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PHOSPHONIC ACID AND PHOSPHINIC ACID TRIPEPTIDES AS INHIBITORS OF GLUTATHIONYLSPERMIDINE SYNTHETASE

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Abstract: A series of phosphonic and phosphinic acid derivatives of glutathione were synthesized as potential inhibitors of glutathionylspermidine synthetase, an essential enzyme in the biosynthesis of trypanothione in trypanosomatids. The compounds showed moderate activity.

Trypanothione[1] (N^1,N^8-bis(glutathionyl)spermidine) together with trypanothione reductase[2] (EC 1.6.4.8) play an essential role in maintenance of intracellular thiol redox and in defence against oxidant stress in protozoa of the genera *Trypanosoma* and *Leishmania*[3]. Since the trypanothione system is unique to these parasites and substitutes for many of the functions of the glutathione/glutathione reductase system of the mammalian host, it presents an attractive target for the development of new antitrypanosomal and antileishmanial drugs[3].

Glutathionylspermidine synthetase and trypanothione synthetase catalyze the final two steps in trypanothione biosynthesis in which glutathione is coupled to the N^1 and N^8 primary amines of spermidine. Both enzymes have been purified and characterized from the insect trypanosomatid *Crithidia fasciculata*[4]. As carbon-nitrogen ligases, both enzymes are proposed to use ATP to form an acylphosphate at the glycine carboxylate of glutathione, activating it for nucleophilic attack by the primary amine(s) of the spermidine moiety.

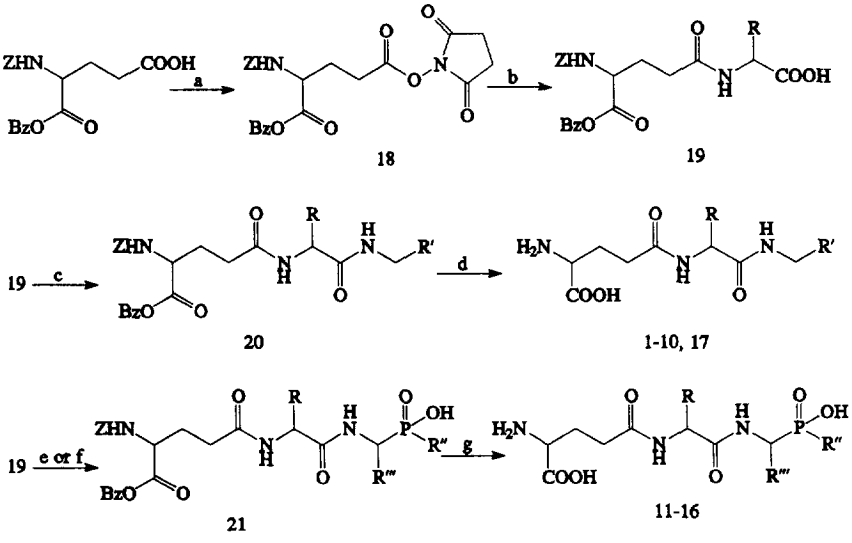
Substituting the activated carboxylic acid group of the substrate for a phosphonic or phosphinic acid function has afforded inhibitors of other enzymes belonging to this class such as *D*-Ala-*D*-Ala ligase,[5,6] glutamine synthetase,[7] glutathione synthetase[8] and *meso*-diaminopimelic acid adding enzyme[9].

Another (P)-acid approach involves the use of the acylphosphate as target. In this case the carbonyl-oxygen group (CO-O) is substituted for a keto- ($CO-CH_2$), an alcohol- ($CH(OH)-CH_2$), an amino- ($CH(NH_2)-CH_2$) or an alkylene group ($(CH_2)_2$ or $(CH_2)_3$). This approach has been applied to *D*-Ala-*D*-Ala ligase[10] and glutamine synthetase[11].

The activity of these (P)-amino acids and (P)-pseudopeptides on carbon-nitrogen ligases

prompted us to prepare a series of *L*-γ-glutamyltripeptides, derived from glutathione, in which the glycyl carboxylic acid function is substituted with a group containing phosphonic acid or phosphinic acid. In other studies (to be published elsewhere) we found *L*-Val and *L*-Leu suitable replacements for *L*-Cys, thereby facilitating the synthetic pathways to the desired compounds. The compounds are listed in table 1. **1-10** are phosphonic analogues of glutathione or its acylphosphate. **11-16** are phosphinic acid analogues. **12** and **18** are *L*-Ala-derivatives. **17** is the diphenylester of **2**.

Scheme 1

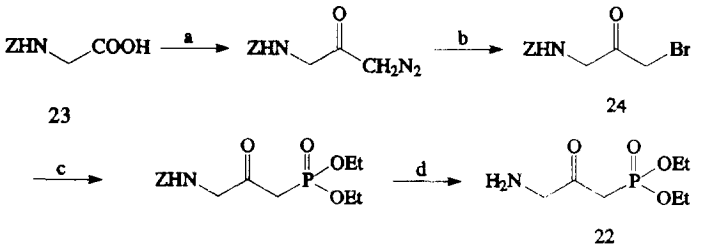


a) *N*-OHSuccinimide/dioxane, 4°C, DCC, 12h, yield 94% **b)** Dioxane, *L*-Val (R:$CH(CH_3)_2$) or *L*-Leu (R:$CH_2CH(CH3)_2$)/$NaHCO_3/H_2O$, rt, 1h, yield 97% **c)** 1. THF, *N*-methylmorpholine, isobutylchloroformate, -15°C 2. phosphonic ester/DMF, yields 50 to 70% (R':table 1-corresponding ethyl esters) **d)** 1. HBr/AcOH, 3h 2. propylene oxide/MeOH, HPLC, yields 30 to 50% (R':table 1) **e)** Solution A: CH_2Cl_2, DIEA, BOP, 0°C, 30min, solution B: phosphonous acid, DMF, DIEA, 0°C, 30min; add A to B, 0°C, 12h **f)** DMF, DPPA, 0°C, 24h then DPPA, H_2O, 4°C, 3 d, yield 70% (R'' and R''':table 1) **g)** $H_2/Pd/C$, MeOH, HPLC, yields 20 to 40%.

These compounds were synthesized (scheme 1) from γ-*N*-hydroxysuccinimide-activated, Z-protected *L*-γ-glutamic acid α-benzyl ester **18** and *L*-Val or *L*-Leu. The dipeptide **19** obtained was coupled using a mixed anhydride method to the phosphonic moieties protected as esters (for compounds **1-10, 17**) or to the free phosphinic acid moieties with the aid of BOP[12] (for **11-15**) or DPPA[9] (for **16**). Deprotection of the intermediate phosphonic (**20**)- and phosphinic (**21**) esters was done with usual methods.

Starting phosphonic esters were obtained as follows. Diethyl esters of Gly^P, ß-Ala^P and $Gaba^P$ were synthesized by an Arbuzov reaction from *N*-phthalyl protected ω-bromoalkane amines.[13] Diethyl p-aminobenzylphosphonate is commercially available. Gly^P diphenylester was prepared from paraformaldehyde and triphenylphosphite.[14] Diethyl 3-amino-2-oxo-propylphosphonate **22** was prepared (scheme 2) from Z-Gly **23**. Mixed anhydride activation with isobutyl chloroformate/*N*-methylmorpholine, reaction with diazomethane and subsequent addition of hydrobromic acid in ether afforded the corresponding 1-benzyloxycarbonylamino-3-bromo-2-propanone **24**. **22** was obtained by an Arbuzov reaction and deprotection by hydrogenolysis.

Scheme 2



a) 1. isobutylchloroformate/Et_3N, $CHCl_3$, -5°C 2. CH_2N_2/ether, 0°C, yield 65% **b)** HBr/ether, 0°C, yield 95% **c)** $P(OEt)_3$, 100°C, HPLC, yield 56% **d)** H_2, Pd/C, MeOH, yield 95%.

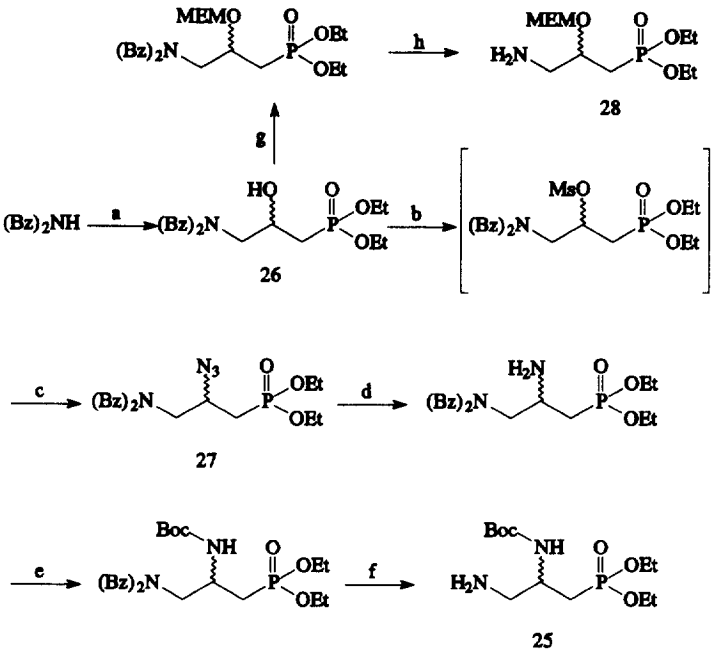
Diethyl 3-amino-2-*tert*-butyloxycarbonylaminopropylphosphonate **25** was prepared (scheme 3) as a racemic mixture from diethyl 2,3-epoxypropylphosphonate. Ring opening with dibenzylamine and subsequent treatment of alcohol **26** with mesylchloride and sodium azide under phase transfer conditions afforded the corresponding azide **27**. Reduction of the latter with sodium borohydride, introduction of the Boc protecting group and deprotection afforded **25** as a racemic mixture.

Diethyl 3-amino-2-hydroxypropylphosphonate could not be obtained in satisfactory yields by reduction of **22**. It was used as MEM-protected compound **28** and obtained (scheme 3) as a racemic mixture, from **26** through MEM protection with MEM chloride and deprotection.

Starting phosphinic acids were obtained as their free acids from trimethylsilyl phosphonite as described.[15,16] Some spectroscopic data are listed as a note.[17]

All compounds were tested as inhibitors at 5mM for glutathionylspermidine synthetase, purified from *C. fasciculata* as described.[4] Hydrolysis of ATP during the synthetase reaction is measured spectrophotometrically from oxidation of NADH, coupled by means of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase. The compounds did not inhibit the enzymes used in the ATP assay.

Scheme 3



a) diethyl 2,3-epoxypropylphosphonate (prepared by an Arbuzov reaction from 1-bromo-2,3-epoxypropane), MeOH, reflux, 6h, yield 90% **b)** MsCl, C_6H_6, Et_3N, 0°C, 30min. **c)** NaN_3/H_2O, tetrabutylN$^+$Br$^-$, 60°C, 3h, yield 60% **d)** $NaBH_4$, THF/MeOH, reflux, yield 65% **e)** $(Boc)_2O$, Et_3N, dioxane, yield 85% **f)** H_2, $Pd(OH)_2/C$, MeOH, yield 88% **g)** MEM-N$^+$(Et)$_3$Cl$^-$/CH_3CN, reflux, 5h, 66% **h)** H_2, $Pd(OH)_2/C$, MeOH, yield 94%.

As shown in table I some compounds show modest inhibitory activity. The most active compound is *L*-γ-Glu-*L*-Leu-Gly^P **2**, with a K_i value of 60 ± 9μM (linear non-competitive inhibition). The diphenyl ester of **2** (**17**) is slightly less active than his parent compound. The *L*-Val (P)-tripeptide **1** is less active (K_i: 290 μM) and chain extension as in compounds **3-6** strongly decreases the inhibitory activity. Acylphosphate analogues **7-10** and phosphinic acids **11-16** are less active. We can conclude that phosphonic acid *L*-γ-Glu-*L*-Leu-Gly^P **2** is a modest inhibitor of glutathionylspermidine synthetase. **2** may be useful as a lead compound for further development of more potent inhibitors for glutathionylspermidine synthetase.

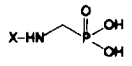
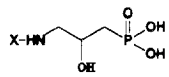
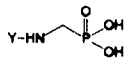
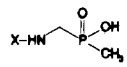
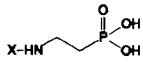
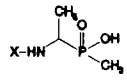
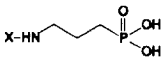
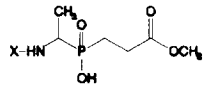
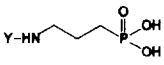
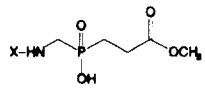
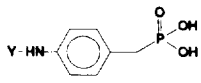
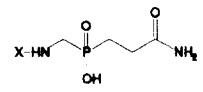
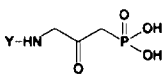
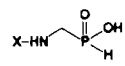
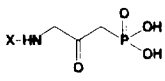
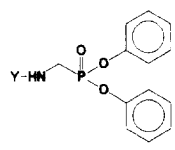
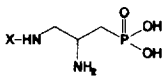
	Compound	%Inh		Compound	%Inh
1		78	10		NI
2		86	11		26
3		NI	12		14
4		NI	13		15
5		24	14		71
6		NI	15		11
7		62	16		50
8		NI	17		76
9		10			

Table 1: Inhibitory properties of **1-17** (at 5 mM) on glutathionylspermidine synthetase in the presence of 5 mM glutathione and spermidine. Other assay conditions were as described previously. (NI=not inhibitory, X= γ-L-Glu-L-Val-, Y=γ-L-Glu-L-Leu-).

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16. Borloo, M.; Jiao, X-Y.; Wojtowicz, H.; Padinchare, R.; Verbruggen, C.; Augustyns, K.; Haemers, A. *Synthesis*, **1995**, 1074-1076.
17. Some spectroscopic data: ^{1}H-NMR (300 MHz) and mass spectra (FAB).
1. (D_2O+MeOD): 0.95 (6H, d, $CH(\mathbf{CH_3})_2$); 2.31 (3H, m, $CH\mathbf{CH_2}$,$\mathbf{CH}(CH_3)_2$); 2.50 (2H, m, $CH_2\mathbf{CH_2}C=O$); 3.45 (2H, d, CH_2P); 4.02 (1H, t, CH-glu); 4.20 (1H, d, CH-val). $[M+H]^+$ = 341.
7. (D_2O): 0.94 (6H, d, $CH(\mathbf{CH_3})_2$); 1.65 (2H, m, $\mathbf{CH_2}CH(CH_3)_3$); 2.16-2.38 (4H, m, CH_2P, $CH\mathbf{CH_2}CH_2$); 2.49 (2H, t, $CH_2\mathbf{CH_2}C=O$); 3.77 (3H, m, $N\mathbf{CH_2}C=O$); 3.87 (1H, t, CH-glu); 4.19 (1H, dd, CH-leu). $[M+H]^+$ = 397.
8. (D_2O): 0.95 (6H, dd, $CH(\mathbf{CH_3})_2$); 2.08-2.43 (5H, m, CH_2P, $\mathbf{CH}(CH_3)_2$, $CH\mathbf{CH_2}CH_2$); 2.53 (2H, t, $CH_2\mathbf{CH_2}C=O$); 3.78 (2H, m, $N\mathbf{CH_2}C=O$); 4.06 (1H, m, CH-glu); 4.27 (1H, dd, CH-val). $[M+H]^+$ = 383.
9. (D_2O): 0.98 (6H, dd, $CH(\mathbf{CH_3})_2$); 2.01 (2H, m, CH_2P); 2.15-2.35 (3H, m, $\mathbf{CH}(CH_3)_2$, $CH\mathbf{CH_2}CH_2$); 2.56 (2H, t, $CH_2\mathbf{CH_2}C=O$); 3.61 (3H, m, $N\mathbf{CH_2}CHNH$); 3.75 (1H, m, CH-glu); 4.17 (1H, d, CH-val). $[M+H]^+$ = 384.
10. (D_2O): 1.07 (6H, dd, $CH(\mathbf{CH_3})_2$); 2.03 (2H, m, CH_2P); 2.18 (1H, m, $\mathbf{CH}(CH_3)_2$); 2.34 (2H, m, $CH\mathbf{CH_2}CH_2$); 2.69 (2H, t, $CH_2\mathbf{CH_2}C=O$); 3.47 (3H, m, $N\mathbf{CH_2}CHOH$); 3.97 (1H, m, CH-glu); 4.19 (1H, m, CH-val). $[M+H]^+$ = 385.
13. (D_2O): 1.07 (6H, d, $CH(\mathbf{CH_3})_2$); 1.45 (3H, dd, $CH\mathbf{CH_3}$); 2.05 (2H, m, $P\mathbf{CH_2}$); 2.32 (3H, m, $\mathbf{CH}(CH_3)_2$, $CH\mathbf{CH_2}CH_2$); 2.68 (4H, m, $CH_2C=O$, $CH_2C=O$); 3.32 (1H, d, HNCHP); 3.43 (3H, s, OCH_3); 3.80 (1H, t, CH-glu); 4.22 (1H, d, CH-val). ^{31}P-NMR : 47.74. $[M+H]^+$ = 425.
14. (D_2O): 1.07 (6H, d, $CH(\mathbf{CH_3})_2$); 1.92 (2H, m, $P\mathbf{CH_2}CH_2$); 2.24 (3H, m, $\mathbf{CH}(CH_3)_2$, $CH\mathbf{CH_2}CH_2$); 2.65 (4H, m, $CH_2C=O$, $CH_2C=O$); 3.52 (2H, d, $HN\mathbf{CH_2}P$); 3.83 (3H, s, OCH_3); 3.89 (1H, t, CH-glu); 4.24 (1H, d, CH-val). ^{31}P-NMR: 39.49. $[M+H]^+$ = 411.
16. (D_2O + DMSO): 0.95 (6H, d, $CH(\mathbf{CH_3})_2$; 1.98 (2H, m, $CH\mathbf{CH_2}CH_2$); 2.15 (1H, m, $\mathbf{CH}(CH_3)_2$); 2.38 (2H, m, $CH_2C=O$); 3.22 (2H, dm, CH_2P, J: 214 Hz); 3.98 (1H, m, CH-glu); 4.17 (1H, d, CH-val); 7.09 (1H, d, J: 588 Hz, P-H); $[M+H]^+$ = 325.

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