k_{on} and k_{off} values was performed by nonlinear regression analysis of the data as described in *Experimental protocols.*

The results we obtained from all the compounds tested are suggestive of slow binding inhibition, but we regard this with caution, since the compounds studied are expected to be in equilibrium between the active aldehyde form, I_A, and the inactive cyclic, I_C, and hydrated, I_H forms.



Thus, the binding of glucosamine derivative to cathepsin B and papain may show a lag phase due, for example, to the poor availability of the active form rather than to slow-binding inhibition. According to this hypothesis, the enzyme initially reacts with I_A and consequently the formation of I_A from I_{tot} becomes rate-limiting for inhibition under conditions of $[I_A] \ll [E] \ll [I_{tot}]$. However, this first explanation seems unlikely as we never observed instantaneous inhibition by I_A even though a discrete amount of I_A is present at zero time, perhaps 2.6 x 10^{-5} fold I_{tot} assuming a similitude with D-glucose equilibrium [31].

This lack of instantaneous inhibition by the initial concentration of I_A may be due to a bimolecular enzymatic reaction at enzyme and inhibitor concentrations in the range of 10^{-9}–10^{-10} M. Within this range of concentrations collision rates are slow because the rate constant for formation of EI complex from E and I_A is related to $k_{on}[I_A]$ [34]. Under the conditions we used, enzyme concentration ranged from 6 x 10^{-9} to ≈ 1 x 10^{-8} M and $[I_A]$ varied from 260–10 nM. Fast second-order rate constants for enzyme and substrate association are usually in the range of 1 x 10^7 M^{-1} s^{-1} to 1 x 10^8 M^{-1} s^{-1} [37]. Multiplication of these values by the concentration of I_A, 50 nM (about the middle of the range used in this study) corresponding to 2 mM I_{tot}, yields pseudo first-order constants, k_{on} $[I_A]$, of 5–0.5 s^{-1}. These values correspond to $t_{1/2}$ of 0.14 to 1.4 s for the reaction of I_A with cathepsin B. These calculated values for $t_{1/2}$ are smaller than the value obtained from progress curves since **3a** requires half-times of ≈ 4–10 min to achieve a steady state inhibited velocity under the conditions used.

One of the reasons for the observed lag phase prior to the inhibition could be that there is a slow interconversion rate between the inactive forms I_C and I_H and the aldehydic form I_A of the inhibitor. ^{13}C NMR spectrum of the D-glucosamine derivative **7i** in D_2O showed C-1 signals at 91.60 (α anomer) and 95.56 δ (β anomer) in the estimated ratio 73/27 after 60 min of accumulation. This finding confirms the expected equilibration between the cyclic forms through the

aldehyde form, but kinetic data could not be obtained by NMR spectroscopy. However, in the case of leupeptin it has been shown [6] that, even at nanomolecular concentration of leupeptin and enzyme, achievement of the equilibrium between I_H and I_A is sufficiently rapid so that the concentration of I_A in the presence of thiol protease will always be close to equilibrium concentration of I_A. In our case, assuming for a value $[I_A]$ of 2.6×10^{-5} $[I_{tot}]$ with enzyme concentration ranging from 6×10^{-9} to 1×10^{-8} M, $[I_A]$ is enough larger than [E] for all the inhibitors tested, with the exception of few progress curves corresponding to 0.4 and 0.25 mM $[I_{tot}]$.

Besides these important considerations on the nature of the phenomena observed, we quantitatively described them by using terminology and equations for slow-binding inhibition. Tables I and II summarize the kinetic parameters for the D-glucosamine derivatives we tested. The most effective appear to be **7c** and **7f** for both cysteine-proteases. The serine-proteases chymotrypsin, trypsin, and porcine pancreatic elastase were unaffected by all the compounds **7c–7i** as well as human leukocyte elastase by **7m** and **7n** under the conditions described in the *Experimental protocols*.

Discussion

Simple *N*-acyl derivatives like *N*-acetyl and *N*-*Z*-D-glucosamine **7a** and **7b** are devoid of inhibiting activity against papain at 25 and 10 mM concentration respectively. Introduction of peptidyl recognizing units useful for binding at the active site of the enzyme, lead to the reversible inhibitors **7c–7i** with K_i ranging from 67–860 μM (table I). Furthermore, no inhibiting activity has been observed for the tetracetyl derivative **6c**, where the hemiacetal function is blocked, while the corresponding *N*-peptidyl-D-glucosamine **7c** is one of the most active derivatives. These results suggest that the simultaneous presence of the recognizing unit and of the aldehyde group released by the hemiacetal function is required for the onset of biological activity. Increase of the K_i observed with **7o** by changing from L to D the configuration of the phenylalanine unit of **7c** is also in accordance with the proposed mechanism.

All the peptide chains used to improve binding contain at least a Phe or Leu unit owing to the strong preference of papain as well as cathepsin B for substrates having L-phenylalanine at P_2 [38]. The highest inhibiting activity (K_i = 67 μM) has been obtained by employing the dipeptidyl residue Ac-Leu-Leu which characterizes leupeptin, but the same result was achieved by the use of a single aminoacyl unit, Ac-Phe, in **7c**. Acetyl derivatives at the amino terminal group gave regularly better inhibitors than the corresponding Z-derivatives as a consequence, probably, of steric restrictions at subsite S_3 for the aminoacyl derivative **7d** and at subsite S_4 for the dipeptidyl derivatives **7g** and **7i**.

The *N*-peptidyl-D-glucosamines with the highest inhibiting activity for papain were tested on cathepsin B. In accordance with the results obtained for other peptidyl aldehydes and of the resemblance of the active site of these proteases, the inhibiting constants are similar.

Table I. Inhibition of papain by D-glucosamine and *N*-peptidyl-D-glucosamines. 100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (v/v); [E] = 6×10^{-9} M; [S] = 125 μM ($K_m \times 12.5$). Replicate determinations indicate standard deviation for kinetic parameters less than 20%.

Compound	*Range (mM)*	k_{on} *(M^{-1} min^{-1})*	k_{off} *(min^{-1})*	k_i *(μM)*	$(K_i)_{cor}$ *(nM)* [a]
D-glucosamine·HCl	5.3–1.1	55	0.016	291	
7a	25			NI[b]	
7b	10			NI[b]	
7c	2.5–0.55	210	0.014	67	1.7
7d	2.5–0.55	190	0.037	195	5.1
7e	10–2.5	36	0.031	860	22.4
7f	1.25–0.25	520	0.035	67	1.7
7g	2.5–0.55	250	0.017	70	1.8
7h	5.0–1.25	50	0.012	240	6.2
7i	2.5–1.0	90	0.027	300	7.8
7o	3.75–0.55	360	0.046	128	3.3

[a]Calculated on the basis of a fractional concentration of the aldehyde form of 2.6×10^{-5} determined for D-glucose [31]; [b]no inhibition observed at the concentration indicated.

Table II. Inhibition of cathepsin B by D-glucosamine and *N*-peptidyl-D-glucosamines. 100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (V/V); [E] = 8×10^{-9} M; [S] = 125 μM ($K_m \times 4.2$); replicate determinations indicate standard deviation for the kinetic parameters less than 20%.

Compound	*Range (mM)*	k_{on} *(M^{-1} min^{-1})*	k_{off} *(min^{-1})*	k_i *(μM)*	$(K_i)_{cor}$ *(nM)* [a]
D-glucosamine·HCl	5.3–1.1	22	0.025	1136	
7c	2.5–0.4	270	0.03	111	2.9
7f	2.5–0.25	400	0.05	125	3.2
7g	4.5–1.25	50	0.05	1000	26
7h	10–2.5	25	0.06	2400	62
7o	3.75–0.55	110	0.065	591	15

[a]Calculated on the basis of a fractional concentration of the aldehyde form of 2.6×10^{-5} determined for D-glucose [31].

In spite of the high effectiveness of the Z_2-Lys-Val-Pro sequence incorporated in the trifluoromethylketone Z_2-Lys-Val-Pro-Val-CF_3 which was recently reported by Stein *et al* [23] to have a K_i value < 0.1 nM, the corresponding D-glucosamine derivative **7n**, as well as **7m**, showed no inhibiting activity against HLE at 1 mM concentration. The lack of activity of these D-glucosamine derivatives designed as inhibitors of HLE, with respect to **7c–7i** tested against cysteine-proteases, may probably reflect the difference in the primary specificity between the 2 enzyme families. In serine-proteases primary specificity is essentially controlled by the S_1 subsite and HLE, with increased hydrophobicity of the active site prefers Val at P_1 [39]. The peptidyl derivatives **7m** and **7n**, presenting the strongly hydrophilic glucosamine residue at P_1, will fail to establish the optimal binding at the S_1 subsite and will find, in addition, steric restrictions more important than the positive contributions of the P_2-P_5 residues. For papain, on the contrary, the primary specificity is controlled by the hydrophobic subsite S_2, and positive contributions to binding of Phe or Leu in P_2 will result more important than possible unfavourable interactions of the D-glucosamine residue in P_1.

Inhibition of serine proteases was also tested for all D-glucosamine derivatives **7c–7i**. No inhibition of PPE, trypsin and chymotrypsin was observed at 5 mM and 3 mM concentration of **7c–7h** and **7i** respectively. This finding appears to be in accordance with the previous discussion and indicates that *N*-peptidyl-D-glucosamines **7c–7i**, contrary to other natural and synthetic peptidyl aldehydes which inhibit both serine and cysteine-proteases, are selective reversible inhibitors of cysteine-proteases.

On the basis of the assumption that only the carbonyl form of the inhibitor binds directly to the enzyme, it was proposed [23] that the observed values of K_i and k_{on} should be corrected for the fraction of total inhibitor that exists in the hydrate, unreactive form, if these parameters are to reflect the interaction of enzyme and inhibitor carbonyl form. Based on a highly accurate study [6] on the active and inactive forms, $(K_i)_{cor}$ of 2.2 x 10^{-11} M and 1 x 10^{-10} M to papain and cathepsin B have been calculated for leupeptin. The fractional concentration of the active form of the inhibitor, 2 x 10^{-2} was obtained by ^{1}H NMR measurements. Fractional concentrations of the aldehyde form for carbohydrates are by far lower and require a polarographic method of determination. The observed value for D-glucose was 2.6 x 10^{-5} [31]. With the hypothesis that equilibria between aldehyde and pyranosic forms should not be substantially modified by substitution of the C-2 hydroxyl group for peptidyl chains, the value of 2.6 x 10^{-5} has been assumed as the approximate fractional concentration of the aldehyde form of the *N*-peptidyl-D-glucosamines for the calculation of their $(K_i)_{cor}$ (tables I and II). The value obtained for papain inhibition with **7f**, bearing the Ac-Leu-Leu unit characteristic of leupeptin, is 1.7 x 10^{-9} M, some 17-fold greater than that of leupeptin. The relative proximity of these values seems to support our hypothesis on the mechanism of inhibition of cysteine-proteases by *N*-peptidyl derivatives of D-glucosamine and confirm the reduced importance of the hydrophilic chain of the amino sugar at P_1 site in this case.

Experimental protocols

Chemical synthesis

Melting points (Büchi oil bath apparatus) are uncorrected. IR spectra were obtained with a Perkin–Elmer 521 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian EM 390 spectrometer (for compounds **6c–6o**); ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 spectrometer (for compounds **7c–7o**); in all cases TMS was used as internal standard. Optical rotations were determined with a Schmidt-Haensch 1604-polarimeter. Elemental microanalyses were within ± 0.4% of the calculated values.

N-Acylation of 2-amino-1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucose. General procedure

Method A. A solution of the required protected amino acid or peptide (1 mmol) and *N*-methylmorpholine (1 mmol) in anhydrous THF (2 ml) was cooled to –15°C and *i*-butylchloroformate (1 mmol) was added dropwise under stirring. After 30 min a solution of 2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucose hydrochloride (1 mmol) and *N*-methylmorpholine (1 mmol), in anhydrous THF (2 ml) was added slowly, while a temperature of –15°C was maintained. The resulting reaction mixture was stored for 15 h at 4°C in a refrigerator, allowed to warm to room temperature and filtered. Solution was concentrated at reduced pressure and the residue dissolved in EtOAc (30 ml), washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4 the solvent was removed under reduced pressure to give the crude product.

Method B. A solution of the required protected amino acid or peptide (1 mmol), 2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucose hydrochloride (1 mmol) and triethylamine (1 mmol) in anhydrous CH_2Cl_2 (8 ml) was cooled to –15°C and a solution of dicyclohexylcarbodiimide (1 mmol) in anhydrous CH_2Cl_2 was added dropwise under stirring at the same temperature. After stirring for 3 h at –15°C the reaction mixture was stored in a refrigerator overnight. Precipitated dicyclohexylurea was filtered and the solvent evaporated. The residue was dissolved in EtOAc (30 ml) and washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4 the solvent was removed under reduced pressure to give the crude product.

2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6c***

N-Acetyl-L-phenylalanine (1.2 g, 5.8 mmol) was coupled by *Method A*. Crystallization from AcOEt gave the pure product, 2.43 g (85%): mp = 225–227°C; $[\alpha]_D^{22} = -12°$ (1.0; $CHCl_3$). IR ($CHCl_3$): main peaks at 3621, 3434, 2974, 2875, 1754, 1672, 1389, 1244, 1047 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.87, 1.93, 2.00, 2.05 and 2.07 (5 s, 15H, CH_3CO), 3.20 (bs, 2H, Phe CH_2), 3.74–3.96 (m, 1H, H-5), 3.98–4.35 (m, 3H, H-2,6,6'),

4.36–4.66 (m, 1H, Phe CH), 5.05 (t, 1H, J = 9 Hz, H-4), 5.20–5.44 (m, 1H, H-3), 5.88 (d, 1H, J = 9 Hz, H-1), 7.30 (s, 5H, C_6H_5), 7.96 (bs, 2H, NH).

2-(N-Carbobenzyloxy)-L-phenylalanylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6d***

N-Carbobenzyloxy-L-phenylalanine (1.5 g, 5.0 mmol) was coupled by *Method A*. The crude product was crystallized from MeOH, to give 2.7 g (86%): mp = 208–209°C; $[\alpha]_D^{22}$ = –13.5° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3689, 3412, 2997, 2957, 1755, 1710, 1366, 1244, 1081 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.94, 1.97, 2.02 and 2.04 (4 s, 12H, CH_3CO), 2.65–3.35 (m, 2H, Phe CH_2), 3.71–3.98 (m, 1H, H-5), 4.10–4.50 (m, 4H, Phe CH and H-2,6,6'), 5.02 (s, 2H, Z CH_2), 5.10–5.24 (m, 2H, H-3,4), 5.80 (d, 1H, J = 9 Hz, H-1), 6.55–6.84 (m, 1H, NH), 7.08–7.42 (m, 10H, aromatics).

2(N-Carbobenzyloxy)-L-leucylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6e***

Obtained by acylation with *N*-carbobenzyloxy-L-leucine (0.936 g, 3.82 mmol) by *Method A*. The crude product was crystallized from EtOH, to give 1.95 g (89%): mp = 208–210°C; $[\alpha]_D^{22}$ = –13.5° (1.0; $CHCl_3$): main peaks at 3687, 3431, 2958, 1754, 1513, 1369, 1242, 1080 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.95 (d, 6H, J = 4.5 Hz, Leu CH_3), 1.40–1.75 (m, 3H, Leu βCH$_2$γCH), 1.96 and 2.14 (2 s, 12H, CH_3CO), 3.75–4.00 (m, 1H, H-5), 4.02–4.48 (m, 4H, Leu αCH and H-2,6,6'), 5.12 (s, 2H, Z CH_2), 5.17–5.45 (m, 2H, H-3,4), 5.84 (d, 1H, J = 9 Hz, H-1), 6.63 (d, 1H, J = 9 Hz, NH), 7.45 (s, 5H, C_6H_5).

2-[N-(N-Acetyl)-L-leucyl]-L-leucylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6f***

Obtained from [*N*-(*N*-acetyl)-L-leucyl]-L-leucine (1.5 g, 5.2 mmol) according to *Method A*. Silica gel chromatography ($CHCl_3$/EtOAc 7:3) and crystallization from EtOH gave the pure product: 2.5 g (78%): mp = 232–234°C; $[\alpha]_D^{22}$ = –30° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3687, 3432, 3321, 2960, 1754, 1672, 1504, 1369, 1243, 1079 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.90 (d, 12H, J = 4.5 Hz, Leu CH_3), 1.52 (bs, 6H, Leu βCH$_2$γCH), 2.04 and 2.06 (2 s, 15H, CH_3CO), 3.74–4.00 (m, 1H, H-5), 4.10–4.68 (m, 5H, Leu αCH and H-2,6,6'), 5.12 (t, 1H, J = 9 Hz, H-4), 5.44 (t, 1H, J = 9 Hz, H-3), 5.79 (d, 1H, J = 9 Hz, H-1), 6.65 (d, 1H, J = 7.5 Hz, NH), 7.12 (d, 1H, J = 7.5 Hz, NH), 7.62 (d, 1H, J = 9 Hz, NH).

2-[N-(N-Carbobenzyloxy)-L-leucyl]-L-leucylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6g***

Obtained by acylation with [*N*-(*N*-carbobenzyloxy)-L-leucyl]-L-leucine (2.1 g, 5.7 mmol) by *Method A*. The crude product was purified by silica gel chromatography ($CHCl_3$) and crystallization from EtOH to give 2.8 g (70%): mp = 210–212°C; $[\alpha]_D^{22}$ = –20.5° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3687, 3426, 3337, 2960, 1755, 1709, 1501, 1369, 1241, 1079 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (d, 12H, J = 4.5 Hz, Leu CH_3), 1.32–1.87 (m, 6H, Leu βCH$_2$γCH), 1.98 and 2.15 (2 s, 12H, CH_3CO), 3.75–4.00 (m, 1H, H-5), 4.02–4.53 (m, 5H, Leu αCH and H-2,6,6'), 5.16 (s, 2H, Z CH_2), 5.20 (t, 1H, J = 9 Hz, H-4), 5.50 (t, 1H, J = 9 Hz, H-3), 5.85 (d, 1H, J = 9 Hz, H-1), 6.53 (d, 1H, J = 7.5 Hz, NH), 7.19 (d, 1H, J = 9 Hz, NH), 7.35 (s, 5H, C_6H_5).

2-[N-(N-Acetyl)-glycyl]-L-phenylalanylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6h***

Obtained from [*N*-(*N*-acetyl)-glycyl]-L-phenylalanine (0.8 g, 3.0 mmol) according to *Method A* (THF containing 20% DMF was required to dissolve the starting material in this case). The crude product was purified by crystallization from EtOH and gave 1.6 g (90%): mp = 207–209°C; $[\alpha]_D^{22}$ = + 15° (1.0; MeOH); IR ($CHCl_3$): main peaks at 3275, 3087, 1748, 1642, 1553, 1370, 1226, 1072 cm^{-1}; ^1H NMR (CD_3OD) δ 1.94, 1.96, 2.00 and 2.03 (4 s, 15H, CH_3CO), 2.77–3.14 (m, 2H, Phe CH_2), 3.84–3.98 (m, 1H, H-5), 4.00 (bs, 2H, Gly CH_2), 4.10–4.33 (m, 2H, H-6,6'), 4.34–4.68 (m, 2H, Phe CH and H-2), 5.00 (t, 1H, J = 9 Hz, H-4), 5.35 (t, 1H, J = 9 Hz, H-3), 5.85 (d, 1H, J = 9 Hz, H-1), 7.28 (s, 5H, C_6H_5).

2-[N-(N-Carbobenzyloxy)-glycyl]-L-phenylalanylamido-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6i***

Obtained from [*N*-(*N*-carbobenzyloxy)-glycyl]-L-phenylalanine (1.07 g, 3.0 mmol) according to *Method A*. Silica gel chromatography ($CHCl_3$) and crystallization from MeOH gave the pure product, 1.23 g (60%): mp = 178–180°C; $[\alpha]_D^{22}$ = –11° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3687, 3413, 3010, 1754, 1692, 1512, 1367, 1241, 1078 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.93, 1.96, 2.00 and 2.05 (4 s, 12H, CH_3CO), 2.80–3.24 (m, 2H, Phe CH_2), 3.68–3.95 (m, 3H, Gly CH_2 and H-5), 4.00–4.44 (m, 3H, H-2,6,6'), 4.46–4.76 (m, 1H, Phe CH), 4.96–5.50 (m, 2H, H-3,4), 5.13 (s, 2H, Z CH_2), 5.67–5.94 (m, 2H, H-1 and NH), 6.75 (d, 1H, J = 7.5 Hz, NH), 7.12 (m, 6H, Z C_6H_5 and NH), 7.38 (s, 5H, Phe C_6H_5).

2-(N-Carbobenzyloxy)-L-prolylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6l***

N-Carbobenzyloxy-L-proline (1.25 g, 5.0 mmol) was coupled according to *Method A*. The pure product was obtained by trituration of the crude solid with anhydrous Et_2O, 2.77 g (96%): mp = 170–172°C; $[\alpha]_D^{22}$ = –42° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3621, 3468, 2975, 1754, 1693, 1508, 1409, 1240, 1079 cm^{-1}; ^1H NMR (DMSO–d_6) δ 1.86 (bs, 4H, Pro βγCH$_2$), 1.98 and 2.03 (2 s, 12H, CH_3CO), 3.26–3.66 (m, 2H, Pro δCH$_2$), 3.84–4.29 (m, 4H, H-2,5,6,6'), 4.30–4.46 (m, 1H, Pro CH), 4.89 (t, 1H, J = 9 Hz, H-4), 5.10 (s, 2H, Z CH_2), 5.30 (t, 1H, J = 9 Hz, H-3), 5.80 (d, 1H, J = 9 Hz, H-1), 7.40 (s, 5H, C_6H_5), 7.88–8.30 (m, 1H, NH).

2-[N-(N-Carbobenzyloxy)-L-valyl]-L-prolylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6m***

[*N*-(*N*-Carbobenzyloxy)-L-valyl]-L-proline (1.9 g, 5.4 mmol) was coupled according to *Method B*. The crude product was purified by silica gel chromatography (EtOAc/$CHCl_3$ 1:1)) and triturated with hexane, to give 2.6 g (72%): mp = 169–170°C; $[\alpha]_D^{22}$ = –39° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3620, 3426, 2974, 1754, 1512, 1390, 1241, 1045 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.94 and 0.98 (2 d, 6H, J = 6 Hz, Val CH_3), 1.86, 2.01, 2.06 and 2.11 (4 s, 12H, CH_3CO), 2.01 (bs, 5H, Val βCH and Pro βγCH$_2$), 3.75 (bs, 3H, Pro δCH$_2$ and H-5), 4.06–4.47 (m, 5H, Val and Pro αCH and H-2,6,6'), 4.97–5.48 (m, 2H, H-3,4), 5.13 (s, 2H, Z CH_2), 5.73 (d, 1H, J = 9 Hz, NH), 5.91 (d, 1H, J = 9 Hz, H-1), 6.87 (d, 1H, J = 9 Hz, NH), 7.45 (s, 5H, C_6H_5).

2-{N-[N-(N,N'-Dicarbobenzyloxy)-L-lysyl]-L-valyl}-L-prolylamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucose ***6n***

Obtained by acylation with *N*-[*N*-(*N*,*N*'-dicarbobenzyloxy-lysyl)-valyl]-proline (0.77 g, 1.4 mmol) by *Method A*. The pure product was obtained by silica gel chromatography (EtOAc) and crystallization from $CHCl_3$/*i*Pr–ether 2:1, 1.2 g (96%): mp = 168°C; $[\alpha]_D^{22}$ = –20° (1.0; $CHCl_3$); IR ($CHCl_3$): main peaks at 3620, 3431, 2975, 1754, 1499, 1447, 1368, 1245 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.85 and 0.88 (2 d, 6H, J = 6 Hz, Val CH_3), 1.33 (bs, 6H, Lys βγsCH$_2$), 1.90, 1.95, 1.98 and 2.05 (4 s, 12H, CH_3CO), 1.68–2.28 (m, 5H, Pro βγCH$_2$ and Val βCH), 2.98 (bs, 2H, Lys εCH$_2$), 3.66 (bs, 3H, Pro δCH$_2$ and H-5), 3.90–4.48 (m, 6H, Lys, Val, Pro αCH and H-2,6,6'), 4.78–5.35

(m, 2H, H-3,4), 5.04 (s, 4H, Z CH_2), 5.78 (d, 1H, J = 9 Hz, H-1), 7.25 (bs, 2H, NH), 7.45 (s, 10H, Z C_6H_5), 7.84 (d, 1H, J = 9 Hz, NH), 8.10 (d, 1H, J = 9 Hz, NH).

Deacetylation of 1,3,4,6-tetra-O-acetyl-2-deoxy-β-D-glucose peptidyl derivatives. General procedure
Method C. To a solution of tetra-*O*-acetyl derivative (1 mmol) in anhydrous THF (10 ml) cooled to –10°C, 1 M sodium methoxide, freshly prepared from dry CH_3OH (4 mmol), was added slowly under stirring. After 10 min at –10°C and 20 min at room temperature, pH of the reaction mixture was adjusted to 5–6 by careful addition of 6 N H_2SO_4. Solvents were removed under reduced pressure. The product was extracted from crude residue with boiling EtOH and obtained as a crystalline solid by cooling, except where otherwise indicated.

2-(N-Acetyl)-L-phenylalanylamido-2-deoxy-β-D-glucose ***7c***
Tetra-*O*-acetyl derivative **6c** (1.07 g, 2 mmol) was deacetylated according to *Method C*. Crystallization from EtOH gave the pure product, 0.49 g (67%): mp = 204–207°C; $[\alpha]_D^{22}$ = + 44° (1.0; DMF); IR (KBr): main peak at 3286, 2921, 1626, 1547, 1373, 1113 cm^{-1}; ^1H NMR (DMSO–d_6) δ 1.68–2.00 (m, 3H, CH_3CO), 2.95–3.23 (m, 2H, Phe CH_2), 3.40–3.65 (m, 6H, H-2,3,4,5,6,6'), 4.45–4.70 (m, 1H, Phe CH), 4.95 (s, 1H, H-1), 7.08–7.33 (m, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 22.35 (CH_3), 37.92 (Phe CH_2), 53.60 (Phe CH), 54.32 (C-2), 61.00 (C-6), 70.83 and 70.98 (C-3, C-4), 71.93 (C-5), 90.61 (C-1), 125.96–138.12 (aromatics), 168.84 (AcCO), 171.61 (Phe CO).

2-(N-Carbobenzyloxy)-L-phenylalanylamido-2-deoxy-β-D-glucose ***7d***
Tetra-*O*-acetyl derivative **6d** (0.5 g, 0.8 mmol) was deacetylated according to *Method C*. Product was recrystallized from MeOH to give 0.26 g (70%): mp = 205–206°C; $[\alpha]_D^{22}$ = + 45° (1.0; DMF); IR (KBr): main peaks at 3338, 2919, 1677, 1535, 1265, 1106 cm^{-1}; ^1H NMR (DMSO–d_6) δ 3.03–3.13 (m, 2H, Phe CH_2), 3.40–3.65 (m, 6H, H-2,3,4,5,6,6'), 4.36–4.68 (m, 1H, Phe CH), 4.91–4.97 (m, 3H, Z CH_2 and H-1), 7.18–7.35 (m, 10H, aromatics); ^{13}C NMR (DMSO–d_6) δ 37.88 (Phe CH_2), 54.15 (Phe CH), 55.82 (C-2), 60.92 (C-6), 64.98 (Z CH_2), 70.39 and 70.89 (C-3, C-4), 72.02 (C-5), 90.57 (C-1), 125.99–138.24 (aromatics), 155.58 (Z CO), 171.58 (Phe CO).

2-(N-Carbobenzyloxy)-L-leucylamido-2-deoxy-β-D-glucose ***7e***
Tetra-*O*-acetyl derivative **6e** (0.5 g, 0.84 mmol) was deacetylated according to *Method C*. Crystallization from EtOH gave 0.18 g (50%): mp = 171–172°C, Lit [13] 188°C; $[\alpha]_D^{22}$ = + 45° (1.0; DMF); IR (KBr): main peaks at 3307, 2956, 1690, 1535, 1263, 1116 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.75–0.92 (m, 6H, Leu CH_3), 1.38–1.50 (m, 2H, Leu CH_2), 1.52–1.67 (m, 1H, Leu γCH), 3.40–3.76 (m, 6H, H-2,3,4,5,6,6'), 4.00–4.18 (m, 1H, Leu αCH), 4.93 (s, 1H, H-1), 4.92–5.08 (m, 2H, Z CH_2), 7.35 (s, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 21.18 and 23.04 (Leu CH_3), 24.11 (Leu γCH), 40.88 (Leu CH_2), 52.91 (Leu αCH), 54.13 (C-2), 60.82 (C-6), 65.33 (Z CH_2), 70.15 and 70.71 (C-3, C-4), 71.87 (C-5), 90.38 (C-1), 127.50–136.87 (aromatics), 155.82 (Z-CO), 172.65 (Leu CO).

2-[N-(N-Acetyl)-L-leucyl]-L-leucylamido-2-deoxy-β-D-glucose ***7f***
Tetra-*O*-acetyl derivative **6f** (0.5 g, 0.81 mmol) was deacetylated according to *Method C*. Crystallization from EtOH gave 0.23 g (63%): mp = 175–178°C (dec); $[\alpha]_D^{22}$ = + 10° (1.0; DMF); IR (KBr): main peaks at 3289, 2937, 1633, 1551, 1369, 1035 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.53–0.94 (m, 12H, Leu CH_3), 1.41 (bs, 4H, Leu CH_2), 1.58 (bs, 2H, Leu γCH), 1.84 (s, 3H, CH_3CO), 3.18–3.71 (m, 6H, H-2,3,4,5,6,6'), 4.14–4.40 (m, 2H, Leu αCH), 4.92 (s, 1H, H-1); ^{13}C NMR (DMSO–d_6) δ 21.40–23.11 (Leu CH_3), 22.33 ($AcCH_3$), 24.00 and 24.11 (Leu γCH), 40.35 and 40.95 (Leu CH_2), 50.73 and 51.20 (Leu αCH), 54.22 (C-2), 60.87 (C-6), 70.17 and 70.74 (C-3, C-4), 71.94 (C-5), 90.34 (C-1), 169.45 (AcCO), 171.77 and 172.03 (Leu CO).

2-[N-(N-Carbobenzyloxy)-L-leucyl]-L-leucylamido-2-deoxy-β-D-glucose ***7g***
Tetra-*O*-acetyl derivative **6g** (0.5 g, 0.7 mmol) was deacetylated according to *Method C*. Crystallization from EtOH gave 0.24 g (63%): mp = 190–191°C, Lit [13] 202°C; $[\alpha]_D^{22}$ = + 15° (1.0; DMF); IR (KBr): main peaks at 3291, 2957, 1690, 1642, 1536, 1139 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.95 and 0.98 (2 d, 12H, J = 9 Hz, Leu CH_3), 1.35–1.52 (m, 4H, Leu CH_2), 1.53–1.70 (m, 2H, Leu γCH), 3.42–3.70 (m, 6H, H-2,3,4,5,6,6'), 4.30–4.60 (m, 2H, Leu αCH), 4.92 (bs, 1H, H-1), 5.05 (s, 2H, Z CH_2), 7.33 (s, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 21.36–23.20 (Leu CH_3), 23.96 and 24.16 (Leu γCH), 40.66 and 41.40 (Leu CH_2), 50.72 and 53.33 (Leu αCH), 54.29 (C-2), 61.07 (C-6), 65.33 (Z CH_2), 70.48 and 71.11 (C-3, C-4), 72.04 (C-5), 90.55 (C-1), 127.55–136.97 (aromatics), 155.86 (Z CO), 171.78 and 172.06 (Leu CO).

2-[N-(N-Acetyl)-glycyl]-L-phenylalanylamido-2-deoxy-β-D-glucose ***7h***
Tetra-*O*-acetyl derivative **6h** (0.54 g, 1.0 mmol) was deacetylated according to *Method C* (20% anhydrous DMF in CH_3OH was required to dissolve the starting material in this case). Crystallization from EtOH gave 0.22 g (51%); mp = 208–209°C; $[\alpha]_D^{22}$ = + 146° (1.0; DMF); IR (KBr): main peaks at 3312, 2958, 1656, 1554, 1242, 1033 cm^{-1}; ^1H NMR (DMSO–d_6) δ 1.83 (s, 3H, CH_3CO), 2.97–3.23 (m, 2H, Phe CH_2), 3.44–3.73 (m, 8H, H-2,3,4,5,6,6' and Gly CH_2), 4.43–4.63 (m, 1H, Phe CH), 4.94 (s, 1H, H-1), 7.21 (bs, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 22.15 ($AcCH_3$), 37.84 (Phe CH_2), 41.70 (Gly CH_2), 53.59 (Phe CH), 54.30 (C-2), 60.81 (C-6), 70.11 and 70.52 (C-3, C-4), 71.85 (C-5), 90.40 (C-1), 126.24–137.48 (aromatics), 168.63 (AcCO), 170.38 and 171.29 (Gly and Phe CO).

2-[N-(N-Carbobenzyloxy)-glycyl]-L-phenylalanylamido-2-deoxy-β-D-glucose ***7i***
Tetra-*O*-acetyl derivative **6i** (0.5 g, 0.73 mmol) was deacetylated according to *Method C*. Crystallization from EtOH gave 0.16 g (42%): mp = 190–192°C (dec); $[\alpha]_D^{22}$ = + 40° (1.0; DMF); IR (KBr): main peaks of 3299, 2923, 1642, 1531, 1264, 1036 cm^{-1}; ^1H NMR (DMSO–d_6) δ 3.00–3.23 (m, 2H, Phe CH_2), 3.42–3.73 (m, 8H, Gly CH_2 and H-2,3,4,5,6,6'), 4.65 (bs, 1H, Phe CH), 4.96 (s, 1H, H-1), 5.03 (s, 2H, Z CH_2), 7.13–7.40 (m, 10H, aromatics); ^{13}C NMR (DMSO–d_6) δ 38.10 (Phe CH_2), 43.07 (Gly CH_2), 53.41 (Phe CH), 54.25 (C-2), 60.86 (C-6), 65.43 (Z CH_2), 70.21 and 70.72 (C-3, C-4), 71.94 (C-5), 90.48 (C-1), 126.10–137.55 (aromatics), 156.35 (Z CO), 168.53 and 171.11 (Gly and Phe CO).

2-(N-Carbobenzyloxy)-L-prolylamido-2-deoxy-β-D-glucose ***7l***
Tetra-*O*-acetyl derivative **6l** (0.87 g, 1.5 mmol) was deacetylated according to *Method C*. Recrystallization from MeOH gave 0.48 g (78%): mp = 184–186°C, Lit [13] 193°C; $[\alpha]_D^{22}$ = + 39° (1.0; DMF); IR (KBr): main peaks at 3391, 2951, 1697, 1659, 1547, 1422, 1134, 1021 cm^{-1}; ^1H NMR (DMSO–d_6) δ 1.68–1.92 (m, 4H, Pro βγCH_2), 3.08–3.15 (m, 2H, Pro δCH_2), 3.42–3.70 (m, 6H, H-2,3,4,5,6,6'), 4.26–4.33 (m, 1H, Pro CH), 4.90 (bs, 1H, H-1), 5.06 (s, 2H, Z CH_2), 7.27–7.40 (m, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 22.90 (Pro γCH_2), 30.21 (Pro βCH_2), 46.46 (Pro δCH_2), 54.02 (C-2), 59.15 (C-6), 60.01 (Pro

CH), 65.43 (Z CH_2), 70.57 and 70.93 (C-3, C-4), 72.03 (C-5), 90.85 (C-1), 126.70–136.96 (aromatics), 153.78 (Z CO), 171.89 (Pro CO).

2-[N-(N-Carbobenzyloxy)-L-valyl]-L-prolylamido-2-deoxy-β-D-glucose ***7m***

Tetra-*O*-acetyl derivative **6m** (1.0 g, 1.5 mmol) was deacetylated according to *Method C*. The crude product was triturated with anhydrous Et_2O to give 0.54 g (70%): mp = 165–166°C; $[\alpha]_D^{22} = -40°$ (1.0; DMF); IR (KBr): main peaks at 3395, 2964, 1632, 1530, 1451, 1036 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.87 and 0.91 (2 d, 6H, *J* = 6 Hz, Val CH_3), 1.73–2.02 (m, 5H, Pro βγCH_2 and Val βCH), 3.08–3.17 (m, 2H, Pro δCH_2), 3.45–3.72 (m, 6H, H-2,3,4,5,6,6'), 4.03 (m, 1H, Val αCH), 4.39 (m, 1H, Pro CH), 4.90 (bs, 1H, H-1), 4.94–5.10 (m, 2H, Z CH_2), 7.33 (s, 5H, aromatics); ^{13}C NMR (DMSO–d_6) δ 18.27 and 18.81 (Val CH_3), 24.28 (Pro γCH_2), 29.06 (Val βCH), 29.69 (Pro βCH_2), 47.10 (Pro δCH_2), 53.93 (Val αCH), 57.74 (C-2), 59.47 (Pro CH), 60.80 (C-6), 65.44 (Z CH_2), 70.58 and 70.64 (C-3, C-4), 71.87 (C-5), 90.58 (C-1), 127.51–136.76 (aromatics), 156.16 (Z CO), 170.43, 171.45 (Val and Pro CO).

2-{N-[N-(N,N'-Dicarbobenzyloxy)-L-lysyl]-L-valyl}-L-prolylamido-2-deoxy-β-D-glucose ***7n***

Tetra-*O*-acetyl derivative **6n** (0.96 g, 1.0 mmol) was deacetylated according to *Method C*. The crude product, obtained by removal of EtOH, was crystallized from CH_2Cl_2/Et_2O 1:1 to give 0.58 g (71%): mp = 168–170°C; $[\alpha]_D^{22} = -26°$ (1.0; MeOH); IR (KBr): main peaks at 3321, 2940, 1704, 1631, 1531, 1254, 1042 cm^{-1}; ^1H NMR (DMSO–d_6) δ 0.84 (bs, 6H, Val CH_3), 1.41 (bs, 6H, Lys βγδCH_2), 1.89 (bs, 5H, Pro βγCH_2 and Val βCH), 2.95 (bs, 2H, Lys εCH_2), 3.04–3.20 (m, 2H, Pro δCH_2), 3.42–3.90 (m, 6H, H-2,3,4,5,6,6'), 4.42 (bs, 3H, Lys, Val Pro αCH), 4.90 (s, 1H, H-1), 5.00 (s, 4H, Z CH_2), 7.30 (s, 10H, aromatics); ^{13}C NMR (DMSO–d_6) δ 18.07 and 19.11 (Val CH_3), 22.70 (Lys γCH_2), 24.32 (Pro γCH_2), 28.94 (Lys βCH_2), 29.08 (Pro βCH_2), 30.06 (Val βCH), 31.46 (Lys δCH_2), 38.37 (Lys εCH_2), 47.08 (Pro δCH_2), 53.97, 54.48 (Lys and Val αCH), 55.31 (C-2), 59.30 (Pro CH), 60.94 (C-6), 65.08 and 65.36 (Z CH_2), 70.66 and 70.75 (C-3, C-4), 72.06 (C-5), 90.74 (C-1), 127.61–137.11 (aromatics), 155.86, 156.06 (Z CO), 169.80, 171.55, 171.84 (Lys, Val, Pro CO).

2-(N-Acetyl)-D-phenylalanylamido-2-deoxy-β-D-glucose ***7o***

Prepared from the corresponding tetra-*O*-acetyl derivative (0.58 g, 1.08 mmol) according to *Method C*. Recrystallization from EtOH gave the pure product, 0.2 g (50%); mp = 124–126°C; $[\alpha]_D^{22} = +46°$ (1.0; DMF); IR (KBr): main peaks at 3298, 2927, 1631, 1549, 1373, 1105 cm^{-1}; ^1H NMR (DMSO–d_6) δ 1.75 (s, 3H, CH_3CO), 2.94–3.14 (m, 2H, Phe CH_2); 3.36–3.71 (m, 6H, H-2,3,4,5,6,6'), 4.35–4.76 (m, 1H, Phe CH), 4.96 (s, 1H, H-1), 7.13–7.33 (m, 5H, C_6H_5); ^{13}C NMR (DMSO–d_6) δ 22.39 (CH_3), 37.93 (Phe CH_2), 53.53 (Phe CH), 54.32 (C-2), 60.99 (C-6), 70.29 and 70.99 (C-3, C-4), 71.98 (C-5), 90.24 (C-1), 126.05–137.90 (aromatics), 168.95 (AcCO), 171.32 (Phe CO).

Enzymatic procedures

Papain assay

Papain EC 3.4.22.2 was obtained from Sigma, product No P-4762, and solutions were made fresh daily by incubating the enzyme (1.5–2 mg) for 45 min at room temperature in 25 ml of 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 0.5 mM L-cysteine according to Thompson [40] with minor modifications. Enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM^{-1} cm^{-1}) [41]. The activated enzyme solution was stored at 4°C and papain fully retained its activity for at least 10 h. All reagents used were from Sigma unless otherwise indicated. All buffers and solutions were made with ultra high quality water (Elga UHQ).

The rate of hydrolysis of Z-Gly-ONp was monitored continuously at 405 nm [42] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing buffer and substrate was used in the reference cell to correct for non-enzymatic hydrolysis of the substrate. The final concentration of the organic solvent in the activity mixture was 12% (v/v) acetonitrile. This concentration of organic solvent is ineffective towards kinetic parameters of papain catalysed hydrolysis of Z-Gly-ONp [43]. Injection of enzyme solution initiated the reaction. A substrate concentration of 125 μM (K_m x 12.5) avoided substrate depletion during all the experiments due to spontaneous and enzymatic hydrolysis.

Cathepsin assay

Cathepsin B EC 3.4.22.1 from bovine spleen, product No C-6286, was purchased from Sigma and solutions were made fresh daily by incubating the enzyme under the same conditions as for papain. Enzyme concentration was determined from absorbance at 280 nm ($E^{1\%}$ = 20 cm^{-1}) [44]. Cathepsin B activity was tested as described for papain [45].

Chymotrypsin and trypsin assay

Chymotrypsin EC 3.4.21.1 from bovine pancreas, product No 27270, was obtained from Fluka. Enzyme was dissolved in 50 mM phosphate buffer pH 6.8 and concentration determined from the absorbance at 280 nm (E = 50 mM^{-1} cm^{-1}) [46]. Chymotrypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at 256 nm [47].

Trypsin EC 3.4.21.4 from bovine pancreas, code TRTPCK, was from Worthington Enzyme (Cooper Biomedical). The enzyme was dissolved in 0.001 N HCl and concentration determined from the absorbance at 280 nm [48]. Trypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at 253 nm [49]. Preincubation (30 min) of D-glucosamine hydrochloride at 5.3 mM and D-glucosamine derivatives **7c–7h** and **7i** at 5.0 mM and 3.0 mM concentration respectively with both the enzymes did not inhibit their activity.

Porcine pancreatic elastase assay

Porcine pancreatic elastase EC 3.4.21.11, product No E-0127, was obtained from Sigma. Enzyme was dissolved in 50 mM phosphate buffer pH 7.0 and concentration determined from $E^{1\%}$ = 22 cm^{-1} at 280 nm [50]. Activity was monitored by following the hydrolysis of Boc-L-Ala-ONp at 347 nm [51], in 50 mM phosphate buffer pH 7.0 at 25°C. 1 mM of Z_2-Lys-Val-Pro and Z-Val-Pro-D-glucosamine derivatives **7m** and **7n** yielded < 20% inhibition of the enzyme which was steady over a 30-min period.

Human leukocyte elastase assay

Human leukocyte elastase EC 3.4.21.37, product No E-1508, was purchased from Sigma. Enzyme was dissolved in 0.1 M phosphate buffer pH 7.6, 0.5 M NaCl and tested in the same buffer according to [52] with minor modifications. 1 mM of Z_2-Lys-Val-Pro and Z-Val-Pro D-glucosamine derivatives **7m** and **7n** did not inhibit HLE activity towards MeOSuc-Ala-Ala-Pro-Val-pNA.

Kinetic calculations and data analysis

Progress curves for slow-binding inhibition are fitted, by non linear regression analysis, to the integrated equation:

$$P = v_s t + (v_o - v_s)\,(1 - e^{-k_{obs}t})/k_{obs} + d \qquad (3)$$

where P is the product concentration, in this case the absorbance at 405 nm, v_o is the initial velocity at $t = 0$, v_s is the final steady-state velocity, k_{obs} is the observed first-order rate constant for the approach to steady-state and d is the displacement of absorbance from zero at $t = 0$. The equation we used is a general expression and described any type of inhibition in which the steady-state is reached by means of a first-order process.

Absorbances were continuously measured and stored in the computerized spectrophotometer. Progress curves were composed of 180 to 360 (absorbance, time) points. Absorbance data points were transferred to a Data System 450 personal computer (Kontron) equipped with a math coprocessor and fitted to eqn 3 by using Enzfitter, a nonlinear regression data analysis programme [53] from Elsevier-Biosoft (Cambridge). Software for collection and handling of progress curves data on computer was supplied by Kontron.

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