α-Halo [(Phenylphosphinyl)methyl]phosphonates as Specific Inhibitors of Na^+-Gradient-Dependent Na^+–Phosphate Cotransport across Renal Brush Border Membrane†

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Certain phosphonocarboxylate analogues of phosphate are known to inhibit Na^+-phosphate (P_i) cotransport in renal brush border membrane (BBM), but previously tested potential inhibitors incorporating structurally versatile aryl functionality were inactive. In this work, a series of novel α-halogenated [(phenylphosphinyl)methyl]phosphonates [PhpXYMP: X, Y = H, F (2); F, F (3); H, Cl (6); Cl, Cl (4); H, Br (7); Br, Br (5); and Cl, Br (8)] were prepared via synthesis of the corresponding triethyl esters, acid hydrolysis, and isolation as pyridine salts. The compounds were evaluated as inhibitors of Na^+-gradient-dependent $^{32}P_i$ uptake by rat renal cortex BBM vesicles (BBMV) in vitro. The PhpFMP racemate 2 had higher activity (−49% Δ inhibition) than other members of the series (−22 to −39% Δ inhibition). pK_a values of 1.5–2.0, 2.7, and 7.1 were estimated for 2 using a ^{31}P δ vs pH plot, indicating that in the activity assays it exists as both dianion and trianion, with the latter form predominant. PhpFMP had no significant inhibitory effect on Na^+-gradient-dependent uptake of D-glucose or L-proline in the same BBMV, and did not inhibit BBM alkaline phosphatase. Kinetic analysis showed that PhpFMP acts as a strictly competitive inhibitor of Na^+–P_i cotransport with $K_i = 0.358 \pm 0.021$ mM ($n = 3$). The racemate 2 was resolved as its (−)-quinine salt into enantiopure (+)-2 [Na^+ salt, $[\alpha]^{25}_D = +6°$ (aqueous MeOH)] and a Na^+ salt of 2 enriched in (−)-2. The two compounds did not differ significantly as inhibitors of Na^+-gradient dependent $^{32}P_i$ uptake by rat renal cortex BBM vesicles (BBMV) in vitro. The results are discussed in terms of structural requirements for inhibition.

Introduction

Two phosphonocarboxylates known to possess antiviral properties,[2] phosphonoacetic acid (PAA) and phosphonoformic acid (PFA), are also specific competitive inhibitors of Na^+–phosphate (P_i) cotransport across the luminal brush border membrane (BBM) of renal[3,4] and intestinal epithelia.[5] In search of more potent and potentially irreversible inhibitors of the Na^+–P_i cotransporter in renal BBM, several halogenated PAA derivatives were subsequently investigated.[6,7] Systematic introduction of different halogen atoms, or different combinations of halogen atom pairs, has afforded a simple means of varying the basicity, steric profile, and other properties of parent pyrophosphate analogues such as PAA and methanediphosphonate (MDP).[2] α-Halogenated PAA and MDP derivatives exhibit significant differences in binding specificity for viral and human DNA polymerases.[2,8]

Recently, we reported that bromochlorophosphonoacetate (ClBrPAA) competitively inhibits Na^+/Pi cotransport in renal BBM with a K_i of 62 ± 16 μM, 26-fold lower than the K_i of PAA.[6] The greater potency of ClBrPAA vs PAA in cotransport inhibition contrasts with its ≥7-fold lower activity vs PAA in inhibition of in vitro DNA synthesis by human α DNA polymerase,[2] thus demonstrating that appropriate α-halogenation produces divergent changes in PAA specificity for the two systems. Such divergence is desirable for Na^+–P_i cotransport antagonists to limit toxicity arising from DNA polymerase inhibition.

PFA and PAA have been useful probes in initial studies of the Na^+–P_i cotransporter.[3,4,6,9–11] However, design of Na^+–P_i cotransport antagonists better suited for more detailed elucidation of both the renal and intestinal

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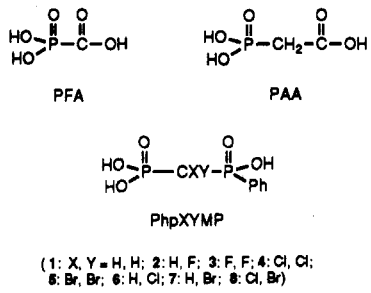
Scheme I



cotransporters would be aided by the discovery of inhibitor lead structures affording greater latitude for derivatization, e.g. by incorporation of a covalently reactive group. Ideally, new inhibitor prototypes should also be more susceptible to pharmacological manipulation and tissue-targeting in vivo. Two previously tested phosphate analogues incorporating an aryl moiety, phenylphosphonate and α-(*p*-chlorophenyl)phosphonoacetate, were ineffective as inhibitors.[10]

[(Hydroxyphenylphosphinyl)methyl]phosphonic acid (1) resembles PAA insofar as it is triprotic and possesses an α-CH_2 accessible to halogen substitution, but its aryl moiety introduces a new locus for derivatization, e.g. with chemically reactive or photoactive functionality. The phenyl group should also enhance lipophilicity, although the influence of this property on cotransport inhibition has not been studied systematically, and in addition provides an analytically useful chromophore. We describe here a new category of phosphonate analogues, α-halo [(phenylphosphinyl)methyl]phosphonates (PhpXYMP) (Scheme I) which represent the first aryl-containing inhibitors of Na^+–P_i cotransport in renal BBM. (Following the preliminary communication of this work,[1] additional examples of aryl phosphonates exhibiting inhibition of Na^+–P_i cotransport in BBM have been reported.[12,13])

The monohalo derivatives **2** (X, Y = H, F), **6** (X, Y = H, Cl), and **7** (X, Y = H, Br) and also the heterodihalo derivative **8** (X, Y = Cl, Br) have a chiral α-C atom and were evaluated as racemates. (In aqueous solutions of compounds **2–8**, the phosphinyl phosphorus atom is effectively prochiral, whereas it is chiral in the triester precursors **12**, **12′–18**, **18′**, which we synthesized nonstereospecifically.) The possibility of stereochemical specificity in phosphate analogues which inhibit Na^+–P_i cotransport has not been previously explored. Accordingly, we also prepared the enantiomerically pure (+)- and enantiomerically enriched (−)-enantiomers of **2**, the most active compound in the series **2–8**, to determine whether they displayed any detectable difference in inhibiting Na^+–P_i cotransport in renal BBM.

Chemistry

The α-halo PhpMP analogues **2–8** were synthesized by adapting methods previously used to prepare corresponding phosphonoacetate[2,14,15] and methanediphosphonate[16,17] derivatives to halogenation and dealkylation of the triethyl ester of **1**, **9**. Synthetic routes are summarized in Scheme II. The ester **9** was prepared by condensation of diethyl phenylphosphonite **10** with diethyl (chloromethyl)phosphonate **11**.[18] The fluorinated derivatives **12**, **12′**, and **13** were prepared by treatment of the K^+ carbanion of **9** with perchloryl fluoride.[16] [Compounds with paired *N*,*N′* numbers represent binary mixtures of diastereomers, which are racemates except for **19**,**19′** (Scheme II; vide infra).]

The dichloro (**14**) and dibromo (**15**) esters were obtained by treatment of **9** with ClO^- and alkaline aqueous Br_2, respectively, and the corresponding monohalo esters **16**,**16′** and **17**,**17′** were made by stoichiometric reduction of the corresponding dihalo derivatives. The bromochloro ester **18** was prepared by bromination of **16**,**16′**. The PhpXYMP acids **2–8** were obtained by hydrolysis (aqueous HCl) of the corresponding triethyl esters followed by isolation as crystalline pyridine salts.

The esters **12**,**12′–18**,**18′** were characterized by ^1H, ^{13}C, ^{31}P, and (where applicable) ^{19}F NMR. ^{31}P chemical shifts of diethyl [(ethoxyphenylphosphinyl)methyl]phosphonate and its α-halo derivatives ranged from δ 25.3 ($PhpF_2MP$, **13**) to δ 33.6 (PhpMP, **9**) for the phosphinyl (p) group and from δ 4.6 ppm (**13**) to δ 19.6 (**9**) for the phosphonyl (P) group. Higher shielding of both types of phosphorus nucleus was observed in all the α-halo PhpMP derivatives relative to the parent **9**. A similar effect has been reported for α-halo PAA and MDP derivatives.[15,17] Conversely, as expected, more electronegative halogen substitution decreased shielding of the α-^{13}C nucleus, producing a chemical shift span of about 58 ppm for the esters **12**,**12′–18**,**18′**. However, the diagnostic value of these resonances was limited by the difficulty of observing them in **17**,**17′** and in the dihalo derivatives.

The diastereomeric compositions of (**12**,**12′** and **16**,**16′–18**,**18′**) were readily determined by ^{31}P NMR analyses,[19] with both the phosphinyl (p) and phosphonyl (P) resonances of each diastereomer being resolvable (in the case of **12**,**12′** and **16**,**16′**, distinct diastereomer ^{13}C resonances were also observed). Although their separate isolation was irrelevant to ultimate synthesis of **2**, the precursor triethyl ester diastereomers (**12**,**12′**) were separated by flash chromatography to clarify NMR assignments. The more polar (TLC) diastereomer **12′** had ^{31}P NMR resonances at δ = 29.37 ppm (p) and 11.85 ppm (P), while in **12**, the phosphinyl ^{31}P resonance was shifted upfield by about 0.80 ppm. A similar but smaller ^{31}P chemical shift difference was observed for the phosphonyl signal. The phosphinyl and phosphonyl ^{31}P–^{19}F coupling constants differed only by 1–2 Hz between diastereomers, whereas the corresponding ^{31}P–^{31}P coupling constants were significantly different (8 Hz for **12**, 17 Hz for **12′**). A ^{19}F

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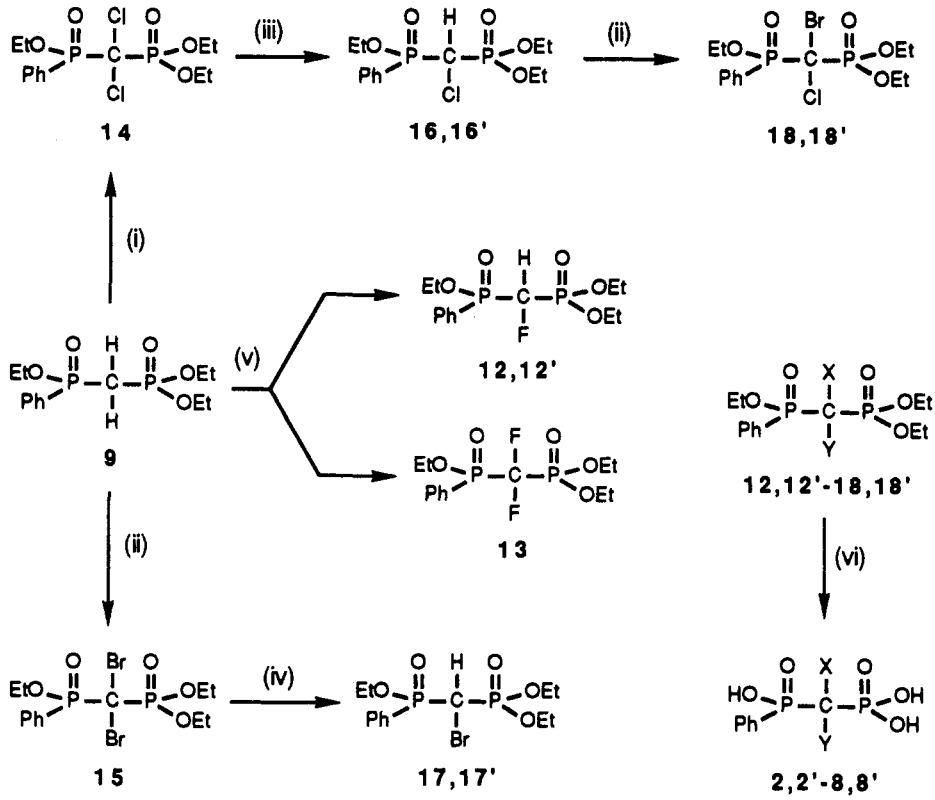
Scheme II



chemical shift difference of 0.97 ppm for the two diastereomers was also noted: the PhpFMP diastereomer (12′) giving rise to the more downfield ^{31}P (p) signal also had the more downfield ^{19}F NMR signal. The ^1H-coupled ^{19}F NMR spectrum showed similar J_{FH} coupling values (46 Hz) for the two diastereomers.

The pertinent charge state(s) of Na^+–P_i cotransport inhibitors, and indeed of substrate phosphate itself, is of interest. It appears likely that $H_2PO_4^-$ and/or HPO_4^{2-} are important transported forms of phosphate if the local cotransporter pH reflects an external pH near 7, given that the pK_a of $H_2PO_4^-$ is 7.1. It is not yet known whether one or both anionic forms is transported. We therefore undertook a preliminary estimation of pK_a values for PhpFMP 2. The dependence of ^{31}P NMR chemical shift values on pH for aqueous solutions of α-halo MDP and related compounds[16,20] prompted a similar approach in measuring the acidity of 2. Plots of δ ^{31}P (both p and P resonances) vs pH for a sample of 2.7% (w/v) 2 triacid in 7:1 H_2O/D_2O titrated with 1 M NaOH are presented in Figure 1. In general, the phosphinyl resonance was more sensitive to pH (maximum Δδ of ~4 ppm; Figure 1a) than the phosphoryl resonance (maximum Δδ of 1.4 ppm; Figure 1b). Analysis of inflection points in the plots indicates that PhpFMP has a pK_1 of 1.5–2, and a pK_2 of 2.7; its phosphonic acid group has a pK_3 value of 7.1, the latter value being obtained from both plots. Ignoring pK effects arising from minor differences in ionic strength and temperature, these results indicate that at the cotransporter inhibition assay pH of 7.5, 2 predominantly exists in the trianion form A, but with a significant amount of the dianion B present (Scheme III).

Scheme III

It is noteworthy that dissociation of the remaining phosphonic acid proton from B, which increases shielding of the phosphonyl nucleus with an attendant upfield shift of 1 ppm in the ^{31}P spectrum (Figure 1b), has the opposite effect on the phosphinyl resonance, which is significantly downshifted. The observed deshielding of the phosphinyl nucleus presumably reflects local polarization changes as charge symmetry in the molecule is altered.

As stated in the Introduction, we considered it desirable to attempt resolution of the monofluoro analogue 2 as it was found to be the most effective inhibitor of renal BBM Na^+–P_i cotransporter in the group of compounds tested (2–8; see Results section). Partial resolution of [fluoro(hydroxyphenylphosphinyl)methyl]phosphonic acid (2) was accomplished by fractional crystallization of its diastereomeric (−)-quinine salts, yielding 19 (bis-salt), >98% diastereopure by both ^{31}P and ^{19}F NMR and 19′ (mono-salt), ~67% diastereopure by both ^{31}P and ^{19}F NMR (Figure 2). Efforts to resolve brucine salts of 2 were unsuccessful. Compounds 19 and 19′ were converted into the corresponding Na^+ salts of (+)-2 and (predominantly) (−)-2 by brief treatment with dilute base, extraction, and

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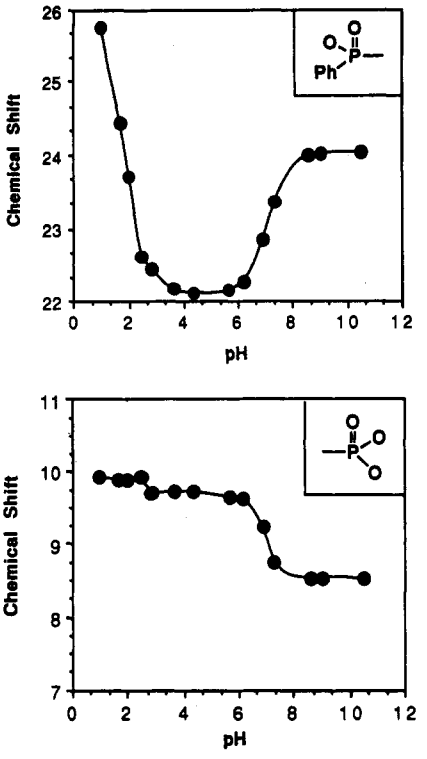

Figure 1. ^{31}P NMR chemical shift vs pH plots for [fluoro(hydroxyphenylphosphinyl)methyl]phosphonic acid (**2**, 12 mg in 0.4 mL of H_2O and 0.05 mL of D_2O) titrated with NaOH (1 M): (a) data for phosphinyl (p) resonance, and (b) data for phosphonyl (P) resonance. Spectra were measured on a Bruker WP-270 SY spectrometer operated at 109.3 MHz, on samples at ca. 24 °C. Chemical shifts are reported relative to 85% phosphoric acid as an external reference.

repeated precipitation from aqueous MeOH-acetone. After characterization by ^{31}P, ^{13}C, and ^{19}F NMR and by spectropolarimetry, the Na^+ salts were tested to determine possible α-C stereospecificity in cotransporter inhibition by **2** enantiomers.

Experimental Section

Synthetic Chemistry. Halocarbon grease and oil for use with perchloryl fluoride (Ozark-Mahoning, Tulsa, OK) were obtained from Halocarbon Products Corporation, Hackensack, NJ. For general safety precautions in handling perchloryl fluoride see ref 21, and Bulletins DC-1819 and I-1819, Pennsalt Chemicals Corp., Philadelphia, PA. All solvents and reagents were analytical grade. Chloromethylphosphonic dichloride and dichlorophenylphosphine were purchased from Aldrich Chemical Co. Clorox bleach obtained from a local market was used as the source of sodium hypochlorite (5.25%). Brucine and quinine were purchased from Aldrich Chemical Co. Proton (^1H, 270.13 MHz or 360.13 MHz), carbon ($^{13}C\{^1H\}$, 67.92 MHz or 90.56 MHz), phosphorus ($^{31}P\{^1H\}$, 109.35 MHz or 145.73 MHz), and fluorine (^{19}F, 338.9 MHz) NMR spectra were measured on a Bruker WP-270 SY or a Bruker AM-360 spectrometer. NMR ester samples were ca. 5% v/v in $CDCl_3$. NMR salt samples were ca. 10% v/v (loose solid) in D_2O. Chemical shifts are reported relative to TMS (internal $CHCl_3$: ^1H, δ = 7.24; ^{13}C, δ = 77.0 [esters]; external benzene: ^{13}C, δ = 128.0 [salts]), external 85% H_3PO_4 (^{31}P, δ = 0.0), or external $CFCl_3$ (^{19}F, δ = 0.0). Phosphonyl and phosphinyl groups are designated respectively as P and p in assignments. Thin-layer chromatography (TLC) was carried out on plastic sheets precoated with a 0.2-mm layer of fluorescent silica gel 60 (Merck 5735). Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. pH measurements were carried out using a Corning 125 pH meter equipped with a Beckman combination AgCl electrode calibrated with standard buffers between pH 4 and 10. Optical rotations were measured (in triplicate, unless noted otherwise, error of mean ≤ 0.5°) on a Perkin-Elmer 241 polarimeter. Elemental analyses were performed by Galbraith Laboratories.

(*S*/*R*)-Diethyl [(Ethoxyphenylphosphinyl)methyl]phosphonate (9). The method of Park[18] was used with minor modifications on a 25× larger scale. A mixture of **10** (127 g, 641 mmol), prepared by reaction of dichlorophenylphosphine with excess NaOEt,[22] and **11** (117 g, 627 mmol), prepared by reaction of chloromethylphosphonic dichloride with EtOH,[18] was placed in a 500-mL round-bottomed flask fitted with a reflux condenser connected to a nitrogen bubbler. Heat was applied and the temperature maintained at 170 °C over 16 h. The product (70% by ^{31}P NMR) was vacuum distilled to give 98.8 g (49.2%) of a colorless, viscous oil: bp 130–132 °C (0.002 mmHg); ^1H NMR δ 7.88–7.78, 7.57–7.42 (m, 5.1 H, C_6H_5), 4.19–3.85, (m, 6.4 H, OCH_2CH_3), 2.59 (m, 2.6 H, pCH_2P), 1.20, 1.18, 1.05 (3t, $^3J_{HH}$ = 7 Hz, 9.3 H, OCH_2CH_3); $^{13}C\{^1H\}$ NMR δ 131.90 (d, $^4J_{Cp}$ = 3 Hz, *p*-C_6H_5), 131.24 (d, $^2J_{Cp}$ = 10 Hz, *o*-C_6H_5), 127.90 (d, $^3J_{Cp}$ = 13 Hz, *m*-C_6H_5), *ipso*-C_6H_6 not fully resolved, 61.85, 61.59 (2d, $^2J_{CP}$ = 6.5 Hz, $POCH_2CH_3$), 60.73 (d, $^2J_{Cp}$ = 7 Hz, $pOCH_2CH_3$), 28.81 (dd, pCP), 15.80, 15.66, 15.54 (3d, *J* = 6 Hz, OCH_2CH_3); $^{31}P\{^1H\}$ NMR δ 33.57 (d, $^2J_{pP}$ = 7.5 Hz), 19.56 (d, $^2J_{Pp}$ = 7.5 Hz).

(*S*/*R*)-Diethyl [Dichloro(ethoxyphenylphosphinyl)methyl]phosphonate (14). To a solution of 5.25% sodium hypochlorite (665 g, 469 mmol), adjusted to pH 7.25 (1 M HCl), at 0 °C was added dropwise with vigorous stirring 15 g (47 mmol) of **9**. Stirring was continued for 4 h at room temperature. The turbid solution was then extracted with three 100-mL portions of Et_2O. The combined organic phases were dried ($MgSO_4$) and the solvent removed by rotary evaporation (Buchi Rotavapor, water pump) at 30 °C. The product (11.25 g, 62%) was obtained by vacuum distillation as a colorless oil: bp 144–148 °C (0.002 mmHg); ^1H NMR δ 7.98–7.91, 7.60–7.54, 7.48–7.41 (3m, C_6H_5), 4.45–4.12 (m, OCH_2CH_3), 1.38, 1.32, 1.23 (3t, $^3J_{HH}$ = 7 Hz, OCH_2CH_3); $^{13}C\{^1H\}$ NMR δ 134.45 (d, $^2J_{Cp}$ = 9 Hz, *o*-C_6H_5), 133.31 (d, $^4J_{Cp}$ = 3 Hz, *p*-C_6H_5), 127.86 (d, $^3J_{Cp}$ = 14 Hz, *m*-C_6H_5), 125.40 (d, $^1J_{Cp}$ = 149 Hz, *ipso*-C_6H_5), 66.00, 65.89 (2d, $^2J_{CP}$ = 7 Hz, $POCH_2CH_3$), 64.45 (d, $^2J_{Cp}$ = 7 Hz, $pOCH_2CH_3$), 16.44, 16.28, 16.18 (3d, *J* = 6, 6, 7 Hz, OCH_2CH_3); δ pCP not observed; $^{31}P\{^1H\}$ NMR δ 29.04 (d, $^2J_{pP}$ = 17 Hz), 9.00 (d, $^2J_{Pp}$ = 17 Hz). Anal. ($C_{13}H_{20}Cl_2O_5P_2$) C, H.

(*S*/*R*)-Diethyl [Dibromo(ethoxyphenylphosphinyl)methyl]phosphonate (15). A solution of sodium hydroxide (7.50 g, 188 mmol) in H_2O (20 mL) was prepared in a 100 mL, three-necked round-bottomed flask fitted with a thermometer and an addition funnel. The solution was cooled to 0 °C (ice–salt bath) and bromine (4.8 mL, 15 g, 94 mmol), followed by **9** (8.6 g, 27 mmol), was slowly added at ca. −5 °C with vigorous stirring. The reaction mixture was immediately extracted with $CHCl_3$ (2 × 15 mL). The combined extracts were washed (H_2O, 2 × 15 mL), dried over $MgSO_4$, and evaporated (Rotavapor, water pump) to give 8.8 g (68%) of crude product. ^{31}P NMR analysis of the residue showed 95% **16** and 5% of an unidentified compound, δ 10.5 ppm.

Purification of **15** was accomplished by flash chromatography on a 30 × 6.25-cm column of 60–200-mesh silica gel (Baker) eluted with ethyl acetate/pentane (3:1 v/v). Thirty 50-mL fractions were collected and analyzed by ^{31}P NMR analysis. Fractions 14–30 were combined and rechromatographed similarly to give the product in >99% purity by ^{31}P NMR: colorless oil which decomposed during attempted vacuum distillation; ^1H NMR δ 7.98–7.90, 7.55–7.50, 7.43–7.36 (3m, C_6H_5), 4.43–4.08 (m, OCH_2CH_3), 1.31, 1.26, 1.18 (3t, $^3J_{HH}$ = 7 Hz, OCH_2CH_3); $^{13}C\{^1H\}$ NMR δ 134.68 (d, $^2J_{Cp}$ = 9 Hz, *o*-C_6H_5), 133.14 (d, $^4J_{Cp}$ = 3 Hz, *p*-C_6H_5), 127.66 (d, $^3J_{Cp}$ = 14 Hz, *m*-C_6H_5), 125.88 (d, $^1J_{Cp}$ = 149 Hz, *ipso*-C_6H_5), 66.11, 65.99 (2d, $^2J_{CP}$ = 7 Hz, $POCH_2CH_3$), 64.37 (d, $^2J_{Cp}$ = 7 Hz, $pOCH_2CH_3$), 16.32, 16.12, 16.02 (3d, *J* = 6, 6.5, 6.5 Hz, OCH_2CH_3), δ pCP not observed; $^{31}P\{^1H\}$ NMR: δ 28.58 (d, $^2J_{pP}$ = 13 Hz), 9.23 (d, $^2J_{Pp}$ = 13 Hz). Anal. ($C_{13}H_{20}Br_2O_5P_2$) C, H.

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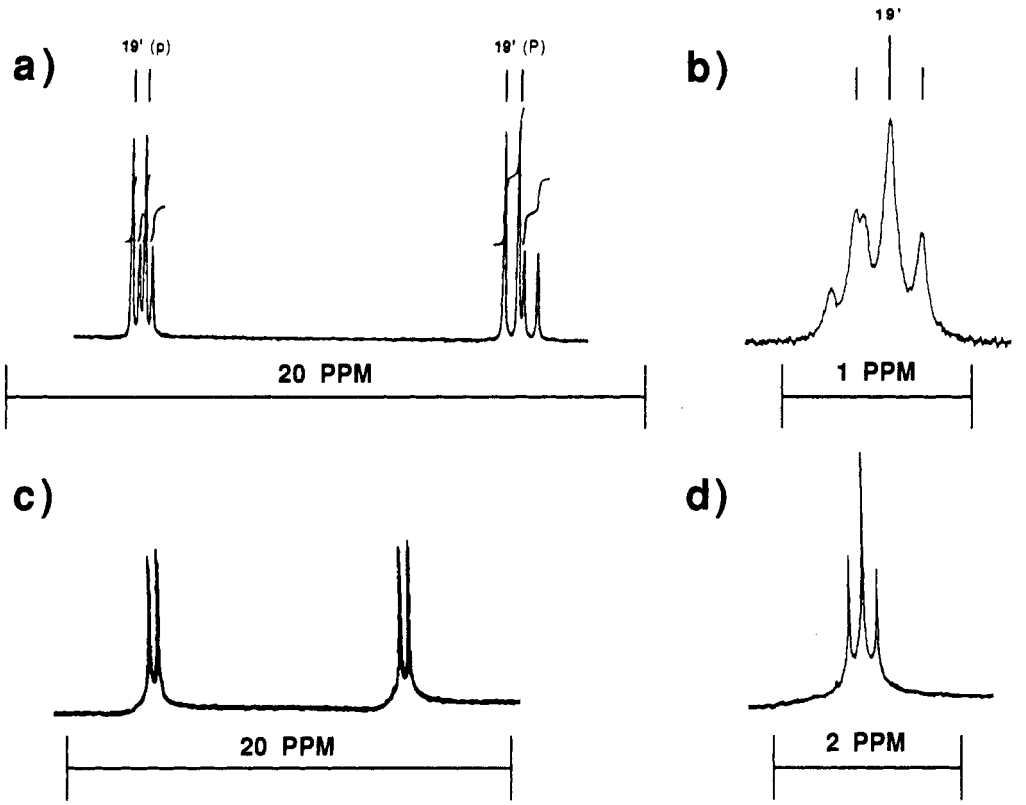

Figure 2. $^{31}P\{^1H\}$ and $^{19}F\{^1H\}$ NMR spectra of [fluoro(hydroxyphenylphosphinyl)methyl]phosphonic acid (−)-quinine salts measured on a Bruker AM-360 spectrometer operated at 145.73 (^{31}P) or 338.9 (^{19}F) MHz. Chemical shifts are reported relative to 85% phosphoric acid (^{31}P) or $CFCl_3$ (^{19}F) as external references. Each sample contained 5–10% v/v (loose solid) of salt in MeOH-d_4 at ca. 24 °C: (a) ^{31}P NMR spectrum of recrystallized salt from final head crop (cf. Experimental Section), (−)-quinine salt of (−)-**2** (**19'**, labeled resonances at δ_p 21.7 (d), δ_P 10.2 (d), partially resolved from (−)-quinine salt of (+)-**2** (**19**, unlabeled resonances at δ_p 21.3 (d), δ_P 9.7; (b) ^{19}F NMR of sample from a, **19'**, labeled resonance at δ −221.3 (~t), **19**, unlabeled resonance at δ −221.15 (~t); (c) ^{31}P NMR spectrum of recrystallized salt from final tail crop (cf. Experimental Section), (−)-quinine salt of (+)-**2**, **19**, resonances at δ_P 21.3 (d), δ_P 9.7 (d); (d) ^{19}F NMR of sample from c, **19**, resonance at δ −220.3 (~t).

(*S*/*R*,*S*/*R*)-Diethyl [Chloro(ethoxyphenylphosphinyl)methyl]phosphonates (16,16'). A solution of sodium sulfite (20 g, 159 mmol) in H_2O (150 mL) was added dropwise to **14** (6.42 g, 16.0 mmol) in acetone (70 mL) while the temperature was maintained near 0 °C. When addition was complete, the cooling bath was removed and stirring was continued for 70 min. The reaction mixture was then extracted with chloroform (3 × 100 mL), the combined organic layers were dried ($MgSO_4$), and the solvent was removed by rotary evaporation (water pump) to give 4.79 g (84%) of crude product. Vacuum distillation gave a colorless oil: bp 145–150 °C (0.002 mmHg); ^1H NMR δ 7.85–7.80, 7.56–7.36 (2m, C_6H_5), 4.32–3.97 (m, OCH_2CH_3, CHCl), 1.35–1.12 (m, OCH_2CH_3); $^{13}C\{^1H\}$ NMR δ 133.03–132.67 (m, *o*-C_6H_5, *p*-C_6H_5), 128.34–128.07 (m, *m*-C_6H_5), 64.20–63.86, 63.03–62.93, 62.58–62.48, (3m, OCH_2CH_3), 47.12 (dd, CHCl), 46.32 (dd, CHCl), 16.40–16.06 (m, OCH_2CH_3); $^{31}P\{^1H\}$ NMR δ 31.23 (d, $^2J_{pP}$ = 5 Hz), 30.38 (d, $^2J_{pP}$ = 4 Hz), 13.98 (d, J_{Pp} = 4 Hz), 13.86 (d, $^2J_{Pp}$ = 5 Hz).

(*S*/*R*,*S*/*R*)-Diethyl [Bromo(ethoxyphenylphosphinyl)methyl]phosphonates (17,17'). To **15** (2.79 g, 5.84 mmol) in acetone (20 mL) and H_2O (40 mL) was added with cooling (~0 °C) a solution of $SnCl_2{\cdot}2H_2O$ (1.32 g, 5.85 mmol) in H_2O (20 mL). Stirring was continued for 16 h at room temperature. The mixture was extracted with ether (50 mL). The ether phase was dried ($MgSO_4$) and the solvent removed in vacuo, leaving 1.75 g (75%) of product as an undistillable oil, >99% pure by ^{31}P NMR. Further purification was carried out by flash chromatography on a column of silica gel (15 × 6 cm), eluted with ethyl/pentane (3:1): ^1H NMR δ 7.91–7.82, 7.60–7.42 (2m, C_6H_5), 4.38–3.94 (m, OCH_2CH_3, CHBr), 1.44–1.16 (m, OCH_2CH_3); $^{13}C\{^1H\}$ NMR δ 134.73 (d, $^2J_{Cp}$ = 9 Hz, *o*-C_6H_5), 133.17 (d, $^4J_{Cp}$ = 4 Hz, *p*-C_6H_5), 127.61 (d, $^3J_{Cp}$ = 14 Hz, *m*-C_6H_5), 125.93 (d, $^1J_{Cp}$ = 149.3 Hz, *ipso*-C_6H_5), 66.15, 66.04, (2d, $^2J_{CP}$ = 7.5 Hz, $POCH_2CH_3$), 64.41 (d, $^2J_{Cp}$ = 7 Hz, $pOCH_2CH_3$), 16.35, 16.16, 16.05 (3d, J = 6, 7, 7 Hz, OCH_2CH_3), δ pCP not observed; $^{31}P\{^1H\}$ NMR δ 30.65 ($^2J_{pP}$ = 2.5 Hz), 29.71 (d, $^2J_{pP}$ = 4.5 Hz), 13.81 (d, $^2J_{Pp}$ = 5 Hz), 13.78 (d, $^2J_{Pp}$ = 2.5 Hz).

(*S*/*R*,*S*/*R*)-Diethyl [Bromochloro(ethoxyphenylphosphinyl)methyl]phosphonates (18,18'). A solution of sodium hydroxide (1.2 g, 30 mmol) in H_2O (80 mL) was cooled to −2 °C and bromine (2.5 g, 0.8 mL, 16 mmol) was added while the temperature was maintained near 0 °C. The ester **16,16'** (1.00 g, 2.82) was then added dropwise and the resulting mixture immediately extracted with chloroform (80 mL) to give 1.02 g (83%) of crude oil. The product was isolated as described in the preceding preparation as a thick colorless oil (>99% pure by ^{31}P NMR), which decomposed on attempted vacuum distillation: ^1H NMR δ 7.78–7.70, 7.37–7.22 (2m, C_6H_5, 5.0 H), 4.18–4.01 (m, OCH_2CH_3, 6.1 H), 1.17–1.00 (m, OCH_2CH_3, 9.5 H); ^{13}C NMR δ 134.06 (d, $^2J_{Cp}$ = 9 Hz, *o*-C_6H_5), 132.92 (d, $^4J_{Cp}$ = 3 Hz, *p*-C_6H_5), 127.35 (d, $^3J_{Cp}$ = 14 Hz, *m*-C_6H_5), 125.42 (d, $^1J_{Cp}$ = 149 Hz, *ipso*-C_6H_5) 65.73–65.50 (m, $POCH_2CH_3$), 64.03–63.91 (m, $pOCH_2CH_3$), 64.23–60.64 (m, pCP), 16.03–15.62 (m, OCH_2CH_3); ^{31}P NMR δ 28.85 (d, $^2J_{pP}$ = 14 Hz), 28.36 (d, $^2J_{pP}$ = 16 Hz), 9.00 (d, $^2J_{Pp}$ = 14 Hz), 8.92 (d, $^2J_{Pp}$ = 16 Hz).

(*S*/*R*)-Diethyl [(Ethoxyphenylphosphinyl)difluoromethyl]phosphonate (13) and (*S*/*R*,*S*/*R*)-Diethyl [(Ethoxyphenylphosphinyl)fluoromethyl]phosphonates (12,12'). A 1-L, three-necked, round-bottomed flask (joints greased with Halocarbon Series 25-5S grease), fitted with a thermometer and addition funnel (with exit bubbler) and connected via an inlet bubbler (Halocarbon Series 27 oil in both bubblers) and a T-valve to N_2 and a cylinder of perchloryl fluoride, was charged under N_2 with *t*-BuOK (3.6 g, 32 mmol) and freshly distilled dry toluene (600 mL). The solution was cooled to 5 °C and **9** (8 g, 25 mmol) in toluene (120 mL) was added via the addition funnel. The solution became light brown. After 30 min at 0–5 °C the solution was allowed to warm to 10 °C (removal of ice bath), and perchloryl fluoride was passed in until absorption (bubbler) ceased (40 min). The system was purged with N_2 (5 min), following which the reaction mixture was extracted with H_2O (2 × 50 mL). The toluene layer was dried ($MgSO_4$) and evaporated (Rotavapor,

Table I. Characterization of PhpXYMP Pyridine Salts

pyridine salt of PhpXYMP (X,Y)	mp (°C)	NMR data (Bruker WP-270 SY) δ^{31}P (ppm) [J (Hz)]	NMR data (Bruker WP-270 SY) δ^{13}C (ppm) [J (Hz)][a]	analyses
2 (H,F)	120.5–121.5	25.0 (bd, $^2J_{pF}$ = 61) 9.7 (bd, $^2J_{PF}$ = 63)	132.8 (d, J = 3) 132.1 (d, J = 10) 131.9 128.8 (d, J = 13) 89.0 (ddd)	($C_{12}H_{14}FNO_5P_2$·H_2O) C, H, N
3 (F,F)	192.5–194	20.0 (td, $^2J_{pF}$ = 79, $^2J_{pP}$ = 49) 3.9 (td, $^2J_{PF}$ = 84, $^2J_{pP}$ = 47)	133.2 (d, J = 10) 133.0 130.8 (d, J = 137) 128.7 (d, J = 13)	($C_{12}H_{13}F_2NO_5P_2$) C, H, N
4 (Cl,Cl)	224–225.5	24.5 (b), 8.7 (b)	134.6 (d, J = 9) 132.9 130.0 (d, J = 145) 128.1 (d, J = 13) 77.9 (dd)	($C_{12}H_{13}Cl_2NO_5P_2$) C, H, N
5 (Br,Br)	216–217.5	23.7 (b), 8.5 (b)	135.1 (d, J = 9) 132.9 129.7 (d, J = 147) 127.9 (d, J = 14) 56.2 (dd)	($C_{12}H_{13}Br_2NO_5P_2$) C, H, N
6 (H,Cl)	140.5–142	26.7 (b), 11.7 (b)	133.1–132.6 (m) 131.0 128.8 (d, J = 14) 49.4 (dd)	($C_{12}H_{14}ClNO_5P_2$·0.25H_2O) C, H, N
7 (H,Br)	120–121	26.8 (b), 11.6 (b)	132.5–132.8 (m) 130.8 128.6 (d, J = 13) 37.6 (dd)	($C_{12}H_{14}BrNO_5P_2$·0.5H_2O) C, H, N
8 (Cl,Br)	218–219	24.1 (b), 8.7 (b)	134.9 (d, J = 10) 132.9 129.9 (d, J = 147) 128.1 (d, J = 13) 68.4 (dd)	($C_{12}H_{13}BrClNO_5P_2$) C, H, N

[a] Pyridine resonances at ~ δ 147.5, 141.5, 128 ppm omitted.

water pump) to leave 6.88 g of a yellowish oil containing (^{31}P NMR) **13** (50%), **12,12′** (30%), **9** (15%), and two other phosphorus-containing components (5%). Evaporation of the aqueous residue gave an oil (0.5 g), analyzing (^{31}P NMR) for 90% **9**, with **13** and **12,12′** comprising the remainder. Isolation of the difluoro and monofluoro products in the organic extract was accomplished by flash chromatography on a 20- × 6.25-cm column of silica gel (Merck Kieselgel 60, 230–400 mesh) eluted with ethyl acetate/pentane (3:1). Forty 30-mL fractions were collected and analyzed by TLC [R_f(**13**) = 0.49; R_f(**12**) = 0.10; R_f(**12′**) = 0.06; R_f(impurities) = 0.7, 0.2] and ^{31}P NMR. Fraction 8 contained **13** (98% pure by NMR), fraction 18 contained >95% of one monofluoro diastereomer (**12**, δ_p 28.8), and fractions 29–40 had >95% of the other monofluoro diastereomer (**12′**, δ_p 29.4). Intermediate fractions yielded the bulk of monofluoro products as mixtures.

13: ^{1}H NMR δ 7.92–7.86, 7.63–7.56, 7.50–7.42 (3m, C_6H_5, 5.1 H), 4.37, 4.28 (2p, $^3J_{HP}$, $^3J_{HH}$ = 7 Hz, POCH_2CH$_3$), 4.19 (m, pOCH_2-CH$_3$) total 6.0 H, 1.37, 1.29, 1.23 (3t, $^3J_{HH}$ = 7 Hz, OCH$_2$CH_3); ^{13}C{^{1}H} NMR δ 133.65 (d, $^4J_{Cp}$ = 3 Hz, p-C_6H_5), 133.18 (d, $^3J_{Cp}$ = 10 Hz, o-C_6H_5), 128.41 (d, $^3J_{Cp}$ = 14 Hz, m-C_6H_5), $ipso$-C_5H_6 not observed, 65.08, 64.92 (2d, $^2J_{CP}$ = 7 Hz, POCH$_2$CH$_3$), 63.78 (d, $^2J_{Cp}$ = 7 Hz, pOCH$_2$CH$_3$), 16.43, 16.23, 16.15 (3d, J = 5.5 Hz, OCH$_2$CH$_3$), δ pCP not observed; ^{31}P{^{1}H} NMR δ 25.25 (ddd, $^2J_{pF}$ = 84, 76 Hz, $^2J_{pP}$ = 56 Hz), 4.56 (ddd, $^2J_{PF}$ = 88, 87 Hz, $^2J_{Pp}$ = 56 Hz); ^{19}F NMR δ-181 (m).

12: ^{1}H NMR δ 7.92–7.83, 7.60–7.41 (2m, C_6H_5), 5.01 (ddd, $^2J_{HF}$ = 46 Hz, CHF), 4.35–4.08 (m, OCH_2CH$_3$), 1.39, 1.33, 1.25 (3t, $^3J_{HH}$ = 7 Hz, OCH$_2$CH_3); ^{13}C{^{1}H} NMR δ 133.08 (d, $^4J_{Cp}$ = 3 Hz, p-C_6H_5), 132.25 (d, $^2J_{Cp}$ = 9 Hz, o-C_6H_5), 128.44 (d, $^3J_{Cp}$ = 14 Hz, m-C_6H_5), $ipso$-C_6H_5 not observed, 86.65 (octet, CHF), 63.75, 63.66 (2d, $^2J_{CP}$ = 7 Hz, POCH$_2$CH$_3$), 62.85 (d, $^2J_{Cp}$ = 6.5 Hz, pOCH$_2$-CH$_3$), 16.39, 16.31, 16.16 (3d, J = 6, 6, 6.5 Hz, OCH$_2$CH$_3$); ^{31}P{^{1}H} NMR δ 28.56 (dd, $^2J_{pF}$ = 56 Hz, $^2J_{pP}$ = 8 Hz), 11.90 (dd, $^2J_{PF}$ = 65 Hz, $^2J_{Pp}$ = 8 Hz); ^{19}F NMR δ −226.3 (ddd, CHF, $^2J_{FP}$ = 65 Hz, $^2J_{Fp}$ = 55 Hz, $^2J_{FH}$ = 46 Hz).

12′: ^{1}H NMR δ 7.90–7.42, 7.62–7.43 (2m, C_6H_5), 5.14 (ddd, $^2J_{HF}$ = 46 Hz, CHF), 4.28–4.00 (m, OCH_2CH$_3$), 1.36, 1.26, 1.18 (3t, $^3J_{HH}$ = 7 Hz, OCH$_2$CH_3); ^{13}C{^{1}H} NMR δ 133.18 (d, $^4J_{Cp}$ = 3 Hz, p-C_6H_5), 132.81 (d, $^2J_{Cp}$ = 9.5 Hz, o-C_6H_5), 128.38 (d, $^3J_{Cp}$ = 13.5 Hz, m-C_6H_5), $ipso$-C_6H_5 not observed, 85.95 (octet, CHF), 63.90, 63.56 (2d, $^2J_{CP}$ = 7, 7 Hz, POCH$_2$CH$_3$), 62.19 (d, $^2J_{Cp}$ = 6 Hz, pOCH$_2$CH$_3$), 16.43, 16.26, 16.16 (3d, J = 6.5 Hz, OCH$_2$CH$_3$); ^{31}P{^{1}H} NMR δ 29.37, (dd, $^2J_{pF}$ = 59 Hz, $^2J_{pP}$ = 17 Hz), 11.85 (dd, $^2J_{PF}$ = 64 Hz, $^2J_{Pp}$ = 17 Hz); ^{19}F NMR δ −225.3 (ddd, CHF, $^2J_{FP}$ = 64 Hz, $^2J_{pF}$ = 59 Hz, $^2J_{FH}$ = 46 Hz).

Preparation of Pyridine Salts of 2–8. Esters **12,12′–16,16′** and **18,18′** were refluxed in excess concentrated HCl for 3–9 h; esters **17,17′** was refluxed in 10% HCl for 12 h to avoid possible halogen exchange as observed in hydrolysis of triethyl bromophosphonoacetate.[8] After hydrolysis, HCl was removed at reduced pressure (water pump), H_2O (50 mL) added, and rotary evaporation repeated. After at least four repetitions of the latter step, pyridine (5–10 mL) was added to the evaporate and the mixture left in a freezer (−10 °C) until crystallization was complete. Excess pyridine was pumped off (<1 mmHg) and the product dissolved in MeOH. On recrystallization in MeOH/acetone, the products were obtained as white crystalline or powderlike solids. Characterization data are summarized in Table I.

Preparation of [Fluoro(hydroxyphenylphosphinyl)methyl]phosphonic Acid (2). The mixture of diastereomers **12,12′** (0.37 g, 1.09 mmol) was refluxed in concentrated HCl for 9 h. Excess HCl was removed in vacuo; the sticky off-white solid obtained was repeatedly dissolved in H_2O/MeOH and the solvent removed (at least 5 times), leaving 0.23 g (0.89 mmol, 82%) of the desired triacid: ^{13}C NMR (D_2O, pH 0.81) δ 132.57 (d, $^4J_{pC}$ = 2 Hz, p-C_6H_5), 131.79 (d, $^2J_{pC}$ = 10 Hz, o-C_6H_5), 130.61 (d, $^1J_{pC}$ = 137 Hz, $ipso$-C_6H_5), 128.33 (d, $^3J_{pC}$ = 13 Hz, m-C_6H_5), 88.11 (ddd, J values not assigned, CHF); ^{31}P NMR (D_2O, pH 0.81): δ 25.3 (broad d, $^2J_{pF}$ = 58 Hz), 9.48 (broad d, $^2J_{PF}$ = 61 Hz).

Synthesis and Attempted Fractional Crystallization of [Fluoro(hydroxyphenylphosphinyl)methyl]phosphonic Acid Brucine Salts. To a solution of anhydrous brucine (106 mg, 0.28 mmol) in hot acetone (20 mL), the **2** triacid (72.0 mg, 0.28 mmol) was added with stirring. The mixture was gently heated for about 2 min while a white precipitate formed. The crude product was isolated by suction filtration and washed several times with acetone. Attempted fractional crystallizations with

various combinations of methanol/acetone yielded several crops of microcrystals. Optical rotations measured for these crops ranged from $[\alpha]^{25}_D = -2.5°$ ($c = 0.006$, 95% aqueous ethanol) to $[\alpha]^{25}_D = -4.6°$ ($c = 0.005$, 90% aqueous ethanol): $^{31}P\{^1H\}$ NMR δ 23.0 ppm (d, $^2J_{PF} = 61$ Hz), 10.0 ppm (d, $^2J_{PF} = 63$ Hz); ^{19}F NMR δ −228.5 ppm (td, $^2J_{PF} = 63$ Hz, $^2J_{PH} = 47$ Hz).

Synthesis and Resolution of [Fluoro(hydroxyphenylphosphinyl)methyl]phosphonic Acid Quinine Salts (19,19′). To a solution of quinine base (169 mg, 5.21 mmol) in hot acetone was slowly added with stirring the triacid **2** (134 mg, 5.26 mmol). The reaction mixture was gently heated for about 10 min before the precipitate was collected by suction filtration and washed several times with acetone (217 mg, 70%). Reprecipitation (8×) with methanol/acetone gave a product with $[\alpha]^{25}_D = -57.7°$ ($c = 0.007$ M, methanol) which was recrystallized from methanol/acetone (35 mL/10 mL respectively) to constant $[\alpha]^{25}_D$: white needles, decomposition ca. 190 °C, $[\alpha]^{25}_D = -48°$ ($c = 0.012$ M, methanol). ^{31}P NMR analysis revealed 2 sets of double doublets in a ratio of 1 (**19**) to 2 (**19′**) (integrated peaks; Figure 2a). Similarly, ^{19}F NMR showed two partially resolved sets of triplets, again indicative of two isomers, in approximately the same ratio (peak heights; Figure 2b): $^{31}P\{^1H\}$ NMR (**19**) δ 21.7 ppm (broad d, $^2J_{PF} = 61$ Hz), 10.2 ppm (dd, $^2J_{PF} = 62$ Hz, $^2J_{PH} = 12$ Hz); and (**19′**) δ 21.5 ppm (broad d, $^2J_{PF} = 60$ Hz), 9.7 ppm (dd, $^2J_{PF} = 61$ Hz, $^2J_{PH} = 11$ Hz); $^{19}F\{^1H\}$ NMR (**19**) δ −221.2 ppm (broad t, $^2J_{FP} = 60$ Hz) and (**19′**) δ −221.1 ppm (broad t, $^2J_{FP} = 57$ Hz). Anal. ($C_{27}H_{33}FP_2N_2O_7$) C: calcd, 56.06; found, 56.58; H: calcd, 5.70; found, 5.70. After 4 days, a new crop of crystals was isolated from the main mother liquor $[\alpha]^{25}_D = -74°$ ($c = 0.026$ M, methanol). Recrystallization to constant $[\alpha]^{25}_D$ gave microprisms, mp 168–169 °C (**19**) with $[\alpha]^{25}_D = -79°$ ($c = 0.018$ M, methanol). ^{31}P NMR analysis showed two multiplets (**19**, 98%): δ 21.2 ppm (broad d, $^2J_{PF} = 58$ Hz), δ 9.9 ppm (sharp ddd, $^2J_{PF} = 60$ Hz, $^2J_{PH} = 11$ Hz, $^2J_{PP} = 6$ Hz); ^{19}F NMR (**19**, 98%) δ −220.5 ppm (dt, $^2J_{FP} = 60$ Hz, $^2J_{FH} = 47$ Hz). Anal. ($C_{47}H_{57}FP_2N_4O_9 \cdot 2MeOH$) C, H.

Preparation of (+)-[Fluoro(hydroxyphenylphosphinyl)methyl]phosphonic Acid Sodium Salt (Na^+ Salt of (+)-2). The quinine salt **19** was dissolved in H_2O and the pH adjusted to 11 with NaOH. [Absence of hydrogen exchange at the chiral α-carbon (which would result in racemization) was verified by ^1H NMR analysis of a sample similarly treated in D_2O.] The mixture was immediately extracted three times with chloroform, then again three times with ether. The aqueous layer was evaporated in vacuo, leaving a white solid which was reprecipitated 4 times from a mixture of water, methanol, and acetone (1:2:3): Na^+ salt of (+)-**2**, mp > 250 °C, verified to be free of residual quinine by ^{13}C NMR; $[\alpha]^{25}_D = +6° \pm 0.5$ ($c = 0.019$ M, 54% aqueous methanol); $^{13}C\{^1H\}$ NMR δ 135.6 ppm (d, $^1J_{Cp} = 128$ Hz, *ipso*-C_6H_5), 131.7 ppm (d, $^3J_{Cp} = 10$ Hz, *o*-C_6H_5), 127.8 ppm (d, $^2J_{Cp} = 12$ Hz, *m*-C_6H_5), δ 130.8 ppm (d, $^4J_{Cp} = 2$ Hz, *p*-C_6H_5), δ 92.9 ppm (ddd, *J* values unassigned, CHF); $^{31}P\{^1H\}$ NMR δ 23.8 ppm (dd, $^2J_{pF} = 63$ Hz, $^2J_{pP} = 7$ Hz), 7.6 ppm (broad d, $^2J_{PF} = 56$ Hz).

Preparation of (−)-[Fluoro(hydroxyphenylphosphinyl)methyl]phosphonic Acid Sodium Salt (Na^+ Salt of (−)-2) from its Partially Resolved Quinine Salt. The procedure described above was applied to the quinine salt mixture of **19** and **19′**. The white solid thereby obtained was quinine-free by ^{13}C NMR: Na^+ salt of (−)-**2**, (33% ee based on NMR analysis of **19,19′** precursor); mp > 250 °C; optical rotation measurement $[\alpha]^{25}_D = -1° \pm 0.5°$ ($c = 0.012$, 80% aqueous methanol) (due to limited sample and the small observed rotation, a more accurate measurement using matched solvent (see previous paragraph) was not possible). $^{13}C\{^1H\}$ NMR δ 135.9 ppm (d, $^1J_{Cp} = 128$ Hz, *ipso*-C_6H_5), 131.4 ppm (d, $^2J_{Cp} = 9$ Hz, *o*-C_6H_5), 130.9 ppm (d, $^4J_{Cp} = 2$ Hz, *p*-C_6H_5), 128.1 ppm (d, $^3J_{Cp} = 12$ Hz, *m*-C_6H_5), 92.2 ppm (ddd, *J* values unassigned, CHF); ^{31}P NMR δ 23.7 ppm (dd, $^2J_{pF} = 63$ Hz, $^2J_{pP} = 6$ Hz), 7.8 ppm (broad dd, $^2J_{PF} = 56$ Hz, $^2J_{Pp} = 6$ Hz).

Biochemical Studies. Brush border membrane vesicles (BBMV). BBMV were prepared from the cortex of kidneys freshly dissected from adult male Sprague-Dawley rats. BBMV were prepared using the magnesium-precipitation procedure described in our previous reports.[3,9] All transport measurements were conducted using BBMV freshly prepared on the same day.[3] The rest of the BBMV preparation was aliquoted, quickly frozen in dry ice, and stored at −70 °C. Frozen aliquots of BBMV were employed for enzyme measurements. It was previously established that frozen storage does not alter alkaline phosphatase or leucine aminopeptidase activities.[3] Uptake of $^{32}P_i$, [^{3}H]-D-glucose, and [^{3}H]-L-proline was measured by the rapid filtration technique described in our previous communications.[3,6,9] Uptake of solute was measured in the presence of an [outside > inside] 100 mM Na^+ gradient after a 5-s interval, when the Na^+-gradient uptake was linearly proportional to the time.[3,6,23] The solute uptake was measured in quadruplicate and the mean of replicate values was entered as $n = 1$ for statistical evaluation.[3,6,7,9,24] The solute uptake rate was expressed in picomoles of solute taken up in 5 s per milligram of protein of BBMV[4,11–14,16–19]

The uptake of $^{32}P_i$, [^{3}H]-D-glucose, and [^{3}H]-L-proline in the absence of Na^+ in the initial (5 s) time phase of uptake, was negligible (less than 5%), hence this component was not measured and subtracted in these experiments.[3,25] The inhibitory effect of the compounds is expressed as percent (%) inhibition, compared to the control without added test compound or else as a K_i value. Inhibition kinetics were measured over a ^{32}Pi concentration range of 75–400 μM Pi for compound **2**, or for PFA as a reference, relative to a control assay with no added inhibitor; kinetics results were graphically evaluated by a Lineweaver–Burk plot.[3,6,7] Inhibition by the individual pairwise enantiomers of **2** was measured as a dose-response effect at 1 mM (PFA control) in two separate BBMV preparations. Sample amount limitations precluded full kinetic evaluation.

Alkaline phosphatase and leucine aminopeptidase activities were determined by use of chromogenic substrates, as described previously.[3,6] The protein content in BBMV preparations was measured by the method of Lowry et al.[26] with minor modifications described previously.[23,24]

Pharmacological Results and Discussion

α-Mono and α,α-dihalosubstituted ([phenylphosphinyl)methyl]phosphonates (PhpXYMP) in the form of pyridine salts were investigated for their effect on [Na^+ extravesicular > Na^+ intravesicular] Na^+–gradient-dependent uptake of $^{32}P_i$ phosphate by BBM vesicles freshly prepared from the rat renal cortex in vitro.[3,6,7,9]

The activities of the new compounds were compared with that of the archetypal inhibitor, PFA.[3,9] Pyridine alone at the test concentrations had no significant effect on Na^+–P_i cotransport. However, for assurance, pyridine was included at equimolar concentration in all control incubations (without tested compound). As shown in Table II, all of the PhpXYMP derivatives **2–8** inhibited Na^+–P_i cotransport to some degree. The monofluoro-substituted racemate **2** (PhpFMP) was the most potent inhibitor within this group. Consequently **2** was investigated in greater detail for its substrate specificity, kinetics, and potency as an inhibitor of Na^+–P_i cotransport.

When tested for its effect on two other Na^+-gradient-dependent cotransporters within BBM, specific for transport of [^{3}H]-D-glucose and [^{3}H]-L-proline,[3,6,24] **2** had neither inhibitory nor stimulatory effects, thus indicating

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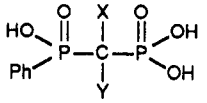
Table II. Halogenated PhpXYMP Inhibition of Na^+-Gradient-Energized $^{32}P_i$ Uptake by BBMV



compound	X	Y	% Δ inhibition: ±SEM (n)[a]
PhpFMP (2)	H	F	−49 ● 5 (6)
$PhpF_2MP$ (3)	F	F	−39 ● 7* (3)
PhpClMP (6)	H	Cl	−30 ± 11 (3)
$PhpCl_2MP$ (4)	Cl	Cl	−39 ● 4* (3)
PhpClBrMP (8)	Cl	Br	−31 ● 6* (3)
$PhpBr_2MP$ (5)	Br	Br	−22 ● 6 (3)
PFA			−63 ± 3* (6)

[a] Degree of inhibition relative to control at 1 mM compound and 0.1 mM P_i. Statistically significant inhibition (by paired t test) at $p < 0.05$ (*) or higher level of significance; n = number of experiments.

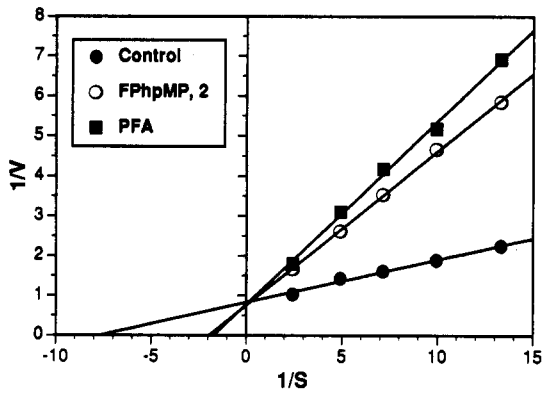


Figure 3. Representative Lineweaver–Burk reciprocal plot of inhibition data for BBMV Na^+-gradient-dependent $^{32}P_i$ uptake by PhpFMP (2, pyridine salt) and for comparison, by PFA. Effect of pyridine alone was negligible at equivalent concentration (data not shown). V = P_i uptake in nmoles/5 s per mg of protein; S = 75–400 μM P_i. Inhibition by both compounds was strictly competitive. The average K_i for compound 2 = 0.358 ± 0.021 mM, n = 3; the average K_i for PFA = 0.273 ± 0.040 mM, n = 3.

its substrate specificity for interaction with the Na^+–P_i cotransporter (data not shown).

As some phosphate analogues interact with other components of BBM, (e.g. 1-hydroxyethane-1,1-diphosphonate (EHDP) which strongly inhibits phosphoesterases[3]), we explored the effect of 2 on the activity of alkaline phosphatase and another intrinsic BBM enzyme, leucineaminopeptidase. At 1 mM concentration, 2 showed no inhibitory effects on alkaline phosphatase or leucine aminopeptidase; whereas 1 mM EHDP, included as a control, showed significant (−60%) inhibition of alkaline phosphatase, as in our previous study.[3] Inhibition of Na^+–P_i cotransport by 2 is strictly competitive (Figure 3). The inhibitory constant (K_i) of 2, 0.358 ± 0.021 mM (mean ± SEM, n = 3; see Figure 3 for representative plot; the K_i value given here replaces the slightly lower value in our preliminary communication[1]) was similar to the K_i of PFA[3,6,9] and about 6-fold smaller than that previously measured for PAA.[3,7] Hence, introduction of the phenyl ring by phosphinyl substitution was accomplished with an increase rather than loss of affinity.

The α-halo [(phenylphosphinyl)methyl]phosphonates 2–8 represent the first phosphonate inhibitors of the Na^+–P_i cotransporter possessing an aromatic ring in their structure.[3,27] In preceding studies, compounds such as phenylphosphonic acid[3] or α-(*p*-chlorophenyl)phosphonoacetic acid[10] were found to be inactive. Although the quantitation of inhibition in the whole group of derivatives (Table II) was evaluated only approximately, it appears that substitution with various halogens had a different effect in this type of compound than in halogenated derivatives of PAA.[6,7,10] In a previous study of α-halo PAA analogues, the highest inhibitory effect (highest affinity for transporter, i.e. lowest K_i) was seen in bromo-substituted derivatives[6,10] and α-fluoro PAA compounds were the least inhibitory (Dousa and McKenna, unpublished results); in contrast, in the present studies, fluorine appeared to be most effective as a substituent (Table II). A somewhat similar trend is observed in α-halo methanediphosphonates (MDP): in an evaluation of Cl-, Br-, ClBr-, Br_2-, FCl- and FMDP[16,17] at 1 mM under the same assay conditions reported here, the α-monofluoro derivative FMDP (corresponding structurally to 2 in the MpP series) gave the highest % inhibition (−39%; other compounds gave −5% (MDP itself) and −14% to −31% (α-halo MDP samples) (data not shown). These patterns remain unexplained at the present time, although we note that the second pK_a value we have estimated for the phosphonic acid moiety of 2 and that of the substrate anion $H_2PO_4^-$ are very similar.

The possibility that α-carbon chirality might affect inhibitor binding was explored in a comparison of inhibition by (+)- and (−)-enantiomers of FMpP (2). The (+)-enantiomer was tested in essentially enantiopure form, whereas the (−)-enantiomer was tested as a moderately enriched sample. The results, based on two independent experiments, indicate no significant variation in activity (SE of averages (7–10%) exceeded difference of averages (3%) by ≥2; data not shown). Thus the orientation of the H–C–F dipole has little or no apparent influence on inhibitor binding to the cotransporter. This finding is consistent with the relatively modest differences in potency of dihalo vs monohalo inhibitors in the group 2–8 (Table II).

α-Halo PhpXYMP derivatives such as compound 2 may be amenable to further modification to yield inhibitors of Na^+–P_i cotransport with specifically designed and desired properties for site-directed effects.[3,27] The presence of the phenyl ring provides a convenient potential site for reactive or photoreactive groups suited for covalent modification of BBM,[6] as well as for labeling with radioisotopes, e.g. ^3H. Hence, 2 and related derivatives offer a new lead in the search for useful probes for therapeutic manipulation of Na^+–P_i cotransporter in mammalian epithelia.[3,5,27]

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Note Added in Proof: During the course of this work, the Ozark-Mahoning Company ceased supply of perchloryl fluoride. This reagent has now become available again from Mercator, Inc., 560 Sylvan Ave., Englewood Cliffs, NJ 07632.

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