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NOVEL INHIBITORS OF TRYPANOTHIONE BIOSYNTHESIS: SYNTHESIS AND EVALUATION OF A PHOSPHINATE ANALOG OF GLUTATHIONYL SPERMIDINE (GSP), A POTENT, SLOW-BINDING INHIBITOR OF GSP SYNTHETASE

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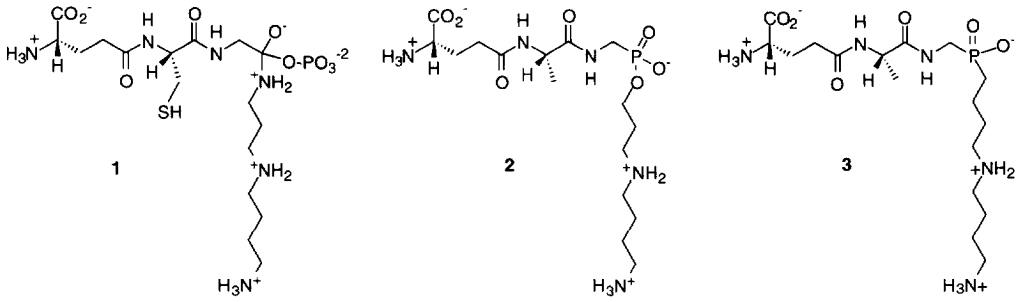
Abstract: A phosphinate analog of glutathionyl spermidine (Gsp), **3**, has been synthesized and evaluated as an inhibitor of Gsp synthetase (GSPS). In addition, a non-spermidine-containing derivative (**4**) was synthesized in order to evaluate the role of the polyamine moiety in ligand binding. Compound **4** showed almost no inhibitory activity against *E. coli* GSPS while **3** was found to be a potent, slow-binding inhibitor of this enzyme, with the collisional E•I complex isomerizing to an E•I* complex with 410-fold higher affinity.

Similar to aerobic cells, protozoal parasites of the genera *Trypanosma* and *Leishmania* are confronted with so-called oxidative stress or oxidative siege[1] from various reactive oxygen species (ROS). Endogenous antioxidants play important roles in defenses against ROS and the associated lethal consequences to the organisms. Since the discovery of the unique trypanothione (TSH) antioxidant system in parasites,[2] TSH biosynthesis has become an attractive target for antiparasitic drug design.[3] In fact, it has been postulated that several experimental and therapeutic drugs such as pentamidine, berenil, difluoromethylornithine (DFMO), and buthionine sulfoximine exert their trypanocidal activities by obstructing various steps of TSH biosynthesis.[4] Recently, we have been involved in research to investigate the enzymology associated with glutathione-spermidine conjugates and to develop antiparasitic agents by the design and synthesis of enzyme inhibitors designed to block the biosynthesis of trypanothione.[5-7] An obvious enzyme of interest is glutathionyl spermidine (Gsp) synthetase (GSPS), an ATP-dependent ligase which participates in amide bond formation between the ubiquitous redox cofactor, glutathione, and the polyamine, spermidine, at the penultimate step of TSH biosynthesis. Although the exact mechanism for the GSPS-catalyzed reaction is not well established, we have proposed a tetrahedral intermediate (Figure 1, structure **1**) derived from an acyl phosphate based on extensive literature precedents of other ATP-dependent ligases/synthetases.[5]

Figure 1

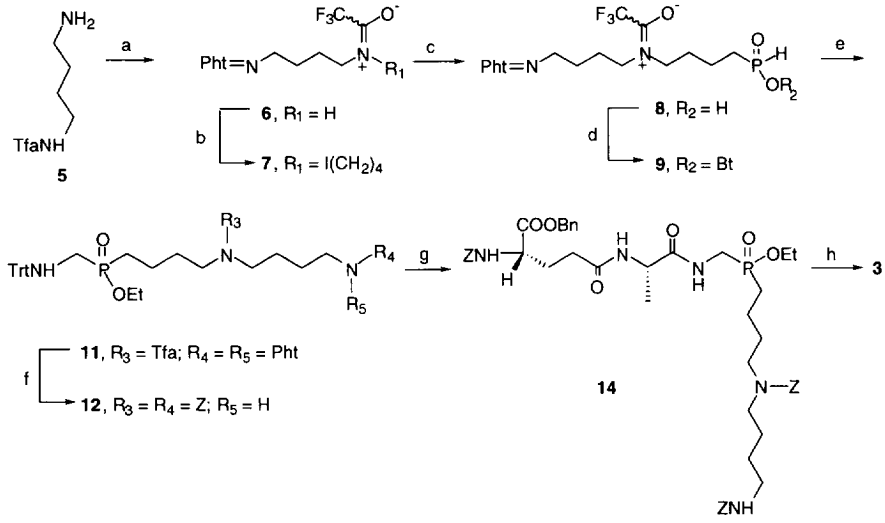


Phosphonates, phosphonamidates, and phosphinates have been employed successfully in protease and ligase enzyme inhibitor design. These stable tetrahedral phosphorous species are thought to act by mimicking the tetrahedral intermediates of the reaction catalyzed by this group of enzymes.[8] Using this concept, potent phosphorus containing inhibitors for D-alanine:D-alanine ligase[9-12] and glutamine synthetase[13-15] have been developed and studied in detail. In previous research,[7] we have shown that a phosphonate tetrahedral mimic (Figure 1, structure **2**) of the proposed intermediate, **1**, is indeed an excellent inhibitor of GSPS with an inhibition constant of 6 μM for the inhibitor binding to the free enzyme (K_i). However, no time-dependence was observed for the inhibition of GSPS by **2**. Prompted by the recent publication on the use of phosphinic acids as inhibitors of GSH synthetase,[16] γ-glutamylcysteine synthetase[17] and GSPS,[18] we wish to communicate our results on synthetic and preliminary biochemical studies of a phosphinate mimic (Figure 1, structure **3**) of the proposed tetrahedral intermediate (**1**). The synthesis and evaluation of a non-polyamine-containing phosphinate, **4**, for use in ascertaining the contribution of the spermidine-like moiety of **3** to ligand binding, is also described.

Chemistry

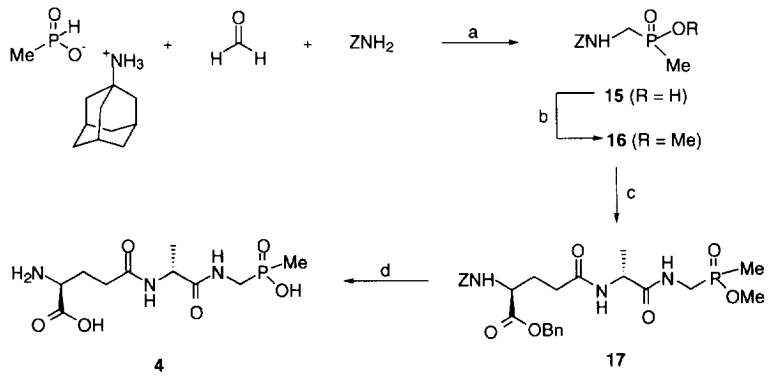
Synthesis of the target phosphinate **3** is outlined in Scheme 1. Starting from mono-Tfa-protected putrescine (**5**),[19] the polyamine building block **7** was prepared in two steps in moderate yield. As evidenced by its carbon and fluorine NMR, compound **7** exists as an equimolar mixture of its *Z* and *E* isomers. This *Z/E* mixture was subjected to an Arbuzov-type reaction with bistrimethylsilyl hydrogen phosphonite (BTH).[20] The formation of the corresponding polyamine-bearing phosphonous acid **8** was not observed when the reaction of **7** and BTH was carried out in either dichloromethane[20] or THF at rt or under reflux. Satisfactory results were achieved only when the reaction was carried out in toluene at 105 °C. Initially, we tried to construct the other P-C bond directly from **8**, through the corresponding in situ-generated trimethylsilyl phosphonite,[20] and the Schiff base, **10**, derived from trityl amine and formaldehyde, but without success. Therefore, **8** was converted to the corresponding ethyl ester (**9**) by coupling with ethanol in the presence of DCC and DMAP[21,22] or preferably using PyBOP as the coupling reagent.[23] The phosphonite **9** could be purified readily by flash chromatography on silica gel using an aprotic solvent (EtOAc); it was later found to be hydrolytically labile even in the absence of an acid or a base catalyst. Abramov-type reaction of **9** with **10**, afforded the key phosphinate intermediate

Scheme 1



Reagents and conditions: (**a**) N-carbethoxyphthalimide, TEA, THF, reflux, 93%; (**b**) 1,4-diiodobutane, KH, 18-C-6, THF, 55%; (**c**) (i) $H_2PO_2^-$ NH_4^+, HMDS, 110 °C, 1.5 h; (ii) toluene, 105 °C, 12 h; (**d**) PyBOP, EtOH, DIEA, 76% (from **7**); (**e**) $Ph_3CN{=}CH_2$ (**10**), $BF_3{\bullet}Et_2O$, toluene, reflux, 81%; (**f**) (i) $NH_2NH_2{\bullet}H_2O$, MeOH; (ii) ZCl, DIEA, CH_2Cl_2, 82%; (**g**) (i) 1 M HCl/MeOH, reflux, 20 min; (ii) Z-Glu (Ala-OH)-OBn (**13**), PyBOP, DIEA, CH_2Cl_2, 80%; (**h**) (i) 30% HBr/AcOH, rt, 48 h; (ii) AG 50W-X2 cation exchange resin, 0.1 M NH_4HCO_3 buffer, 77%.

Scheme 2



Reagents and conditions: (**a**) AcCl, rt, 6 h then H_3O^+, yield 67%; (**b**) CH_2N_2, 100%; (**c**) (i) H_2, 10% Pd/C; (ii) BOP, Z-Glu(Ala-OH)-OBn, DIEA, 85% (from **16**); (**d**) (i) H_2, 10% Pd/C; (ii) 30% HBr in AcOH, rt, 94% (from **17**); or 1 N LiOH, rt, 12 h and then H_2, 10% Pd/C, 20% (from **17**).

(**11**) in excellent yield. In order to avoid any possible deprotection problems in the final steps leading to **3**, the phthaloyl and Tfa protecting groups in **11** were removed simultaneously by hydrazinolysis and the resulting free amines were reprotected as benzyl carbamates to provide **12** in good overall yield. No detectable hydrolysis of

the ethyl ester occurred during the hydrazinolysis process leading to the conclusion that the ethyl ester in **11** is much more stable than in **9**. Selective removal of the trityl group in **12** followed by PyBOP-mediated coupling of the resultant amine and protected dipeptide **13** provide the protected target phosphinate **14** in good yield. The carboxyl benzyl ester, phosphorus ethyl ester, and the three benzyloxyl carbonyl groups in **14** were subsequently removed simultaneously by treatment with 30% hydrogen bromide in acetic acid at rt. The target phosphinic acid (**3**) was obtained in satisfactory yield (77%).[24]

In order to evaluate the role of the polyamine portion in the inhibition of GSPS by phosphapeptides such as **2** and **3**, a truncated phosphinate (**4**)[25] was synthesized from methyl α-benzoxylcarbonylaminomethane-phosphinic acid (**15**)[26] (Scheme 2).

Enzyme Inhibition Study

Enzyme kinetic data were obtained for the inhibition of *E. coli* GSPS[6] by **3** and **4** and analyzed in a similar way to that reported for D-alanine:D-alanine ligase[10] using a pyruvate kinase/lactate dehydrogenase coupled assay. Compound **3** showed potent inhibitory activity whereas the non-polyamine-containing derivative, **4**, was devoid of significant inhibitory activity at concentrations up to 7 mM (Table 1). In contrast to phosphonate, **2**, a reversible, non-competitive inhibitor,[7] phosphinate **3** is a time-dependent inhibitor of GSPS (Figure 2). As a linear mixed-type inhibitor, phosphinate **3** has an inhibition constant of 3.2 μM for inhibitor

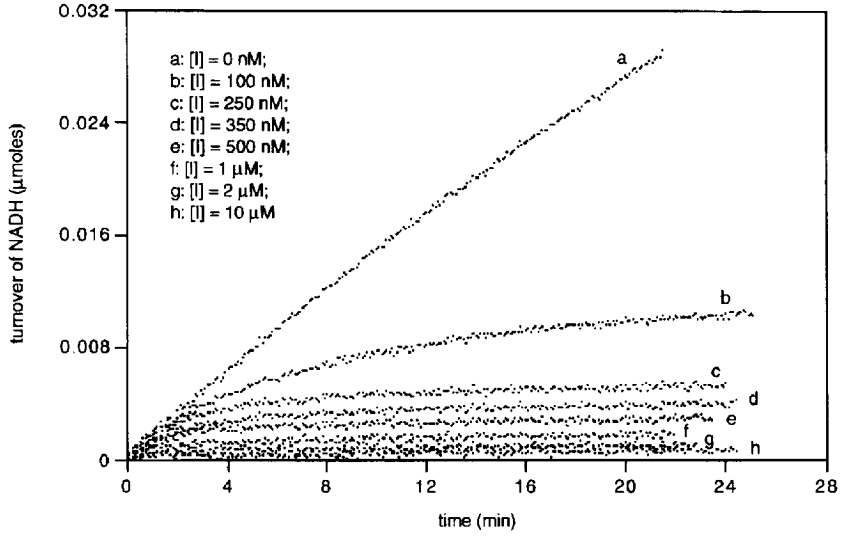


Figure 2. Progress curves for the inhibition of Gsp synthetase by phosphinate **3**. The study was assayed spectrophotometrically by coupling the hydrolysis of ATP to the pyruvate kinase/lactate dehydrogenase reactions, and initiated by adding Gsp synthetase to an assay mixture containing glutathione (1.56 mM), spermidine (10 mM), ATP (2 mM), and **3** (0.1-10 μM) in 50 mM NaPIPES (pH 6.8) at 37 °C.

binding to free enzyme (K_i) and tightens over time, in a typical E•I $\rightleftharpoons$ E•I* slow-binding isomerization[27] to a 410-fold more potent inhibitor, at 7.8 nM for binding to enzyme-substrate complex (K_i*) (Table 1). Preliminary data indicate that the inhibition of GSPS by **3** is ATP-dependent and that recovery from inhibition is slow (C-H. Lin, unpublished results); such a mechanism was excluded for the phosphonate inhibitor **2**.[7]

In a very recent paper,[18] the synthesis of several tripeptidic phosphonic and phosphinic acids lacking a polyamine moiety (**18**-**20**, Table 1) and their inhibition of GSPS were reported. The most potent of the series, L-γ-Glu-Leu-Glyp (**18**), is a linear non-competitive inhibitor, with an inhibition constant of 60 μM. Replacement of Leu by Val results in a slight decrease of inhibition potency (**19**, K_i = 290 μM). The corresponding polyamine-containing phosphonic acid tripeptide **2** previously reported by us[7] is only a slightly more potent inhibitor (K_i = 6 μM) for GSPS compared to the simpler phosphapeptides **18** and **19**. All compounds in Table 1 are non-competitive inhibitors. Although not established for the phosphonate series (e.g., **2**) the potent inhibition by phosphinic acid inhibitors such as **3**, seems to require the polyamine portion; that is, the truncated analogs of **3** (**4** and **20**, Table 1) showed extremely poor inhibitory activity towards GSPS. This requirement for a spermidine-like moiety in potent GSPS inhibitors reflects the structure requirements for spermidine substrates in GSPS catalysis.[6] The polyamine-containing phosphinic peptide **3** is the most potent inhibitor of GSPS reported to date and the only one to show slow-binding, progressive time-dependent conversion to a tightened complex. The difference between the initial K_i of 3.2 μM and the K_i* of 7.8 nM reflects a 410-fold gain in potency from traversing this additional binding mode. Further efforts to improve on the inhibitory potency of **3** and to develop similar inhibitors of related enzymes involved in trypanothione biosynthesis are currently in progress.

Table 1. Inhibition of GSPS by phosphapeptides: AA-NHCH$_2$-P(=O)(OH)XR

Compds	AA	X	R	K_i (μM)	K_i* (μM)	References
2	γ-Glu-Ala	O	$(CH_2)_3NH(CH_2)_4NH_2$	6.0	14	7
3	γ-Glu-Ala	CH_2	$(CH_2)_3NH(CH_2)_4NH_2$	3.2	0.0078	This work
4	γ-Glu-Ala	CH_2	H	a	a	This work
18	γ-Glu-Leu	O	H	60	-	19
19	γ-Glu-Val	O	H	290	-	19
20	γ-Glu-Leu	CH_2	H	b	b	19

[a]6.7% Inhibition at 7 mM.

[b]26% Inhibition at 5 mM.

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24. Spectroscopic data for **3**: ^{1}H NMR (D_2O) δ 1.36 (d, 3H, J = 7.2), 1.41-1.59 (m, 4 H), 1.62-1.78 (m, 6H), 2.00-2.12 (m, 2H), 2.43 (t, 2H, J = 6.5), 2.90-3.17 (m, 6H), 3.35 (d, 2H, J = 9.3), 3.68 (t, 1H), 4.24 (q, 1H); ^{13}C NMR (CD_3OD) δ 176.5, 175.8, 175.1, 56.1, 51.2, 48.2, 48.1, 40.5 (d, $^1J_{C\text{-}P}$ = 98 Hz), 40.2, 32.9, 30.4, 28.6 (d, $^1J_{C\text{-}P}$ =93 Hz), 28.3 (d, $^2J_{C\text{-}P}$ = 16 Hz), 26.1, 24.2, 20.1, 17.6; ^{31}P NMR (D_2O) δ 39.7; MS (FAB) *m/z* 438 (MH^+, 89), 155 (24), 121 (12), 89 (100); HRMS (FAB) calcd for $C_{17}H_{36}N_5O_6PH$: 438.2481. Found: 438.2482; Reversed-phase HPLC[28] t_R = 7.7 min.
25. Spectroscopic data for **4**: ^{1}H NMR (D_2O) δ 1.18 (d, 3H, J = 14), 1.37 (d, 3H, J = 7), 2.08-2.12 (m, 2H), 2.40-2.50 (m, 2H), 3.35 (d, 2H, J = 10), 3.79-3.78 (q, 1H), 4.23-4.28 (q, 1H); ^{13}C NMR (CD_3OD) δ 176.4, 176.3, 175.7, 55.9, 51.8, 42.1 (d, J = 100), 32.8 (d, J = 27), 27.9, 18.3, 15.8 (d, J = 94); ^{31}P NMR (D_2O) δ 38.3; MS (FAB) *m/z* 310 (MH, 100), 172 (41), 110 (33); HRMS (FAB) calcd for $C_{10}H_{20}N_3O_6PH$: 310.1168. Found: 310.1176.
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