Synthesis and Evaluation of Multisubstrate Inhibitors of an Oncogene-Encoded Tyrosine-Specific Protein Kinase. 2

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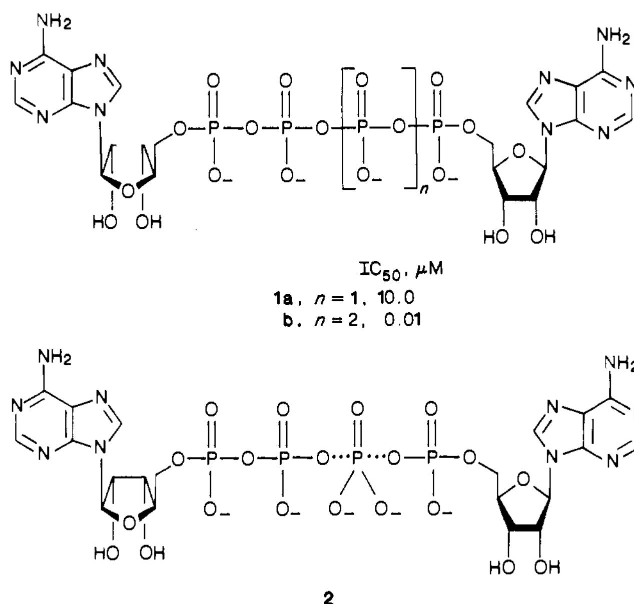
Tyrosine-specific protein kinases that transfer the terminal phosphate from ATP to protein acceptors are associated with certain transforming viruses and cell surface growth factor receptors. Here we describe the synthesis and testing of potential multisubstrate inhibitors of this class of enzymes. The inhibitors were prepared by covalent attachment of the terminal phosphate of ATP or its tetraphosphate analogue to tyrosine mimics. Testing against $p60^{v\text{-}abl}$, the tyrosine kinase from the Abelson murine leukemia virus, showed that the series of inhibitors was moderately potent (IC_{50} values as low as 13 μM). However, structural modification of the tyrosine mimic, including replacement with a serine-like moiety, had little effect on potency. It is therefore concluded that the ATP moiety is largely responsible for binding and that the enzyme requires additional structural features for recognition of the tyrosine-containing substrate.

In the preceding paper[1] we presented a rationale for the design of multisubstrate inhibitors of tyrosine-specific protein kinases. Our chemical strategy assumed that the enzyme's catalytic mechanism proceeds through a direct transfer of the γ-phosphate of ATP to a tyrosine acceptor rather than through a phosphorylated enzyme intermediate. Several compounds were described that retained the adenosine moiety of ATP but in which the triphosphate group had been replaced with the nonionic *p*-sulfonylbenzoyl functionality of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), an ATP binding site affinity ligand.[2] This change was made to render potential inhibitors membrane permeable so that they could be evaluated in cell culture systems. However, to evaluate the influence of the charged triphosphate linkage in determining inhibitor affinity and recognition, we now describe the design and synthesis of multisubstrate inhibitors containing a polyphosphate linkage.

Ap_5A (**1b**), a potent inhibitor of adenylate kinase, is a good example of a multisubstrate kinase inhibitor containing a polyphosphate linkage.[3] Adenylate kinase catalyzes the transfer of the γ-phosphate of ATP to AMP to form two ADP molecules. The transition state can be depicted as **2**. Compared either to **2** or to Ap_4A (**1a**), the latter of which might be expected to be a closer analogue of the transition state, **1b** has an additional phosphate group that lengthens the distance between the adenosine residues by approximately 3 Å. This results in a 1000-fold increase in inhibitor potency. This is consistent with X-ray crystallographic and NMR evidence, which suggest that substrate binding sites for related kinases are more distant than implied by transition state **2**.

Multisubstrate analogues of hexokinase containing polyphosphate linkages, **3a** and **3b**, are only weak inhibitors of this enzyme.[4,5] X-ray crystallography has shown that hexokinase undergoes a significant conformational change following substrate binding.[6] When binding to glucose, one enzyme lobe is rotated 12° compared to the apoenzyme resulting in a cleft closure and burial of the sugar. When the enzyme is crystallized in the presence of xylose (6-deshydroxymethylglucose) and ADP, however, the cleft remains open.[7] This suggests that the 6-hydroxyl group of glucose, which hydrogen bonds to aspartic acid and serine residues at the enzyme active site, is essential for inducing the conformational change that brings the substrate binding sites into closer proximity.

Analogues **3a** and **3b** lack a free 6-hydroxyl group, and this may explain their poor inhibitory properties. That these inhibitors are competitive with respect to ATP but are uncompetitive for glucose is consistent with this interpretation.



The goal of the present study was to explore a similar strategy in probing the distance between the substrate binding sites (ATP and tyrosine) in a tyrosine-specific protein kinase. The geometry of the transition state for the phosphorylation of tyrosine residues (**4**) is based on known enzymatic mechanisms[8] and X-ray[6,7] and spectroscopic analyses for related kinases.[6,7] To evaluate the triphosphate linkage in substrate recognition and to study the distance between the substrate binding sites, inhibitors incorporating polyphosphate chains of varying length were prepared (Table I). The importance of the tyrosine

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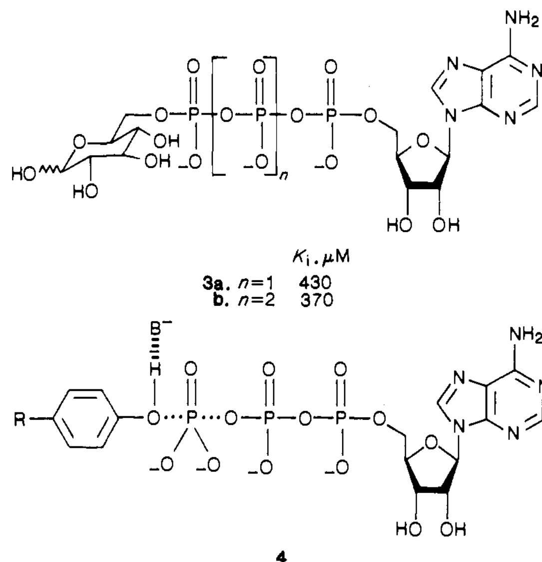


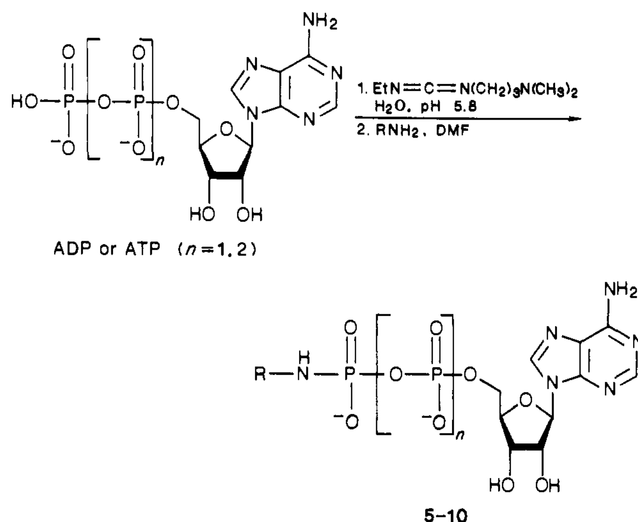
phenolic hydroxyl in substrate recognition was examined by testing a series of phosphoramidate inhibitors. The phosphoramidate NH of inhibitors **5–10** may provide hydrogen-bonding capability that mimics the tyrosine hydroxyl. Since serine and threonine residues are not phosphorylated by tyrosine-specific protein kinases, the influence of the aromatic ring in substrate recognition was probed by examining simple aniline (**5–7**) and phenol (**11** and **12**) derivatives. An analogue lacking the aromatic ring, **8**, was also prepared. Blocked tyrosine analogues derived from *p*-amino-*N*-acetylphenylalanine amide were incorporated into inhibitors **9** and **10** to evaluate the importance of the tyrosine amide functionalities in enzyme recognition.

Synthesis of Inhibitors. The phosphoramidates (**5–10**) were prepared as described[9] except that the amines employed were water insoluble and therefore were added as DMF solutions (Scheme I). A general procedure is given in the Experimental Section. Purification by anion exchange chromatography on DEAE-cellulose gave the desired compounds as ammonium salts. Amines used in preparing the phosphoramidates were obtained commercially. *p*-Amino-*N*-acetylphenylalanine amide was prepared by nitrating phenylalanine by using a procedure reported by Rapoport.[10] The nitro compound **17** was then elaborated as described in the Experimental Section by standard procedures to give **13** (Scheme III). The phosphate esters **11** and **12** were synthesized by using a procedure described by Eckstein for the corresponding guanosine analogues,[11] which utilized the imidazolate method developed by Hoard and Ott[12] (Scheme II).

Enzyme Assays. Tyrosine Kinase Activity. The transforming gene product (p60$^{v\text{-}abl}$) of the Abelson murine leukemia virus (A-MuLV) served as a source of tyrosine kinase activity.[1] p60$^{v\text{-}abl}$ was expressed in and extracted from *Escherichia coli* as described.[13] Compounds were tested in duplicate for their ability to inhibit enzyme-mediated incorporation of γ-^{32}P-labeled ATP into α-casein by using the assay conditions described earlier.[1] Duplicate values were within 5%.

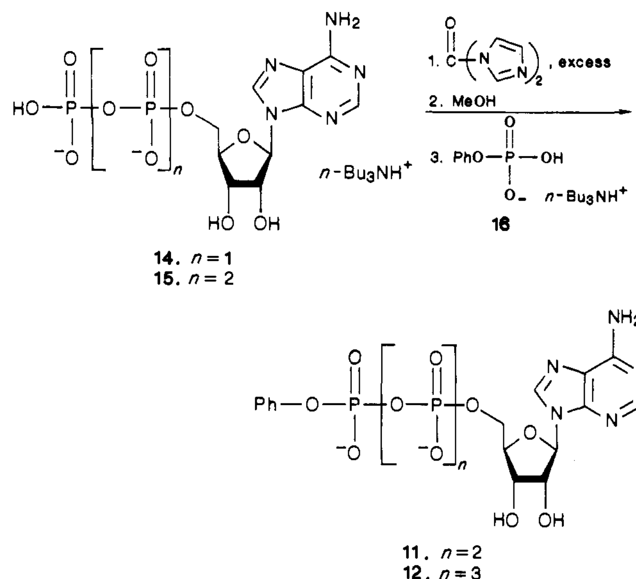
Time Course of Inhibition. Previous studies established[13] that p60$^{v\text{-}abl}$-mediated phosphorylation of α-casein was linear with time. Inhibition of this reaction by 5′-FSBA and compounds **5**, **6**, **8** and **11** was examined following preincubation of inhibitors with enzyme (Figure 1).

Scheme I. Preparation of Phosphoramidates[a]



[a] For R and *n*, see Table I.

Scheme II. Preparation of Phosphate Esters



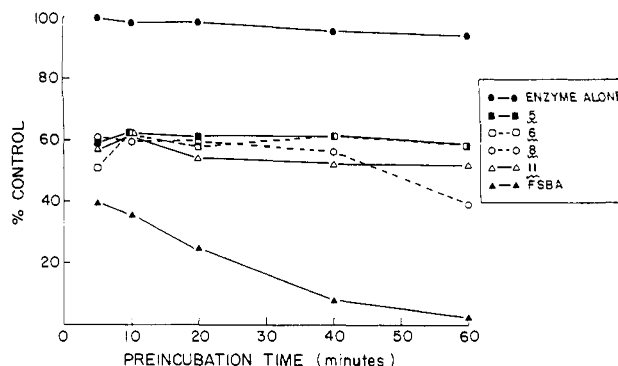


Figure 1. Inhibition of p60$^{v\text{-}abl}$ tyrosine kinase activity: effect of preincubation time. Compounds were assayed in duplicate; values were within 5%.

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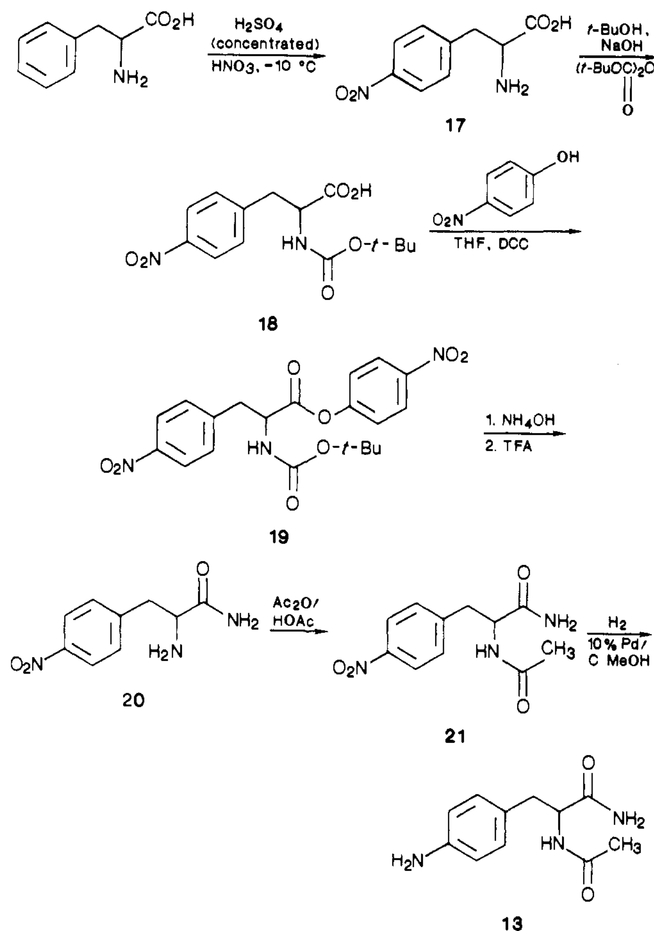
Table I. Inhibition of p60$^{v\text{-}abl}$ Tyrosine Kinase Activity

compd	R	n	IC_{50}, μM
5	HN, Me	1	150
6	HN, Me	2	16
7	H NCH2, OMe	1	90
8	$NHCH_2CH_2NHC(O)Me$	1	25
9	HN, NH2, HN, Me, O	1	44
10	HN, NH2, HN, Me, O	2	110
11	O	1	13
12	O	2	41

Scheme III. Preparation of 4-Amino-*N*-acetylphenylalanine Amide



Inhibitors were preincubated with enzyme 5–60 min (Figure 1). Assay conditions were otherwise identical with those described previously.[1]

Results and Discussion

The IC_{50} values for inhibitors **5–7** (Table I) indicate that an aromatic ring and an acidic hydrogen analogous to the tyrosine phenolic hydroxyl are insufficient structural features to guarantee recognition at the tyrosine binding site of p60$^{v\text{-}abl}$. Removal of the acidic hydrogen in compounds containing an aromatic ring (**11** and **12**) did not influence inhibitory potency. In addition, the inhibitory potency of compound **8**, which contains a serinelike moiety, was essentially indistinguishable from compounds bearing both an acidic hydrogen and an aromatic ring (**5–7**, **9**, and **10**). The distance between the adenosine and aromatic ring likewise failed to have a consistent effect on potency (compare **5**, **9**, and **11** with **6**, **10**, and **12**, respectively). These data indicate that the enzyme requires additional structural features for efficient substrate recognition.

Endowing more peptidelike character to the tyrosine portion of the inhibitors by incorporating amide functionalities (**9** and **10**) failed to increase potency. Although p60$^{v\text{-}abl}$ specifically phosphorylates tyrosine residues within polypeptide substrates the structural features required for recognition have not been established. Several tyrosine-containing peptides of known sequence have been investigated as tyrosine kinase substrates,[14] but the best K_m values are in the millimolar range. A consensus amino acid sequence flanking the target tyrosine residue has yet to be identified, suggesting that higher orders of protein structure may be involved in substrate recognition and binding.

The similar IC_{50} values displayed by the present inhibitors and those[1] in which the triphosphate linkage was replaced with a *p*-sulfonylbenzoyl linkage implies that compound recognition is occurring only at the enzyme ATP binding site.

Preincubation with enzyme failed to increase inhibitory potency, suggesting that progressively tighter binding between compound and enzyme did not occur. Compounds **5**, **6**, and **11** showed virtually no change in inhibitory potency with increased preincubation time (Figure 1), while compound **8** displayed slight increase in inhibition at the longest preincubation times. However, on the basis of the structure of this compound, it is difficult to see why its inhibitory effect should be time dependent.

The results of this study clearly indicate that for the present strategy to be successful more structural information on relevant tyrosine substrates is needed before potent multisubstrate p60$^{v\text{-}abl}$ kinase inhibitors can be rationally designed. We should also point out that we have not eliminated the possibility of an ordered binding mechanism in which initial recognition of peptide substrate induces a conformational change in the enzyme or that a phosphorylated enzyme intermediate is involved.

Experimental Section

Materials and Methods. Solvents were dried and/or purified by standard procedures.[15] Water was glass distilled. All starting

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materials were obtained from the Aldrich Chemical Co. Nucleotide starting materials were checked for purity by HPLC with an Altex Ultrasphere 5-μm C-18 column with an ion pairing buffer system as eluant (0.03 M KH_2PO_4, 0.01 M tetra-*n*-butylammonium phosphate, 19% CH_3CN, pH 2.65, 1 mL/min). Thin-layer chromatography was done on Analtech Uniplate 250-μm silica gel plates or PEI-cellulose F plates purchased from E. Merck. Baker 40 μm flash silica gel was used for flash chromatography. DEAE-cellulose D52 was purchased from Whatman. Infrared (IR) spectra were recorded on a Perkin-Elmer 783 spectrophotometer as Nujol mulls. NMR spectra were obtained either as D_2O or $CDCl_3$ solutions with a Varian EM390 spectrometer, and chemical shifts were recorded relative to tetramethylsilane. Fast atom bombardment mass spectra were obtained with a VG ZAB IF-HF spectrometer equipped with a standard FAB ion source with a glycerol matrix. Optical rotations were obtained with a Perkin-Elmer 241MC Polarimeter at 23 °C and were recorded in the indicated solvents. Elemental analyses for C, H, and N were performed by the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories. Analytical values were within 0.4% of the calculated values unless otherwise noted.

Carrier-free [^{32}P]orthophosphoric acid was purchased from Amersham Corp. [γ-^{32}P]ATP was prepared using the Gamma Prep System from Promega Biotec, and product purity was checked by thin-layer chromatography with PEI-cellulose plates purchased from E. Merck, eluted with 1.6 M LiCl. Protein was determined by the method of Bradford[16] with bovine serum albumin as a standard. Octylglucopyranoside and dithiothreitol were obtained from Calbiochem Behring. Coomassie G250 stain was purchased from Serva Fine Chemicals. Other reagents used in the isolation and assay of enzymes were purchased from either Fisher Chemical Co., Sigma Chemical Co., or J. T. Baker. Counts incorporated into the enzyme substrates were measured with a Beckman LS2800 Liquid Scintillation Counter. The Mono Q column and FPLC system were purchased from Pharmacia.

***P*^{1}-Adenosine-5′ *P*^{3}-*p*-Toluidine Triphosphate (5).** A solution of the disodium salt of adenosine-5′ triphosphate (250 mg, 0.45 mmol) in H_2O (7 mL) was cooled to 15–20 °C and adjusted to pH 5.8. To this solution was added 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (435 mg, 227 mmol) as a solid; then, *p*-toluidine (243 mg, 2.27 mmol) in DMF (1.5 mL) was added dropwise. The pH maintained between 5.65 and 5.85 by the periodic addition of 0.1 N HCl while the cooled solution was stirred for 2.5 h; TLC [silica; isobutyric acid–NH_4OH–H_2O (66:1:33)] showed that the ATP had been consumed. The pH was adjusted to 7.3 by the addition of NaOH, and the reaction mixture was evaporated at 25 °C. The residue was taken up in H_2O (10 mL) and applied to a DEAE-cellulose column (50 g), which had been preequilibrated with NH_4HCO_3. The column was washed with several column volumes of H_2O followed by elution of product with a linear gradient (2 L) of NH_4HCO_3, 0–0.4 M, pH adjusted to 8.5 with ammonium carbonate. UV-absorbing fractions were evaluated by TLC (PEI cellulose; 1.6 M LiCl); pure fractions were combined and lyophilized, yielding 190 mg (65%) of product as diammonium salt: MS-FAB, *m/e* 597 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 4.1–4.6 (m, 5 H), 6.05 (d, 1 H), 6.85 (s, 4 H), 8.25 (s, 1 H), 8.50 (s, 1 H). Anal. ($C_{17}H_2N_6O_{12}P_3 \cdot 2NH_3 \cdot H_2O$) C, H, N.

4-Amino-*N*-acetylphenylalanine Amide (13). To a solution, prepared by slowly adding L-phenylalanine (4.6 g, 28 mmol) to concentrated H_2SO_4 (7.5 mL), was added concentrated HNO_3 (1.8 mL) dropwise at −10 °C. After 3.5 h at this temperature, the solution was poured into ice water (75 mL), and the crude product was precipitated by neutralization with concentrated NH_4OH (20 mL). The precipitate was recrystallized repeatedly from boiling water to remove the contaminating ortho isomer yielding 4-nitrophenylalanine 17 (1.5 g, 24%), mp 250–251 °C. A solution of this material (6.6 mmol) in *tert*-butyl alcohol (30 mL) was treated with a solution of NaOH (0.26 g, 6.6 mmol) in H_2O (6 mL). After 1 h *tert*-butoxycarbonyl anhydride (1.58 g, 7.2 mmol) and *tert*-butyl alcohol (30 mL) were added, and the mixture was stirred at 25 °C overnight. The solution was diluted with H_2O and washed with pentane. The aqueous layer was acidified with $KHSO_4$ (1.92 g, 6.8 mmol) and extracted with EtOAc. The extract was washed (H_2O), dried (Na_2SO_4), and concentrated to give crude *p*-nitro-*N*-*t*-BOC-phenylalanine (18) (1.17 g, 58%). This material (3.8 mmol) in THF (10 mL) was added to *p*-nitrophenol (0.56 g, 4.0 mmol). The mixture was then chilled to 5 °C and treated with DCC (0.78 g, 3.8 mmol). After being stirred overnight at 25 °C, the solution was filtered. The residue was concentrated and triturated with 2-propanol and with petroleum ether to give *p*-nitro-*N*-*t*-BOC-phenylalanine-ON-*p* (19) (0.99 g, 61%), mp 193–194.5 °C. The *p*-nitrophenol derivative 19, (2.3 mmol) was dissolved in THF (20 mL), and concentrated NH_4OH (1.5 mL) was added dropwise. After being stirred overnight at 25 °C, the reaction mixture was evaporated and the residue was triturated with Et_2O to yield 4-nitro-*N*-*t*-BOC-phenylalanine amide (0.68 g, 95%). The amide (2.2 mmol) was stirred for 1 h in TFA (4 mL). The mixture was evaporated, and the residue was triturated with Et_2O; the resulting solid amine **20** was mixed with glacial HOAc (60 mL) and heated to 90 °C. After dropwise addition of acetic anhydride (10 mL), the mixture was heated for 15 min, cooled, and evaporated to give 4-nitro-*N*-α-acetylphenylalanine amide (21) (0.20 g, 53%). This material (1.2 mmol) in MeOH (160 mL) was treated with 10% palladium on carbon (0.135 g) and hydrogenated (Paar) for 2 h. After filtration, concentration gave 13 (1.26 g, 100%, 4.3% from the L-phenylalanine). IR, NMR, and mass spectral data were consistent with the structure. The 4-amino-*N*-acetylphenylalanine amide (13) was then used in the preparation of compounds **9** and **10**.

***P*^{1}-Adenosine-5′ *P*^{3}-Phenyl Triphosphate (11).** The disodium sallt of ADP (3.0 g, 7.0 mmol) was converted to the corresponding tri-*n*-butylammonium salt (14) by percolating its solution in water (25 mL) through a column of AG50-2 resin (20 mL, pyridinium form). All UV-positive fractions were combined and concentrated in vacuo to leave a clear glass. The glass was dissolved in water (20 mL) and treated with tri-*n*-butylamine (3.37 mL, 14 mmol). After being stirred for 1 h, the reaction mixture was concentrated to leave 14 as a light yellow oil.

A solution of 14 (630 mg, 0.68 mmol) in dry acetone (3 mL) containing powdered, dried 4A molecular sieves (0.5 g) at room temperature under argon was treated with an excess of carbonyldiimidazole (324 mg, 2.0 mmol, 3 equiv) and allowed to stir for 5 h. The mixture was treated with dry MeOH (200 μL, 4.93 mmol) to destroy the excess reagent. After 1.5 h the mixture was concentrated and taken up in dry DMF (8 mL). After treatment with 16 in dry DMF (2 mL), the reaction was allowed to stir overnight. Filtration and concentration gave a light yellow oil, which was shown by TLC on silica gel (acetone–H_2O–NH_4OH, 7:2:1) and PEI cellulose plates (1.6 M LiCl) to have one new component. The mixture was chromatographed on DEAE-cellulose (50 g), which had been preequilibrated with NH_4HCO_3. The column was washed with water (200 mL), and the product was eluted with a linear gradient (1 L) of 0–1 M $(NH_4)_2CO_3$, which the pH adjusted to 8.5 with ammonium carrbonate. UV-positive fractions were assayed by TLC on PEI-cellulose plated with 1.6 M LiCl as eluant. Appropriate fractions were combined and lyophilized to give the diammonium salt of 11 as the trihydrate (160 mg, 17%): MS-FAB, *m/e* 582 (M^+ + H); NMR (D_2O) δ 8.50 (s, 1 H), 8.20 (s, 1 H), 7.3–6.3 (m, 5 H), 6.05 (d, 1 H), 4.2–4.6 (m, 5 H). Anal. ($C_{16}H_{20}N_5O_{13}P_3 \cdot 2NH_3 \cdot 3H_2O$) C, H, N.

***P*^{1}-Adenosine-5′ *P*^{4}-Phenyl Tetraphosphate (12).** With use of the tri-*n*-butylammonium salts of phenyl phosphate (16) and ATP (15), the phenyl ester of adenosine tetraphosphate 12 was prepared in a similar manner (3% yield): NMR (D_2O) δ 8.65 (s, 1 H), 8.40 (s, 1 H), 7.3–7.1 (m, 5 H), 6.20 (d, 1 H), 4.3–4.7 (m, 5 H).

Synthesis of Phosphoramidates 5–10. Compounds 5–10 were prepared as described[9] except that the amines employed were added as DMF solutions. Purification by DEAE-cellulose afforded the desired compounds as ammonium salts.

5: 65% yield; MS-FAB, *m/e* 597 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 4.1–4.6 (m, 5 H), 6.05 (d, 1 H), 6.85 (s, 4 H), 8.25 (s, 1 H), 8.50 (s, 1 H). Anal. ($C_{17}H_{23}N_6O_{12}P_3 \cdot 2NH_3 \cdot H_2O$) C, H, N.

6: 54% yield; MS-FAB, *m/e* 677 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 4.4–4.6 (m, 5 H), 6.18 (d, 1 H), 6.88 (s, 4 H), 8.20 (s, 1 H), 8.58 (s, 1 H). Anal. ($C_{17}H_{24}N_6O_{15}P_4 \cdot 2NH_3 \cdot 4.25H_2O$) C, H, N.

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7: 21% yield; MS-FAB, m/e 627 (M^+ + H); NMR (D_2O) δ 3.70 (s, 3 H), 3.8–4.0 (d, 2 H), 4.2–4.6 (m, 5 H), 6.06 (d, 1 H), 6.62 (d, 2 H), 7.05 (d, 2 H), 8.15 (s, 1 H), 8.48 (s, 1 H). Anal. ($C_{18}H_{25}N_6O_{13}P_3 \cdot 2NH_3 \cdot 3H_2O$) C, H, N.

8: 20% yield; MS-FAB, m/e 592 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 3.0–3.5 (m, 4 H), 4.2–4.6 (m, 5 H), 6.20 (d, 1 H), 8.44 (s, 1 H), 8.68 (s, 1 H). Anal. ($C_{14}H_{24}N_7O_{13}P_3 \cdot 2NH_3 \cdot 1.5H_2O$) C, H, N.

9: 61% yield; MS-FAB, m/e 711 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 2.72 (t, 2 H), 4.2–4.6 (m, 6 H), 6.05 (d, 1 H), 6.98 (s, 4 H), 8.22 (s, 1 H), 8.52 (s, 1 H). Anal. ($C_{21}H_{29}N_8O_{14}P_3 \cdot 2NH_3 \cdot 4H_2O$) C, H, N.

10: 60% yield; MS-FAB m/e 791 (M^+ + H); NMR (D_2O) δ 2.00 (s, 3 H), 2.85 (m, 2 H), 4.3–4.6 (m, 5 H), 6.13 (d, 1 H), 6.98 (s, 4 H), 8.28 (s, 1 H), 8.60 (s, 1 H). Anal. ($C_{21}H_{30}N_8O_{17}P_4 \cdot 3NH_3 \cdot 4.5H_2O$) C, H, N.

Registry No. **5**, 115094-21-8; **6**, 115094-22-9; **7**, 115094-23-0; **8**, 115094-24-1; **9**, 115117-16-3; **10**, 115094-25-2; **11**, 115094-26-3; **12**, 115117-17-4; **13**, 115094-27-4; **14**, 90290-56-5; **15**, 56-65-5; **15**·2Na, 987-65-5; **16**, 90290-56-5; **17**, 949-99-5; **18**, 33305-77-0; **19**, 66163-66-4; **20**, 81677-61-4; **21**, 61925-80-2; tyrosine-specific protein kinase, 80449-02-1; p-toluidine, 106-49-0; L-phenylalanine, 63-91-2; 4-nitro-N-t-BOC-phenylalanine amide, 66163-67-5.

Phosphinic Acid Inhibitors of D-Alanyl-D-alanine Ligase

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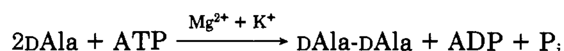
We report the synthesis of a series of phosphinic acid dipeptide analogues, $NH_2CH(R^1)PO(OH)CH_2CH(R^2)CO_2H$, related to DAla-DAla. The best of these compounds are potent, essentially irreversible inhibitors of DAla-DAla ligase, and their preferred stereochemistry was shown by chiral synthesis of (1(S)-aminoethyl)(2(R)-carboxy-1-n-propyl)phosphinic acid, **12b**, and by X-ray crystallography of its derivative benzyl [1(S)-[(benzyloxycarbonyl)-amino]ethyl](2(R)-carbomethoxy-1-propyl)phosphinate, **13**, to correspond to the stereochemical configuration of DAla-DAla at both centers. A mechanism for the inhibition of DAla-DAla ligase by these compounds is proposed to involve an ATP-dependent formation of phosphorylated inhibitor within the enzyme's active site. The antibacterial activities of these compounds are modest although their spectra include both Gram-positive and Gram-negative susceptible organisms. The best antibacterial activity was shown by (1(S)-aminoethyl)[2-carboxy-2(R)-(methylthio)-1-ethyl]phosphinic acid, **3e**, whose MIC's range from 4–128 μg/mL on nine of a panel of 11 bacterial organisms. Combination of one of the more active phosphinic acids **12b** with the alanine racemase inhibitor fluoro-D-alanine enhances the antibacterial spectrum of the latter on several strains of bacteria and inhibits fluoro-D-alanine's self-reversal, which normally occurs at concentrations several fold higher than its MIC level. This inhibition of fluoro-D-alanine self-reversal is consistent with an involvement of DAla-DAla ligase inhibition in the antibacterial activity of these compounds.

Most therapeutically useful antibacterial agents inhibit the biosynthesis or function of target structures present only in bacteria. One of these targets, peptidoglycan, is a cross-linked cell wall polymer that plays an essential role in protecting bacteria from lysis. Its assembly is blocked by numerous antibacterials that are enzyme inhibitors, including β-lactams, D-cycloserine, fluoro-D-alanine, alafosfalin, moenomycin, and fosfomycin.[1]

Peptidoglycan biosynthesis is initiated with the construction of uridine-5′-diphosphate–N-(acetylmuramyl)-LAla-DGlu-Dap(Lys)-DAla-DAla, which is synthesized via a multienzyme pathway culminating in the addition of D-alanyl-D-alanine to a uridine-5′-diphosphate–N-acetylmuramyl tripeptide. D-Alanyl-D-alanine is in turn synthesized by D-alanyl-D-alanine ligasae [D-alanyl-D-alanine synthetase (ADP); EC 6.3.2.4].[1,2] It seems quite probable that failure to assemble and incorporate DAla-DAla into the bacterial cell wall will result in bacterial lysis since racemase inhibitors such as fluoro-D-alanine by inhibiting DAla formation are antibacterial as are β-lactam antibiotics, which inhibit transpeptidation involving the DAla-DAla unit. D-Cycloserine, which is transported across the cell wall via the D-alanine/glycine transport system, is a fair inhibitor of this enzyme, and it is an effective antibiotic presumably because it also inhibits alanine racemase and D-amino acid transaminase as well.[2,26] However, apart from cycloserine analogues,[3] surprisingly little has been done in the synthesis of D-alanyl-D-alanine ligase inhibitors, and the ability to establish if ligase inhibition is lethal or not has never been fully accomplished. Some dipeptide analogues[4] of DAla-DAla are modest inhibitors of the ligase as is (1-aminoethyl)phosphonic acid.[5,6] We report here the synthesis and properties of a series of phosphinic acids, the most active of which are tight-binding inhibitors of the enzyme.

Design Considerations. The chemistry catalyzed by DAla-DAla ligase can be summarized as follows:[7,8]



$$2\text{DAla} + \text{ATP} \xrightarrow{Mg^{2+} + K^+} \text{DAla-DAla} + \text{ADP} + P_i$$

The stoichiometry of this reaction is similar to that of glutamine and γ-glutamylcysteine synthetases in which the γ-carboxyl group of glutamic acid is activated by ATP for

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