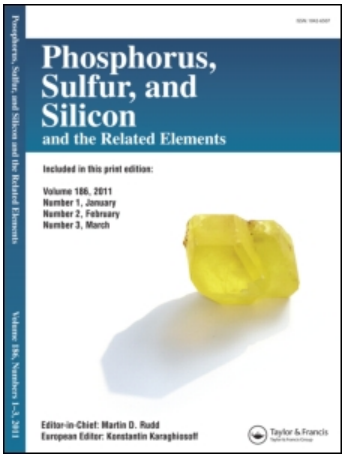

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SYNTHESIS OF PHOSPHONO DIPEPTIDES, INHIBITORS OF CATHEPSIN C

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SYNTHESIS OF PHOSPHONO DIPEPTIDES, INHIBITORS OF CATHEPSIN C

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Phosphono dipeptides containing glycine, glycylglycine or *L*-alanine at N-termini and racemic phosphonic acid analogues of aromatic amino acids, as well as racemic alicyclic aminophosphonates, exhibit moderate inhibitory activity towards cathepsin C. This activity is probably due to the binding of the phosphonate moiety by a positively charged part of the enzyme which is complementary to the carboxylate part of the synthetic dipeptide products of the enzymatic reaction.

Keywords: phosphonic acid analogues; protease inhibitors; substrate analogues

INTRODUCTION

Cathepsins are lysosomal proteases that play an important role in turnover of intracellular proteins. [1] However, extracellularly they have also been implicated in tumor invasion and metastasis. [2, 3] The ability of tumour cells to invade into the extracellular matrix has been attributed to cathepsins released by tumour cells or associated with the plasma membrane tumour cells. [4, 5] Thus, the search for the inhibitors of these enzymes is of interest since they may constitute a new group of anticancer agents.

Cathepsin C, also known as dipeptidyl peptidase I (DPPI, EC 3.4.14.1), is an oligomeric protease that removes dipeptides from the unblocked N-termini of proteins and peptides. It is perhaps the most abundant lysosomal cysteine

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protease. Cathepsin C is implicated in the pathogenesis of rheumatoid arthritis, muscular dystrophy and tumour metastasis. [6, 7]

Substrates of the enzymatic reaction, dipeptides composed of N-terminal glycine or alanine and C-terminal aromatic amino acid are well recognised as the weak inhibitors of cathepsin C. [8] In this paper we report our preliminary studies on the design of the inhibitors of cathepsin C, namely the synthesis and evaluation of inhibitory activity of a series of phosphono dipeptides, analogues of weak dipeptide inhibitors of the enzyme, obtained by replacement of the C-terminal carboxylic moiety of the peptide by the phosphonic acid group. These compounds were synthesized on the premise that the binegative phosphonate anion will be bound by the positively charged carboxylate-binding site of the enzyme. It is well recognised that strong electrostatic binding of phophonate dianion by the appropriate portion of the enzyme accounts significantly for the inhibitory action of many of the phosphonic acid analogues of amino acids. [9]

RESULTS AND DISCUSSION

The design of selective inhibitors of cysteine proteases has been the subject of considerable research effort in recent years. As far as we are aware, the replacement of C-terminal group of short peptides which are the synthetic substrates of these enzymes by phosphonic acid moiety has not been used previously for the design of inhibitors of these proteases. The choice of phosphono peptides is based on the recognition that ionic interactions are an important component of enzymatic specificity. A cationic group of the enzyme should be complementary to the carboxylate and thus analogues of carboxylate should also contain delocalized charge. Phosphonates provide an analogy in this regard, however, they are stereoelectronically distinct since the functional group is tetrahedral.

Although all the studied phosphono peptides were only moderate inhibitors of DPPI some relationship between structure and inhibitory potency of these compounds may be drawn.

None of the tested compounds appear to act as competitive inhibitor of the enzyme indicating that these compounds do not act as simple analogues of the synthetic substrates. Most of the inhibitors exhibited mixed type of inhibition (compounds **3**, **4**, **6**, **7** and **8**), compound **2**, of relatively low potency, acted as non-competitive inhibitor, whereas the strongest inhibitor—peptide **9**—was uncompetitive. This findings suggests that the mode of inhibition of DPPI by phosphono peptides is quite complex. The decrease of the percent of inhibition observed for compounds **1** and **5** versus increase of the duration of the

preincubation of inhibitor with the enzyme suggests that these peptides may also serve as false, slowly hydrolysing, substrates of DPPI.

The most active peptide (compound **9**) was obtained by introduction of amino(adamantyl)methylphosphonic acid into the C-terminus of the dipeptide. This indicates that the hydrophobic binding site for aromatic amino acid side chain is more spacious than the required to bind the flat aromatic part of the substrate. This finding was also confirmed by relatively strong inhibition of DPPI by compounds **4** and **7**.

The findings described above indicate that replacement of a carboxylic group of synthetic substrates of DPPI by a phosphonic acid moiety may be considered as a promising way for the design of new inhibitors of this enzyme, as well as other cysteine proteases.

EXPERIMENTAL

Chemicals

Phosphono dipeptides were synthesized using the standard procedure. [10, 11] Their yields and physicochemical data are given in Table I. All the peptides contain C-terminal aminophosphonic acids as racemate. H-NMR spectra were recorded using a Tesla instrument operating at 100MHz. IR spectra were recorded in KBr pallets with a Perkin-Elmer 377 spectrometer. Microanalyses were made by the Central Analytical Laboratory of the Institute of Organic Chemistry, Biochemistry and Biotechnology.

Enzyme Preparations

Enzyme was isolated from bovine spleen by the standard procedure. [12]

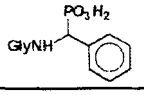
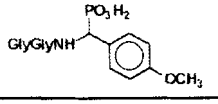
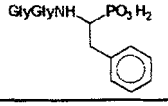
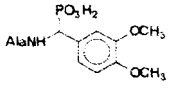
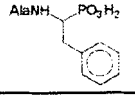
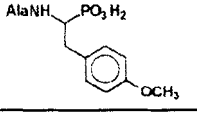
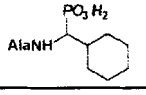
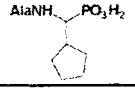
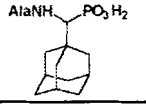
Percentage of Inhibition

Percentage of inhibition was measured after 2 min, 30 min and 1 h of incubation of the 25 μl of DPPI (0.77 mU) solution with 1 ml of inhibitor (0.5 mM final concentration) in 0.2 M Tris-HCl buffer, pH 6.0, containing 2-mercaptoethanol (5 mM final concentration) and sodium chloride (10 mM final concentration) at 37°C. After incubation 1 ml of the synthetic substrate (glycyl-*L*-phenylalanine-β-naphtylamide, 0.5 mM final concentration) was added and the reaction continued for 5 min at 37°C. The enzyme was deactivated by addition of 3 ml of 1:1 (v/v) mixture of *p*-dimethylaminobenzaldehyde (1% in methanol) and 0.5 M KCl-HCl buffer, pH 1.4. After 20 min the enzyme activity was measured following the change of absorbance at 450 nm. The data are given in Table II.

Kinetic Assays

DPPI was assayed at 37°C in 0.25 ml of 0.2 M Tris-HCl buffer, pH 6.0, containing 2-mercaptoethanol (5 mM final concentration) and sodium chloride (10 mM final concentration) at 37°C. The assay mixture contained 5 μl of DPPI (0.35 mU), and 0.20 ml of synthetic substrate-glycyl-*L*-phenylalanine-*p*-nitroanilide (1 mM-7 mM final concentration) and inhibitor (0.5 mM-2 mM final

Table 1. Phosphonopeptides

Compound	Structure	Yield (%)	M.p. (°C) decomp.	$[\alpha]^{20}_{578}$ (c 1, H_2O)
I		75	260-270	-
II		33.5	219-221	-
III		29	264-265	-
IV		41	273-275	-6° a
V		75	261-264	+ 4°
VI		52	285-287	+11° b
VII		61	278-280	+11°
VIII		55	245-251	+15°
IX		30.5	276-279	+12° a

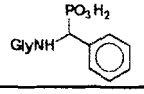
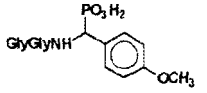
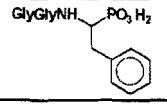
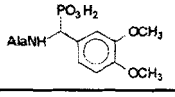
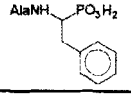
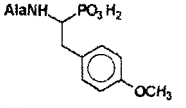
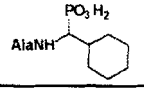
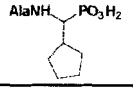
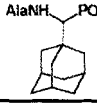
[a] (c 1, 1M NaOH) , [b] (c 1, 5M HCl)

concentration). The final volume was adjusted to 0.25 ml with the assay buffer. The hydrolysis was monitored following the change of absorbance at 385 nm. The K_m value was found to be 5 mM.

Evaluation of Kinetic Parameters

Kinetic parameters (K_m and K_i), as well as the inhibition types were determined using the LIBURK computer programme worked out by Dr W. Rode et al. from

Table 2. Inhibition of DPPI by phosphono peptides.

Peptide	% of inhibition			K_i	K_m/K_i	type of inhibition
	2 min	30 min	1 h	mM		
	40	19	2	ND		
	35	41	88	0.97	5.2	non-competitive
	-[a]	44	61	4.09	1.2	mixed
	42	54	87	0.28	17.8	mixed
	47	30	12	ND		
	63	100	100	0.70	7.1	mixed
	32	57	81	0.46	10.1	mixed
	70	74	96	1.29	3.9	mixed
	64	87	100	0.05	100	uncom-petitive

[a] stimulation of enzymatic activity was observed

the Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw (Poland).

Spectroscopic Characteristics of Compounds Listed in Table I

I: Anal. Calcd. for $C_9H_{13}N_2O_4P$: P, 12.70, N, 11.48, Found: P, 12.90, N, 11.45. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 4.34 (s, 2H, NCH_2), 5.82 (d, J_{PH} = 20.5Hz, 1H, CHP), 7.86 (s, 5H, C_6H_5). IR (KBr, ν, cm^{-1}): 3260 (NH), 1670 (CO), 1545 (NH), 1155 and 1060 (PO_3H^-).

II: Anal. Calcd. for $C_{12}H_{18}N_3O_6P \times 1.5H_2O$: P, 8.64, N, 11.73, Found: P, 8.84, N, 11.47. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 3.89 (s, 3H, OCH_3), 4.02 and 4.19 (s, 2H each, NCH_2), 5.47 (d, J_{PH} = 21.0Hz, 1H, CHP), 7.1-7.8 (m, 4H, C_6H_4). IR (KBr, ν, cm^{-1}): 3270 (NH), 1680 and 1640 (CO), 1545 (NH), 1170 and 1040 (PO_3H^-).

III: Anal. Calcd. for $C_{12}H_{18}N_3O_5P$: P, 9.83, N, 12.69, Found: P, 9.94, N, 12.86. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 2.7-3.75 (m, 2H, CH_2CHCO), 4.08 (bs, 4H, 2 × NCH_2), 4.35-5.1 (m, 1H, CHP), 7.50 (bs, 5H, C_6H_5). IR (KBr, ν, cm^{-1}): 3280 (NH), 1675 and 1640 (CO), 1535 (NH), 1150, 1040 and 1010 (PO_3H^-).

IV: Anal. Calcd. for $C_{12}H_{19}N_2O_6P \times H_2O$: P, 9.21, N, 8.33, Found: P, 9.25, N, 8.29. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 1.92 and 2.07 (d, J = 7.0Hz, 1.5H each, $CHCH_3$), 4.18 and 4.24 (s, 3H each, OCH_3), 4.77 (q, 1H, J = 7.0Hz, $CHCH_3$), 5.85 (bd, J_{PH} = 22.0Hz, 1H, CHP), 7.25-7.6 (m, 3H, C_6H_3). IR (KBr, ν, cm^{-1}): 3270 (NH), 1680 and 1640 (CO), 1545 (NH), 1170 and 1040 (PO_3H^-).

V: Anal. Calcd. for $C_{10}H_{17}N_2O_4P \times 2H_2O$: P, 10.47, N, 9.46, Found: P, 10.36, N, 9.77. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 1.31 and 1.77 (d, J = 7.0Hz, 1.5H each, $CHCH_3$), 2.9-3.8 (m, 2H, $CHCH_2$), 4.23 (q, 1H, J = 7.0Hz, $CHCH_3$), 4.5-5.1 (m, 1H, CHP), 7.5-7.8 (m, 5H, C_6H_5). IR (KBr, ν, cm^{-1}): 3290 (NH), 1665 (CO), 1565 (NH), 1155, 1115 and 1030 (PO_3H^-).

VI: Anal. Calcd. for $C_{12}H_{19}N_2O_5P \times 1.5H_2O$: P, 9.37, N, 8.48, Found: P, 9.38, N, 8.62. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 1.17 and 1.63 (d, J = 7.0Hz, 1.5H each, $CHCH_3$), 3.0-3.35 (m, 2H, $CHCH_2$), 3.83 (s, 3H, OCH_3), 4.15 (q, 1H, J = 7.0Hz, $CHCH_3$), 4.15-4.9 (m, 1H, CHP), 6.75-7.4 (m, 4H, C_6H_4). IR (KBr, ν, cm^{-1}): 3300 (NH), 1655 (CO), 1565 (NH), 1185, 1120 and 1050 (PO_3H^-).

VII: Anal. Calcd. for $C_{10}H_{21}N_2O_4P \times 3H_2O$: P, 9.75, N, 8.80, Found: P, 9.54, N, 8.64. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 0.9-2.45 (m, 11H, 5 × CH_2 and CHCHP), 1.68 (d, J = 7.0Hz, 3H, $CHCH_3$) 3.65-4.85 (m, 2H, 2 × NCH). IR (KBr, ν, cm^{-1}): 3300 (NH), 1650 (CO), 1530 (NH), 1135 and 1075 (PO_3H^-).

VIII: Anal. Calcd. for $C_9H_{19}N_2O_4P \times 5H_2O$: P, 9.12, N, 8.23, Found: P, 9.28, N, 8.24. ^{1}H-NMR (D_2O + D_2SO_4, δ ppm): 1.7-2.85 (m, 9H, 4 × CH_2 and

CHCHP), 1.70 (d, J = 7.3Hz, 3H, CHC**H**$_3$) 4.35-4.8 (m, 2H, 2 × NC**H**). IR (KBr, ν, cm^{-1}): 3275 (NH), 1655 (CO), 1535 (NH), 1155, 1125 and 1075 (PO_3H^-).

IX: Anal. Calcd. for $C_{14}H_{25}N_2O_4P \times 9H_2O$: P, 6.48, N, 5.86, Found: P, 6.52, N, 5.57. ^{1}H-NMR ($D_2O + D_2SO_4$, δ ppm): 1.5-2.45 (m, 15H, 6 × C**H**$_2$ and 3 × **CH**), 1.78 (d, J = 7.0Hz, 3H, CHC**H**$_3$) 3.7-4.25 (m, 2H, 2 × NC**H**). IR (KBr, ν, cm^{-1}): 3295 (NH), 1660 (CO), 1540 (NH), 1100 and 1080 (PO_3H^-).

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