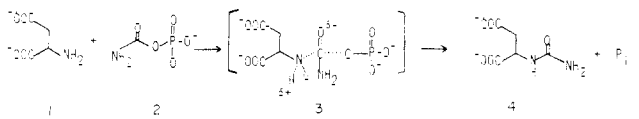
Design and Synthesis of New Transition-State Analogue Inhibitors of Aspartate Transcarbamylase

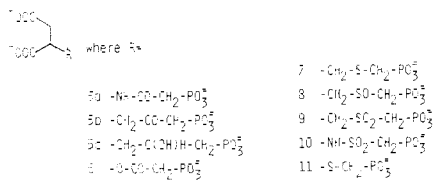
G. King Farrington, Alok Kumar, and Frederick C. Wedler*

Department of Molecular and Cell Biology, P. M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. 16801. *Received March 11, 1985*

Six transition-state or bisubstrate analogue inhibitors (**6–11**) have been designed, synthesized, and tested against aspartate transcarbamoylase (ATCase). Several of these inhibitors, **7–9**, were designed as analogues of *N*-(phosphonoacetyl)-L-aspartate (PALA, **5a**) and incorporated a tetrahedral sulfur group (–S–, –SO–, $-SO_2-$) α to a phosphonic acid moiety. Synthesis of **7–9** was accomplished with a new reagent, diethyl (mercaptomethyl)phosphonate (**19**). Thiol addition of **19** to diethyl itaconate or other olefins proves a new general synthetic route to (thiomethyl)-phosphonate analogues of acyl phosphates or diphosphate anhydrides. Analysis of the observed inhibition kinetics with ATCase and structural modeling studies indicate that increased steric size of the sulfur moieties in the sulfide **7**, sulfoxide **8**, sulfone **9**, and sulfonamide **10** may cause these compounds to be less potent inhibitors of *Escherichia coli* ATCase than *N*-(phosphonoacetyl)-L-aspartate (PALA, **5a**). The pK_a of the carbonyl groups (or S-analogue thereof) may be a key factor in determining the affinity of ATCase for inhibitor. The distance from the α-carbon to the phosphorus atom was judged to be a less important factor in determining the tightness of inhibitor binding since no significant change in the inhibition constant (K_i) occurred upon elimination of the α-methylene group in sulfide **7** to give sulfide **11**. The ester analogue of PALA (**5a**), *O*-(phosphonoacetyl)-L-malic acid (**6**), exhibited a K_i of 2×10^{-6} M.

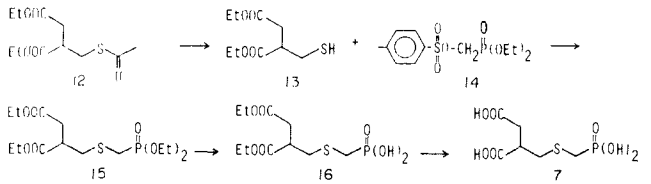
Aspartate transcarbamylase (EC 2.1.3.2, ATCase) catalyzes the formation of *N*-carbamyl-L-aspartate (**4**) from L-aspartate (**1**) and carbamyl phosphate (**2**), the first committed step in de novo pyrimidine biosynthesis. In mammals ATCase exists as a multienzyme complex that includes carbamyl phosphate synthetase and dihydroorotase.[1,2] The transition-state analogue *N*-(phosphonoacetyl)-L-aspartic acid, PALA (**5a**), is a potent inhibitor of ATCase with demonstrated antitumor activity.[3-5] Thus, ATCase is a proven target for anticancer drugs.





The present research has two goals: First was the development of new structural analogues of the ATCase transition state (**3**), to explore and further elucidate those structural features necessary for optimal binding to the enzyme active site. Second was the application of these new ATCase inhibitors as anticancer therapeutic agents. This report considers only the first of these two objectives: synthesis of new inhibitors and in vitro inhibition kinetics with ATCase from *Escherichia coli*. In the proposed transition state **3** the carboxylates of L-aspartate are oriented cis to one another in the active site, and the carbonyl group of carbamyl phosphate is presumed to exist as the tetrahedral adduct.[6] To mimic transition-state **3**, our

Scheme I



newly designed inhibitors incorporated several different tetrahedral sulfur groups at the carbonyl position of PALA (**5a**), as in **7–10**. A previously synthesized tetrahedral analogue, (4,5-dicarboxy-2-hydroxypentyl)phosphonate, DIHOP (**5c**), was a less potent inhibitor of ATCase than PALA or the corresponding ketone (4,5-dicarboxy-2-ketopentyl)phosphonate, DIKEP (**5b**). Those differences were attributed to either steric hindrance or the tetrahedral geometry of the alcohol.[7] PALA (**5a**), with –NHCO– in place of the $-CH_2CO-$ of DIKEP (**5b**), binds 7 times more tightly to the active site than DIKEP. Roberts et al.[7] have argued that the higher pK_a of the carbonyl of PALA (**5a**) is responsible for the tighter binding of PALA (**5a**) vs. DIKEP (**5b**). This may reflect a requirement for protonation of the carbonyl by a general acid group on the enzyme, to neutralize the partial negative charge that develops on this oxygen upon formation of **3**, the postulated transition state.[8]

Among the several new synthetic analogues that incorporate a sulfur atom at the carbonyl position, the tetrahedral sulfoxide group of **8** has several properties important for tight binding to the active site: an oxygen with a high pK_a[9] and less steric hindrance than the alcohol of DIHOP (**5c**). To further explore the extent to which steric restrictions in the active site control inhibitor binding, sulfide **11** in which the overall length of **7** was decreased by one methylene was synthesized. In addition, the oxygen analogue of PALA (**5a**), the trigonal phosphonoacetate ester analogue **6**, was synthesized, since it (like PALA) had

(1) Hoogenrad, N. J.; Levine, R. L.; Kretchmer, N. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 981.
(2) Shoaf, W. T.; Jones, M. E. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 796.
(3) Johnson, R. K.; Inowye, T.; Goldin, A.; Stark, G. R. *Cancer Res.* **1976**, *36*, 2720.
(4) Weber, G. *New Engl. J. Med.* **1977**, *296*, 486.
(5) Swyryd, E. A.; Seaver, S. S.; Stark, G. R. *J. Biol. Chem.* **1974**, *249*, 6945.
(6) Davies, G. E.; Vanaman, T. C.; Stark, G. R. *J. Biol. Chem.* **1970**, *245*, 1175.
(7) Roberts, M. F.; Opella, S. J.; Schaffer, M. H.; Phillips, H. M.; Stark, G. R. *J. Biol. Chem.* **1976**, *251*, 5976.
(8) Schmidt, P. G.; Stark, G. R.; Baldeschwieler, J. D. *J. Biol. Chem.* **1969**, *244*, 1860.
(9) Truce, W. E.; Klinger, T. C.; Brand, W. W. In "Organic Chemistry of Sulfur"; Oae, S., Ed.; Plenum Press: New York, 1977; p 527.

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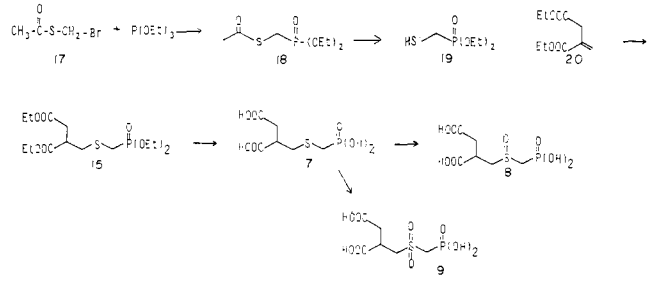
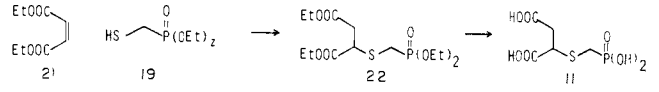
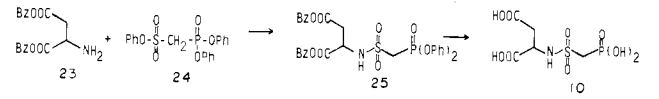
Scheme II



Scheme III



Scheme IV



the potential to mimic the tight, nonproductive binding to the active site of carbamyl phosphate and succinate.[10,11]

Synthesis of Inhibitors

Simple α-phosphoryl sulfoxides were previously synthesized by Mikolajczyk and Zatorski[12] as reagents for Horner–Wittig reactions. These compounds were prepared by Arbuzov reactions of triethyl phosphites with the corresponding α-chloromethyl sulfides, followed by oxidation of the sulfide. The α-phosphoryl sulfide **7**, the precursor of **8** and **9**, was synthesized by two methods:

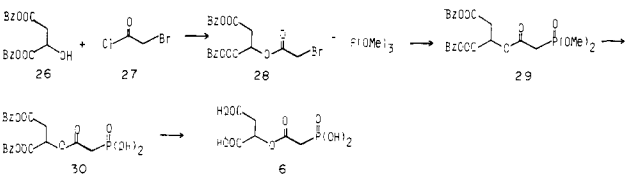
Scheme I. Alkylation of thiol **13** by reaction with tosylate **14** in the presence of 1 equiv of base gave the sulfide **15**. Due to low overall yield (ca. 3%) of **7** by this route and difficulties in purifying the intermediates and final product, it was necessary to devise an alternate scheme.

Scheme II. A second strategy that avoids several of the deficiencies of the previous reaction sequence was developed. Arbuzov reaction of bromide **17** and triethyl phosphite gave (diethylphosphono)methyl acetyl sulfide (**18**) in 65% yield. Treatment of **18** with sodium ethoxide gave the key intermediate diethyl (mercaptomethyl)-phosphonate (**19**) in 85% yield. Thiol **19** was used without purification in the subsequent Michael addition to diethyl itaconate (**20**) to give sulfide **15**. The carboxylate and phosphonate groups of **15** were deblocked in a single step by refluxing in 6 N HCl to give the α-phosphonic sulfide **7** with 20% overall yield. Oxidation of the sulfide **7** with hydrogen peroxide gave either the sulfoxide **8** or the sulfone **9** in high yield (80–98%).

As shown in Scheme III the synthesis of α-phosphoryl sulfide **11** was accomplished by an analogous series of reactions in which diethyl fumarate (**21**) was substituted for diethyl itaconate (**20**) in the Michael addition. The overall yield of this scheme was ca. 25%.

Sulfonamide **10** had been synthesized previously by Hunt[14] but had not been obtained in pure form. Synthesis of **10** was completed by a more direct two-step synthetic

Scheme V



route shown in Scheme IV. Triphenyl (sulfonylmethyl)phosphonate (**24**) prepared by the method of Fild and Reich[15] was refluxed with dibenzyl L-aspartate (**23**) in toluene to give sulfonamide **25**. After purification by chromatography on silica gel, the blocking groups of **25** were removed by treatment with dilute base instead of the two-step procedure employed by Hunt.[14] Sulfonamide **10** was acidified to pH 2 with HCl, salt was removed by passage through a column of Dowex 50 (H^+), and the product was then crystallized as the cyclohexylamine salt.

The malate ester analogue **6** of PALA (**5a**) was prepared as shown in Scheme V. Bromoacetyl chloride (**27**) was esterified with dibenzyl malate (**26**) to give bromoacetyl ester **28**. The Arbuzov reaction of trimethyl phosphite with alkyl bromide **28** yielded acetylphosphonate **29**. The phosphonate ester was deblocked by treatment with bromotrimethylsilane,[13] and the benzyl groups of the carboxylate esters in **30** were removed by hydrogenation to give **6** in a 15% overall yield.

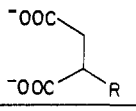
The purity and stability of phosphonoacetate ester **6** were determined by use of malic enzyme,[16] to assay for the presence of malic acid produced from hydrolysis of the phosphonoacetate ester **6** by observing $NADP^+$ reduction at 340 nm. Assays of **6** were performed before and after hydrolysis of the phosphonoacetate ester with dilute base. Prior to hydrolysis less than 3% malate impurity was detected, and hydrolysis yielded >99% malate. Incubation of phosphonoacetate ester **6** in 100 mM HEPES buffer (pH 7.4), 100 mM KCl at 37 °C, indicated that less than 3% spontaneous hydrolysis occurred over 3 days. The malic enzyme assay was also used to determine the exact concentration of inhibitor **6** in stock solutions after base hydrolysis of an aliquot.

Enzyme Inhibition Studies

In order to avoid complications in interpretation of the inhibition kinetics due to homotropic interactions within the native enzyme (C_6R_6), in vitro inhibition studies were carried out for each synthetic analogue with the catalytic subunit (C_3), prepared from native enzyme according to the methods of Gerhart and Holoubek.[17,18] Activity assays were carried out by measuring the conversion of L-[^{14}C]-aspartate to *N*-carbamyl-L-[^{14}C]aspartate, separated on Dowex 50 (H^+) according to the methods of Porter et al.[19] The effect of each inhibitor at fixed levels was determined vs. varied concentrations of carbamyl phosphate, the first substrate to bind in the compulsory order kinetic mechanism of ATCase.[19–21] If the inhibitor bound to the car-

(10) Collins, K. D.; Stark, G. R. *J. Biol. Chem.* **1971**, *246*, 6599.
(11) Jacobson, G. R.; Stark, G. R. "The Enzymes", 3rd ed.; Academic Press: New York, 1973; Vol. IX, p 225.
(12) Mikolajczyk, M.; Zatorski, A. *Synthesis* **1973**, 669.
(13) Morita, T.; Okamoto, Y.; Sakurai, H. *Tetrahedron Lett.* **1978**, *28*, 2523.
(14) Hunt, J. T. Ph.D. Thesis, University of California, Berkley, 1974.
(15) Fild, M.; Reich, H.-P. *Chem. Ber.* **1980**, *113*, 142.
(16) Hsu, R. Y.; Lardy, H. A. "Methods in Enzymology"; Lowenstein, J. M., Ed.; Academic Press: New York, 1969; Vol. 13, p 230.
(17) Gerhart, J. C.; Holoubek, H. *J. Biol. Chem.* **1967**, *242*, 2886.
(18) Gerhart, J. C.; Pardee, A. B. *J. Biol. Chem.* **1962**, *237*, 891.
(19) Porter, R. W.; Modebe, M. O.; Stark, G. R. *J. Biol. Chem.* **1969**, *244*, 1846.
(20) Wedler, F. C.; Gasser, F. J. *Arch. Bioch. Biophys.* **1974**, *163*, 57.
(21) Stuper, A. J.; Brugger, W. E.; Jurs, P. C. "Computer Assisted Studies of Chemical Structure and Biological Function"; Wiley-Interscience: New York, 1979.

Table I. Inhibition Data and α-Carbon to Phosphorus (α-C-P) Bond Distances for New Synthetic Analogue Inhibitors of ATCase



compd	R	K_i,[a] μM	type of inhibn[b]	α-C-P dist, Å
5a	$NHCOCH_2PO_3^{2-}$	0.03	C	4.64
6	$OCOCH_2PO_3^{2-}$	2	C	4.55
7	$CH_2SCH_2PO_3^{2-}$	430	NC	5.61
8	$CH_2SOCH_2PO_3^{2-}$	2300	C	5.60
9	$CH_2SO_2CH_2PO_3^{2-}$	650	C	5.60
10	$NHSO_2CH_2PO_3^{2-}$	500	C	5.60
11	$SCH_2PO_3^{2-}$	1100	C	4.60

[a] Vs. carbamyl phosphate. [b] Key: C = competitive; NC = noncompetitive.

bamyl phosphate site, it was expected to show a competitive kinetic pattern vs. carbamyl phosphate. In order to vary the concentration of carbamyl phosphate near its K_m (1.4×10^{-5} M) and in order to obtain sufficient conversion of [^{14}C]-L-Asp to carbamyl-L-aspartate, the concentration of aspartate also had to be in the range of 10^{-4}–10^{-5} M, well below the K_m for L-Asp (ca. 10 mM at pH 7.8, 28 °C). This low aspartate concentration relative to its K_m served to overcome several other problems as well:[19] (1) Due to the high V_{max} of the catalytic subunit, if [L-Asp] ≥ K_m, a sizable fraction (>50%) of the carbamyl phosphate pool would be consumed before the reaction could be stopped (within 5–15 s), and linear initial velocity kinetics would not be obtained, since velocity would be linearly dependent on [carbamyl phosphate] near its K_m value. (2) An alternative way to slow down the reaction velocity would be to lower [E]. To do so would require an [E] below 1 μg/mL, a condition at which rapid inactivation of the catalytic subunit occurs.[19] (3) The pool size of L-aspartate should not be so much larger than that of carbamyl phosphate that fewer than 500 cpm of [^{14}C]-labeled *N*-carbamyl-L-aspartate are produced from L-[^{14}C]aspartate in any reaction. The other requirement rigidly satisfied in all these experiments was that less than 10–12% of the limiting L-Asp pool was consumed in any reaction within the time period of the assay, since velocity was directly proportional to [Asp], due to the condition of [Asp] ≪ K_m.

The experimental protocol used for accurate determination of K_m and K_i values under these conditions has been derived previously by Porter et al.[19] for studies with PALA. The observed inhibition patterns and calculated K_i values for each synthetic analogue are presented in Table I. The phosphonoacetate ester **6** exhibited competitive kinetics vs. carbamyl phosphate (Figure 1) and a K_i value of 2 μM, well below the K_m value of 14 μM for carbamyl phosphate. Although the sulfoxide **8**, sulfone **9**, sulfonamide **10**, and sulfide **11** were all competitive inhibitors, it was surprising that the sulfide **7** exhibited pure noncompetitive kinetics vs. carbamyl phosphate (Figure 2). The K_i values for the tetrahedral analogues **7–10** were in excess of 100 μM.

Structure-Function Correlations

In order to correlate structural features of the synthetic analogues **6–11** with the observed inhibition constants, we have utilized a computer-based structural analysis system (see the Experimental Section). This program (ADAPT) was used to calculate bond lengths and angles for PALA and for each of the new synthetic analogues **6–11**. With these parameters we also included in our analysis several important previously existing criteria related to binding of inhibitors to the active site of ATCase: (1) There are steric restrictions at the active site, as set forth by Stark in the postulated "compression" mechanism for ATCase.[8,19] (2) The carbonyl oxygen of carbamyl phosphate contributes significantly to the strength of binding. (3) The pK_a of this carbonyl oxygen is important, possibly due to protonation by a general-acid group of the enzyme.[7,8]

First, steric effects were evaluated. As shown in Table I, the distance from the α-carbon to the phosphorus atom (α-C-P) calculated for the sulfoxide **8** and sulfone **9** were virtually identical with that for sulfide **7**. Since the K_i values for these compounds were all within 430–2300 μM, it is unlikely that the unusual noncompetitive kinetics exhibited by **7** is due to the fact that **7** exceeds the steric constraints of the active site and is therefore unable to occupy the carbamyl phosphate site on ATCase. Rather, it may result from the absence of an oxygen on sulfur—see criterion 2 above. Deletion of one methylene group from the sulfide **7** decreases the α-C-P distance by ca. 1.0 Å. The resulting shorter sulfide **11** shows competitive kinetics

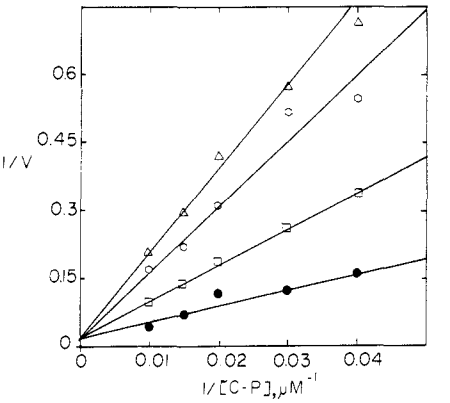


Figure 1. Competitive inhibition of the ATCase (catalytic subunit) by *O*-(phosphonoacetyl)-L-malic acid (**6**) at pH 7.8, vs. carbamyl phosphate. Assay mixtures contained 100 mM HEPES, 10 mM KCl, 80 μM 0.033 μCi L-[^{14}C]aspartate, and 2.2 μg/mL of enzyme. Carbamyl phosphate was varied from 20 to 100 μM. In the figure, velocity V is expressed as micromoles of carbamyl-L-aspartate produced per minute. The maximum conversion of L-aspartate or carbamyl phosphate did not exceed 12% in any experiment. Inhibitor (μM): (●) zero; (□) 2.4; (○) 4.8; (△) 7.2.

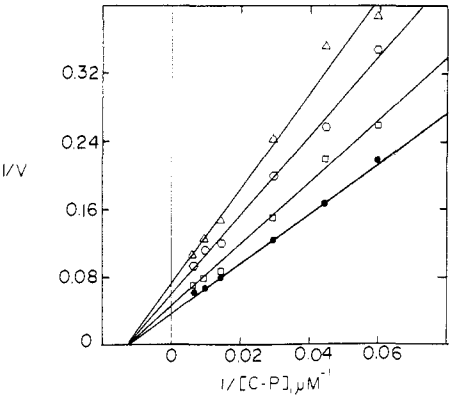


Figure 2. Noncompetitive inhibition of ATCase (catalytic subunit) by 2-[[(phosphonomethyl)thio]methyl]succinic acid (**7**) at pH 7.8, vs. carbamyl phosphate. Assay mixtures contained 100 mM HEPES, 10 mM KCl, 80 μM 0.033 μCi μCi L-[^{14}C]aspartate, and 2.2 μg/mL of enzyme. Carbamyl phosphate was varied from 16.6 to 150 μM. In the figure, velocity V is expressed as of micromoles of carbamyl-L-aspartate produced per minute. The maximum consumption of L-aspartate or carbamyl phosphate did not exceed 12% in any experiment. Inhibitor (μM): (●) zero; (□) 100; (○) 300; (△) 400.

(22) Zanotti, G.; Monaco, H. L.; Fotte, J., personal communication.

vs. carbamyl phosphate (Table I), without any substantial change in K_i value. Again, the data seem to indicate that an oxygen on the sulfur is necessary for sulfoxide **8** and sulfone **9** to occupy the carbamyl phosphate site, indicated by a change from noncompetitive to competitive inhibition. The actual basis for the noncompetitive inhibition of ATCase by **7** remains unknown at present. Introduction of oxygen on sulfur may alter the binding affinity of analogues **8** and **9** in two opposite ways: (a) in a *positive* manner due to better mimicry of the transition state, perhaps allowing H-bond formation but (b) in a *negative* manner due to increased steric size of the sulfoxide or sulfone relative to carbonyl.

Since the phosphonoacetate ester **6** has an α-C–P distance that approximates that of PALA (**5a**), increased overall length cannot explain the weaker binding of **6** relative to PALA. This brings us to consider in more detail the third criterion, protonation or H bonding of the oxygen on carbonyl or analogues thereof. One explanation for the 100-fold higher affinity of the enzyme for PALA (**5a**) than **6** may be the lower pK_a of the carbonyl oxygen group of the ester. To estimate a value for the pK_a of the carbonyl in the ester **6**, we started with the pK_a of ethyl acetate (−2.0) in H_2SO_4.[23] To this value were added the inductive effects due to the phosphonate (−1.7) and the α-carboxyl (−0.9), to give a final value of −4.6.[7,24] A direct comparison of the pK_a of **6** and that of PALA (**5a**) is not possible because the pK_a of PALA (**5a**) was determined in DCl/H_2O, not H_2SO_4, and different acidity functions are used. A further correction was made, therefore, assuming that the difference of 2.2 units in the pK_a of butyramide in DCl vs. H_2SO_4 would also be valid for PALA (**5a**).[7,24] Thus, we estimate the pK_a of the carbonyl of PALA (**5a**) in H_2SO_4 to be about −3.3. The difference in the pK_a values of PALA (**5a**) vs. the ester **6** is estimated to be greater than 1.0 pH unit. This difference may be responsible in large part for the ca. 100× lower value of K_i for PALA (**5a**) vs. **6** with ATCase.

Conclusions

One of the principal contributions of the present work is the synthesis of a new intermediate, the α-phosphoryl thiol **19**, here demonstrated to be a potentially useful and versatile synthetic reagent. New synthetic routes are now open that involve simple, direct addition of the α-phosphoryl thiol **19** to olefins in high yield. This approach may be useful in the synthesis of new biologically active, chemically stable inhibitors designed to mimic diphosphate anhydrides or tetrahedral adducts of acyl phosphate.

From these analyses and comparisons we conclude that the steric size of the carbonyl analogue group and the pK_a of this group are apparently important to the optimal design of inhibitors for *E. coli* ATCase. Inhibitors that incorporate large tetrahedral groups bind less well to ATCase than PALA, due to steric restrictions of the active site. Changes in the α-C–P bond distance and properties of the group bridging between the α-C and the carbonyl moiety (NH, CH_2, O) appear to be of relatively minor importance. The small difference in K_i values of sulfonamide **11** vs. sulfone **9**, or of PALA (**5a**) vs. DIKEP (**5b**) and the phosphonoacetate ester **6**, support this latter view.

Although only one inhibitor, **7**, among those synthesized was especially effective against *E. coli* ATCase, the potencies of these new compounds with ATCase from mammalian tissues or other sources has yet to be determined in vitro or in vivo. Thus, the potential usefulness of these newly synthesized analogues of PALA as chemotherapeutic agents will be the goal of future research efforts.

Experimental Section

Enzymatic Assays. ATCase, isolated from *E. coli* by the method of Gerhart and Holoubeck,[17] was a gift from Dr. H. K. Schachman. The enzyme had been isolated from a diploid strain of *E. coli* that lacks orotidylate decarboxylase and requires uracil.[17] Absence of uracil derepresses the synthesis of ATCase in *E. coli*, causing overproduction of ATCase by these cells. The enzyme was separated into catalytic and regulatory subunits according to the procedure of Gerhart and Holoubeck.[17] L-[^{14}C]Aspartic acid was purchased from Amersham and purified before use as described by Porter et al.[19] Each reaction assay contained 100 mM HEPES buffer (pH 7.8), 10 mM KCl, 80 μM L-aspartate, 0.033 μCi of L-[^{14}C]aspartate, and 2.2 μg/mL catalytic subunit. Carbamyl phosphate concentration was varied from 17.6–150 μM for the sulfide **7** and 20–100 μM for all others. Enzyme, carbamyl phosphate, and the inhibitor were preincubated in buffer at least 5 min in a water bath at 28 °C prior to initiation of the reaction by addition of 80 μM unlabeled L-aspartate containing 0.033 μCi of L-[^{14}C]aspartate. After 15 s the reactions were stopped by addition of 1.0 mL of 0.2 N acetic acid. A 1.0-mL sample of this mixture was then applied directly to the top of the Dowex 50 (H^+) column (1 × 5 cm), and carbamyl-L-aspartate was eluted with 5 mL of water. Data were analyzed and K_i values calculated for each inhibitor as described by Porter et al. for PALA.[19]

Assays for malate using malic enzyme were carried out according to the method of Hsu and Lardy,[16] at room temperature, in 100 mM HEPES buffer, pH 7.4, with a 10-fold excess of $NADP^+$ relative to the amount of malic acid present. Each assay contained 1 unit of enzyme. The formation of NADPH was monitored at 340 nm. The absorbance changes for known concentrations of malic acid were used as a standard curve from which unknown amounts of malic acid could be determined. Malic enzyme and the necessary reagents were purchased from Sigma.

Structural Calculations. Structural modeling of these new synthetic compounds was performed using the ADAPT (automated data analysis and pattern recognition toolkit) chemical software system.[21] Calculations were performed on a PRIME 750 computer containing two megabytes of main memory, running PRIMOs Revision 18.3, located in the Chemistry Department, The Pennsylvania State University. Atoms between the α-carbon and the phosphorus atom were maximaly overlapped prior to α-C–P distance calculations. Distance calculations are based on idealized bond lengths and angles, except for C–P bond distances, which were obtained from the X-ray crystallographic structure of PALA (**5a**).[22]

Syntheses. All compounds exhibited NMR spectra consistent with their respective structures. ^{1}H NMR spectra were determined with a Varian EM-360-A spectrophotometer at 60 MHz, using $CDCl_3$, acetone-d_6, or D_2O as sample solvents. Chemical shifts are reported in parts per million (δ) downfield from Me_4Si or 3-(trimethylsilyl)propionate-2,3,3,3-d_4 (TSP). Mass spectra were recorded on a KRATOS-MS9/50 spectrometer at 70 eV. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed with Baker Si250 TLC plates. Preparative high-pressure liquid chromatography (HPLC) was carried out on a Waters Prep-500 containing two silica gel columns in sequence. Other silica gel column purifications were performed by the flash chromatography method of Still et al.[25] using Baker silica gel for flash chromatography. Distillations were carried out with a Kontes short-path distillation apparatus. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN. Chemical reagents and starting materials were obtained from Aldrich Chemical Co. or Alfa Products and were used without further purification, unless otherwise noted. All trialkyl phosphites were distilled from sodium prior to use.

Diethyl [(*p*-Tolylsulfonyl)methyl]phosphonate (13). Sodium (3.0 g, 0.13 mol) was dissolved in 100 mL of absolute ethanol under a nitrogen atmosphere, and diethyl 2-[(acetylthio)methyl]succinate[26] (**12**; 28.2 g, 0.11 mol) was slowly added.

(23) Lane, C. A. *J. Am. Chem. Soc.* **1964**, *86*, 2521.
(24) Armstrong, V. C.; Moodie, R. B. *J. Chem. Soc. B* **1968**, 275.
(25) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

The mixture stirred for 1 h at room temperature. Ethanol was removed in vacuo, 100 mL water was added, and the mixture was acidified to below pH 3.0 with 0.5 N HCl. The mixture was extracted with ether (2 × 150 mL) and the ether phase dried over Na_2SO_4. After in vacuo removal of solvent the residue was distilled to give 14.8 g (63%) of a light yellow oil: bp 71–72 °C (0.03 mm); NMR ($CDCl_3$) δ 1.3 (t, 6 H, J = 6 Hz, OCH_2CH_3), 1.5 (t, 1 H, J = 6 Hz, CH_2SH), 2.6–3.1 [m, 5 H, C(OOEt)CH_2CH(COOEt)-CH_2SH], 3.9–4.4 (dq, 4 H, J = 2 Hz and J = 8 Hz, OCH_2CH_3), ($C_9H_{16}O_4S$).

Diethyl [(*p*-Tolylsulfonyl)methyl]phosphonate (14). *p*-Toluenesulfonyl chloride (11.3 g, 0.06 mol) was dissolved in 50 mL of freshly distilled anhydrous pyridine, and the mixture was placed in a three-neck flask under nitrogen atmosphere and cooled to 0 °C. Diethyl(hydroxymethyl)phosphonate[27] (5.0 g, 0.03 mol) was added dropwise with constant stirring over a 30-min period. After 48 h at −20 °C the reaction mixture was poured into ice water (150 mL). The ester was then extracted from the aqueous phase with ether (150 mL), followed by ether extraction (2 × 100 mL). The ether fractions were combined, washed with ice-cold 6 N HCl (2 × 100 mL) and then water (150 mL), and dried over Na_2SO_4. In vacuo removal of ether gave 3.7 g (38.6%) of a clear oil: NMR ($CDCl_3$) δ 1.3 (t, 6 H, J = 6 Hz, OCH_2CH_3), 2.4 (s, 3 H, CH_3Ph), 4.2 (d, 2 H, J_{H-P} = 13 Hz, CH_2P), 3.8–4.4 (m, 4 H, J_{H-P} = 10 Hz, J = 6 Hz, $POCH_2CH_3$), 7.5 (dd, 4 H, J = 24 Hz and J = 8 Hz, aromatic H), ($C_{12}H_{19}O_6PS$).

Diethyl 2-[[[(Diethylphosphono)methyl]thio]methyl]-succinate (15). Under nitrogen atmosphere sodium (1.43 g, 62 mmol) was dissolved in 70 mL of absolute ethanol. To the solution was added thiol **13** (13.7 g, 62 mmol) with stirring, followed by slow addition of tosylate **14** (20.0 g, 62 mmol). The reaction mixture was heated to 60 °C for 8 h and filtered and solvent removed in vacuo. The resulting oil was dissolved in ethyl acetate/hexane (7:3) and chromatographed on a Waters Prep 500 HPLC, using the same solvent mixture as the eluent. The solvent was removed in vacuo to give 6.0 g (26%) of a clear oil: NMR ($CDCl_3$) δ 1.3 (q, 12 H, J = 6 Hz, OCH_2CH_3), 2.75 (d, 2 H, J_{H-P} = 12 Hz, SCH_2P), 3.9–4.5 [m, 8 H, C(O)OCH_2CH_3, P(O)-OCH_2CH_3]; mass spectrum, m/e 370.1214 (M^+), ($C_{14}H_{27}O_7PS$).

Diethyl 2-[[(Phosphonomethyl)thio]methyl]succinate (16). To 25 mL of stirred dry acetonitrile under nitrogen atmosphere were added in the following order ester **15** (2.9 g, 7.9 mmol), NaI (3.6 g, 24 mmol), and chlorotrimethylsilane (2.9 g, 27 mmol). The stirred mixture was heated to 50 °C for 1 h and filtered, and the solvents were removed in vacuo. To the reaction mixture was added water (10 mL), followed by neutralization with a solution of 1 N Na_2CO_3. The aqueous mixture was extracted with ether (2 × 20 mL) to remove the iodine, and the aqueous layer was applied to a column (1.5 × 22 cm) of Dowex 1 AG-11-X8 (Cl^- form, 100–200 mesh). Elution was performed with a linear gradient of HCl (0–0.2 N). Fractions were assayed for phosphorus by the method of Lanzetta et al.,[28] positive fractions were combined, and the HCl was removed *in vacuo*. The resulting oil was diluted with water (10 mL), frozen, and lyophilized to give 1.35 g (52%) of a clear oil: NMR (acetone-d_6) δ 1.25 (m, 6 H, J = 6 Hz, OCH_2CH_3), 2.4–3.2 (m, 7 H, $CH_2CHCH_2SCH_2P$), 3.9–4.4 [m, 4 H, C(O)-OCH_2CH_3], ($C_{10}H_{19}O_7PS$).

Alternative Method. Our experience with deblocking of **30** to yield **6** suggests that use of bromotrimethylsilane with **15** would yield **16** in higher yield than the above procedures. This remains to be proven experimentally.

2-[[(Phosphonomethyl)thio]methyl]succinic Acid (7). Diester **16** (1.35 g, 4.3 mmol) was added to 40 mL of 0.5 N NaOH and the resultant mixture stirred 2 h at room temperature. The solution was acidified by batchwise addition of Dowex 50-X8 (H^+ form, 100–200 mesh), then filtered, and lyophilized. The solid was dissolved in acetone and precipitated with ether to give 0.93 g (86%) of an acid **7** as a white solid: mp 127–128 °C; NMR (D_2O) δ 2.85 (d, 2 H J_{H-P} = 13 Hz, SCH_2P), 2.7–3.1 [m, 5 H, $DOOCCH_2CH(COOD)CH_2SCH_2P$], ($C_6H_{11}O_7PS$).

(Diethylphosphono)methyl Acetyl Sulfide (18). Bromomethyl acetyl sulfide[29] (17; 26.8 g, 0.16 mol) and triethyl phosphite (28.4 g, 0.17 mol) were combined in a flask fitted with a Dean–Stark trap. The mixture was stirred at 130 °C for 2.5 h, during which time ethyl bromide collected in the Dean–Stark trap. The product was distilled to give 23.5 g (65%) of a clear oil: bp 105–106 °C (0.03 mm); NMR ($CDCl_3$) δ 1.25 (t, 6 H, J = 6 Hz, $POCH_2CH_3$), 2.4 [s, 3 H, $C(O)CH_3$], 3.25 (d, 2 H J_{H-P} = 13 Hz, PCH_2), 4.25 (m, 4 H, J_{H-P} = 10 Hz and J = 6 Hz, $POCH_2CH_3$), ($C_7H_{15}O_4PS$).

Diethyl (Mercaptomethyl)phosphonate (19). Thioacetyl **18** (33.9 g, 0.15 mol) was added to a solution of sodium ethoxide, freshly prepared from Na (3.4 g, 0.15 mol) dissolved in 50 mL of absolute ethanol. The mixture was stirred 1 h at room temperature, followed by in vacuo removal of solvent. The residue was dissolved in water (50 mL) and the solution acidified to pH 2 with 1 N HCl. The product was extracted with ether (3 × 150 mL), the ether phase dried over Na_2SO_4 and filtered, and ether removed in vacuo. The residue was distilled to give 23.5 g (85%) of phosphonate **19** as a clear oil: bp 69–71 °C (0.03 mm); NMR ($CDCl_3$) δ 1.3 (t, 6 H, J = 6 Hz, $POCH_2CH_3$), 1.85 (dt, 1 H, J_{H-P} = 5 Hz and J = 7 Hz, $HSCH_2P$), 2.75 (dd, 2 H, J_{H-P} = 13 Hz and J = 6 Hz, $HSCH_2P$), 3.9–4.4 (quint, 4 H, J_{H-P} = 6 Hz and J = 6 Hz, $POCH_2CH_3$), ($C_5H_{13}O_3PS$).

Diethyl 2-[[[(Diethylphosphono)methyl]thio]methyl]-succinate (15). Thiol **19** (5.0 g, 27.2 mmol) was added to a solution of sodium ethoxide, freshly prepared from sodium (0.1 g, 4.3 mmol) dissolved in 100 mL of absolute ethanol. Diethyl itaconate (**20**; 5.1 g, 27.4 mmol) was then added and the mixture refluxed for 17 h. Ethanol was removed in vacuo, water (50 mL) added, and the solution acidified with 1 N HCl to pH 3.0. The product was extracted with ether (3 × 100 mL), the pooled ether extracts were dried over Na_2SO_4 and filtered, and ether was removed in vacuo. The residue was distilled to give 5.0 g (50%) of tetraester as a clear oil: bp 162–163 °C (0.04 mm); NMR ($CDCl_3$) δ 1.25 (q, 12 H J = 6 Hz, OCH_2CH_3), 2.7 (d, 2 H, J_{H-P} = 13 Hz, SCH_2P), 3.9–4.5 (m, 8 H, $COCH_2CH_3$, $POCH_2CH_3$); mass spectrum, m/e 370.1214 (M^+) ($C_{14}H_{27}O_7PS$).

2-[[(Phosphonomethyl)thio]methyl]succinic Acid (7). Tetraester **15** (5.0 g, 13.5 mmol) was refluxed 36 h in 6 N HCl (75 mL). The product was treated with Norit A charcoal, and then HCl and water were removed in vacuo to give 3.22 g (92%) of a white solid: mp 127–128 °C; NMR (D_2O) δ 2.8 [m, 7 H, $DOOCCH_2CH(COOD)CH_2SCH_2P$]. Anal. ($C_6H_{11}O_7PS$) C, H, S, P. For mass spectral analysis the carboxylate groups of **7** were esterified by refluxing with ethanol and benzene for 6 h to give **16**. Mass spectrum: m/e 314.0580 (M^+).

2-[[(Phosphonomethyl)sulfinyl]methyl]succinic Acid (8). Sulfide **7** (0.5 g, 1.9 mmol) was dissolved in 2.0 mL of water, containing 0.22 mL (1.2 equiv) of 30% H_2O_2 solution. The reaction was stirred for 3 h at 50 °C and then frozen and lyophilized. Residual solvent was removed from the product oil in vacuo over 24–48 h, with intermittent scratching, to yield 0.39 g of solid (72%); dec pt 150–152 °C; NMR (D_2O) δ 2.7–3.0 (m, 2 H, $CHCH_2COOD$), 3.25 [d, 2 H, J_{H-P} = 13 Hz, $S(O)CH_2P$], 3.2–3.4 [m, 3 H, $DOOCCH(CH_2COOD)CH_2S(O)CH_2P$]. Anal. ($C_6H_{11}O_8PS$) C, H.

2-[[(Phosphonomethyl)sulfonyl]methyl]succinic Acid (9). The sulfide **7** (0.2 g, 0.8 mmol) was dissolved in 4 mL of a 15% solution of H_2O_2 and refluxed for 24 h. The solvent was removed in vacuo to give 0.2 g (86%) of a white solid: dec pt 205–207 °C; NMR (D_2O) δ 2.7–3.0 (m, 2 H, $CHCH_2COOD$), 3.45 [d, 2 H, J_{H-P} = 13 Hz, $S(O_2)CH_2P$], 3.3–3.9 [m, 3 H, $DOOCCH(CH_2COOD)CH_2S(O_2)CH_2P$]. Anal. ($C_6H_{11}O_9PS$) C, H.

Diethyl 2-[[(Diethylphosphono)methyl]thio]succinate (22). Sodium (40 mg, 1.7 mmol) was dissolved in absolute ethanol (25 mL), to which thiol **19** (8.3 g, 45 mmol) was added followed by diethyl fumarate **21** (7.76 g, 45 mmol). The mixture was stirred 12 h at room temperature, solvents were removed in vacuo, and the residue was taken up in ether (50 mL). After filtration, ether was removed in vacuo, and the oil was distilled to give 8.1 g (50%)

(26) Bohme, H.; Kreitz, K.; Nurnberg, E. *Arch. Pharmacol.* **1959**, *292*, 456.
(27) Abramov, V. S. *J. Gen. Chem.* **1952**, *22*, 647.
(28) Lanzetta, P. A.; Alvarez, L. J.; Reinach, P. S.; Candia, O. A. *Anal. Biochem.* **1979**, *100*, 95.
(29) Bohme, H.; Bezzenberger, H.; Clemnent, M.; Dick, D.; Nurnberg, E.; Schlephack, W. *Liebigs Ann. Chem.* **1959**, *623*, 92.
(30) Zervas, L.; Winitz, M.; Greenstein, J. P. *J. Org. Chem.* **1957**, *22*, 1515.

of a clear oil: bp 165–170 °C (0.04 mm); NMR ($CDCl_3$) δ 1.1–1.6 (m, 12 H, OCH_2CH_3), 2.8–3.2 [m, 9 H, $CH_3CH_2O(O)CCH_2CH$-$(O)OCH_2CH_3$, $P(O)OCH_2CH_3$], ($C_{13}H_{25}O_7PS$).

2-[(Phosphonomethyl)thio]succinic Acid (11). To ester **22** (2.0 g, 5.6 mmol) was added 6 N HCl (35 mL), and the solution was refluxed for 36 h. After treatment with charcoal (Norit A), water was removed in vacuo, leaving a thick oil that over 3 days in vacuo solidified to give 1.0 g (73%) of a white solid: mp 140–141 °C; (D_2O) δ 2.6–2.8 (d, 2 H, J_{H-P} = 13 Hz, SCH_2P), 2.6–2.7 [m, 2 H, $DOOCH_2CH(COOD)S$], 3.5–3.8 [t, 1 H, J = 6 Hz, $DOOCCH_2CH(COOD)$]. Anal. ($C_5H_9O_7PS$) C, H.

Dibenzyl *N*-[[(Diphenylphosphono)methyl]sulfonyl]aspartate (25). The *p*-toluenesulfonic acid salt of dibenzyl L-aspartate was converted to the free amine **23** by dissolving the salt in 150 mL of water and adjusting the pH to above 9.0 with a saturated solution of Na_2CO_3. This solution was then extracted with ether (2 × 150 mL), and the combined ether fractions were dried over Na_2SO_4. Diphenyl [(phenoxysulfonyl)methyl]-phosphonate (**24**), synthesized by the method of Fild and Riech,[15] (2.06 g, 5.1 mmol) was combined with the free base form of dibenzyl L-aspartate (**23**; 1.6 g, 5.1 mmol) in 20 mL of toluene and refluxed for 16 h. The products were applied to a silica gel column and separated by elution with EtOAc/hexanes (1:3). After removal of solvent, the product was crystallized from EtOAc/hexane to give 1.6 g (44%) of white crystals: mp 72–73 °C; NMR ($CDCl_3$) δ 2.8–3.1 (m, 2 H, CH_2COOBz), 4.2 (d, 2 H, J_{H-P} = 13 Hz, SCH_2P), 4.6–4.8 [m, 1 H, $(BzOOC)CHNHS(O_2)CH_2COOBz$], 5.5 (d, 4 H, J = 2 Hz, $COOCH_2Ph$), 6.3–6.5 [m, 1 H, $NHS(O)CH_2P$], 7.1–7.5 (m, 20 H, aromatic H); mass spectrum, *m/e* 623.1366 (M^+), ($C_{31}H_{30}NO_9PS$).

***N*-[(Phosphonomethyl)sulfonyl]aspartic Acid (10).** Tetraester **25** (0.73 g, 1.2 mmol) was refluxed for 4.5 h in 10% NaOH (20 mL). After extraction with ether (20 mL), the aqueous solution was passed through a Dowex 50-X8 column (1.5 × 40 cm, H^+ form, 100–200 mesh) and the product eluted with water. The acidic fractions were pooled and evaporated to dryness in vacuo. The solid residue was dissolved in acetone, to which cyclohexylamine (81 μL, 1.1 mmol) was added with vigorous stirring. Filtration of the precipitate gave 0.35 g (72%) of a white solid: dec pt 185–187 °C; NMR (D_2O) δ 2.7–2.8 (m, 2 H, $DOOCCH_2CHCOOD$), 3.5 [2 d, H, J_{H-P} = 15 Hz, $S(O_2)CH_2P$], 4.0–4.3 [q, 1 H, J = 6 Hz, $CH_2CH(COOD)ND$], monocyclohexylamine salt. Anal. ($C_5H_{10}NO_9PS$) C, H.

Dibenzyl *O*-(Bromoacetyl)malate (28). Dibenzyl L-malate[31] (**26**; 6.0 g, 19 mmol) and triethylamine (1.92 g, 19.2 mmol) were combined in ether (25 mL) at room temperature, and bromoacetyl chloride (**27**; 3.0 g, 19.2 mmol) in ether (50 mL) was added dropwise over 20 min. The products were filtered to remove triethylamine hydrochloride, and solvent was removed in vacuo. The residue was column chromatographed on silica gel with petroleum ether/ether (3:1) to give 3.2 g (38.5%) of a clear oil: NMR ($CDCl_3$) δ 2.95 (d, 2 H, J = 6 Hz, CH_2COOBz), 3.8 [s, 2 H, C-$(O)CH_2Br$], 5.2–5.3 (d, 4 H, J = 2 Hz, OCH_2Ph), 5.6 (t, 1 H, J = 6 Hz, $CHCOOBz$], 7.3 (s, 10 H, aromatic H), ($C_{20}H_{19}BrO_6$).

Dibenzyl *O*-[(Dimethylphosphono)acetyl]malate (29). To the bromide **28** (3.2 g, 7.3 mmol) was added trimethyl phosphite (1.1 g, 8.6 mmol) and the mixture rapidly heated to reflux for 1 h. Products were purified by column chromatography on silica gel, eluted with hexane/EtOAc (3:7), to give 2.1 g (60%) of a clear oil: NMR ($CDCl_3$) δ 2.9 (d, 2 H, J = 6 Hz, CH_2COOBz), 3.1 [d, 2 H, J_{H-P} = 12 Hz, $C(O)CH_2P(O)(OCH_3)_2$], 3.7 [d, 6 H, J_{H-P} = 12 Hz, $P(O)(OCH_3)_2$], 5.2 (d, 4 H J = 2 Hz, OCH_2Ph), 5.6 (t, 1 H, J = 6 Hz, $CHCOOBz$), 7.3 (s, 10 H, aromatic H); mass spectrum, *m/e* 464.1220 ($C_{22}H_{25}O_9P$).

Dibenzyl *O*-(Phosphonoacetyl)malate (30). The ester **29** (1.0 g, 2.2 mM) was combined with bromotrimethylsilane (0.78 mL, 5.9 mM) in 10 mL of chloroform and the reaction stirred for 20 min. The solvents and volatile constituents were removed on the rotoevaporator, the remainder was diluted with 25 mL of ether, treated with charcoal Norit A, and filtered. Water (0.2 mL) was then added, the ether was removed on the rotoevaporator, and an additional 10 mL of water was added. The pH of the solution was adjusted to 7.0 with 0.1 N NaOH; lyophilization of the water gave 0.61 g (79%) of a solid: dec pt 230–232 °C; NMR (D_2O) δ 2.6–3.1 [m, 4 H, $BzO(O)CCH_2C(H)OC(O)CH_2P$], 5.1 (d, 4 H J = 2 Hz, OCH_2Ph), 5.35 (t, 1 H, J = 6 Hz, $BzOOCCH_2CHCOOBz$), 7.2 (s, 10 H, aromatic H), ($C_{20}H_{19}Na_2O_9P$).

***O*-(Phosphonoacetyl)-L-malic Acid (6).** The benzyl ester **30** (0.86 g, 1.8 mmol) was dissolved in water (25 mL), and 0.3 g of 10% Pd on charcoal was added. The solution was hydrogenated for 45 min on a Parr apparatus under 45 psi of H_2. The solution was filtered, the pH adjusted to 7.0 with 0.1 N NaOH, and the solution lyophilized to yield 0.5 g (83%) of a crystalline solid: dec pt 250–252 °C; NMR (D_2O) δ 2.7–3.2 [m, 4 H, $CH_2CHOC(O)$-CH_2P], 5.0 (s, HDO), 5.0–5.3 (t, 1 H, $CH_2CHCOOD$). Anal. ($C_6H_5Na_4O_9P$) C, H, P. For mass spectral analysis, **6** was derivatized with diazomethane to give the corresponding tetramethyl esters. Mass spectrum: *m/e* found 312.0609 (M^+).

Acknowledgment. Support for this research was provided in part by a grant from the National Institutes of Health (Grant GM26582). F.C.W. also acknowledges support of a Faculty Research Award (Award FRA-209) from the American Cancer Society. We are grateful to T. R. Stouch for his help with the computer-modeling studies.

Registry No. **6**, 97998-19-1; **7**, 97998-20-4; **8**, 97998-21-5; **9**, 97998-22-6; **10**, 98013-92-4; **11**, 97998-23-7; **12**, 97998-24-8; **13**, 97998-25-9; **14**, 31618-90-3; **15**, 97998-26-0; **16**, 97998-27-1; **17**, 54820-26-7; **18**, 70660-08-1; **19**, 70660-05-8; **20**, 2409-52-1; **21**, 623-91-6; **22**, 97998-28-2; **23**, 2791-79-9; **24**, 73300-72-8; **25**, 97998-29-3; **26**, 85719-40-0; **27**, 22118-09-8; **28**, 97998-30-6; **29**, 97998-31-7; **30**, 97998-32-8; *p*-toluenesulfonyl chloride, 98-59-9; diethyl (hydroxymethyl)phosphonate, 3084-40-0; triethyl phosphite, 122-52-1; trimethyl phosphite, 121-45-9; aspartate transcarbamylase, 9027-25-2.

(31) Smith, S. L.; Brown, E. S.; Smith, J. D.; Andrako, J. *J. Pharm. Sci.* **1965**, *54*, 1269.

Antitrichomonal Activity of Mesoionic Thiazolo[3,2-*a*]pyridines[1]

Keith A. M. Walker,* Eric B. Sjogren,[2] and Thomas R. Matthews

Syntex Research, Palo Alto, California 94304. *Received January 31, 1985*

Screening of mesoionic compounds as potential electron acceptors by analogy with metronidazole led to the finding of in vitro antitrichomonal activity for anhydro-2-phenyl-3-hydroxythiazolo[3,2-*a*]pyridinium hydroxide (1). In a series of analogues, potent in vitro activity was found to be associated with amino substitution; however, such activity was dependent on specific structural features and not on the reduction potential. The most active compounds showed only poor in vivo activity.

For many years the chemotherapy of trichomonal (*Trichomonas vaginalis*) infections has been dominated by the nitroheterocycles, particularly in more recent times by the nitroimidazoles. Studies on metronidazole[3-5] (I) and

(1) Contribution No. 679 from the Institute of Organic Chemistry, Syntex Research, Palo Alto, CA 94304.

(2) Present address: Department of Chemistry, Harvard University, Cambridge, MA 02138.

0022-2623/85/1828-1673$01.50/0