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Synthesis and activity of phosphinic tripeptide inhibitors of cathepsin C

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Abstract—Phosphinic tripeptide analogues Gly-Xaaψ[P(O)(OH)CH_2]-Gly have been developed as inhibitors of cathepsin C (DPP I), a lysosomal, papain-like cysteine protease. The target compounds were synthesised by addition of methyl acrylate to the appropriate phosphinic acids followed by the *N*-terminus elongation using mixed anhydride procedure. The latter step has been demonstrated to be a suitable method for *N*-terminal extension of the phosphinic pseudopeptide analogues without requirement of hydroxyphosphinyl protection. The title compounds appeared to be moderate inhibitors of the cathepsin C. However, although designed as transition state analogues, they surprisingly exhibited noncompetitive mode of binding to cathepsin C. Differences in kinetics of *C*-terminal acids and esters have been additionally observed.

1. Introduction

Cathepsin C or dipeptidyl peptidase I (DPP I, E.C. 3.4.14.1) is a lysosomal cysteine protease representing the most numerous papain-like class of enzymes, that are expressed throughout the animal and plant kingdom as well as in viruses and bacteria. Cathepsins represent promising drug targets as their key proteolytic activities have been implicated in degenerative, invasive and immune system related disorders.[1–4] In mammals, multiple functions have been associated to cathepsin C. It removes dipeptides from the nonsubstituted *N*-terminus of polypeptide substrates with broad specificity thus exhibiting the exopeptidase activity and like other lysosomal cysteine proteases, it is involved in nonspecific intracellular protein degradation. Beyond this function, DPP I is recognised to be crucial for activation of a number of granular serine proteases, granzymes A and B, cathepsin G, neutrophil elastase and chymase.[5] It has been also suggested to be involved in several pathological disorders, like Duchene muscular dystrophy[6,7] and basal cell carcinomas.[8] Mutation in DPP I gene have been shown to result with autosomal-recessive palmoplantar keratoderma Papillon–Lefévre and Haim–Munk syndromes.[9,10]

DPP I differs from the most other relatives of papain family in its large molecular size, oligomeric structure, retention of a large part of the propeptide in the mature form, and requirements for halide ions for activity.[11,12] Cathepsin C is inhibited by natural protein inhibitors from the cystatine superfamily.[13–15] Regarding low molecular, irreversible, synthetic inhibitors, DPP I is specifically inhibited by Gly-Phe-diazomethane[16] and weakly by E-64. Recently, novel, competitive inhibitors based on oligoarginines were developed using peptide combinatorial chemistry approach.[17] Among phosphorus containing compounds *C*-terminal phosphono peptides exhibited relatively low potency towards cathepsin C acting additionally with the enzyme in quite complex mode.[18]

The application of phosphinic analogues of peptides as inhibitors of proteases is based on the concept of the resemblance of a phosphorus moiety to the high-energy tetrahedral transition state of the amide bond hydrolysis.[19–23] This idea has been particularly attracting in construction of a wide range of potent and selective inactivators of metalloproteases that have been reviewed recently.[24–26] However, a phosphorus containing moiety

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is additionally able to coordinate the zinc atom present in the active sites of metalloproteases and to block its function in the process of hydrolysis. This fact can be considered as the major reason of such successful applications for metallo-dependent enzymes. In cases of the other protease families no similarly spectacular achievements have been obtained. A single interesting exception represents bis(α-aminoalkyl)phosphinic acids, a modification of phosphinic peptidomimetics, which formula offers a possibility of the transition state acting core situated inside the symmetrical peptide environment. Nevertheless, the idea of such inhibitor geometry was not inspired by the structure of the natural substrates, but by the symmetry of enzymes, and it resulted in strong inhibitors of the aspartic protease of the human immunodeficiency virus type 1—the etiologic agent of AIDS.[27–30]

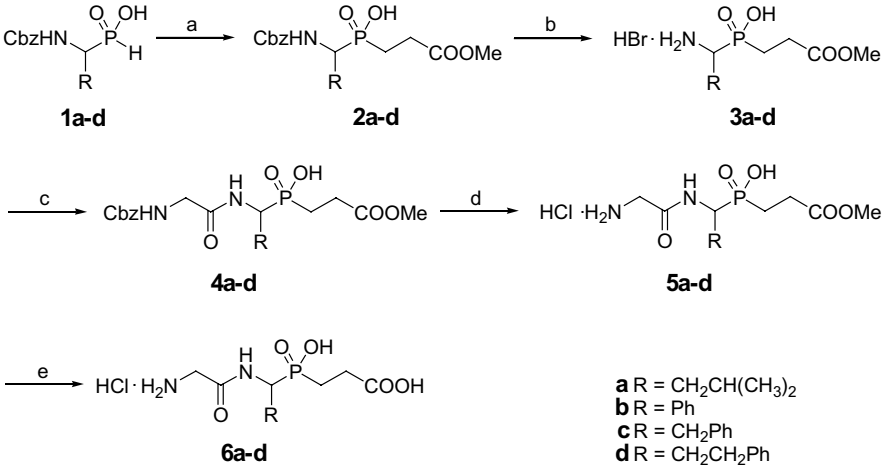
Organophosphorus compounds studied so far appeared to be surprisingly weak inhibitors of cysteine proteases.[31] In this paper we report the synthesis and evaluation of phosphinic analogues of tripeptides designed as inhibitors of cathepsin C. Although they appeared to be moderate inhibitors of the enzyme, their binding mode turned up to be somewhat surprising.

2. Chemistry

The starting phosphinic amino acid analogues **1** were synthesised in the reaction of an appropriate aldehyde with salt of diphenylmethylamine and hypophosphorous acid, followed by hydrolysis of the adduct and *N*-terminal protection with benzyl chloroformate, similarly as described in the literature.[32] Two typical synthetic procedures are usually applied to convert phosphinic acids into pseudodipeptides: addition of an acrylate to the aminophosphinic substrate preactivated into the form of its trivalent silyl ester[33–35] or corresponding addition of to the appropriate ester in the presence of a strong base.[36,37] In this work the first procedure have been chosen (Scheme 1). An *N*-benzyloxycarbonylaminophosphinic acid **1** was activated by heating with hexamethyldisilazane and then methyl acrylate was added. After workup and recrystallisation (ethyl acetate/methanol, 95:5) the protected pseudopeptides **2** were separated in good yields (65–80%). The benzyloxycarbonyl group was removed by action of HBr in AcOH and the products **3** were separated upon treatment with ether. They were added as a solid to the solution of the *N*-benzyloxycarbonylglycine preactivated into its mixed anhydride by means of isobutyl chloroformate.

This procedure appeared to be a simple and satisfactory method for the *N*-terminus elongation of the individual phosphinic dipeptides in solution. Synthesis on solid phase of 20 member libraries of analogous tripeptides has been reported in the literature.[38] But combinatorial chemistry on solid phase demands introduction of very specific protection groups to the phosphinic dipeptide building block (namely *N*-Fmoc and phosphinyl adamantyl ester) to be compatible with the Fmoc SP methodology and to suppress side reactions.[34] Several examples of successful application of this approach for development of potent and selective inhibitors of zinc metalloproteases has been reviewed recently.[24–26] On the contrary to *C*-terminal extension of phosphinic dipeptides by carboxylate activation, that can be assisted by phosphonamidate side product formation, no necessity of protection of the phosphinate function has been evidenced in our case. After standard work-up and purification on column chromatography (chloroform/methanol, 100:0 → 90:10) the tripeptide analogues **4** were separated in satisfactory yields (50–60%).

To obtain esters **5** the benzyloxycarbonyl group was removed by action of HBr in AcOH. After evaporation to dryness the residue was dissolved in 10% HCl and benzyl bromide was washed out by extraction with ether. Final evaporation gave compounds **5**. The methyl



Scheme 1. Reagents and conditions: (a) hexamethyldisilazane, 100–110 °C, 3 h, then $H_2C{=}CHCOOMe$, 80–90 °C, 3 h, then MeOH; (b) 33% HBr/AcOH, rt, 2 h; (c) $CbzNHCH_2COOH$, isobutyl chloroformate, NEt_3, 0 °C → rt; (d) 33% HBr/AcOH, rt, 2 h; then evaporation, 10% HCl and washing with ether; (e) bromotrimethylsilane, 10 equiv, 3 d, rt, then H_2O, then concd HCl.

esters were hydrolysed by use of bromotrimethylsilane in dichloromethane. After evaporation of volatile components the residue was treated consecutively with water and then with concd HCl. Final evaporation gave the target compounds as their hydrochlorides **6**. Both steps of deprotection proceeded practically quantitatively (with yields exceeding 95%).[39]

3. Activity

As seen from Table 1 all the studied compounds appeared to be reversible, noncompetitive and slow-binding inhibitors of cathepsin C. The equilibrium in the inhibitor binding was reached within several minutes after addition of the enzyme to the mixture of substrate and inhibitor. Inhibition constants were determined from Lineweaver–Burk plots of steady-state portion of the reaction plots. Data presented in Table 1, although consider only for eight compounds, enable to coil some general conclusions.

Table 1. Inhibition of cathepsin C by phosphinic tripeptide analogues[40]

Ester	K_i [mM]	Acid	K_i [mM]
5a	0.040	**6a**	0.039
5b	0.188	**6b**	0.176
5c	0.514	**6c**	0.429
5d	0.312	**6d**	0.187

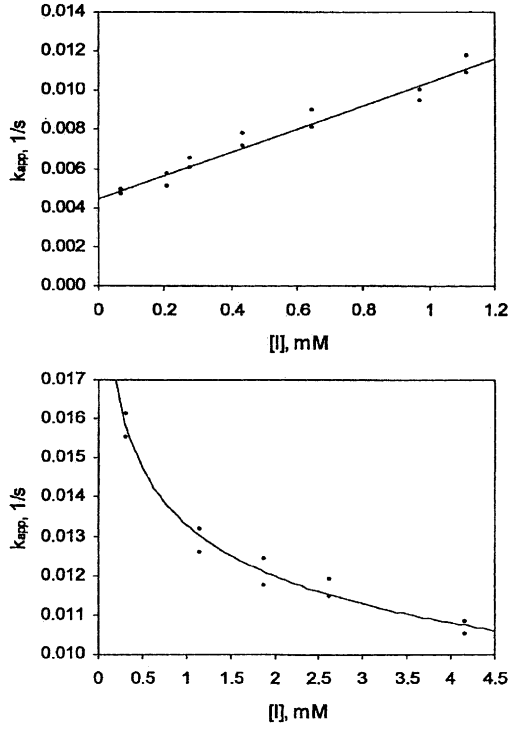


Figure 1. Linear dependence of the apparent first-order rate constant versus inhibitor concentration determined for the ester inhibitors (upper diagram, compound **5c**, substrate concentration 2.00 mM) and hyperbolic dependence for the corresponding acids (lower diagram, compound **6c**, substrate concentration 1.47 mM).

Noncompetitive character of inhibition suggests that all the studied compounds interact with the regulatory rather than with the active site of the enzyme and thus do not serve as transition-state analogues.

Cathepsin C moderately favours acids **6** against esters **5**. However, the differences in binding affinities are very small suggesting that this part of the molecule may not play significant role in inhibition. On the other hand this is, most likely, not true since there is a significant difference in the mechanism of slow-binding process if considering these two groups of compounds (Fig. 1). Thus, the mechanism of binding of esters **5** is a slow one-step process, as indicated from the linear dependence of the apparent first-order rate constant for slow binding versus inhibitor concentration.[41] On the contrary, binding of acids **6** was achieved accordingly to a two-step mechanism, in which the enzyme upon binding of inhibitor undergoes slow isomerisation to form slowly dissociating complex. This is clearly seen from hyperbolic dependence of the apparent rate constant versus inhibitor concentration.[41] Thus, esters **5** and acids **6** seem to constitute two different classes of inhibitors of cathepsin C.

Acknowledgements

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39. The representative spectroscopic data obtained for the phosphinic tripeptide inhibitors **5** and **6**. *Methyl glycyl*-3-[(1-*amino*-3-*methylbutyl*)*hydroxyphosphinyl*]*propionate hydrochloride* (**5a**). ^{31}P NMR (121.50 MHz, D_2O): δ 49.61; ^{1}H NMR (300.13 MHz): δ 0.77 and 0.85 (d each, $J = 5.8$ Hz, 3H and 3H, $2\times CH_3$), 1.53 (m, 3H, CH_2CH), 1.95 (m, 2H, PCH_2), 2.56 (m, 2H, CH_2COO), 3.63 (s, 3H, OCH_3), 3.79 (s, 2H, NCH_2), 4.18 (dd, $J_{PH} \approx J_{HH} \approx 10$ Hz, 1H, NCHP). *Glycyl*-3-[(1-*amino*-3-*methylbutyl*)*hydroxyphosphinyl*]*propionic acid hydrochloride* (**6a**). ^{31}P NMR (121.50 MHz, D_2O): δ 50.30; ^{1}H NMR (300.13 MHz): δ 0.76 and 0.84 (d each, $J = 5.9$ Hz, 3H and 3H, $2\times CH_3$), 1.49 (m, 3H, CH_2CH), 1.95 (m, 2H, PCH_2), 2.54 (m, 2H, CH_2COO), 3.78 (s, 2H, NCH_2), 4.19 (dd, $J_{PH} \approx J_{HH} \approx 10$ Hz, 1H, NCHP).
40. Assay of Inhibitory Activity: Cathepsin C was isolated and purified according to literature procedure.[42] Its activity was equal 0.8 mU. Enzymatic reaction was assayed at 25 °C in 0.2 M acetate buffer (pH 4.5) containing 2-mercaptoethanol (5 mM final concentration) and sodium chloride (10 mM final concentration). The assay mixture contained 50 μL of cathepsin C, 2.0 mL of synthetic substrate (glycyl-L-phenylalanine-*p*-nitroanilide, 0.2–3.0 mM final concentration, $K_M = 2.23$ mM) and 0.4 mL of inhibitor (0.2–2.0 mM final concentration). The total volume was adjusted to 2.45 mL with the assay buffer. The course of reaction was monitored following the change in absorbance at 400 nm with Specord M40 (Carl Zeiss Jena).
Kinetic parameters were determined using the computer programme worked-out for slow-binding kinetics. The programme is available upon request.
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