SYNTHESIS AND PROTEINASE INHIBITORY PROPERTIES OF DIPHENYL PHOSPHONATE ANALOGUES OF ASPARTIC AND GLUTAMIC ACIDS

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Abstract: The synthesis of diphenyl phosphonate analogues of aspartic and glutamic acid, and their inhibitory activity against S. aureus V8 protease and granzyme B, is described. The study has revealed difficulties with protecting group compatibility in the synthesis of these analogues. Two analogues, Acetyl.Asp^P(OPh)$_2$ and Acetyl.Glu^P(OPh)$_2$ were found to function as irreversible inactivators of V8 proteinase, yet exhibit no activity against granzyme B. © 1998 Elsevier Science Ltd. All rights reserved.

Introduction:

α-Aminoalkylphosphonic acids are analogues of natural aminoacids and as such have been the subject of much research effort over past years[1]. The ability of these compounds to act as enzyme inhibitors[2] and exhibit other diverse biological activity[3] has added to their interest and intensified efforts to establish their actual or potential pharmaceutical value.

It has now been shown by us[4] and others[5] that the diphenyl esters of α-aminoalkylphosphonic acids are particularly potent and show high selectivity as irreversible inhibitors of serine proteinases, a large group of enzymes with a wide range of important biological functions[6]. Thus far, α-aminoalkylphosphonic acid ester analogues of a number of aliphatic- and aromatic aminoacids have been prepared including valine, phenylalanine, tryptophan, and tyrosine,[4] and the basic aminoacids ornithine, lysine, etc[7]. However, to date, no similar α-diphenyl phosphonate analogues of the acidic aminoacids, aspartic and glutamic have been reported. Such derivatives might prove to be effective inactivators of those serine proteinases exhibiting a P_1 specificity for aspartate and glutamate (nomenclature of Schecter and Berger[8]). Examples of such proteinases are S. aureus V8 protease, which exhibits dual specificity for both Asp and Glu at P_1[9], and granzyme B, which exhibits an almost exclusive specificity for Asp[10]. Both of these proteinases are of biomedical importance. For example, the former is known to cleave α_1-antichymotrypsin[11] and might thus contribute to the known pathophysiological role of Staph. aureus in cystic fibrosis[12]. Likewise, granzyme B is an effector proteinase, produced by cytotoxic T lymphocytes, which has been demonstrated to activate pro-caspase 3, via limited proteolysis, thus generating one of the central cysteine proteinases implicated in apoptosis (programmed cell death) [13,14].

A considerable number of preparations of analogues (1) with ω-substituted phosphinate and phosphonate groups have been published[15] which for the glutamic acid analogue give structures related to phosphinothricin (1, R=Me, n=2) which has antibiotic, fungicidal and herbicidal properties. The α-substituted analogue (2, R=H, n=1) of aspartic acid and its trimethyl ester have also been reported[16], but the procedures used could not easily be adapted to our use.

(1)   (2)

We now wish to report the synthesis of the diphenyl phosphonate analogues (2, R=Ph) of Asp and Glu and their inhibitory activities against *S. aureus* V8 protease and granzyme B.

Chemistry:

The synthetic strategy chosen for their preparation was based on a previously reported phase transfer alkylation procedure[17], but modified by us to provide diphenyl esters under non-phase transfer conditions (see **Scheme 1**). The required imine diphenyl N-(diphenylmethylene)aminomethylphosphonate (4) was prepared via an initial Arbusov reaction between diphenylethyl phosphite[18] and N-(bromomethyl)phthalimide (3). Deprotection of the phthalimido group with hydrazine hydrate/acetic acid in refluxing THF, followed by treatment with benzophenone imine, at room temperature in dry dichloromethane overnight, provided (4) in 78% yield.
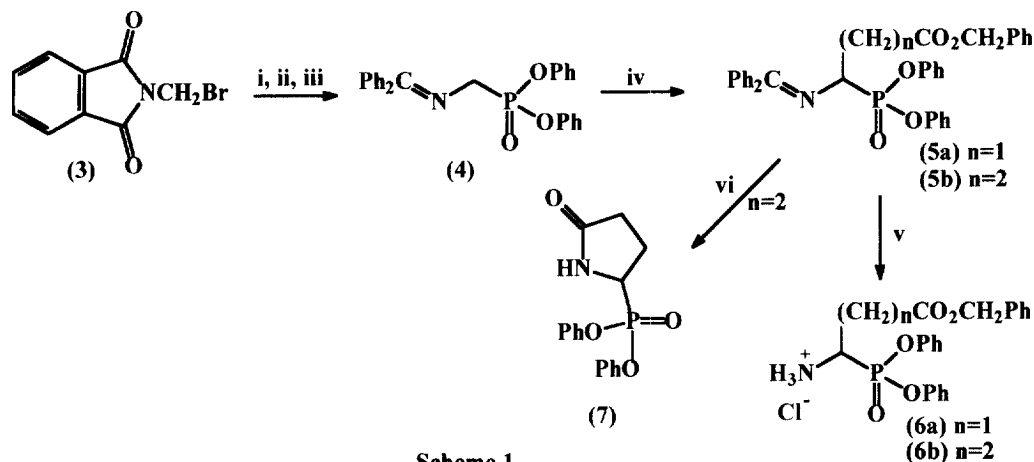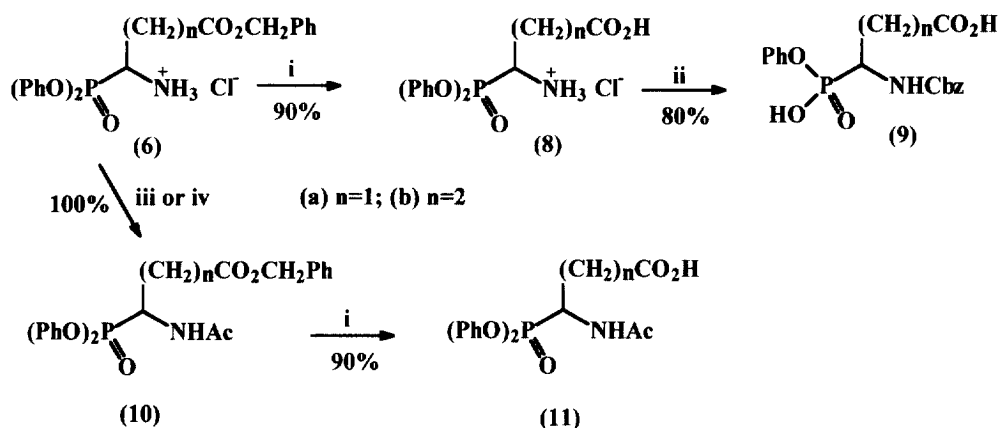

Scheme 1

i, (PhO)$_2$POEt, xylene, reflux, 5 days; ii, NH$_2$NH$_2$.H$_2$O, AcOH, THF, reflux, 4h.; iii, Ph$_2$C=NH, CH$_2$Cl$_2$, R.T.:
iv, KHMDS, Br(CH$_2$)$_n$CO$_2$CH$_2$Ph, THF, -78°C; v, dil. HCl, ether; vi, TFA, CH$_2$Cl$_2$

The anion of (4) was generated using KHMDS (potassium hexamethyldisilazide) in dry THF at -78°C and alkylated with benzyl bromoethanoate and benzyl-3-bromopropanoate to give (5a) (72%) and (5b) (75%), respectively. Acid hydrolysis with ethereal dilute HCl of (5a) and (5b) resulted in complete and selective removal of the benzophenone protecting group to give (6a) and (6b), respectively. However, when deprotection was attempted using TFA in dichloromethane (5b) gave the cyclized pyroglutamic acid derivative (7) in 85% yield[19]. A similar cyclization occurs when a related (+) - camphor glutamic acid analogue is hydrolysed in aqueous acetic acid[20]. Presumably the stronger acid (HCl) used in our case suppresses equilibrium with the free base and hence the cyclisation reaction (**Scheme 2**).
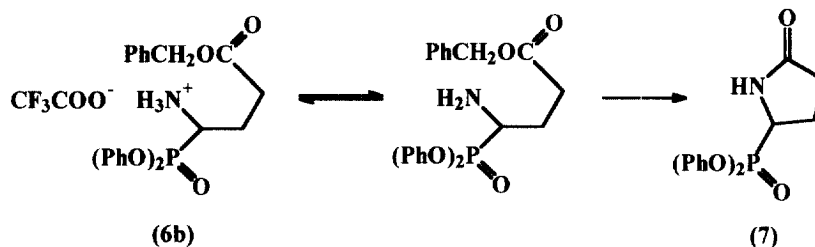
Scheme 2

We now envisaged hydrogenolysis of (6a) and (6b) to remove the benzyl group followed by CBZ protection at nitrogen to complete the synthesis (Scheme 3).
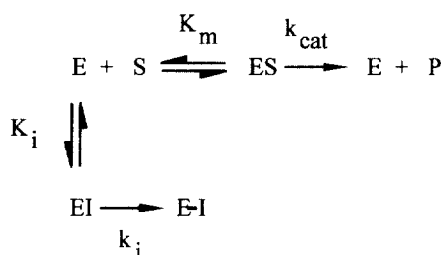

Scheme 3

i, H$_2$, Pd/C, THF; ii, CbzCl/pH 9.5; iii, AcCl, Et$_3$N; iv, Ac$_2$O

However, following hydrogenation, attempts to N-protect the free acid (8a) with Cbz led to the loss of a phenoxy group at phosphorus to form (9a),[21] even under very mild basic conditions. Since simple diphenyl phosphonate esters are stable under much more basic conditions, this suggests that the carboxylate anion generated during Cbz protection is participating in the hydrolysis reaction. Bruice et al. has observed enhanced rates of ester hydrolysis for o–carboxyl substituted-phenyl phosphodiesters[22] and attributed them to similar anchimeric assistance from the carboxylate group. Work is now in hand to examine the kinetics of this reaction.

Unfortunately, initial Cbz protection of (6a) offered no advantage, as the conditions needed for debenzylation, i.e. hydrogenolysis or acid treatment, also removed the Cbz group. An alternative approach using the Cbz protected t-butyl ester of (6a) was also unsuccessful since the minimum acidic conditions necessary for removal of the t-butyl group led to N-deprotection. To obviate these difficulties, acetylated derivatives were prepared (either with acetyl chloride/triethylamine or neat acetic anhydride). These acetylated compounds (10a) and (10b) could then be readily deprotected by hydrogenolysis over Pd/C to give analogues (11a)[23] and (11b),[23] respectively.

Enzymes Inhibition:[24]

We examined the ability of Acetyl-$Asp^P(OPh)_2$ (**11a**) and Acetyl-$Glu^P(OPh)_2$ (**11b**) to block the enzyme-catalysed hydrolysis of the appropriate fluorogenic substrate for each proteinase. **Figure 1** shows the progress curves obtained for the V8 proteinase-catalysed hydrolysis of Cbz-Leu-Leu-Glu-β-naphthalamide, carried out in the presence of varying amounts of Acetyl-$Glu^P(OPh)_2$. These progress curves are typical of the action of an active-site-directed irreversible inhibitor, operating via the mechanism listed below[25,26]. Similar progress curves were obtained with Acetyl-$Asp^P(OPh)_2$ (data not shown). However, neither of the two diphenyl phosphonates caused any inhibition of granzyme B.

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \overset{k_{cat}}{\longrightarrow} E + P$$

$$K_i \updownarrow$$

$$EI \overset{}{\underset{k_i}{\longrightarrow}} E\text{-}I$$

In this scheme, the proteinase **E** catalyses the hydrolysis of substrate **S**, to generate the fluorescent product **P**. This reaction is characterised by the standard kinetic constants K_m and k_{cat}. In the presence of competing inhibitor **I**, the proteinase binds the inhibitor to form the Michaelis complex **EI** (the affinity of this interaction is characterised by the inhibitor constant K_i), which is then covalently modified with first-order rate constant k_i, to form the inactivated complex **E-I**.

Data from progress curves such as those shown in **Figure 1**, were fitted, using non-linear regression analysis [25], to the integrated rate equation **1**.

$$[P] = A. (1 - \exp(-k_{app}.t)) \tag{1}$$

This rate equation represents a first-order process of apparent rate constant k_{app} and amplitude A, for the formation of product **P** as a function of time[25]. The values of A and k_{app} for 5 different inhibitor concentrations were then determined and, from these, the inhibitor specificity constant k_i / K_i was evaluated for each of the diphenyl phosphonates[25]. These are recorded in **Table 1**.
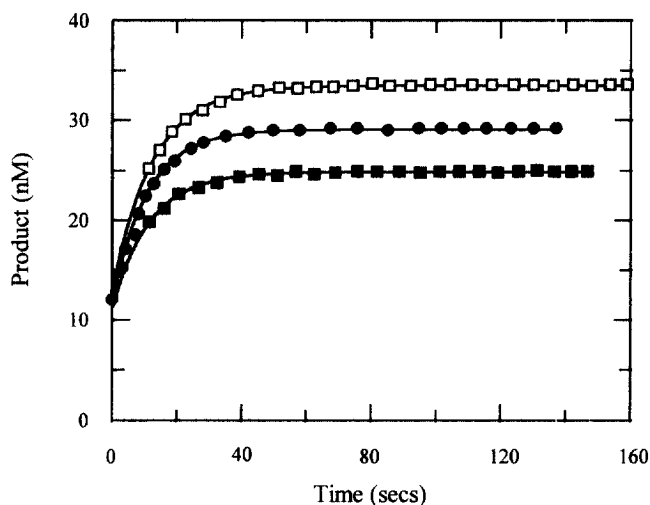
Figure 1. Progress curves for the formation of β-naphthylamine generated by the *Staph aureus* V8 proteinase-catalysed hydrolysis of Cbz-Leu-Leu-Glu-β-naphthalamide (50 μM) in the presence of 5 μM (□), 10 μM (●) and 50 μM (■) Acetyl-Glu^P(OPh)$_2$.

Diphenyl phosphonate analogue	Apparent second-order rate constant (k_i / K_i)	
	S. aureus V8 proteinase	Granzyme B
Acetyl-Glu^P(OPh)$_2$	$5.3 \pm 0.5 \times 10^3$ M^{-1}.s^{-1}	N.I.
Acetyl-Asp^P(OPh)$_2$	$5.0 \pm 0.5 \times 10^2$ M^{-1}.s^{-1}	N.I.

Table 1. Second-order rate constants for the inactivation of *S. aureus* V8 proteinase with aspartate- and glutamate diphenyl phosphonates. The inactivation studies were performed in 100 mM phosphate buffer, pH 7.4, maintained at 25°C. N.I. = not inhibited (no effect was observed using inhibitor concentrations as high as 150 μM).

Conclusions: This study has presented details of the first synthesis of diphenyl phosphonate analogues of aspartic and glutamic acids and has demonstrated that they function as irreversible inactivators of *S. aureus* V8 proteinase. The 10-fold greater inhibitory activity of the glutamic acid analogue is in keeping with the known relative effectiveness of substrates containing Asp and Glu at P$_1$.

References and Notes:

1. Engel, R. (Ed), *Handbook of Organophosphorus Chemistry*, Marcel Decker, Inc., New York, 1992; Kukhar, V. P.; Solodenko, V. A. *Russ. Chem. Revs.*, **1987**, *56*, 860; Petrov, K. A.; Chauzov, V. A.; Erokhina, T. S. *Russ. Chem. Revs.*, **1974**, *43*, 984.

2. Giannousis, P.P.; Bartlett, P.A. *J. Med. Chem.*, **1987**, *30*, 1603; Allen, M. C.; Fuhrer, W.; Tuck, B.; Wade, R.; Wood, J. M. *J. Med. Chem.*, **1989**, *32*, 1652; Logush, E. W.; Walker, D. M.; McDonald, J. F.; Leo, G. C.; Franz, J. E. *J. Org. Chem.* **1988**, *53*, 4069.

3. Kafarski, P.; Mastalerz, P. *Aminophosphonates: Natural Occurence, Biochemistry and Biological Properties*, Beitr. Wirkstafforschung, 1984, No. 21.

4. Bergin, C.; Hamilton, R.; Walker, B.; Walker, B. J. *J. C. S. Chem. Commun.*, **1996**, 1155; Bergin, C.; O'Matiu, E.; Hamilton, R.; Walker, B.; Walker, B. J. *Phosphorus, Sulfur and Silicon*, **1996**, *111*, 90; Hamilton, R.; Kay, G.; Shute, R. E.; Travers, J.; Walker, B.; Walker, B. J. *Phosphorus, Sulfur and Silicon*, **1993**, *77*, 387.

5. Oleksyszyn, J.; Powers, J. C. *Biochemistry*, **1991**, *30*, 485; Fastrez, J.; Jespers, L.; Lison, D.; Renard, M.; Sonveaux, E. *Tetrahedron Letts.*, **1989**, *30*, 6861; Oleksyszyn, J.; Boduszek, B.; Kam, C-M.; Powers, J. C., *J. Med. Chem.*, **1994**, *37*, 226.

6. Reich, E.; Rifkin, D. B.; Shaw, E. (Eds) in *"Proteases and Biological Control"*, Cold Spring Harbor, New York, 1975.

7. Hamilton, R.; Walker, B.; Walker, B. J. *Tetrahedron Letts.*, **1993**, *34*, 2847; Wang,C-L. J.; Mical, A. J.; Spitz, S.; Reilly, T. M. *Tetrahedron Letts.*, **1992**, *33*, 7667.

8. Schecter, I; Berger, A. Biochem. *Biophys. Res. Commun.* **1968**, *32*, 888.

9. Sorensen, S.B; Sorensen, T.L; Breddam, K. *FEBS Letts.* **1991**, *294*, 195.

10. Caputo, A; James, M.N.G; Powers, J.C; Hudig, D; Bleackly, R.C. *Nature Struct. Biol.* **1994**, *1*, 364.

11. Catanese, J; Kress, L.K. *Biochim. Biophys. Acta* **1984**, *789*, 37.

12. Branger, C; Gardye, C; Lambert-Zechovsky, N. *J. Med. Microbiol.* **1996**, *45*, 294.

13. Darmon, A.J; Nicholson, D.W; Bleackly, R.C. *Nature* **1995**, *377*, 446.

14. Quan, L.T; Tewari, M; O'Rourke, K; Dixit, V; Snipas, S.J; Poirier, G.G; Ray, C; Pickup, D.J; Salvesen, G.S. *Proc. Natl. Acad. Sci. USA.* **1996**, *93*, 1972.

15. Maier, L.; Lea, P. J., *Phosphorus Sulfur and Silicon*, **1983**, *17*, 1.

16. Campbell, M. M.; Carruthers, N. I.; Mickel, S. J. *Tetrahedron*, **1982**, *38*, 2513; Soroka, M.; Mastalerz, P. *Roczniki Chem.*, **1976**, *50*, 661.

17. Genet, J. P.; Uziel, J.; Port, M.; Touzin, A. M.; Roland, S.; Thorimbert, S.; Tanier, S., *Tetrahedron Letts.*, **1992**, *33*, 77.

18. Griffin, B. S.; Burger, A. *J. Am. Chem. Soc.*, **1956**, *78*, 2336.

19. ^{1}H NMR data for (7) [CDCl$_3$, 300MHz]; & 2.5 [m, 4H, (CH_2CH_2)], 4.19 [m, 1H, (N-CH-P)], 7.07 - 7.48 [m, 11H, (2xOPh + NH)],

20. Groth, U.; Richter, L.; Schollkopf, U. *Tetrahedron*, **1992**, *48*, 117.

21. ^{1}H NMR data for (9a) [D$_2$O, 300MHz]; δ 2.6 [m, 1H, (CH_a-CO$_2$H)], 2.84 [m, 1H, (CH_b-CO$_2$H)], 4.36 [m, 1H, (N-CH-P)], 5.06 [s, 2H, (O-CH_2Ph)], 7.04 - 7.45 [m, 10H, (O-*Ph* + OCH$_2$*Ph*)].

22. Bruice, T.C.; Blasko', A.; Petyak, M. E. J. Am. Chem. Soc., **1995**, *117*, 12064; Bruice, T.C.; Blasko', A.; Arasasingham, R. D.; Kim, J. S. *J. Am. Chem. Soc.*, **1995**, *117*, 12070.

23. ^{1}H NMR data for (11a) [CDCl$_3$ +DMSO, 300MHz]; δ 1.95 [s, 3H, (CH_3CON)], 2.8 - 3.05 [m, 2H, (CH_2-CO$_2$H)], 5.2 - 5.4 [m, 1H, (N-CH-P)], 7.1 - 7.45 [m, 10H, (2xO*Ph*)], 7.55 [d, 1H,(Ac-NH)], ^{1}H NMR data for (11b) [CDCl$_3$ +DMSO, 300MHz]; δ 1.95 [s, 3H, (CH$_3$CON)], 2.12 [m, 1H, (CH_a-CH$_2$CO$_2$H)], 2.38 [m, 1H, (CH_b-CH$_2$CO$_2$H)], 2.47 [m, 2H, (CH_2CO$_2$H)], 4.86 [m, 1H, (N-CH-P)], 7.12 - 7.24 [m, 10H, (2xO*Ph*)], 7.6 [d, 1H, (AcNH)].

24. **Biology:**   *S. aureus* V8 protease was purchased from Pierce (catalogue No. 20195) and granzyme B was a kind gift from Dr J. Trapani, University of Melborne. The activity of the former was assayed using the fluorogenic substrate Cbz-Leu-Leu-Glu-β-naphthalamide (Sigma Chemical Co. Poole, Dorset); whereas the latter was assayed using the chromogenic, thiol benzyl ester substrate Acetyl-Ala-Ala-Asp-S-Bzl (Bachem, Bubendorf, Switzerland).

25. Walker, B.; Elmore, D.T. *Biochem. J.* **1984**, *221*, 277.

26. Tian, W-X.; Tsou, C-L. *Biochemistry*, **1982**, *21*, 1028.