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A nonhydrolyzable analogue of phosphotyrosine, and related aryloxymethano- and aryloxyethano-phosphonic acids as motifs for inhibition of phosphatases

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Abstract—Nonhydrolyzable analogues of both stereoisomers of phosphotyrosine, and a series of related aryloxy (or thio) methyl and aryloxy (or thio) ethyl phosphonic acids of the general formula $RX–(CH_2)_n–PO_3H_2$ (where X = O or S and $n = 1$ or 2), have been tested as nonhydrolyzable mimetics of phosphatase substrates. These compounds were tested against a panel of phosphatases (two alkaline phosphatases, a protein–tyrosine phosphatase, and two serine/threonine phosphatases) with different active site motifs. The compounds exhibit competitive inhibition toward all enzymes tested, with the best inhibition expressed toward the Ser/Thr phosphatases. The stereoisomers of the phosphotyrosine analogues exhibited an unexpected difference in their inhibitory properties toward the protein–tyrosine phosphatase from *Yersinia*. The K_i for the D isomer is 33-fold lower than that of the L isomer, and is more than an order of magnitude lower than the reported K_m of the substrate L-phosphotyrosine.

The phosphorylation of certain amino acid residues,[‡] controlled by protein phosphatases and kinases, regulates a number of processes in living organisms, including metabolic pathways, membrane transport, gene transcription, and motor mechanisms. The therapeutic potential of phosphatase inhibition creates an interest in the design of potent and selective inhibitors, in mechanistic studies, and structural investigations. We have sought nonhydrolyzable substrate analogues for use in structural studies with phosphatases, particularly metallophosphatases.

Simple phosphonic acids are nonhydrolyzable analogues of phosphate esters in which the P–O ester bond is replaced with a nonhydrolyzable P–C bond ($RO–PO_3H_2$ vs $R–PO_3H_2$). Such compounds are generally moderate inhibitors of phosphatases; for example, K_i values of phosphonates toward *E. coli* alkaline phosphatase (EcAP) are in the high micromolar to the millimolar range.[1] Phosphonic acids (which exist at physiological pH as their salts, called phosphonates) continue to be the focus of recent active academic research[2–4] and patents[5–8] involving the potential use of such compounds in several diseases, including cancer and HIV.

However, structural studies show that phosphonates bind in unusual manners. Structures have been reported for EcAP with bound mercaptomethylphosphonic acid ($K_i = 600\,\mu M$) and with bound phosphonoacetic acid ($K_i = 5.5\,mM$).[9] In the former case, the inhibitor binds with the sulfur atom coordinated to zinc, and the phosphonate group is directed away from the active site. Phosphonoacetic acid binds with the phosphonate moiety in the active site, but exhibits an unconventional hydrogen bond between an arginine nitrogen atom and a hydrogen of the methylene group.[9]

α-Fluorophosphonates have been widely examined as inhibitors of phosphatases, and structural elaborations of such compounds are potent inhibitors of a number of phosphatases, particularly members of the protein–tyrosine phosphatase (PTP) family.[10–13] However, as with other phosphonate inhibitors, X-ray structural analyses have shown that such compounds bind in a fashion different from that of a substrate. A good example has been documented with PTP 1-B. The X-ray structure of the substrate phosphotyrosine, bound to a

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catalytically disabled form of PTP 1-B in which the nucleophilic Cys was mutated to Ser, revealed a normal binding mode.[14] In contrast, structures of the native enzyme complexed with α-fluoro phosphonates show that the fluorine atoms result in altered conformations of the active site residues that bind the phosphoryl group (Asp 181 and Phe 182).[12,13] As a result, the catalytic general acid is in a position that would be nonfunctional in a catalytic complex.

These unusual binding modes suggest that the presence of the ester (P–OR) oxygen atom in the substrate is important for proper binding in the catalytic complex. The compounds studied here were designed as substrate mimics, incorporating the presence of an electronegative heteroatom (O or S) in a position approximating that of the ester oxygen atom, that might afford sufficient inhibition to be used for future structural analysis studies. On this basis, we explored compounds of the general structure $RX–(CH_2)n–PO_3^{2-}$ (where X = O or S and $n = 1$ or 2). We also synthesized and tested the D and L isomers of the nonhydrolyzable analogues of phosphotyrosine, where R = D or L tyrosine and $n = 1$. Figure 1 shows the compounds that were synthesized and tested.

Another advantage of aryloxymethyl and aryloxyethyl phosphonic acids over simple phosphonic acids is the reduction in pK_a values of the phosphonyl moiety. Simple aliphatic phosphonic acids exhibit second pK_a values about one-half unit higher (~7.0) than those of phosphate esters (~6.5). For those phosphatases that hydrolyze phosphate esters in their dianion form, which includes all of those in this study, this change in pK_a will result in a greater proportion of the inhibitor present in the dianion form at physiological pH. The second pK_a values of alkyl–O–CH_2–PO_3H_2 or alkyl–S–CH_2–PO_3H_2 alkyl–S are lowered relative to those of alkyl phosphonic acids, to values close to those of phosphate esters.[15] This was confirmed by measurement of the second pK_a from a plot of ^{31}P NMR chemical shift versus pH for one of our compounds, entry **5** in Figure 1, which bears an aryl–O moiety. The second phosphonic acid pK_a of **5** was determined to be 6.52 ± 0.01.

The general synthetic routes leading to the phosphonic acids are shown in Figure 2. Syntheses of $RX–(CH_2)_2–PO_3H_2$ start from appropriate phenolates or thiolates, which are coupled to 1,2-dibromoethane in a phase-transfer catalysis version of the Williamson synthesis. The alkyl bromides are then reacted with triethyl phosphite in a Michaelis–Arbuzov reaction to form phosphonoesters, which are then deprotected with HCl. The $RO–CH_2–PO_3H_2$ compounds are synthesized using the reagent diethyl 4-chlorophenylsulfonyloxymethylphosphonate developed by Conforth and Wilson.[16] Figure 3

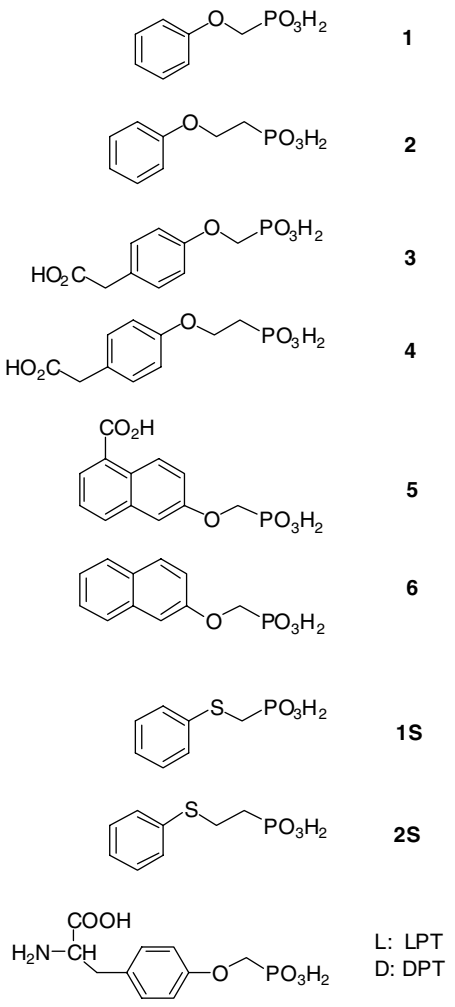

Figure 1. Inhibitors synthesized for this work.

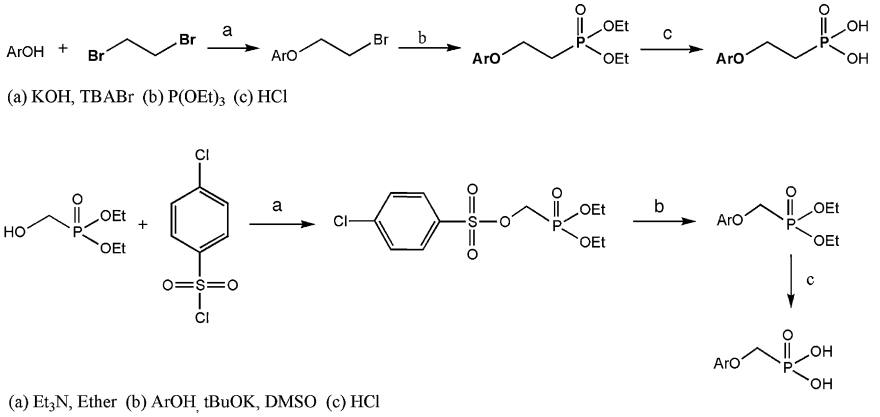

Figure 2. General synthetic routes to aryloxyethyl (top) and aryloxymethyl (bottom) phosphonic acids.

shows the synthetic schemes used to prepare the sulfur analogues **1S** and **2S**. The phosphotyrosine analogues were prepared by reacting the protected tyrosine[17] with diethyl 4-chlorophenylsulfonyloxymethylphosphonate in the presence of potassium *t*-butoxide in DMSO, followed by deprotection with HCl. All compounds were fully characterized by proton and phosphorus NMR, and had elemental analyses within 0.4% of the calculated values.

The compounds in Figure 1 were tested as inhibitors with human placental alkaline phosphatase (PLAP), *E. coli* alkaline phosphatase (EcAP), protein–tyrosine phosphatase from *Yersinia* (YOP), and serine/threonine protein phosphatases 2C (PP2C) and λ (λPP). These phosphatases represent three different classes of active sites and catalytic mechanisms. The alkaline phosphatases have two zinc ions at their active sites, and catalyze phosphoryl transfer by a two-step mechanism with a phospho-serine intermediate.[19,20] The *Yersinia* enzyme has the active site typical of PTPases, which lack a metal cofactor, and accomplish phosphoryl transfer by a two-step mechanism via a phospho-cysteine intermediate.[21] The serine/threonine protein phosphatases utilize a dinuclear metal center, and catalyze direct phosphoryl transfer to a metal-bound water molecule.[22]

Inhibition experiments were conducted using *p*-nitrophenyl phosphate as the substrate at 25 °C. Reaction initial rates were measured spectrophotometrically as changes of absorbance at 400 nm, associated with the formation of *p*-nitrophenolate anion. Lineweaver–Burk plots were used to assess the type of inhibition. Inhibition constant values were calculated by nonlinear least squares fit to the competitive inhibition Michaelis–Menten equation (Eq. 1).

$$\frac{v}{V_{\max}} = \frac{[S]}{K_{\mathrm{m}}\left(1 + \frac{[I]}{K_{\mathrm{i}}}\right) + [S]}. \quad (1)$$

Conditions used for kinetic measurements were as follows:

For PLAP and EcAP: TRIS (100 mM), $ZnCl_2$ (1 mM), $MgCl_2$ (1 mM), pH = 9.0. For YOP: succinate (100 mM), NaCl (150 mM), pH = 6.6. For PP2C: TRIS (100 mM), $MnCl_2$ (5 mM), DTT (1 mM), pH = 7.5. In case of compounds that precipitated at $[Mn^{2+}] = 5$ mM, the kinetics were carried out at $[Mn^{2+}] = 1$ mM. For λPP: MOPS (100 mM), $MnCl_2$ (1 mM), DTT (1 mM), pH = 7.3. The results are shown in Table 1. All inhibitors in this study were found to exhibit competitive inhibition.

After this project was underway, we became aware of a previous, but more limited, study of aryloxymethyl

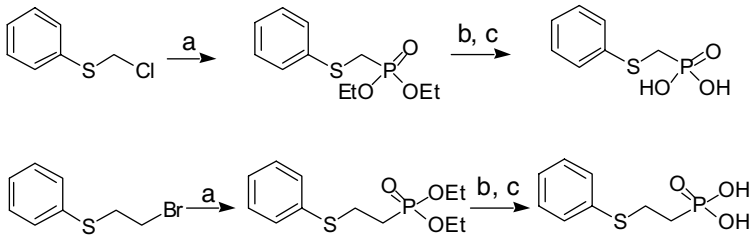

Figure 3. Synthetic scheme used to prepare compounds **1S** and **2S**. Chloromethyl phenyl sulfide was obtained commercially; 1-bromo-2-phenyl thioethane was prepared by a literature procedure.[18] Reagents and conditions: (a) $P(OEt)_3$, 125 °C; (b) TMSI, CH_2Cl_2, 0 °C to rt; (c) MeOH.

Table 1. Inhibition constants (mM) for the phosphotyrosine analogues LPT and DPT, compounds **1**–**6**, and the thio analogues **1S** and **2S**, with the phosphatases examined in this study

	Alkaline phosphatases		PTPase	Ser/Thr PPases	
	PLAP (pH = 9.0)	EcAP (pH = 9)	YOP (pH = 6.6)	PP2C (pH = 7.5)	λPP (pH = 7.3)
1	0.9 ± 0.1	No inhibition	5 ± 1	0.32 ± 0.05*	0.27 ± 0.03
2	0.118 ± 0.004	0.102 ± 0.003	2.17 ± 0.22	0.40 ± 0.09*	0.05 ± 0.01
3	No inhibition	5.1 ± 0.6	12 ± 1	0.13 ± 0.01	0.19 ± 0.03
4	0.14 ± 0.03 (pH = 8.0)	0.6 ± 0.1	19 ± 3	0.23 ± 0.02	0.12 ± 0.06
5	3.4 ± 0.5	4.2 ± 0.8	2.2 ± 0.2	0.067 ± 0.005	0.037 ± 0.002
6	1.1 ± 0.1	4.0 ± 0.8	6.3 ± 0.7	Precipitation	Precipitation
1S	0.131 ± 0.007	0.04 ± 0.04	4.6 ± 0.5	0.17 ± 0.03*	0.33 ± 0.06
2S	0.69 ± 0.03	0.9 ± 0.2	10 ± 2	0.3 ± 0.1*	0.14 ± 0.09
LPT	1.7 ± 0.3	5 ± 1	21 ± 4	0.76 ± 0.05	0.022 ± 0.009
DPT	1.85 ± 0.02	4.8 ± 0.9	0.63 ± 0.06	0.34 ± 0.04	0.033 ± 0.006

'No inhibition' indicates that no reduction in rate was observed in the presence of 5 mM of the inhibitor. For PP2C, $[Mn^{2+}] = 5$ mM except where noted with asterisks, where $[Mn^{2+}] = 1$ mM. The K_m for the substrate *p*NPP is the same within experimental error for PP2C whether $[Mn^{2+}] = 5$ mM or 1 mM (see text), thus, the phosphonic acids are not expected to show appreciably different affinities for PP2C as a function of $[Mn^{2+}]$.

phosphonic acids ($ROCH_2PO_3H_2$) as inhibitors of protein–tyrosine phosphatases.[23] In that study, several compounds were tested as inhibitors of PTP1B and VHR, which share the PTPase catalytic site motif. The reported inhibition constants were generally in the millimolar range,[23] similar to those found in this study with YOP. The present study differs in several significant ways. The prior study tested only two enzymes, both PTPases; no metallophosphatases were examined. No amino acid analogues were prepared, while in the present study, the synthesis and inhibition properties of novel nonhydrolyzable analogues of both the D and L forms of phosphotyrosine are described. In fact, an unexpected difference between the inhibition properties of these stereoisomers provided one of the most intriguing results obtained in this study. The prior study had no chiral compounds of any kind, and the results in this study are the first report of this structural motif incorporated into an amino acid and tested as a phosphatase inhibitor. Finally, the results presented here show that aryloxymethano- and aryloxyethano-phosphonic acids provide significantly better inhibition for metallophosphatases than for PTPases; to our knowledge, these data are the first regarding the inhibition of metallophosphatases by compounds of this type. Only two of the 10 compounds in the present study were examined in the prior one, and none of the phosphatases used in this study are in common.

1. Alkaline phosphatases

For both of the alkaline phosphatases examined, the aryloxyethyl phosphonic acids **2** and **4** are notably better inhibitors than the aryloxymethyl compounds **1**, **3**, **5**, and **6**. The most dramatic examples are **1**, which shows no inhibition of EcAP, while for the ethyl analogue **2**, $K_i = 102\,\mu M$; and **3**, which shows no inhibition of PLAP, while for the ethyl analogue **4**, $K_i = 140\,\mu M$.

The substitution of sulfur for oxygen in the ether position has opposite effects on inhibition depending upon its position in the molecule. Comparison of data for **1** and **2** with **1S** and **2S** show that with the methylene spacer, sulfur substitution significantly improves inhibition for both alkaline phosphatases, while with the ethylene spacer, inhibition is poorer for the sulfur analogue.

Under the conditions of the inhibitions studies (pH = 9.0), the K_m for *p*NPP was 15 μM for EcAP and 25 μM for PLAP. None of the inhibitors exhibited K_i values this low, the closest being the 40 μM K_i exhibited by **1S** toward EcAP.

2. Ser/Thr phosphatases

The Ser/Thr phosphatases PP2C and λPP were the most strongly inhibited of the phosphatases examined in this study. These phosphatases have been most often studied kinetically in the presence of Mn^{2+}. With PP2C a Mn^{2+} concentration of 5 mM has been shown to be necessary for full activity. Phosphonic acids are known to form insoluble polymeric complexes with a number of divalent cations, and a number of compounds in Figure 1 precipitate in the presence of 5 mM Mn^{2+}. Compounds substituted with the carboxyl group (**3**, **4**, **5**) did not precipitate under these conditions, but the manganese concentration was lowered to 1 mM for the inhibition kinetics of PP2C with **1**, **1S**, **2**, and **2S**. Complexes of the naphthyl phosphonic acid **6** precipitated even under these conditions, precluding K_i measurements.

The carboxynaphthyl moiety provided the best discrimination for the two Ser/Thr phosphatases relative to the other enzymes in this study, and was also the best inhibitor of both PP2C and λPP. Comparisons of the K_i values of **1** with **2**, and of **3** with **4**, show that the length of the spacer (methylene or ethylene) does not make a significant difference in inhibitory ability. The substitution of sulfur for oxygen also results in negligible changes in inhibition with the Ser/Thr metallophosphatases, in contrast to the alkaline phosphatases.

The reported K_m of the substrate *p*NPP with PP2C is 1 mM, at pH 7.0, 5 mM Mn^{2+};[24] we obtained a very similar value (1.1 mM) at pH 7.5 under the conditions of our inhibition studies. This value was the same, within experimental error, when Mn^{2+} concentration was lowered to 1 mM. For the lambda phosphatase, at pH 7.3, the K_m of *p*NPP is 5 mM.[25] Both the aryloxymethyl and aryloxyethyl phosphonic acids exhibit K_i values substantially below this value, with those of the best inhibitors (**2** and **5**) more than two orders of magnitude lower.

The nonhydrolyzable analogues of the D and L forms of phosphotyrosine deserve special mention. They are poor inhibitors of the alkaline phosphatases, but better for PP2C and λPP. For λPP, they are the best inhibitors tested. The results with YOP are particularly interesting. Only with this phosphatase do the two stereoisomers exhibit significantly different K_i values. YOP is the only enzyme in this study for which the K_m for the phosphotyrosine substrate has been reported to our knowledge: 9.5 mM at pH 6.6 for L-phosphotyrosine;[26] K_m for *p*NPP is 1.7 mM.[26] The K_i for the analogue of L-phosphotyrosine (LPT) is 21 mM (Table 2), but the value for the D-isomer is 33-fold lower, 0.6 mM. Significantly, the latter value is an order of magnitude below the reported K_m for the L-phosphotyrosine substrate, despite the fact that it is the D isomer. This result suggests that chirality can significantly affect the inhibition properties of even simple phosphonic acids, and, by extension, the incorporation of such nonnatural stereoisomers into more elaborate molecules or polypeptides may afford a way to control phosphatase selectivity.

In summary, we have found that aryloxymethano- and aryloxyethano-phosphonic acids constitute a motif that preferentially inhibits metallophosphatases over PTPases. Incorporation of the methano-phosphonic acid moiety into D and L tyrosine results in novel nonhydrolyzable phosphotyrosine analogues that maintain the oxygen atom corresponding to the scissile P–O ester

bond of the substrate; the lack of this atom may be responsible for unusual binding modes of simpler phosphonic acid inhibitors. Unexpectedly, the D analogue is a significantly better inhibitor of YopH than the natural L isomer, suggesting that stereochemistry of such compounds may offer an avenue for exploitation.

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References and notes

1. Reid, T. W.; Wilson, I. B. In *The Enzymes*; Boyer, P. D., Ed.; Academic: New York, 1971; pp 373–415.
2. Somogyi, G. B. P.; Bodor, N. *Die Pharmazie* **2004**, *59*, 378–381.
3. Zabell, A. P. R. C. S.; Helquist, P.; Stauffacher, C. V.; Wiest, O. *Bioorg. Med. Chem.* **2004**, *12*, 1867–1880.
4. Moosavi-Movahedi, A. A. H. S.; Chamani, J.; Khodarahmi, G. A.; Hassanzadeh, F.; Luo, F.-T.; Ly, T. W.; Shia, K.-S.; Yen, C.-F.; Jain, M. L.; Kulatheeswaran, R.; Xue, C.; Pasdar, M.; Hakimelahi, G. H. *Bioorg. Med. Chem.* **2003**, *11*, 4303–4313.
5. Deluca, H. F.; Pike, J. W.; Shevde, N.; Plum, L. A.; Clagett-Dame, M. *U.S. Pat. Appl. Publ.* 2004; 17 p CODEN: USXXCO US 2004053813 A1 20040318: U.S. 2004; 17 p.
6. Arimilli, M. N.; Becker, M. M.; Bryant, C.; Chen, J. M.; Chen, X.; Dastgah, A.; Fardis, M.; He, G.-X.; Jin, H.; Kim, C. U.; Lee, W. A.; Lee, C. P.; Lin, K.-Y.; Liu, H.; Mackman, R. L.; Mitchell, M. L.; Nelson, P. H.; Pyun, H.-J.; Rowe, T. D.; Sparacino, M.; Swaminathan, S.; Tario, J. D.; Wang, J.; Williams, M. A.; Xu, L.; Yang, Z.-Y.; Yu, R. H.; Zhang, J.; Zhang, L. *PCT Int. Appl.* 2003; 1727 p. CODEN: PIXXD2 WO 2003090690 A2 20031106, 2003.
7. Birkus, G.; Chen, J. M.; Chen, X.; Cihlar, T.; Eisenberg, E. J.; Hatada, M.; He, G.-X.; Kim, C. U.; Lee, W. A.; McDermott, M. J.; Swaminathan, S. *PCT Int. Appl.* 2003; 814 p. CODEN: PIXXD2 WO 2003090691 A2 20031106, 2003.
8. Leblanc, Y.; Dufresne, C.; Gauthier, J. Y.; Young, R. *U.S. Pat. Appl. Publ.* 2003; 27 p, Cont.-in-part of U.S. Ser. No. 745,220. CODEN: USXXCO US 2003114703 A1 20030619: U.S. 2003.
9. Holtz, K. M.; Stec, B.; Myers, J. K.; Antonelli, S. M.; Widlanski, T. S.; Kantrowitz, E. R. *Protein Sci.* **2000**, *9*, 907–915.
10. Stuckey, J. A.; Schubert, H. L.; Fauman, E. B.; Zhang, Z.-Y.; Dixon, J. E.; Saper, M. A. *Nature* **1994**, *370*, 571–575.
11. Chen, L.; Wu, L.; Otaka, A.; Smyth, M. S.; Roller, P. P.; Burke, T. R., Jr.; den Hertog, J.; Zhang, Z. Y. *Biochem. Biophys. Res. Commun.* **1995**, *216*, 976–984.
12. Burke, T. R., Jr.; Ye, B.; Yan, X.; Wang, S.; Jia, Z.; Chen, L.; Zhang, Z. Y.; Barford, D. *Biochemistry* **1996**, *35*, 15989–15996.
13. Groves, M. R.; Yao, Z. J.; Roller, P. P.; Burke, T. R., Jr.; Barford, D. *Biochemistry* **1998**, *37*, 17773–17783.
14. Jia, Z.; Barford, D.; Flint, A. J.; Tonks, N. K. *Science* **1995**, *268*, 1754–1758.
15. Kim, C. U.; Luh, B. Y.; Misco, P. F.; Bronson, J. J.; Hitchcock, M. J.; Ghazzouli, I.; Martin, J. C. *J. Med. Chem.* **1990**, *33*, 1207–1213.
16. Conforth, J.; Wilson, J. R. H. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1897–1900.
17. McNulty, J.; Still, I. W. J. *Synth. Commun.* **1992**, 979–985.
18. Smith, M. B. *Synth. Commun.* **1986**, *16*, 85–90.
19. Le Du, M. H.; Stigbrand, T.; Taussig, M. J.; Menez, A.; Stura, E. A. *J. Biol. Chem.* **2001**, *276*, 9158–9165.
20. Kim, E. E.; Wyckoff, H. W. *J. Mol. Biol.* **1991**, *218*, 449–464.
21. Zhang, Z.-Y. *CRC Crit. Rev. Biochem. Mol. Biol.* **1998**, *33*, 1–52.
22. Jackson, M. D.; Denu, J. M. *Chem. Rev.* **2001**, *101*, 2313–2340.
23. Ibrahimi, O. A.; Wu, L.; Zhao, K.; Zhang, Z.-Y. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 457–460.
24. Fjeld, C. C.; Denu, J. M. *J. Biol. Chem.* **1999**, *274*, 20336–20343.
25. Hoff, R. H.; Mertz, P.; Rusnak, F.; Hengge, A. C. *J. Am. Chem. Soc.* **1999**, *121*, 6382–6390.
26. Zhang, Z.-Y.; Malochowski, W. P.; Van Etten, R. L.; Dixon, J. E. *J. Biol. Chem.* **1994**, *269*, 8140–8145.