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NEW APPLICATIONS FOR BIOCATALYSIS IN ORGANIC SYNTHESIS

By
G. Neil Stowe

December 2008

Chair: Jon D. Stewart
Major: Chemistry

We synthesized a series of alpha-methyl-beta-ketoesters in two steps. These ketoesters were then reduced to the corresponding alpha-methyl-beta-hydroxyesters by our library of purified ketoreductases from Baker’s Yeast and Baker’s Yeast whole cells. These steps allowed us to probe the reduction capabilities and specificities of our purified ketoreductases and also demonstrated that they yield far greater enantioselectivities in comparison to whole cells.

A phosphonic acid analog of the highly toxic chemical warfare agents Sarin, Soman and VX was synthesized in four steps. This analog was tested as a target against DNA aptamers as a part of a program to develop a rapid test for exposure to these nerve agents in the field. Unfortunately, no aptamers bound to the target.

We chemoenzymatically synthesized alpha-fluoro-phosphonic acid carbohydrates in seven steps from noncarbohydrate precursors. The stereochemistry of the alpha-fluorine was controlled by selective reduction of an alpha-fluorovinylphosphonate using our library of alkene reductases. The alpha-fluorinated phosphonate alkane reduction product could then be used with aldolase and a suitable acceptor aldehyde to make five and six membered alpha-fluorinated phosphonic acid carbohydrates of defined stereochemistry without the need for protection / deprotection steps.
CHAPTER 1
ENZYMATIC ROUTE FOR THE SYNTHESIS OF CHIRAL BUILDING BLOCKS FROM ALPHA-METHYL-BETA-KETOESTERS

Introduction

Selective reductions of β-ketoesters can result in valuable chiral building blocks for organic synthesis. These reductions, which control the stereochemistry of an alpha substituent in addition to the β-alcohol, can be accomplished using Baker’s Yeast whole cells that can be purchased at any supermarket (Figure 1-1). However, multiple reductases with potentially different reduction products are present in whole yeast cells; this can significantly decrease the optical purity of the reduced product.

In 2004, Kaluzna et al. made a library of 18 purified β-ketoreductases from Baker’s yeast which were expressed as fusion proteins with glutathione S-transferase (GST) to allow for simple purification. The library of purified reductases was screened against a variety of ketoester substrates to yield chiral α-substituted-β-hydroxyesters (Figure 1-2).

These reductases were overexpressed in E. coli whole cells, allowing for gram scale, selective reductions of α-chloro-β-ketoesters without the need for expensive cofactor regeneration systems. The resulting chiral α-chloro-β-hydroxyesters were then used to efficiently synthesize both antipodes of the Taxol side chain 9a-b and (-)-Bestatin 10 (Figure 1-3).

We wanted to further determine the influence of the substituent at the α-position by comparing electronic versus steric environments while also expanding our knowledge of substrates for our enzyme library. So, α-methyl-β-ketoesters 11a-d were synthesized and screened (Figure 1-4); these β-ketoesters are similar in size but different electronically to the previously screened α-chloro-β-ketoesters 6a-d.
Previous methods for asymmetric synthesis of \( \alpha \)-methyl-\( \beta \)-hydroxyesters employed biocatalytic reduction of \( \alpha \)-methyl-\( \beta \)-ketoesters\(^9\) in addition to ring opening of optically pure epoxides with Grignard reagents\(^10\) and aldol reactions with chiral oxazaborolidinone reagents\(^11\) (Figure 1-5). These approaches were limited: the biocatalytic method offered a small quantity of reductases which gave a limited number of chiral products while chemical methods required chiral starting materials or reagents. We thought that our library of reductases could prove superior to these methods because we had previously shown with \( \alpha \)-chloroesters \( 6a-d \) that multiple chiral products could be made from racemic starting materials.

Thus, if the \( \alpha \)-chlorine of the previously screened \( \alpha \)-chloro-\( \beta \)-ketoesters was seen by our enzymes as only a steric entity, then we hypothesized that substrate acceptance and product stereochemistry should be similar for the \( \alpha \)-chloro and \( \alpha \)-methyl compounds. However, if our enzymes could discern between the different electronic environments of the sterically similar \( \alpha \)-substituents, then we thought that substrate acceptance and product stereochemistry could differ significantly. We also wanted to compare stereochemistry of the alcohol products of nonsubstituted \( \beta \)-ketoesters \( 3a-b \) against the \( \alpha \)-methyl-\( \beta \)-ketoesters. Finally, \( \alpha \)-methyl-\( \beta \)-ketoesters \( 11a-d \) were tested for reduction with Baker’s yeast whole cells to determine if our purified reductases yield better reduction product selectivity in addition to comparison of whole cell substrate acceptance.

**Ketoester Synthesis**

The \( \alpha \)-methyl-\( \beta \)-ketoesters were made using a two step process. First, ethyl propionate was deprotonated by lithium diisopropylamide (LDA) at \(-78^\circ\) C followed by addition of the appropriate aldehyde at the same temperature to yield alcohols \( 12a-d \) and \( 13a-d \).\(^12\) The alcohols were oxidized to ketoesters \( 11a-d \) via standard Swern conditions or PCC (Figure 1-6).\(^12,13\)
Screening

The α-methyl-β-ketoesters 11a-d were screened against our library of purified reductases and whole yeast cells. Screenings with purified reductases were conducted in phosphate (KP_i) buffer and used NADPH cofactor as the hydride source. The cofactor was regenerated using a glucose-6-phosphate (G6P) / glucose-6-phosphate dehydrogenase (G6PDH) couple (Figure 1-7). Whole cell screening of ketoesters 11a-d incubated Fleischman’s® yeast with shaking in KP_i buffer with addition of sucrose as needed.

Gas chromatography (GC) was used to monitor reaction progress and selectivity. Aliquots of the crude reaction mixture were monitored by achiral GC in order to follow reaction progress. The stereoselectivity of each reaction was determined by chiral GC using trifluoroacetylated derivatives 19a-d and 20a-d of the possible alcohol products 12a-d and 13a-d (Figures 1-8, 1-9).

Results and Discussion

The screenings found that only ketoesters 11a (R = Et) and 11b (R = n-Pr) were substrates for our purified reductases. Both ketoesters were reduced by enzymes encoded by yeast genes YDR541c, YGL039w, YAL060w and YGL157w. Chiral GC analysis of the trifluoroacetylated derivatives found only one of four possible alcohol products was formed from reduction of both ketoesters. NMR analysis of the α-methoxy-α-phenylacetic (MPA) esters of the alcohol showed both reductions yielded α-methyl-β-hydroxy esters with 2(S), 3(S) configurations, corresponding to anti-13a and anti-13b. Whole yeast cells also accepted only 11a and 11b as substrates, but gave a 50:50 enantiomeric mixture of anti-12a-b or anti-13a-b as the major products. (Table 1-1).

The α-chloro-β-ketoesters 6a-d proved far better substrates for our purified enzymes than the α-methyl compounds; both 11a (R = Et, α-methyl) and 11b (R = n-Pr, α-methyl) were accepted by four purified enzymes while 6a (R = Et, α-chloro) and 6b (R = n-Pr, α-chloro) were
accepted by 14 and 7 purified enzymes, respectively. However, the major product stereochemistry with respect to the alcohol remained the same for unsubstituted, α-chloro and α-methyl-β-ketoesters. Thus, the hydride was delivered predominately to the same face of the ketone. The α-substituent of the major product was also in the same position for both α-chloro and α-methyl-β-ketoesters, showing that our purified enzymes preferred to accept the same enantiomer of starting material. (Figure 1-10, Table 1-1, Table 1-2).

From a synthetic perspective, our results from reduction of α-methyl-β-ketoesters 11a-b with purified enzymes were successful since we could produce an optically pure product from racemic starting material. However, our method would prove more versatile if more than one chiral product could be made.

The generalizations made with respect to reduction of unsubstituted, α-chloro and α-methyl-β-ketoesters with our purified enzymes could be made with whole yeast cells in some cases. For example, α-methyl 11a (R = Et) gave a 50 : 50 ratio of anti enantiomeric products in 92% de which was similar to the 38 : 50 anti enantiomeric ratio obtained for α-chloro 6a (R = Et). Whole cells were more diastereoselective for reduction of α-methyl 11b (R = Pr) than α-chloro 6b, but 6b still gave a 53 : 36 enantiomeric mixture of anti alcohols as major products.

However, these generalizations did not always hold true. For example, reduction of unsubstituted β-ketoesters 3a and 3b was selective, especially for 3b, but the hydride was added from the opposite face of the ketone during whole cell reduction when the alpha substituent was not present.

The results of purified enzyme versus whole cell reductions were not surprising since multiple reductases are present in whole cells which often give different products. In fact, during this project we discovered from whole cell screening of α-methyl-β-ketoesters 11a-b that
additional reductases, which could lead to additional optically pure products, are present in the yeast genome. These reductases can be isolated and purified via GST fusion protein methodology in the future. However, our current library has proven an excellent method for the production of valuable chiral products from readily or commercially available, racemic starting materials.

**Experimental Procedures**

**Materials**

All organic chemicals, G6P and G6PDH were purchased from Sigma-Aldrich and used without further purification. Diisopropylamine and triethylamine (NEt₃) were freshly distilled from CaH₂ before use. Dimethylsulfoxide (DSMO) was dried overnight over molecular sieves and then freshly distilled before use. n-Butyllithium concentration was determined by titration with 2,5-dimethoxybenzyl alcohol. THF, ether and CH₂Cl₂ were degassed in 20 L drums and passed through two sequential purification columns (activated alumina) under a positive argon using the GlassContour system (GlassContour, Inc.). Thin Layer Chromatography (TLC) was performed on Merck TLC glass sheets with visualization by UV light or staining using potassium permanganate or vanillin. ¹H (300) and ¹³C NMR (75 MHz) spectra were recorded on a Varian Mercury 300 spectrometer. Chemical shifts (δ) for ¹H and ¹³C NMR are given in parts per million (ppm) relative to TMS and referenced relative to residual protonated solvent (CHCl₃: δ_H 7.27 ppm, δ_C 77.00 ppm or C₆D₆: δ_H 7.16 ppm, δ_C 128.39). Some carbon signals were isochronous for racemic alcohols and α-methyl-β-ketoesters. Compounds were separated via GC with an HP 5890 Series II Gas Chromatograph equipped with an achiral DB-17 column and Chirasil-Dex CB or Chirasil Beta-Dex columns (0.25 mm x 25 m x 0.25 μm thickness). GC / MS used an HP 5890 Series II Gas Chromatograph equipped with an achiral DB-17 column (0.25 mm x 25 m x 0.25 μm thickness).
GC short runs were 60° C for 2 minutes, 10° C per minute ramp to 180° C followed by 10 minutes at 180° C. GC long runs were 60° C for 2 minutes, 1° C per minute ramp to 150° C, 10° C per minute ramp to 180° C followed by 10 minutes at 180° C. GC / MS runs were 60° C for two minutes, 10° C per minute ramp to 250° C followed by 10 minutes at 250° C.

Synthesis

Titration of \(n\)-Butyllithium.

An oven dried round bottom flask was charged with 2,5-dimethoxybenzylalcohol (0.225 g / 1.34 mmol) and 8 mL of dry THF. \(n\)-Butyllithium (583 \(\mu\)L) was then added dropwise at room temperature until a dark brown color persisted. At this point, just over 1 equivalent of \(n\)-Butyllithium had been added, and the concentration of the solution was determined to be 2.3 M.

General Procedure for synthesis of alcohols \textbf{7a-d} and \textbf{8a-d}.

A round bottom flask was flame dried while purging with Argon, cooled, and charged with dry tetrahydrofuran (THF) (20 mL) and diisopropylamine (3.1 mL, 22.1 mmol), then cooled to -78° C. \(n\)-Butyllithium (\(n\)-BuLi) (20.1 mL, 22.1 mmol) was then added dropwise at -78° C and the resulting solution stirred at the same temperature for 30 minutes. Ethyl propionate (2.1 mL, 20.3 mmol) in THF (5 mL) was then added dropwise to the solution of LDA at -78° C and the resulting solution stirred at the same temperature for one hour. The appropriate aldehyde (22.4 mmol) in THF (5 mL) was then added dropwise at -78° C and the resulting solution stirred at -78° C for 30 minutes before the addition of 8 mL of saturated ammonium chloride. After warming to room temperature the mixture was poured into 150 mL of ether and washed with 10 mL of saturated sodium chloride (NaCl). The organic layer was dried with magnesium sulfate (MgSO₄) and the solvent removed under reduced pressure to give the crude product which was purified by flash chromatography.
Purified by flash chromatography (6:1 Hexanes : EtOAc) to give the pure compound as a slightly yellow oil in 75% yield as a 55 : 45 mixture of diastereomers.\(^\text{10}\) \(\text{\textsuperscript{1}H NMR (CDCl}_3\text{)}:\ \delta \) 0.97 (m, 6H). 1.17 (d, 3H, \(J = 7.1\) Hz), 1.15 (d, 3H, \(J = 7.4\) Hz), 1.27 (t, 6H, \(J = 7.1\) Hz), 1.54 (m, 4H), 2.52 (m, 2H), 2.58 (br d, 2H \(J = 7.0\) Hz), 3.58 (m, 1H), 3.81 (m, 1H), 4.17 (q, 4H, \(J = 7.1\) Hz). \(\text{\textsuperscript{13}C NMR (CDCl}_3\text{)}:\ \delta \) 10.01, 10.65, 10.89, 14.44, 14.68, 26.97, 27.83, 44.12, 45.05, 60.77, 60.80, 73.41, 74.92, 176.35, 176.58. FT-IR: (neat) 3500, 2954, 1728, 1182. MS: 145 (M-15, 0.5%), 131 (47.0%), 115 (25.6%), 102 (89%), 85 (66%) 74 (100%), 57 (48.3%).

\[
\begin{align*}
\text{syn-12,13a + anti-12,13a}
\end{align*}
\]

Purified by flash chromatography (8:1 Hexanes : EtOAc) to give the pure compound as a slightly yellow oil in 70% yield as a 53 : 47 mixture of diastereomers.\(^\text{11}\) \(\text{\textsuperscript{1}H NMR (CDCl}_3\text{)}:\ \delta \) 0.92 (t, 6H \(J = 7.4\) Hz), 1.17 (d, 3H, \(J = 7.4\) Hz), 1.23 (d, 3H, \(J = 7.1\) Hz), 1.27 (t, 6H, \(J = 7.1\) Hz), 1.45 (m, 8H), 2.45 (m, 2H), 2.55 (br d, \(J = 7.0\) Hz), 3.59 (m, 1H), 3.90 (m, 1H), 4.17 (q, 4H, \(J = 7.1\) Hz). \(\text{\textsuperscript{13}C NMR (CDCl}_3\text{)}:\ \delta \) 10.97, 14.20, 14.31, 14.49, 14.62, 18.95, 19.86, 36.22, 37.10, 44.59, 45.56, 60.71, 60.82, 71.64, 73.30, 176.49, 176.76. FT-IR: (neat) 3500, 2960, 1732, 1187. MS: 174 (m/z, 0.2%), 159 (1.9%), 131 (26.2%), 102 (100%), 74 (90.5%), 57 (31.8%).

\[
\begin{align*}
\text{syn-12,13b + anti-12,13b}
\end{align*}
\]
Purified by flash chromatography (10:1 Hexanes : EtOAc) to give the pure compound as a slightly yellow oil in 60% yield as a 50 : 50 mixture of diastereomers. \(^{14}\) \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 1.01 (d, 3H, \(J = 7.1\) Hz), 1.14 (d, 3H, \(J = 7.1\) Hz), 1.21 (t, 3H, \(J = 7.2\) Hz), 1.25 (t, 3H, \(J = 7.2\) Hz), 2.75 (m, 2H), 3.05 (br s, 2H), 4.13 (q, 2H, \(J = 7.1\) Hz), 4.19 (q, 2H, \(J = 7.1\) Hz), 4.65 (m, 1H), 5.05 (m, 1H), 7.35 (m, 10H). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 11.08, 14.21, 14.29, 14.63, 46.63, 47.32, 60.87, 60.93, 73.89, 76.49, 126.19, 126.83, 127.61, 128.15, 128.36, 128.60, 141.66, 141.78. FT-IR: (neat) 3450, 3063, 1716. MS: 208 (m/z, 5.3%), 193 (0.6%), 163 (3.7%), 133 (24.7%), 102 (100%), 74 (70.0%), 57 (17.5%).

Purified by flash chromatography (10:1 Hexanes : EtOAc) to give the pure compound as a slightly yellow oil in 50% yield as a 51 : 49 mixture of diastereomers. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 1.27 (m, 12H), 2.65 (m, 4H), 2.79 (m, 4H), 3.90 (m, 1H), 4.18 (m, 5H), 7.25 m, (10H). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 11.20, 14.40, 14.45, 14.62, 40.60, 41.48, 43.82, 44.54, 60.83, 60.93, 72.93, 74.68, 126.79, 128.77, 128.81, 129.53, 129.67, 138.32, 138.40, 176.24. FT-IR: (neat) 3490, 2938, 1731. MS: 204 (M – 18, 31.2%), 159 (14.9%), 131 (100%), 85 (72.6%), 57 (32.0%).

General Procedure for the synthesis of ketones 11a-d.

Swern Oxidation: An oven dried round bottom flask was charged with dimethylsulfoxide (DMSO) (1.27 mL, 17.9 mmol) in CH\(_2\)Cl\(_2\) (20 mL) and cooled to -60° C before the dropwise addition of oxalyl chloride (1.42 mL, 16.5 mmol) in CH\(_2\)Cl\(_2\) (5 mL) at the same temperature. After complete addition, the solution was stirred at -60° C for 10 minutes before dropwise addition of alcohol (14.3 mmol) in 5 mL of CH\(_2\)Cl\(_2\) at -60° C. The solution was stirred at -60° C
for 20 minutes after complete alcohol addition and then quenched by dropwise addition of triethylamine (NEt₃) at the same temperature. The resulting solution was allowed to warm to room temperature over a one hour period before the removal of CH₂Cl₂ under reduced pressure. The resulting residue was dissolved in ether (25 mL), washed with H₂O (5 mL) and brine (5 mL) and the organic layer dried with MgSO₄. The solvent was removed under reduced pressure to give the crude ketone which was purified by flash chromatography.

Pyridinium chlorochromate (PCC) oxidation: A round bottom flask was charged with 50 mL of anhydrous CH₂Cl₂ and PCC (3.93 g, 18.2 mmol). The alcohol (9.1 mmol) in 5 mL CH₂Cl₂ was then added dropwise to the solution (The color of the solution rapidly changed from bright orange to brown). The solution was then stirred for four hours at room temperature before the addition of 50 mL of anhydrous ether. The supernatant was then separated from the black precipitate and the precipitate was washed with three portions of ether (3 x 10 mL). The solvent was removed under reduced pressure, and the product purified by flash chromatography.

Prepared by Swern oxidation in 70% yield as a colorless oil after flash chromatography (8 : 1 Hexanes : EtOAc).¹² ¹H NMR (CDCl₃): δ 1.08 (t, 3H, J = 7.3 Hz) 1.26 (t, 3H, J = 7.2 Hz), 1.36 (d, 3H, J = 7.2 Hz), 2.58 (m, 2H), 3.57 (q, 1H, J = 7.2 Hz), 4.25 (q, 2H, J = 7.2 Hz). ¹³C NMR (CDCl₃): δ 12.3, 14.1, 35.6, 51.0, 61.3, 169.9, 206.5. FT-IR: (neat) 2970, 1741, 1715. MS: 158 (m/z, 3.6%), 129 (7.9%), 113 (11.7%), 102 (37.8%), 74 (28.3%), 57 (100%).

Prepared by Swern oxidation in 70% yield as a colorless oil after flash chromatography (8 : 1 Hexanes : EtOAc).¹² ¹H NMR (CDCl₃): δ 1.08 (t, 3H, J = 7.3 Hz) 1.26 (t, 3H, J = 7.2 Hz), 1.36 (d, 3H, J = 7.2 Hz), 2.58 (m, 2H), 3.57 (q, 1H, J = 7.2 Hz), 4.25 (q, 2H, J = 7.2 Hz). ¹³C NMR (CDCl₃): δ 12.3, 14.1, 35.6, 51.0, 61.3, 169.9, 206.5. FT-IR: (neat) 2970, 1741, 1715. MS: 158 (m/z, 3.6%), 129 (7.9%), 113 (11.7%), 102 (37.8%), 74 (28.3%), 57 (100%).
Prepared by Swern oxidation in 61% yield as a colorless oil after flash chromatography (8 : 1 Hexanes : EtOAc).\textsuperscript{15} \textsuperscript{1}H NMR (CDCl\textsubscript{3}): $\delta$ 0.91 (t, 3H, $J = 7.3$ Hz), 1.27 (t, 3H $J = 7.2$ Hz), 1.34 (d, 3H $J = 7.2$ Hz), 1.61 (m, 2H), 2.55 (m, 2H), 3.50 (q, 1H $J = 7.2$ Hz) 4.20 (q, 2H $J = 7.2$ Hz), 7.45 (m, 2H), 7.59 (m, 1H), 8.0 (m, 2H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): $\delta$ 12.6, 13.9, 16.8, 43.1, 52.6, 52.8, 61.2, 170.5, 205.8. FT-IR: (neat) 2965, 1743, 1716. MS: 172 (m/z, 3.4%), 143 (1.3%), 127 (8.8%), 102 (32.0%), 74 (100%), 57 (12.5%).

![Image](image1)

Prepared by PCC oxidation in 72% yield as a colorless oil after flash chromatography (12 : 1 Hexanes : EtOAc).\textsuperscript{16} \textsuperscript{1}H NMR (CDCl\textsubscript{3}): $\delta$ 1.18 (t, 3H $J = 7.2$ Hz), 1.50 (d, 3H $J = 7.0$ Hz), 4.16 (q, 2H $J = 7.2$ Hz), 4.40 (q, 1H, $J = 7.2$ Hz), 7.45 (m, 2H), 7.59 (m, 1H), 8.0 (m, 2H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): $\delta$ 13.9, 14.2, 48.6, 61.6, 128.8, 128.9, 133.7, 136.1, 171.1, 196.2. FT-IR: (neat) 2985, 1744, 1684. MS: 206 (m/z, 9.9%), 161 (2.5%), 133 (0.8%), 102 (100%), 77 (36.4%).

![Image](image2)

Prepared by PCC oxidation in 50% yield as a colorless oil after flash chromatography.\textsuperscript{17} \textsuperscript{1}H NMR (CDCl\textsubscript{3}): $\delta$ 1.26 (t, 3H $J = 7.2$ Hz), 1.32 (d, 3H $J = 7.2$ Hz), 3.62 (q, 1H $J = 7.2$ Hz), 3.82 (s, 2H), 4.19 (q, 2H $J = 7.2$ Hz), 7.25 (m, 5H). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): $\delta$ 12.9, 14.2, 48.7, 52.0, 61.5, 127.7, 128.8, 129.7, 133.6, 170.5, 203.4. FT-IR: (neat) 2985, 1730, 1700. MS: 220 (m/z, 19.9%), 174 (6.9%), 129 (44.3%), 102 (16.1%), 91 (100%), 74 (10.4%).

General procedure for the synthesis of optically pure alcohols \textit{anti-8a} and \textit{anti-8b}:
A round bottom flask was charged with NADP+ (7 mg), glucose-6-phosphate (86 mg), glucose-6-phosphate dehydrogenase (50 μg), α-methyl-β-ketoester (95 μL) and purified reductase (2.2 mL) in 6 mL of KP\textsubscript{i} buffer. The flask was then incubated with gentle shaking at 30 °C for 36 hours. The reaction mixture was then extracted with ether (3 x 15 mL), the combined organics were dried with MgSO\textsubscript{4}, and the solvent removed under reduced pressure to give the crude product as an oil.

Purification by flash chromatography (12 : 1 Hexanes : EtOAc) gave the pure product as a slightly yellow oil in 80% yield\textsuperscript{18,19} \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 0.96 (t, 3H, J = 7.4 Hz), 1.20 (d, 3H, J = 7.1 Hz), 1.26 (t, 3H, J = 7.1 Hz), 1.51 (m, 2H), 2.50 (dq, 1H, J\textsubscript{1} = 7.0 Hz, J\textsubscript{2} = 7.0 Hz), 2.65 (br d, 1H, J = 7.0 Hz), 3.58 (m, 1H), 4.17 (q, 2H, J = 7.1 Hz). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): δ 10.02, 14.38, 14.53, 27.77, 45.00, 60.73, 74.85, 176.30. FT-IR: (neat) 3500, 2969, 1730, 1185. [α]\textsubscript{D} = +3\textdegree (C = 0.3, CHCl\textsubscript{3}). MS was the same as 12-13\textsubscript{a}.

Purification by flash chromatography (12 : 1 Hexanes : EtOAc) gave the pure product as a slightly yellow oil in 80% yield\textsuperscript{11} \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 0.90 (t, 3H, J = 7.1 Hz), 1.20 (d, 3H, J = 7.1 Hz), 1.24 (t, 3H, J = 7.1 Hz), 1.50 (m, 4H), 2.45 (dq, 1H, J\textsubscript{1} = 7.0 Hz, J\textsubscript{2} = 7.0 Hz), 2.59 (br d, 1H, J = 7.0 Hz), 3.59 (m, 1H), 4.15 (q, 2H, J = 7.1 Hz). \textsuperscript{13}C NMR (CDCl\textsubscript{3}): δ 14.17, 14.37, 14.44, 18.93, 37.05, 45.48, 60.71, 73.25, 176.31. FT-IR: (neat) 3500, 2964, 1730, 1186. [α]\textsubscript{D} = +4\textdegree (C = 0.2, CHCl\textsubscript{3}). MS was the same as 12-13\textsubscript{b}. 21
General Procedure for MPA alcohol derivitization:

An NMR tube was charged with one equivalent of alcohol, CDCl₃ (1 mL), and one equivalent of a mixture of (R)-MPA and (S)-MPA (2 : 1), followed by addition of 1.5 equivalents of dicyclohexylcarbodiimide (DCC) and 0.5 equivalents of N,N-dimethylaminopyridine (DMAP). The crude reaction was monitored by ¹H and 1D TOCSY NMR. The chemical shift differences, listed as the change in chemical shift between (R) and (S) esters (δR,S), are summarized in Table 3.

The chemical shift differences between (R) and (S) MPA esters allowed us to assign (S) absolute stereochemistry for both alcohols. We determined relative configuration for both compounds based on vicinal coupling constants (³JHH) for the H₂ signal (7.0–7.5 for anti and 2.5-3.0 for syn) and comparison of the H₃ chemical shift for anti and syn isomers with literature data. For these β-hydroxyketones (R = Me, Et, i-Pr, Ph, Bn), H₃ is shifted upfield for anti isomers.

For α-methyl-β-hydroxyester 13a (R = Et), ³JHH of 7.0 Hz for H₂ in addition to an H₃ chemical shift identical with literature data (δ 3.58 observed, lit. value: δ 3.58 (anti), 3.84 (syn)) allowed us to conclude anti relative stereochemistry, assigning the full configuration as 2(S), 3(S).

For α-methyl-β-hydroxyester 13b (R = n-Pr), ³JHH of 7.0 Hz for H₂ in addition to an upfield chemical shift for H₃ (δ 3.59 from purified enzyme reduction vs. 3.59 and 3.90 from nonselective aldol reaction) allowed us to conclude anti relative stereochemistry, assigning the full configuration as 2(S), 3(S). This assignment does not agree with Hena et al., who published chemical shifts of H₃ for anti and syn isomers as δ 3.7 and 3.5, respectively. However, Hena et al. did not report any coupling constants for H₂ to support their assignment. Thus, we believe the
configuration was not assigned correctly since other \( \alpha \)-methyl-\( \beta \)-hydroxyesters in this series (\( R = \text{Me, Et, } i\text{-Pr, Ph, Bn} \)) assigned the chemical shift of \( H_3 \) for \textit{anti} compounds upfield from \textit{syn}. And, our coupling constants support \textit{anti} relative stereochemistry, while Hena et al. did not report this data.

General procedure for reductions of \( \alpha \)-methyl-\( \beta \)-ketoesters using Baker’s Yeast whole cells:

Fleishman’s\textsuperscript{®} yeast (10 g) was added to a solution of 45 mL of tap water in a baffled Erlenmeyer flask followed by the addition of 15 g of sucrose. The flask was then shaken at 30\( ^\circ \) C for one hour followed by the addition of 100 \( \mu \)L of substrate. The reaction was then shaken at 30\( ^\circ \) C for 5 days with addition of sucrose as needed. Aliquots of the reaction mixture were analyzed via chiral and achiral GC. After five days, the reaction mixture was filtered over Celite and the aqueous layer extracted with EtOAc (3 x 75 mL). The combined organics were washed with brine (1 x 8 mL), dried with MgSO\(_4\) and the solvent removed under reduced pressure to give the crude compound as a yellow oil which was purified by filtration through a short plug of silica to give the pure compound. For each compound, the major products using a long run were one of two possible peaks on achiral GC and two of four possible peaks on chiral GC.

\[
\text{OH} \quad \text{O} \quad \begin{array}{c} \text{CH}_3 \\ \text{anti-12a} \end{array} + \quad \begin{array}{c} \text{OH} \\ \text{CH}_3 \\ \text{anti-13a} \end{array} \quad \text{O} \quad \text{Et}
\]

Purified by a short flash chromatography column to give a slightly yellow oil in 60% yield.\textsuperscript{24} Alcohols were analyzed as trifluoroacetylated derivatives \textit{anti}-19-20a and \textit{syn}-19-20a. The products from NaBH\(_4\) reduction were separated using an achiral DB-17 column (peaks at 11.14 and 12.50 min, long run) and a chiral Dex column (peaks at 26.39, 27.66, 28.23 and 29.08 min, long run). Trifluoroacetylated derivatives of the reaction products showed peaks at 11.14
and 12.50 min (long run) (1 : 21.5, 91% de, long run) on achiral GC and 27.70, 28.38 and 29.11
min (11.0 : 1.0 : 11.0, 0% ee, long run) on chiral GC. The major products are the anti
enantiomers since the peak at 27.70 min corresponds to the product obtained from purified
enzyme reduction. Our results also agree with the literature.

\[
\text{anti-12b} + \text{anti-13b}
\]

For this compound, alcohols anti-12-13b and syn-12-13b were not derivatized. The
products from NaBH₄ reduction were separated using an achiral DB-17 column (peaks at 22.51
and 24.14 min, long run) and a chiral Dex column (peaks at 53.56, 53.91, 54.58 and 54.90 min,
long run). After reaction, product appeared on achiral GC at 22.41 min (>99% de, long run) and
on chiral GC at 53.66 and 54.32 (1.0 : 1.0, 0% ee, long run). These are the anti enantiomers
since the peak at 53.66 min corresponds to the alcohol from purified enzyme reduction.
Figure 1-1. Selective reduction using Baker’s yeast whole cells

\[
\begin{align*}
\text{Figure 1-2. Substrates screened by Kaluzna et al.}
\]
Figure 1-3. Synthetic applications of chiral products from α-chloro-β-ketoester reductions

Figure 1-4. The α-methyl-β-ketoesters synthesized and possible alcohol products
Figure 1-5. Previous methods for selective reduction of α-methyl-β-ketoesters
Figure 1-6. Synthesis of α-methyl-β-ketoesters

Figure 1-7. Cofactor regeneration with G6PDH
Figure 1-8. Trifluoroacetyl alcohol derivatives

Figure 1-9. Trifluoroacetylation GC values for racemic alcohols
Figure 1-10. Reductions of ketoesters to alcohols with purified enzymes
Figure 1-11. *Anti-12,13a + syn-12,13a*
Figure 1-12. *Anti*-13a, from enzymatic reduction of ketone 11a
Figure 1-13. *Anti-12-13b + syn-12-13b*
Figure 1-14. *Anti*-13b from enzymatic reduction of ketone 11b
Figure 1-15. 1-D TOCSY NMR of crude (R) and (S) MPA esters of anti-$13a$

Figure 1-16. 1-D TOCSY NMR of crude (R) and (S) MPA esters of anti-$13b$

Figure 1-17. Nomenclature used for assignment of configuration
Table 1-1. Comparison of α-methyl and α-chloro-β-ketoester reductions

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<sup>a</sup> <20% conversion after 24 hours"
Table 1-2. Comparison of reduction products for α-methyl and unsubstituted β-ketoesters

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<sup>a</sup> <20% conversion after 24 hours<sup>1,5</sup>  <sup>b</sup> Arbitrary assignment, syn product absolute configuration was not assigned

Table 1-3. Chemical shift difference between (R) and (S) MPA esters (Δδ<sub>R,S</sub>)

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<th>Compound</th>
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<th>dH&lt;sub&gt;1a&lt;/sub&gt;</th>
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<th>dH&lt;sub&gt;Me&lt;/sub&gt;</th>
<th>dH&lt;sub&gt;3&lt;/sub&gt;</th>
<th>dH&lt;sub&gt;4a&lt;/sub&gt;</th>
<th>dH&lt;sub&gt;4b&lt;/sub&gt;</th>
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<sup>a</sup> Not measured  <sup>b</sup> Not applicable
CHAPTER 2
SYNTHESIS OF AN APTAMER TARGET FOR RAPID SCREENING OF EXPOSURE TO CHEMICAL WARFARE AGENTS

Introduction

Chemical warfare (CW) began during World War I with the deployment of toxic gases such as phosgene, chlorine and dichloroethylsulfide (mustard gas). More recently, CW was used by terrorists during the release of Sarin gas in Matsumoto City and Tokyo, Japan. CW agents are toxic at low levels: patients admitted to the Nippon Medical Center after exposure to Sarin gas in the Tokyo incident had ingested estimated levels of 0.13 – 0.25 mg for a comatose patient (who never recovered) to 16-32 μg for patients who were sick but fully recovered. The risk of serious injury, or death, from exposure to CW agents continues to the present day.

Rapid, accurate detection of CW agents is of vital importance in order to minimize their potentially devastating effects. Detection of exposure to CW agents relies upon detection of their degradation products since the intact agent is rapidly hydrolyzed in vivo (Figure 2-1). Current methods for CW agent detection include GC / MS, HPLC / MS or assays detecting inhibition of acetylcholine esterase (AChE). Both of these techniques have disadvantages: GC / MS or HPLC / MS require at least 24 hours after sample acquisition, while AChE inhibition assays will not identify specific CW agents and suffer from false positive results due to AChE inhibition from unrelated chemicals. Clearly, a need persists for rapid and accurate detection of CW agents.

Aptamers for CW Agent Detection

Aptamers are single stranded (ss)DNA or RNA oligonucleotides that can bind to targets due to their tertiary structure. Aptamers are selected using an in vitro process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) which begins with a large random pool of oligonucleotides and, through repeated rounds of selection against a target, identifies a
small number of oligonucleotides that tightly bind. Aptamers can bind tightly to a wide range of targets ranging in size from live cancer cells ($K_d = 0.80 \text{ nM}$) to ethanolamine ($K_d = 6 \text{ nM}$).

We set out to isolate DNA aptamers that could bind to 18, a biotinylated analog of CW agent hydrolysis product 17 (Figure 2-2). These aptamers could, in principle, be isolated since phosphonic acid 17 ($n = 2$) was detected at approximately 100 nM in the urine of a Japanese man one day after Sarin exposure in Matsumoto City. Similar levels of 17 (135 – 25 nM) were found in the serum of victims 1.5 hours after hospital admittance in Sarin attacks in Tokyo and Matsumoto City. These people became ill, but their exposure was not fatal.

We chose to biotinylate our analog to allow for easy separation of binding aptamers in solution using streptavidin coated magnetic nanoparticles. After isolation of the DNA aptamers, a rapid colorimetric test for nerve agent exposure in the field would be developed by ADA Technologies using their proprietary technology.

**Target Synthesis**

Biotinylated phosphonic acid 18 was chemically synthesized in four steps starting from phosphonic dichloride 19. The dichloride was converted to the mixed phosphonate ester 20 by sequential treatment with benzyl alcohol and propylene glycol. Ester 20 was then oxidized to carboxylic acid 21 using TEMPO followed by attachment of polyethylene glycol (PEG) amine 22 with polymer bound EDC to form phosphonate 23. After this step, some of amine 22 remained as an inseparable impurity. The benzyl ester was removed using H$_2$ / Pd to give phosphonic acid 18 plus amine impurity 22 (Figure 2-3).

**Aptamer Selection**

We used a 76 nucleotide ssDNA template with a 40 nucleotide random sequence surrounded by forward and reverse priming regions. The primer sequences were chosen
according to Stoltenburg.\textsuperscript{36} The forward primer contained a fluorescent (FAM) tag at its 5’ end, while the reverse primer had a biotin tag at the 5’ position (Figure 2-4). The fluorescent tag allowed for detection of the DNA and biotin allowed for purification and separation of double stranded DNA using streptavidin coated magnetic nanoparticles.

For the initial selection round, biotinylated target 18 was mixed with streptavidin (SA) coated magnetic nanoparticles (mnp’s) and allowed to stand overnight at 4° C before addition of the template strand. The resulting solution was then allowed to incubate at room temperature with gentle shaking for 30 minutes followed by removal of the nonbinding oligonucleotide containing supernatant from the mnp’s. Bound DNA was eluted from the target by denaturation and the resulting ssDNA was amplified by PCR to give double stranded dsDNA containing a fluorescent tag and biotinylated tag on opposite strands. The dsDNA was then allowed to stand overnight with streptavidin coated mnp’s; ssDNA containing the fluorescent tag was eluted by denaturation to give the starting material for the second selection round. Selection rounds 2-5 followed the same procedure as the first round (Figure 2-5).

Selection round 6 employed a counterselection step to determine if DNA was binding the mnp’s or PEG impurity 22 instead of target 18. The counterselection step used acetylated amine 23 as a mimic for PEG impurity 22 (Figure 2-6). The step was performed by binding acetylated amine 23 to streptavidin coated mnp’s as described previously followed by incubation with isolated DNA from the selection process. As shown in Table 3, counterselection after round 6 resulted in a large decrease in fluorescence of the isolated DNA. Additionally, no DNA was isolated after PCR amplification.

**Results and Discussion**

A steady increase in fluorescence to blank should be observed after the initial selection round if DNA is tightly binding since larger quantities are recovered. We did not observe this
steady increase, allowing us to conclude that DNA was not specifically binding to target 18, but instead to the polyethylene glycol impurity.

Generation of the PEG impurity could be avoided by attachment of the methyl phosphonic acid directly to the mnp’s, as employed by Strehlitz et. al. for their preparation of ethanolamine modified mnp’s. This process would require incubation of phosphonic acid 24 with commercially available tosyl activated mnp’s (Figure 2-7). These phosphonic acid modified mnp’s could then be used for SELEX without any major modifications to our current procedure.

Finally, the similarity of the methyl phosphonic acid and phosphate esters in the DNA backbone should be noted. This could prevent DNA from binding tightly using either procedure since it is possible that DNA can not tell the difference between itself and the target. If this similarity is too great, then the detection of hydrolysis products of CW agents with DNA aptamers is not possible.

**Experimental Procedures**

**General Experimental**

All chemicals and dNTP’s were purchased from Sigma Aldrich or Fisher and used without further purification. Solvents were purified as described in the previous procedure. Biotinylated amine 22 was purchased from Pierce. Taq polymerase was purchased from New England Biolabs. All DNA was synthesized by IDT DNA Technologies. Fluorescence was measured using a Tecan Safire microplate reader with 384 well Corning plates.

**Chemical Synthesis**
An oven dried flask was equipped with a stir bar and charged with 20 mL of CH₂Cl₂, methyl phosphonic dichloride (8.8 mmol, 0.8 mL) and triethylamine (17.6 mmol, 2.45 mL). Benzyl alcohol (8.8 mmol, 0.91 mL) was then added dropwise at room temperature so that a gentle reflux was maintained. The solution was stirred at room temperature for two hours before dropwise addition of propylene glycol (8.8 mmol, 0.64 mL) at room temperature. The reaction was then stirred overnight at room temperature. Triethylammonium salts were removed by filtration, the solvent was removed and the product purified by flash chromatography (EtOAc) to give the product (1.61 g, 75 % yield) as a colorless oil. ¹H NMR: (CDCl₃) δ 1.49 (d, 3H, J = 17.6 Hz), 1.82 (m, 2H), 3.72 (t, 2H, J = 5.7), 4.07 (m, 1H), 4.21 (m, 1H), 5.08 (d, 2H, J = 9.1 Hz), 7.4 (m, 5H); ³¹P NMR: (CDCl₃) δ 33.82.

Aqueous 15 % NaHCO₃ (3 mL) was added to a solution of 20 (1.19 mmol, 0.29 g) in 25 mL of acetone and the solution was cooled to 0°C. Sodium bromide (0.24 mmol, 0.024 g) and TEMPO (0.024 mmol, 0.004 g) were then added, followed by the addition of trichloroisocyanuric acid (2.38 mmol, 0.55 g) over a 20 minute period. The reaction was then stirred at room temperature for two hours before the addition of 1.5 mL of isopropanol. The mixture was filtered over Celite, concentrated under vacuum, and 8 mL of saturated Na₂CO₃ was added. The aqueous phase was washed with EtOAc (2 x 20 mL), then acidified with 1 M HCl and extracted again with EtOAc (3 x 20 mL). The combined organic layers from the second extraction were dried with MgSO₄ and concentrated under vacuum to yield the product as a semicrystalline solid (crude yield 0.26 g, 85 %) which was used without purification in the next
step.  $^1$H NMR: (CDCl$_3$) $\delta$ 1.50 (d, 3H, $J = 17.8$ Hz), 2.68 (t, 2H, $J = 6.12$ Hz), 4.26 (m, 2H), 2.08 (dd, 2H, $J = 3.9, 9$ Hz), 7.36 (m, 5H).  $^{31}$P NMR: (CDCl$_3$) $\delta$ 36.87.

Carboxylic acid 21 (0.06 mmol, 16 mg) and PEG biotinylated linker (0.06 mmol, 25 mg) were combined in an oven dried flask that contained 2 mL of CH$_2$Cl$_2$ and 2 mL of DMF. Polymer bound EDC (100 mg) was then added and the reaction was stirred overnight at room temperature. The solvents were then evaporated and the crude product was purified by flash chromatography (5 : 1 EtOAc : MeOH) to give the product (crude yield 0.025 g, 65 % yield) plus some of the PEG biotinylated linker as an inseparable impurity.  $^1$H NMR: (CD$_3$OD) $\delta$ 1.26 (m, 3H), 1.44 (m, 2H), 1.52 (d, 3H, $J = 15$ Hz), 1.64 (m, 4H), 2.22 (t, 2H, $J = 7.4$ Hz), 2.56 (t, 2H, $J = 6.0$ Hz), 2.70 (m, 1H), 2.93 (m, dd, $J = 5.1, 12.7$ Hz), 3.20 (m, 2H), 3.37 (m, 10H), 3.53 (m, 5H), 3.62 (m, 11H), 4.28 (m, 3H), 4.5 (dd, 1H, $J = 4.5, 8.1$ Hz), 5.07 (dd, 2H, $J = 2.3, 8.5$ Hz), 7.4 (m, 6H);  $^{31}$P NMR: (CD$_3$OD) $\delta$ 33.8.

Biotinylated phosphonate 23 (0.025 g, 0.039 mmol) was added to a flask containing 15 mL of MeOH. A spatula tip full of Pd on Carbon was then added to the flask and the mixture was hydrogenated at room temperature under a balloon of hydrogen overnight. The mixture was then
filtered over Celite and MeOH was removed under reduced pressure. The product was then lyophilized to give yellow crystals (0.022 g, 94 % yield). \(^1\)H NMR: (D\(_2\)O) \(\delta\) 1.10 (m, 6H), 1.30 (s, 1H), 1.50 (m, 5H), 1.75 (m, 2H), 1.95 (s, 1H), 2.15 (m, 1H), 2.42 (t, 2H, \(J = 5.7\) Hz), 2.68 (s, 1H), 2.84 (s, 1H), 3.2 (m, 9H), 3.52 (m, 6H), 4.57 (s, 1H). 3\(^1\)P NMR: (D\(_2\)O) \(\delta\) 28.20. (+) ESI-MS: 584 (M + 16, impurity from oxidation of biotin sulfur to sulfoxide), 568 (m/z for biotinylated methyl phosphonic acid 18, most abundant compound), 418 (m/z for biotin linker impurity 22).

![Image of chemical structure]

PEG biotinylated linker (12 mg, 0.028 mmol), acetic anhydride (5.3 \(\mu\)L, 0.056 mmol) and N,N-dimethylaminopyridine (1 small crystal) were combined in an oven dried flask that contained 6 mL of CH\(_2\)Cl\(_2\) at room temperature and stirred overnight at room temperature. The solvent was removed under reduced pressure and the resulting solid purified by flash chromatography (5 : 1 EtOAc : MeOH) to give 23 (6.4 mg, 50 % yield). \(^1\)H NMR: (CDCl\(_3\)) \(\delta\) 1.45 (m, 2H), 1.70 (m, 4 H), 1.95 (s, 3H), 2.25 (t, 2H, \(J = 6.9\) Hz), 2.75 (m, 1H), 2.92 (dd, 1H, \(J = 5.1, 12.9\) Hz), 3.15 (m, 1H), 3.44 (m, 4H), 3.57 (t, 4H, \(J = 5.1\) Hz), 3.64 (s, 6H), 4.33 (m, 1H), 4.51 (m, 1H), 5.50 (s, 1H), 6.49 (s, 1H), 6.60 (s, 1H), 6.76 (s, 1H).

**PCR Amplification**

The reactants for each PCR round were 0.2 mM dNTP’s, 1 \(\mu\)M forward primer, 1 \(\mu\)M reverse primer, 10 mM KCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 20 mM Tris-HCl pH 8.8, 10 mM MgSO\(_4\), 0.1% Triton X-100. PCR conditions were 5 minutes at 94°C and 30 cycles of 1 min at 94°C, 1 min at 47°C, 1 min at 72°C, then 10 minutes at 72°C after the last cycle. 20 units of Taq polymerase (5...
U / μL) were used for a 100 μL PCR; 10 units were initially added via hot start methodology and 10 units were added after 15 cycles. Electrophoresis on a 2% agarose gel with Hae III phix174 markers were used to determine if the correct size of DNA was produced.

**Magnetic Nanoparticles**

Magnetic nanoparticles were synthesized by Joshua Smith at the University of Florida. An aqueous mechanically stirred solution of ammonium hydroxide (2.5%), ferric chloride hexahydrate (0.5 M), ferrous chloride tetrahydrate (0.25 M) and HCl (0.33 M) was stirred for 10 minutes at 350 RPM. The iron oxide nanoparticles were then washed with water three times and ethanol once. The MNPs were dispersed in an ethanol solution containing ~1.2% ammonium hydroxide at a final concentration of ~7.5 mg / mL to a final volume of ~6 mL.

The magnetite core particles were coated with silica by adding 200 μL tetraethylorthosilicate (TEOS) to the ethanolic solution and sonicating for 90 minutes at room temperature. Additional TEOS (10 μL) was introduced and sonication was continued at room temperature for an additional 90 minutes to post-coat the nanoparticles. The sample was washed three times with ethanol. To introduce surface carboxyl groups, 80 μL of carboxy-silane (N-(trimethoxysilylpropyl)ethylenediamine, triacetic acid trisodium salt, 45% in water) was added to 1 mL of silica-coated magnetic nanoparticles (10 mg / mL suspension in 10 mM phosphate-buffered saline, pH 7.4) and the reaction was vortexed for 4 hours at room temperature. The particles were then washed three times with 10 mM phosphate-buffered saline and stored at room temperature.

To prepare for streptavidin coupling, a 1 mg sample of carboxyl-modified magnetic nanoparticles was washed three times with 250 μL aliquots of 0.5 mM MES, pH 5.0. Protein immobilization was carried out by adding 50 μL of a 20 mg / mL EDC solution to the washed
nanoparticles in 250 μL of 0.5 mM MES, pH 5.0 and incubating at room temperature for 15 minutes before 100-300 μg of streptavidin was added to the reaction mixture. The suspension was incubated for 2-4 h with continuous vortexing. The streptavidin-coated nanoparticles were magnetically extracted and washed three times with 500 μL aliquots of 20 mM Tris-Cl, 5 mM MgCl₂, pH 8, then resuspended at a final concentration of 2 mg / mL in the same buffer and stored at 4°C.

**Round 1 of SELEX**

A 500 μL eppendorf reaction tube containing 3 x 10⁻¹⁸ mole of target biotinylated phosphonic acid (30 μL from a 1 x 10⁻¹² M stock solution), 20 μL of streptavidin coated magnetic nanoparticles and 380 μL of buffer was allowed to stand overnight at 4°C. 3 nanomoles of the template sequence was then added (70 μL from a 1μg / μL stock solution) and the reaction was allowed to incubate at room temperature for 30 minutes with gentle shaking. (Before addition, DNA was denatured and renatured by heating to 80°C for 10 min and then cooling on ice for 10 minutes).

The eppendorf tube was then attached to a magnet and allowed to stand for 5 minutes. The supernatant was removed, 200 μL of fresh buffer was added, the tube was gently vortexed, attached to a magnet and allowed to stand for 5 minutes before removing the supernatant. This procedure was performed three total times.

The DNA bound to the phosphonic acid was eluted by adding 100 μL of fresh binding buffer to the eppendorf tube, attaching the tube to a magnet, binding for 5 minutes and then heating to 94°C with shaking for 8 minutes followed by immediate removal of the supernatant while the solution was still hot. This procedure was performed two total times. After elution, DNA was precipitated with EtOH and 4 M NaCl (EtOH = 2.5 x total volume buffer used, 4 M NaCl = 0.025 x total volume buffer used), and allowed to stand overnight at -20°C. The solution
was then microfuged for 20 minutes and the supernatant was removed to give the eluted DNA. The eluted DNA was PCR amplified using the conditions listed in the general experimental.

After amplification, the ds DNA was purified and separated into ss DNA by adding 20 μL of MNP’s to the PCR vial and allowing to stand at 0°C overnight. The tube was then attached to a magnet, allowed to stand for 5 minutes and the supernatant removed. 100 μL of fresh buffer was added, the tube was vortexed, attached to a magnet and allowed to stand for five minutes followed by removal of the supernatant. This procedure was performed three total times. After washing was complete, 100 μL of fresh buffer was added, the tube was attached to a magnet, bound for 5 minutes, heated to 94°C for 8 minutes while bound to a magnet with shaking and the supernatant was removed while still hot. This procedure was performed two total times to give the purified ss FAM labelled DNA in 200 total μL of buffer. The presence of DNA was monitored by fluorescence and by visualization of the amplified DNA after gel electrophoresis.

Rounds 2-5 of SELEX

For subsequent selex rounds the entire PCR amplified and purified ss FAM labelled DNA from the previous round was used. For example, selex for round 2 contained 200 μL of purified DNA solution, 250 μL of buffer and 30 μL of the phosphonic acid from a 1 x 10^{-12} M stock solution and 20 μL of magnetic nanoparticles.

Round 6 of Selex Plus Counterselection Step

Round 6 introduced a counterselection step to ensure that DNA was binding to the phosphonic acid and not to the magnetic nanoparticles or biotinylated polyethylene glycol linker impurity 1. The selex procedure was carried out as usual to give 200 μL of solution after elution from the phosphonic acid. To this 200 μL solution was added 50 μL of a solution which contained 20 μL of magnetic nanoparticles and 30 μL of a 1 x 10^{-12} M solution of acetylated PEG linker (The acetylated PEG linker and magnetic nanoparticles were allowed to bind
overnight at 0°C before addition). The entire solution was shaken gently for 30 minutes, the tube was attached to a magnet and the supernatant removed. 100 μL of buffer was then added, the tube was gently vortexed, attached to a magnet and the supernatant was removed. The recovered solution was precipitated and subjected to PCR amplification conditions as described previously.
Figure 2-1. Hydrolysis product of various CW agents

Figure 2-2. Chemical warfare hydrolysis products vs. biotinylated analog
Figure 2-3. Synthesis of biotinylated CW agent hydrolysis product
**Template Strand**
5’-ATA CCA GCT TAT TCA ATT N₄₀ AGA TAG TAA GTG CAA TCT-3’

**Forward Primer**
5’-FAM-ATA CCA GCT TAT TCA ATT-3’

**Reverse Primer**
5’-BIOTIN-AGA TTG CAC TTA CTA TCT-3’

Figure 2-4. Template strand, forward and reverse primers

Figure 2-5. Procedure for SELEX
Figure 2-6. Synthesis of acetylated PEG impurity

Figure 2-7. Direct attachment of methyl phosphonic acid to mnp’s

Table 2-1. Selection and counterselection fluorescence data

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CHAPTER 3
CHEMOENZYMATIC ROUTE TO FLUORINATED PHOSPHONIC ACID CARBOHYDRATES OF DEFINED STEREOCHEMISTRY

Introduction

The α-Fluorophosphonates

Phosphate esters and anhydrides are widespread in biological systems. These compounds interact with enzyme binding sites through electrostatic, hydrogen bonding and dipole-dipole interactions. The frequent presence of phosphate esters/anhydrides has fueled research to synthesize molecules that contain these useful functional groups. Unfortunately, phosphate esters/anhydrides are susceptible to hydrolysis in vivo by phosphatases, limiting their potential as successful drugs.

Phosphonates, which differ from phosphates by methylene replacement of one bridging oxygen, have been envisioned as nonhydrolyzable mimics of phosphate esters/anhydrides. Blackburn et al. proposed that α-fluorinated phosphonates might serve as superior phosphate analogs because of increased methylene polarity (Figure 3-1). α-Fluorinated phosphonic acids can also be considered as ‘isopolar’ mimics of phosphates due to the reduced pKa of the free hydroxyl groups (Figure 3-2). Other reasons postulated for the superiority of fluorinated phosphonates include increased bridging atom dihedral angle and the possibility for C-F···H-X hydrogen bonding (Figure 3-3).

Routes to α-Monofluorophosphonates

α-Fluorophosphonates are made using various techniques. Blackburn et al. and Prestwich et al. used the Horner-Wadsworth-Emmons (HWE) reaction of fluorinated bisphosphonate to build vinyl α-fluorophosphonates. The vinyl phosphonates can be converted to alkyl α-fluorophosphonates by reduction with Pd / C at atmospheric pressure (Figure 3-4).
HWE,\textsuperscript{51} Peterson\textsuperscript{52} and alkylation\textsuperscript{53} reactions with dibromofluorophosphonate \textbf{40} were used by Savignac to make vinyl and alkyl $\alpha$-fluorophosphonates. Phosphonate \textbf{40} is the Arbuzov product of triethyl phosphite and tribromofluoromethane.\textsuperscript{54} Savignac’s strategy generally relied upon double lithium halogen exchange using $n$-BuLi with in situ trimethylsilyl (TMS) protection/stabilization of the resulting lithiated anion before reaction with an aldehyde or alkyl halide (Figure 3-5).

Electrophilic fluorination using N-fluorobenzenesulfonimide (NFSI) \textbf{52} or Selectfluor \textbf{53} has been used for the selective fluorination of phosphonates, phosphonoacetates and $\beta$-sulfonophosphonates. NFSI and Selectfluor\textsuperscript{55} are stable, solid, easily handled sources of fluorine that can be used without any special precautions or special training. McKenna et. al\textsuperscript{56} used Selectfluor to build $\alpha$-fluorophosphonoacetates while Wnuk et. al\textsuperscript{57} used Selectfluor to make $\alpha$-fluorosulfones. The choice of counterion (Na$^+$ or K$^+$) can be important when optimizing electrophilic fluorinations.

Finally, (diethylamino) sulfur trifluoride (DAST) \textbf{54} has been employed for the nucleophilic displacement of $\alpha$-hydroxyphosphonates by fluorine. DAST was used by Prestwich et al.\textsuperscript{58} for their synthesis of analogs of lysophosphatidic acid. DAST is thermally unstable, so reactions must be conducted at room temperature or below (Figure 3-6).\textsuperscript{59}

\textbf{Methods for Stereochemical Control of $\alpha$-Monofluorophosphonates}

Optically pure $\alpha$-fluorophosphonates are currently made using chiral Lewis Acid or organometallic complexes. Joergensen et al.\textsuperscript{60} used chiral Lewis acid complex \textbf{57} to fluorinate $\alpha$-substituted $\beta$-ketophosphonates with good enantiomeric excess while Sodeoka et al.\textsuperscript{61} used chiral Pd complex \textbf{60} to carry out enantioselective fluorination (Figure 3-7). However, neither method was reported to selectively fluorinate $\alpha$-unsubstituted phosphonates.
The Importance of Stereochemical Control of α-Monofluorophosphonates

O’Hagan and coworkers synthesized sn-glycerol-3-phosphate (G3P) analogs 61-64 (Figure 3-8) and assayed them for activity against glycerol-1-phosphate dehydrogenase (G1DH) by monitoring the rate of NADH formation (Figure 3-9). O’Hagan found that α-monofluorinated phosphonic acid 63 was a substrate for the enzyme. In fact, both methylene 62 and α-monofluoro 63 were better than the natural substrate, 61. O’Hagan also examined the diastereomers of 63 to see if G1DH preferred one, and discovered that one was consumed 20% faster (Figure 3-10). Unfortunately, he was not able to determine the configuration of the better substrate or detect any accumulation of α-hydroxyketone product 65.

Berkowitz et al.62 constructed phosphonate analogs 66-69 of glucose-6-phosphate (G6P) (Figure 3-11). Berkowitz tested these G6P analogs as substrates for glucose-6-phosphate dehydrogenase (G6PDH) (Figure 3-12), and found that the analogs with the highest (68) and lowest (67) $K_m$ varied only by the stereochemistry of the α-fluorine. In fact, an order of magnitude separated the binding of the two compounds. Clearly, further research on stereochemical control of α-fluorinated phosphonates was warranted from the findings of O’Hagan and Berkowitz.

Aldolases in Synthetic Chemistry

Aldolases are enzymes that selectively catalyze the aldol reaction between a donor substrate and acceptor aldehyde to create two new stereocenters. Aldolases are classified according to their reaction mechanism: class I use a lysine residue in the active site to create a donor substrate enamine which then attacks an acceptor aldehyde, class II use Zn$^{2+}$ to stabilize a donor enolate within the active site before attack on the acceptor aldehyde (Figure 3-13). The donor substrate for most class I and class II aldolases is dihydroxyacetone phosphate (DHAP), 72.
Aldolases have been studied as tools for asymmetric organic synthesis using non-natural phosphonic acid donor substrate 80. Stribling\textsuperscript{63} discovered 80 could substitute for natural phosphate substrate 72 in the fructose 1,6-bisphosphate aldolase (FruA) catalyzed aldol reaction with D-glyceraldehyde 3-phosphate 81 to make phosphonic acid carbohydrate 82 (Figure 3-14).

Fessner et al.\textsuperscript{64} synthesized phosphonic acid sugars 83 and 84 using substrate 80 with aldolases from class I and class II, allowing him to make different diastereomeric products from the same starting materials (Figure 3-15). Thus, we reasoned that optically pure fluorinated DHAP mimics 85a-b could be used with aldolase to synthesize optically active fluorinated phosphonic acid carbohydrates (Figure 3-16).

**Selective Reduction of α-Fluoro-β-Ketophosphonates**

Our initial route to optically pure DHAP mimics 85a-b relied upon selective reduction of α-fluoro-β-ketophosphonate 88. The corresponding alcohol 89 would then be deoxygenated using standard Barton-McCombie conditions, followed by acetonide deprotection and selective oxidation of the secondary alcohol to give phosphonate ester 92. Deprotection of the phosphonate ester would give phosphonic acid DHAP mimics 85a-b (Figure 3-17).

α-Fluoro-β-ketophosphonate 88 was made in two steps via reaction of dimethyl methylphosphonate with acetonide methyl ester 95\textsuperscript{65} followed by electrophilic fluorination of β-ketophosphonate 97 with Selectfluor.\textsuperscript{57} Methyl ester 95 was made by selective protection of D-mannitol\textsuperscript{66} followed by NaIO\textsubscript{4} cleavage of diol 94\textsuperscript{65} and immediate oxidation of the resulting aldehyde to the methyl ester using Br\textsubscript{2} / NaHCO\textsubscript{3} / MeOH. Fluorination of 97 gave low yields of monofluorinated compound in addition to a difficult chromatographic separation of difluorinated and monofluorinated products (Figure 3-18).

The α-fluoro-β-ketophosphonate 88 was then reduced by NaBH\textsubscript{4} to give racemic standard 98. Compound 88 was then screened against our library of purified β-ketoreductases from
Baker’s Yeast using the same conditions as listed in Figure 1-7. The reactions were monitored with gas chromatography. Unfortunately, none of the reductases accepted the ketone. We next attempted to modify the acetonide protecting group of α-fluoro-β-ketophosphonate 88 with the less sterically demanding carbonate 99 (Figure 3-19).

Since quantities sufficient for further synthesis of α-fluoro-β-ketophosphonate 88 were difficult to obtain it was decided not to install the carbonate by acetonide deprotection / carbonate reprotction of 88 to give carbonate 100. Instead, acetonide deprotection of β-ketophosphonate 97, which was available on a multigram scale, using Dowex H⁺ resin to give diol 101 followed by carbonate formation with triphosgene to access carbonate 102 on a gram scale. Unfortunately, all attempts to fluorinate 102 yielded only difluorinated product 103 and recovered starting material (Figure 3-20 and Table 3-1). The synthetic route to α-fluorinated DHAP mimics of defined stereochemistry via reduction of an α-fluoro-β-ketophosphonate followed by Barton-McCombie deoxygenation of the resulting alcohol was not further pursued.

**Selective Reduction of α-Fluorovinylphosphonates**

We next hypothesized that DHAP mimics 85a-b could be synthesized by selective reduction of α-fluorovinylphosphonates 104a-b using our library of enone reductases cloned and purified by Despina Bougioukou (Figure 3-21). Our route to α-fluorovinylphosphonates used Horner-Wadsworth-Emmons (HWE) methodology to install the fluorinated alkene.

The initial synthesis of 104a-b used the HWE reaction of fluorinated bisphosphonate 108 with protected aldehyde 112 to make the E isomer of protected phosphonate 113 as the sole product. The fluorinated bisphosphonate 108 was constructed in two steps by initial synthesis of methylene bisphosphonate 107 from diethyl methylphosphonate 106 and diethyl chlorophosphate followed by electrophilic fluorination with Selectfluor. The protected aldehyde was made by ketalation of 1,3-dihydroxyacetone 109 followed by mono acetyl
protection of the 1,3-diol with *Pseudomonas fluorescens* Lipase\(^72\) and subsequent Swern oxidation to aldehyde 112 (Figure 3-22).

This synthetic route proved laborious due to difficulty purifying fluorinated bisphosphonate 108 via flash chromatography or fractional distillation. Additionally, it was found that the HWE reaction of crude 108 with aldehyde 112 gave an inseparable mixture of products. However, HWE reaction of the in situ generated lithiated 108a gave vinyl α-fluorinated phosphonate 113 in one step starting from α-diethyl-dibromofluoromethyl phosphonate 40 (Figure 3-23).\(^51\)

The dibromofluoromethyl phosphonate was generated by the Arbuzov reaction of triethyl phosphite and tribromofluoromethane.\(^54\) This reaction sequence allowed us to completely circumvent the purification problems associated with α-fluorobisphosphonate 108. After considerable experimentation, it was found that deacetylation of 113 with Amberlyst A-26 resin\(^72\) followed by ketal deprotection with Montmorillonite clay\(^73\) afforded α-fluorinated vinyl phosphonate 104a in decent yield (Figure 3-24).

Racemic standard 115, the reduction product of α-fluorinated vinylphosphonate 104a-b, was prepared before screening vinyl phosphonate 104a against our library of purified en-reductases to allow for reaction monitoring by gas chromatography. Synthesis of the racemic standard began with reduction of α-fluorinated phosphonate 113 with PdOH / C followed by acetyl deprotection using Amberlyst A-26 and deketalation with Montmorillonite clay (Figure 3-25). This route proved cumbersome due to difficulty separating the reduction products of 113, but served to make sufficient quantities of crude 115 for GC analysis.

α-Fluorinated phosphonate 104a was then screened against our library of en-reductases using a glucose-6-phosphate / glucose-6-phosphate dehydrogenase cofactor regeneration system;
the screenings revealed that starting material was consumed, but no product could be detected by GC (Figure 3-26). In order to identify the reaction product(s), we ran small scale enzymatic reactions in D$_2$O with stoichiometric quantities of NADPH.

The $^{19}$F NMR after four hours showed complete consumption of starting material with the production of a single peak corresponding in chemical shift to inorganic fluoride (Figure 3-27). Presumably, the fluorine was eliminated during reduction of the $\alpha$-fluorovinylphosphonate via the mechanism in Figure 3-28 to give unfluorinated vinyl phosphonate 119.

To support this mechanism, we synthesized vinyl phosphonate 119 via the same reaction sequence used to make fluorinated vinyl phosphonate 104a. Thus, bisphosphonate 107 was condensed with aldehyde 112 to give protected vinyl phosphonate 113. Acetyl deprotection with Amberlyst A26 followed by deketalation with montmorillonite clay gave 119 (Figure 3-29). Unfluorinated vinyl phosphonate 119 was not observed as a product of the enzymatic reduction of fluorinated vinyl phosphonate 104a with stoichiometric quantities of NADPH (reaction monitored by $^{31}$P NMR), but 119 was detected as an inseparable by-product from the HWE chemical synthesis of racemic fluorinated phosphonate 115 during ketal deprotection (Figure 3-30).

**Synthesis of Racemic $\alpha$-Fluorinated Phosphonic Acids**

We decided to optimize the synthesis of racemic $\alpha$-fluorinated phosphonate 115 before further investigation on the construction of $\alpha$-fluorinated phosphonic acids of defined stereochemistry 85a-b to determine if the fluorinated phosphonic acids are aldolase substrates. Our HWE route to 115 was not sufficient for this purpose, but recent literature reported that terminal alkenes can be converted to $\alpha$-hydroxyketones in one step using Ruthenium or KMnO$_4$ oxidants.
Thus, unsaturated phosphonate 122 was made via double lithium halogen exchange / in situ trimethylsilyl (TMS) protection of dibromofluorophosphonate 40 followed by nucleophilic attack on allyl iodide to give the TMS protected unsaturated phosphonate. This product was not isolated, but instead deprotected in the same pot using lithium ethoxide to give pure 122 after flash chromatography purification (Figure 3-31).

Unfortunately, oxidation of 122 with KMnO₄ yielded a mixture of α-hydroxyketone 115 plus unfluorinated vinyl phosphonate 119 in roughly a 1 : 1 ratio. Phosphonate deprotection of this mixture led to multiple unidentified phosphorus containing products (Figure 56). Oxidation with RuO₄ gave no defluorination, but instead yielded carboxylic acid 123 in low yield (Figure 3-32).

We then hypothesized that deprotection of unsaturated phosphonate 122 followed by alkene oxidation could lead to racemic phosphonic acid 124 (Figure 3-33). Phosphonate 122 was smoothly deprotected with TMSBr followed by hydrolysis of the TMS protected phosphonic acid with water to give phosphonic acid 125. However, KMnO₄ alkene oxidation was not successful and RuO₄ oxidation gave carboxylic acid 126 as the only reaction product (Figure 3-34). Our results with alkene oxidation revealed that this was not a method for synthesis of racemic α-fluorophosphonic acids.

Our next route to racemic α-fluorophosphonic acids sought to selectivly oxidize the secondary alcohol of diol 120 to α-hydroxyketone 118 using glycerol-1-phosphate dehydrogenase (G1DH) (Figure 3-35). For this procedure we planned to use NADH oxidase from *Lactobacillus sanfranciscensis* for cofactor regeneration.

Our route to diol 63 started from the NaIO₄ cleavage of acetonide protected D-mannitol 94 followed by immediate reduction of the resulting aldehyde to acetonide protected alcohol.
This alcohol was converted to triflate and then condensed with phosphonate to give acetonide protected phosphonate in decent yield. Both protecting groups of were removed by initial treatment of the neat phosphonate with TMSBr followed by addition of water for silyl ether cleavage and acetonide deprotection to give diol as a diastereomeric mixture as determine by F NMR (Figure 3-36).

Phosphonic acid (6 mg / 0.53 μmol) was combined with G1DH (80 Units), NADH oxidase from L. sanfranciscensis (80 U) and NAD⁺ (50 mg / 0.08 mmol) in 50 mM phosphate buffered D₂O and stirred at room temperature for 24 hours. The diastereomeric ratio changed during this time period from 0.8 : 1 to 0.4 : 1, indicating that starting material was consumed. We are currently unsure if product was formed, but this data will be obtained by monitoring the reaction by C NMR to observe ketone formation or by mass spectrometry to observe molecular weight change.

Results and Discussion

While logical, our routes to α-fluorinated DHAP mimics of defined stereochemistry were not successful. We initially believed that our library of purified reductases from Baker’s yeast could accept acetonide protected α-fluoro-β-ketophosphonate due to our previous success with sterically similar furan derivative (Figure 3-37). Unfortunately, acetonide protected phosphonate proved too large for our enzymes, and installation of a less sterically demanding protecting group resulted in difluorinated products.

Our enone reductase route for asymmetric synthesis of α-fluorinated DHAP mimics via vinyl α-fluorophosphonates was not viable due to the unexpected elimination of fluorine after enzymatic reduction. Chemical synthesis of phosphonate or phosphonic acid as racemic mixtures via olefin oxidation also did not produce the pure compound due to fluorine
elimination. But, these methods gave us valuable insight about the sensitive nature of these compounds; any future syntheses must avoid the facile loss of fluorine.

Our current route for the synthesis of α-fluorinated DHAP mimics as a racemic mixture appears promising. Additional data, obtained from $^{13}$C NMR or mass spectrometry, must be collected to ensure α-hydroxyketone formation from oxidation of diol 63, since we currently are unsure of the fate of consumed starting material. If the oxidation is successful, then the α-fluorinated DHAP mimic will not be isolated, but instead reacted in situ with aldolase to form diastereomeric mixtures of α-fluorophosphonic acid carbohydrates. The in situ reaction of α-hydroxyketone 65 should minimize defluorination since the enolizable ketone will be converted to the lactol by aldolase (Figure 3-38).

**Future Work**

Future work will chemoenzymatically synthesize optically pure α-fluorophosphonic acids 85a-b from optically pure α-hydroxyphosphonates 133a-b (Figure 3-39). Alpha-hydroxyphosphonates 133a-b have been made previously from (S)-malic acid 134 by Prestwich et al. as diastereomeric mixture 135. We plan to separate the diastereomers via lipase catalyzed kinetic resolution as shown by Backvall et al. for α-hydroxyphosphonate 136 (Figure 3-40).$^{78}$

Nucleophilic displacement of kinetically resolved alcohol 132a with diethylaminosulfur trifluoride (DAST) will give optically pure α-fluorophosphonate 140b.$^{62}$ Or, the alcohol will be converted to the triflate followed by nucleophilic displacement with cesium fluoride$^{79}$ or tetrabutylammonium fluoride (TBAF)$^{80}$ to give 140b. Deprotection of 140b as previously described will give α-fluorophosphonic acid 63b (Figure 3-41).

Diastereomer 63a will be obtained by removal of the acteyl group under neutral conditions$^{81}$ followed by nucleophilic fluorination and deprotection (Figure 3-42). Optically
pure α-fluorophosphonic acids \textbf{63a-b} will then be selectively oxidized and reacted in situ with aldolase to make α-fluorinated phosphonic acid carbohydrates of defined stereochemistry.

**Experimental Procedures**

**Materials**

All organic chemicals were purchased from Sigma or Fisher and used without further purification. THF was distilled from Sodium metal. Flash chromatography was monitored by TLC with staining of plates by KMnO$_4$.

![Chemical Structure](image)

D-mannitol (28 g / 0.15 mol), dry DMSO (30 mL), 2,2-dimethoxypropane (46 mL / 0.38 mmol), and $p$-TsOH (0.5 g / 2.63 mmol) were added to an oven dried flask and stirred under Argon overnight. The solution was then poured into 3% NaHCO$_3$ (150 mL) and the resulting aqueous solution extracted with CH$_2$Cl$_2$ (3 x 100 mL). The combined organic layers were washed with water (2 x 20 mL) and brine (1 x 20 mL), dried over MgSO$_4$, and the solvent removed under reduced pressure. The resulting product was placed under high vacuum for 1.5 hours to remove residual solvent and the crude product recrystallized from heptane : CHCl$_3$ (10 : 1 volume / volume based on crude mass of product) to give 22 g of the pure product as white needles (56% yield).$^{82}$ Melting point 118 – 120°C; $^1$H NMR: (CDCl$_3$) $\delta$ 1.36 (s, 6H), 1.43 (s, 6H), 2.80 (m, 2H), 3.75 (m, 2H), 3.8-4.3 (m, 6H); $^{13}$C NMR: (CDCl$_3$) $\delta$ 109.3, 75.8, 70.9, 66.7, 26.7, 25.2.
Acetonide 94 (6.07 g / 23.15 mmol) was added to 150 mL of CH₂Cl₂ followed by addition of NaIO₄ (9.89 g / 46.28 mmol) and then 4 mL of aqueous saturated NaHCO₃. The resulting mixture was mechanically stirred at room temperature for 3 hours before the addition of 20 g of MgSO₄, then stirred at room temperature for another 15 minutes. The solution was then filtered, and the solvent removed under reduced pressure to give the crude aldehyde that was dissolved into 100 mL of 9 : 1 MeOH : H₂O which also contained 14.58 g / 173.55 mmol of NaHCO₃. Br₂ (4.8 mL / 93.78 mmol) was then added dropwise to the solution and the resulting orange mixture was stirred overnight at room temperature. Saturated aqueous sodium bisulfite was then added dropwise to the solution until the orange color disappeared. Methanol was removed under reduced pressure, and the remaining aqueous phase was extracted with CH₂Cl₂ to yield the crude product which was purified by flash chromatography to give the pure product as a colorless oil (4.81 g, 65% yield from 94). ¹H NMR: (CDCl₃) δ 1.42 (s, 3H), 1.45 (s, 3H), 3.80 (s, 3H), 4.12 (m, 1H), 4.23 (m, 1H), 4.61 (m, 1H). ¹³C NMR: (CDCl₃) δ 25.66, 26.00, 52.52, 67.40, 74.20, 111.50, 177.78.

A round bottom flask equipped with an addition funnel was flame dried while purging with Argon before the addition of 25 mL of THF and then cooled to -10° C. n-butyllithium (13.34 mL
/ 33.34 mmol from a 2.5 M solution) was then added and the mixture was cooled to -78° C. Dimethyl methylphosphonate 96 (3.58 mL / 31.75 mmol) in 7 mL of THF was then added dropwise to the solution of n-BuLi at -78° C and the resulting white slurry was stirred at -78° C for 30 minutes. The slurry was then transferred via cannula to a separate flask containing methyl ester 95 (4.37 mL / 30.16 mmol) in 15 mL of THF at -78° C. The resulting solution was stirred at -78° C for one hour before allowing to warm to room temperature overnight. Saturated ammonium chloride (10mL) was added dropwise at room temperature to quench the reaction and THF was removed under reduced pressure. The resulting aqueous layer was extracted with EtOAc (3 x 20 mL), the combined organic layers were dried with MgSO₄ and the solvent removed under reduced pressure to give the crude product as an oil which was purified by flash chromatography (3:1 Hex : EtOAc to 3 : 1 EtOAc : Hex) to give the pure product as a colorless oil. (6.89 g, 86% yield). ¹H NMR: (CDCl₃) δ 1.37 (s, 3H), 1.46 s (3H), 3.16 (m, 1H), 3.49 (m, 1H), 3.77 (d, 2H, J = 3.6 Hz), 4.35 (m, 4H), 4.55 (m, 1H). ¹³C NMR: (CDCl₃) δ 25.14, 26.21, 35.81, 37.55, 53.31 (m), 66.18, 80.32, 111.43, 202.39. ³¹P NMR: (CDCl₃) δ 23.26.

A round bottom flask equipped with an addition funnel was charged with NaH (0.15 g / 3.7 mmol of a 30% dispersion) and the NaH was washed two times with 5 mL of pentane before addition of 15 mL of THF. The resulting solution was cooled to 0° C and phosphonate 97 (850 μL / 3.7 mmol) dissolved in 5 mL THF was added dropwise to the flask at 0° C. The resulting solution was stirred at 0° C for 30 minutes and room temperature for one hour before recooling to 0° C. This solution was then transferred via cannula to a suspension of Selectfluor (1.31 g /
3.7 mmol) in dry CH$_3$CN at 0° C and the resulting solution stirred at 0° C for one hour before warming to room temperature overnight. The reaction was quenched with saturated ammonium chloride (7 mL), THF and acetonitrile were removed under reduced pressure and the resulting aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organics were dried with MgSO$_4$ and the solvent removed under reduced pressure to give the crude product as an oil. Purification via flash chromatography (5 : 1 Hex : EtOAc to 1:1 Hex : EtOAc) gave the pure product as a slightly yellow oil. $^1$H NMR: (CDCl$_3$) $\delta$ 1.46 (s, 3H), 1.51 (s, 3H), 3.80 (m, 4H), 4.25 (m, 2H), 4.9 (m, 1H), 5.4 (dd, $J = 47.21$, 60.39 Hz), 5.75 (dd, $J = 47.5$, 47.5 Hz). $^{19}$F NMR: (CDCl$_3$) $\delta$ -215.5 (dd, $J = 70.24$, 70.24 Hz), -216.0 (dd, $J = 69.03$, 70.24 Hz).

A round bottom flask containing NaBH$_4$ (0.017 g / 0.44 mmol) in MeOH (4 mL) was cooled to 0° C before the addition of ketone 88 (0.12 g / 0.44 mmol). The solution was stirred at 0° C for one hour and at room temperature for 4 hours before the reaction was quenched with saturated aqueous ammonium chloride (1 mL). MeOH was then removed under reduced pressure and the resulting aqueous layer extracted with CH$_2$Cl$_2$ (3 x 7 mL). The combined organics were dried with MgSO$_4$, the solvent evaporated under reduced pressure and the crude oil purified by flash chromatography (1 : 1 Hexanes : EtOAc to 4 : 1 Hexanes : EtOAc) to give the product as a colorless oil. $^1$H NMR: CDCl$_3$ $\delta$ 1.38 (m, 6H), 3.80 (m, 10H), 4.70 (m,1H). $^{13}$C NMR: (CDCl$_3$) $\delta$ 25.16, 25.20, 25.23, 26.17, 26.41, 26.70, 26.91, 65.42, 65.80, 66.36, 67.00, 68.71, 69.01, 69.74, 69.98, 69.74, 69.98, 70.87, 71.10, 71.87, 72.13, 73.73, 73.79, 73.88, 74.54, 74.69, 75.26, 75.46, 75.15, 75.61, 75.66, 84.94, 86.14, 87.18, 87.41, 88.37, 88.64, 89.65, 90.87.
Acetonide protected phosphonate 97 (0.41 g / 1.63 mmol) was dissolved in 7 mL of MeOH at room temperature before the addition of 0.6 g of Dowex 50WX8-100 resin at room temperature. The resin was then filtered and washed multiple times with acetone to remove the diol product. Acetone and methanol were removed under reduced pressure and the resulting oil / water mixture was azeotroped several times with CH₂Cl₂ to remove the residual water. The resulting oil was then purified by flash chromatography (EtOAc) to give the pure product as a slightly yellow oil (72 % / 0.25 g). ¹H NMR: (CDCl₃) δ 3.35 (d, 2H J = 3.8 Hz), 3.80, (m, 6H), 3.92 (m, 1H), 4.05 (m, 1H), 4.27 (m, 1H). ¹³C NMR: (CDCl₃) δ 36.00, 37.76, 48.31, 48.61, 50.48, 62.68, 77.89. ³¹P NMR: (CDCl₃) δ 23.74.

A flask was charged with diol 101 (0.44 g / 2.09 mmol) and pyridine (0.51 mL / 6.27 mmol) in CH₂Cl₂ (15 mL) and then cooled to -40° C under Argon. Triphosgene (0.62 g / 2.09 mmol) dissolved in CH₂Cl₂ (5 mL) was then added dropwise at the same temperature. The solution was allowed to warm slowly to room temperature over two hours and then stirred for an additional two hours at room temperature. The resulting solution was washed with 10 % HCl
(10 mL), saturated NaHCO₃ (10 mL) and brine (10 mL), dried with MgSO₄, and the solvent removed under reduced pressure to give the crude product as an oil. The crude product was purified by flash chromatography (2 : 1 Hexanes : EtOAc) to give the pure product as a yellow oil. ¹H NMR: (CDCl₃) δ 3.42 (m, 2H), 3.82 (m, 6H), 4.64 (m 2H), 5.32 (m, 1H). ³¹P NMR: (CDCl₃) δ 21.18.

Dihydroxyacetone dimer (7.5 g / 41.6 mmol), trimethylorthoformate (9 mL / 82.2 mmol), and p-TsOH (0.03 g / 0.16 mmol) and anhydrous MeOH (100 mL) were combined in a flask and stirred under Argon overnight at room temperature. Amberlyst A26 resin (0.28 g) was then added at room temperature and the solution was stirred at room temperature for 15 minutes. The solution was then filtered and the solvent was removed under reduced pressure to give a white solid (5.37 g / 95 % yield) which was pure enough for further use. The solid could be purified by flash chromatography (1 : 1 Hexanes : EtOAc) if desired. ¹H NMR: (CDCl₃) δ 3.25 (s, 6H), 3.70 (s, 4H). MS: 105 (M – 31, 4.5%), 77 (100%), 55 (35.4%).

Protected diol 110 (3.75 g / 27.6 mmol), Pseudomonas fluorescens lipase (0.33 g), vinyl acetate (40 mL) and diisopropyl ether (20 mL) were stirred under Argon overnight at room temperature. The lipase was removed by filtration over Celite and the solvents removed under reduced pressure. The resulting oil was purified by flash chromatography(5 : 1 Hexanes : EtOAc to 3 : 1 Hexanes : EtOAc) to give the pure compound as a colorless oil (2.95 g / 60% yield). ¹H NMR: (CDCl₃) δ 2.18 (s, 3H), 3.25 (s, 6H), 3.60 (s, 2H), 4.20 (s, 2H). ¹³C NMR: (CDCl₃) δ 68
20.47, 48.06, 59.80, 99.81, 170.86. FT-IR: (neat) 3463.7, 295.1, 1711.7. MS: 147 (100%), 129 (1.7%), 105 (62.9%), 87 (28.5%), 73 (35.2%).

An oven dried round bottom flask was charged with DMSO (257 μL / 3.54 mmol) in 15 mL of dry CH₂Cl₂ and cooled to -60°C under Argon. Oxalyl chloride (280 μL / 3.27 mmol) was then added dropwise at -60°C and the resulting solution stirred at this temperature for 10 minutes. Acetylated alcohol 103 (450 μL / 2.83 mmol) was then added dropwise to the solution at -60°C followed by stirring at -60°C for 20 minutes before quenching via dropwise addition of NEt₃ (1.3 mL) at -60°C. The solution was stirred at -60°C for 15 minutes before allowing to warm to room temperature over 45 minutes. Solvents were then removed under reduced pressure and the resulting residue dissolved into half saturated NaHCO₃ (6 mL) and extraction of the aqueous layer with ether (3 x 10 mL). The combined organics were washed with brine (5 mL), dried with MgSO₄, and the solvents removed under reduced pressure to give the crude compound (0.42 g / 85% crude yield) as a slightly yellow oil which was used without further purification. ¹H NMR: (CDCl₃) δ 2.05 (s, 3H), 3.30 (s, 6H), 4.25 (s, 2H), 9.45 (s, 1H). ¹³C NMR: (CDCl₃) δ 20.80, 50.16, 61.11, 66.59, 170.1, 204.12. FT-IR: (neat) 2950.02, 1750.32, 1172.08. MS: 147 (100%), 133 (0.9%), 115 (5.6%), 103 (56.9%), 73 (31.3%).

A flame dried round bottom flask was charged with diisopropylamine (8.85 mL / 63.14 mmol) and THF (15 mL), then cooled to -78°C before the dropwise addition of n-butyllithium.
(60.2 mL / 66.2 mmol) at the same temperature. The anion was allowed to stir at -78° C for 30 minutes before the dropwise addition of diethylmethyl phosphonate (4.5 mL / 30.8 mmol) in 5 mL of THF at -78° C. The resulting solution was stirred at -78° C for 30 minutes before the dropwise addition of diethyl chlorophosphate (4.7 mL / 32.3 mmol) in 5 mL of THF. The resulting solution was stirred for 30 minutes at -78° C before slowly allowing to warm to -35° C over a period of 2 hours. The solution was then cooled to -50° C and quenched by the dropwise addition of 3M HCl (22 mL). The resulting viscous mixture was allowed to warm to room temperature and THF was removed by rotary evaporation. The resulting aqueous layer was extracted with CH₂Cl₂ (3 x 20 mL) and the combined organics were dried with MgSO₄ and the solvent removed under reduced pressure. The resulting oil was dissolved into 60 mL of anhydrous ether and the triethylammonium salts removed by filtration. The ether was removed to give the crude product as a yellow oil. The product was purified by vacuum distillation (165° C bath temperature / 125° C apparatus temperature / 0.05 mm Hg) to give the pure compound as a slightly yellow oil (8.0 g / 90% yield).¹⁰¹ H NMR: (CDCl₃) δ 1.30 (t, 12H, J = 7.0 Hz), 2.4 (t, 2H, J = 21.0 Hz), 4.05 (m, 8H). ¹³C NMR: (CDCl₃) δ 16.42, 23.61, 25.42, 27.24, 62.72. ³¹P NMR: (CDCl₃) δ 20.51. IR: (neat) 2985.30, 1480.10, 1024.76. MS: 288 (m/z, 10.2%), 261 (40.8%), 205 (2.0%), 177 (0.6%), 159 (100%), 125 (65.7%).

Potassium hydride (0.29 g / 2.21 mmol of a 30% dispersion in mineral oil) was washed twice with pentane before the addition of 7 mL of THF. The resulting suspension was then cooled to 0° C before the dropwise addition of bisphosphonate ¹⁰⁷ (0.5 mL / 2.01 mmol) in 5 mL of THF. The resulting solution was stirred for 30 minutes at 0° C and one hour at room
temperature before recooling to 0°C. The anion was then transferred via cannula to a solution of Selectfluor (0.71 g / 2.01 mmol) in CH₃CN (20 mL). The resulting solution was then allowed to warm to room temperature overnight before the addition of saturated NH₄Cl (5 mL). THF and acetonitrile were then removed under reduced pressure and the remaining aqueous layer was extracted with ether (3 x 15 mL). The combined organics were dried with MgSO₄ and the solvent removed under reduced pressure to give the crude compound as an oil. Purification by flash chromatography (4.5 : 1 Hexanes : EtOAc to 2: 1 EtOAc : Hex) gave the pure compound as a colorless oil (1.62 g / 50% yield). CAUTION: If this procedure is used the purification requires a much longer time than for standard organic compounds. 

\[ ^1H\text{ NMR: (CDCl}_3\text{)} \delta 1.20 (t, 12H, J = 7.1 \text{ Hz}), 4.05 (m, 8H), 4.82 (dt, 1H, J = 13.4, 45.9 \text{ Hz}). \]

\[ ^{13}C\text{ NMR: (CDCl}_3\text{)} \delta 16.15, 64.81 (d, J = 30.72 \text{ Hz}), 84.05 (dt J = 156.1, 312.7 \text{ Hz}). \]

\[ ^{31}P\text{ NMR: (CDCl}_3\text{)} \delta 11.05 (d, J = 64.09 \text{ Hz}). \]

\[ ^{19}F\text{ NMR: (CDCl}_3\text{)} \delta 312.6 (dt J = 62.3, 45.9 \text{ Hz}). \]

MS: 306 (m/z, 7.1%), 279 (41.4%), 223 (61.4%), 194 (80.8%), 177 (100%), 143 (50.6%). 

![Chemical Structure](image)

Tribromofluoromethane (1.35 mL / 13.8 mmol), triethyl phosphite (2.36 mL / 13.8 mmol) and THF (12 mL) were combined in a round bottom flask and heated to 50°C and stirred overnight at this temperature. The reaction was then cooled, the solvent removed under reduced pressure and the resulting oil purified by flash chromatography (12 : 1 Hexanes : EtOAc) to give the pure product as a slightly yellow oil (4.30 g / 95% yield). 

\[ ^1H\text{ NMR: (CDCl}_3\text{)} \delta 1.42 (t, 6H, J = 7.2 \text{ Hz}), 4.39 (m, 4H). \]

\[ ^{13}C\text{ NMR: (CDCl}_3\text{)} \delta 16.60 (d, J = 10 \text{ Hz}), 66.99 (d, J = 7.0 \text{ Hz}). \]

\[ ^{31}P\text{ NMR: (CDCl}_3\text{)} \delta 2.55 (d, J = 76.9 \text{ Hz}). \]

\[ ^{19}F\text{ NMR: (CDCl}_3\text{)} \delta 76.50 (d, J = 76.8 \text{ Hz}). \]

IR: (neat)
From purified fluorinated bisphosphonate 108: An oven dried round bottom flask was charged with fluorinated bisphophonate 108 (100 µL / 0.38 mmol) in 6 mL of THF and cooled to -78°C before the dropwise addition of n-BuLi (345 µL / 0.38 mmol from a 1.1 M solution). The resulting solution was stirred at -78°C for 10 minutes before the addition of crude aldehyde 112 (84 µL / 0.52 mmol) at the same temperature. The solution was then allowed to stir at -78°C for 1.5 hour before slowly allowing to warm to room temperature overnight. Water (3 mL) was then added, THF was removed under reduced pressure and the resulting aqueous solution was extracted with ether (3 x 8 mL). The combined organic layers were dried with MgSO4 and the solvent removed under reduced pressure to give the crude product as a yellow oil which was purified by flash chromatography (3 : 1 Hexanes : EtOAc to 1.5 : 1 Hexanes : EtOAc) to give the pure product as a slightly yellow oil (0.088 g / 70% yield). ¹H NMR: (CDCl₃) δ 1.40 (t, 6H, J = 7.0 Hz), 2.05 (s, 3H), 3.25 (s, 6H), 4.15 (m, 4H), 4.25 (s, 2H), 5.85 (dd, J = 9.24, 41.91 Hz, 1H). ¹³C NMR: (CDCl₃) 16.14, 16.22, 20.66, 49.05, 62.80, 63.37 (d, J = 5.7 Hz), 99.65 (d, J = 14.6 Hz), 122.3 (d, J = 27.5 Hz), 152.1 (dd, J = 231.6, 288.6 Hz), 170.0. ³¹P NMR: (CDCl₃) δ 4.7 (d, J = 100.72). ¹⁹F NMR: δ -120.4 (dd, J = 41.52, 99.65). FT-IR: (neat) 2985.09, 1751.85, 1020.20. MS: 297 (M – 31, 7.1%), 277 (3.1%), 235 (48%), 207 (15.5%), 99 (68.8%).

From α-dibromofluorophosphonate 40: A flame dried round bottom flask was charged with THF (7 mL) and n-BuLi (325 µL / 0.52 mmol from a 1.6 M solution) and cooled to -78°C
followed by dropwise addition of phosphonate 40 (100 μL / 0.52 mmol) at -78°C (the solution turned red / brown at this point). The resulting solution was stirred at -78°C for one hour before dropwise addition of aldehyde 112 at the same temperature. The solution was then stirred at -78°C for one hour before allowing to warm to room temperature overnight. Water (5 mL) was then added, THF was removed under reduced pressure and the aqueous layer extracted with ether (3 x 10 mL). The combined organics were dried with MgSO4 and the solvent removed under reduced pressure to give the crude product as a brown oil which was purified by flash chromatography using the same solvent system as listed above to give the product as a slightly yellow oil (55.4 mg / 65% yield). Spectral data were also the same as listed above.

A round bottom flask containing acetylated alcohol 113 (0.65 g / 1.98 mmol) and Amberlys A26 resin in 8 mL MeOH was stirred overnight at room temperature. The resin was removed by filtration then washed with acetone. The resulting solution was concentrated under reduced pressure and the crude oil purified by flash chromatography (2 : 1 Hexanes : EtOAc to 1.5 : 1 EtOAc to Hexanes) to give the pure product as a pale yellow oil (0.38 g / 75% yield). 1H NMR: (CDCl3) δ 1.40 (t, 6 H, J = 7.1 Hz), 3.15 (s, 6H), 3.78 (s, 2H), 4.20 (m, 4H), 5.85 (dd, J = 9.4, 42.51 Hz, 1H). 13C NMR: (CDCl3) δ 18.36, 49.40, 63.37, 63.66 (d, J = 5.4 Hz), 101.47 (d, J = 14.3 Hz), 123.12 (d, J = 27.5 Hz), 152.34 (dd, J = 287.1, 233.0 Hz). 31P NMR (CDCl3) δ 4.8 (d, J = 102.54). 19F NMR (CDCl3) δ -120.85 (dd, J = 41.44, 101.73 Hz). IR (neat) ν 3418.12, 2985.48, 1019.28. MS 255 (M – 31, 66.4%), 235 (46.3%), 207 (14.0%), 179 (23.2%), 167 (17.3%), 117 (30.2%), 99 (100%).
A round bottom flask containing alcohol 113 (0.55 g / 1.93 mmol) and Montmorillonite clay (1 g) in 10 mL of CH₂Cl₂ was stirred at room temperature overnight. The solution was then filtered over Celite, the solvent was removed under reduced pressure and the resulting oil purified by flash chromatography (1 : 1 Hexanes : EtOAc to 3 : 1 EtOAc to Hexanes) to give the pure product as a slightly yellow oil (0.26 g / 65% yield).\(^{85}\) \(^1\)H NMR: (CDCl₃) δ 1.40 (t, 6H, \(J = 7.0\) Hz), 4.20 (m, 4H), 4.58 (s, 2H), 6.35 (dd, \(J = 8.2, 42.51\) Hz). \(^{13}\)C NMR: (CDCl₃) 16.54, 64.55 (d, \(J = 5.8\) Hz), 69.85, 116.85 (d, \(J = 25.9\) Hz), 161.26 (dd, \(J = 307.2\) Hz, 225.6 Hz), 196.10. \(^{31}\)P NMR: (CDCl₃) δ 2.70 (d, \(J = 100.11\) Hz). \(^{19}\)F NMR: (CDCl₃) δ -101.6 (dd, \(J = 43.6, 101.73\) Hz). FT-IR: (neat) 3418, 2897, 1699, 1642, 1019.

A round bottom flask was charged with fluorinated alkene 113 (0.50 g / 1.52 mmol), EtOAc (8 mL) and a spatula tip of PdOH / C before hydrogenation overnight at 1 atm at room temperature. The suspension was then filtered over Celite and the solvents removed under reduced pressure to give the crude product as a slightly yellow oil. The crude product was purified by flash chromatography (6 : 1 Hex : EtOAc to 2 : 1 Hex : EtOAc) to give the pure product as a slightly yellow oil (0.15 g / 30% yield). \(^1\)H NMR: (CDCl₃) δ 1.32 (t, 6H, \(J = 7.2\) Hz), 2.05 (s, 3H), 2.29 (m, 2H), 3.05 (s, 6H), 3.40 (s, 2H), 4.10 (m, 4H), 4.87 (m, 1H). \(^{31}\)P NMR: (CDCl₃) δ 19.02 (d, \(J = 75.69\) Hz). \(^{19}\)F NMR: (CDCl₃) δ -208.3 (m).
A round bottom flask was charged with reduced phosphonate 116 (0.15 g / 0.45 mmol), Dowex A26 resin (0.10 g) and Methanol (6 mL) before stirring overnight at room temperature. The solution was then filtered, and the filtrered resin washed several times with acetone. Solvents were then removed under reduced pressure to give the crude compound as a slightly yellow oil. The crude product was purified by flash chromatography (2 : 1 Hex : EtOAc to 2 : 1 EtOAc : Hex) to give the pure product as a slightly yellow oil (0.097 g / 75% yield). 1H NMR: (CDCl₃) δ 1.40 (t, 6H, J = 7.1 Hz), 2.20 (m, 2H), 3.10 (s, 3H), 3.40 (s, 2H), 4.10 (m, 4H), 4.79 (m, 1H).

A round bottom flask was charged with alcohol 117 (0.097 g / 0.34 mmol), montmorillonite clay (0.10 g) and CH₂Cl₂ (5 mL) before stirring at room temperature overnight. The suspension was then filtered over Celite and the solvents removed under reduced pressure to give the crude product as a yellow oil which contained the desired α-fluorophosphonate as the major product plus defluorinated vinyl phosphonate 119 as an inseparable byproduct (crude yield 80 % / 0.065 g). ¹H NMR: (CDCl₃) δ 1.25 (m, 6H), 3.05 (m, 2H), 4.12 (m, 4H), 4.20 (s, 2H), 5.31 (m, 1H), 6.90 (m, 2H, this signal is from the defluorinated vinyl phosphonate side product). ³¹P NMR: (CDCl₃) δ 17.35 (d, J = 73.25 Hz), 15.35 (s, this signal is from the defluorinated vinyl phosphonate side product). ¹⁹F NMR: (CDCl₃) δ 208.13 (m).
An oven dried round bottom flask was charged with bisphosphonate 107 (646 μL / 2.60 mmol) in THF (10 mL) and cooled to -78° C under Argon. n-Butyllithium (1.04 mL from a 2.5 M solution in hexanes) was then added dropwise at -78° C and the resulting solution stirred at the same temperature for 1 hour. Aldehyde 112 (569 μL / 3.56 mmol) was then added dropwise at -78° C and the resulting solution stirred at the same temperature before allowing to warm overnight. Water (3 mL) was then added, and THF was removed under reduced pressure. The remaining aqueous layer was extracted with ether (3 x 10 mL), the combined organics were washed with brine (4 mL), dried with MgSO4 and the solvent removed under reduced pressure to give the crude product as a slightly yellow oil. The crude product was purified by flash chromatography (4 : 1 Hex : EtOAc) to give the pure product as a colorless oil (0.67 g / 82% yield). 1H NMR: (CDCl3) δ 1.30 (t, 6H, J = 7.0 Hz), 2.05 (s, 3H), 3.20 (s, 6H), 4.05 (m, 4H), 4.08 (s, 2H), 6.15 (dd, 1H, J = 20.3, 17.5 Hz), 6.50 (dd, 1H, J = 22.53, 17.3 Hz). 31P NMR: (CDCl3) δ 18.18.

A round bottom flask was charged with vinyl phosphonate 120 (0.67 g / 2.13 mmol), A26 resin (0.70 g) and methanol (8 mL) and then stirred at room temperature overnight. The product was then filtered, the resin was washed several times with acetone and the solvents evaporated to give the crude product as a slightly yellow oil. The crude product was purified by flash
chromatography (1 : 1 Hex : EtOAc) to give the pure product as a slightly yellow oil (0.44 g / 78% yield). $^1$H NMR: (CDCl$_3$) $\delta$ 1.21 (t, 6H $J$ = 7.1 Hz), 3.05 (s, 6H), 3.50 (s, 2H), 3.98 (m, 4H), 6.25 (m, 2H). $^{31}$P NMR: (CDCl$_3$) $\delta$ 18.71.

\[ \text{HO} \begin{array}{c} \text{P} \\ \text{O} \end{array} \text{OEt} \]

A round bottom flask was charged with alcohol 121 (0.44 g / 1.66 mmol), montmorillonite clay (0.55 g) and CH$_2$Cl$_2$ (7 mL) before stirring overnight at room temperature. The suspension was then filtered over Celite and the solvent removed under reduced pressure to give the crude product as a yellow oil. The crude product was purified by flash chromatography (1 : 1 Hex : EtOAc) to give the pure product as a slightly yellow oil (0.33 g / 90% yield). $^1$H NMR: (CDCl$_3$) $\delta$ 1.25 (t, 6H $J$ = 7.1 Hz), 4.05 (m, 4H), 4.42 (s, 2H), 6.80 (m, 2H). $^{31}$P NMR: (CDCl$_3$) $\delta$ 15.44 (s).

\[ \text{HO} \begin{array}{c} \text{P} \\ \text{O} \end{array} \text{OEt} \]

A three neck round bottom flask equipped with a pressure equalizing dropping funnel was flame dried while purging with Argon before charging the flask with n-Butyllithium (6.9 mL / 17.1 mmol from a 2.5 M solution in hexanes) in THF (20 mL). The dropping funnel was charged with a mixture of dibromofluorophosphonate 40 (1.5 mL / 7.83 mmol) and trimethylsilyl chloride (993 $\mu$L / 7.83 mmol) in THF (10 mL). The flask was then cooled to -78°C and the contents of the funnel were added dropwise at this temperature. The resulting brown solution was stirred at -78°C for 10 minutes before the dropwise addition of allyl iodide (927 $\mu$L / 10.2
mmol) in THF (7 mL) at the same temperature. The resulting solution was stirred at -78° C for 45 minutes, then allowed to warm to 0° C on an ice bath. A solution of lithium ethoxide (prepared by adding 0.3 g of lithium wire to ethanol at room temperature) was then added dropwise at 0° C and the resulting solution was allowed to stir at the same temperature for 1 hour before pouring into 15 mL of 2 M HCl. THF was removed under reduced pressure, and the remaining aqueous solution was extracted with ether (3 x 15 mL). The combined organic phases were washed with a freshly prepared solution of saturated sodium bisulfite (3 x 7 mL) and brine (1 x 7 mL), dried with MgSO4 and the solvent removed under reduced pressure to give the crude product as a dark yellow oil. The crude product was purified by flash chromatography (12 : 1 Hex : EtOAc to 8 : 1 Hex : EtOAc) to give the pure product as a slightly yellow oil (1.40 g / 85% yield).

1H NMR: (CDCl3) δ 1.25 (t, 6H J = 7.05 Hz), 2.6 (m, 2H), 4.10 (m, 4H), 4.72 (m, 1H), 5.14 (m, 2H). 13C NMR: δ 15.42, 34.05, 62.31 (m), 67.46, 67.52, 112.48, 131.12. 31P NMR: (CDCl3) δ 17.8 (d, J = 74.26 Hz). 19F NMR: (CDCl3) δ -200.67 (m).

A round bottom flask was charged with NaHCO3 (0.37 g / 4.5 mmol) followed by addition of a 0.1 M aqueous solution of RuCl3 (139 μL) then CH3CN (8.4 mL) and EtOAc (8.4 mL). Oxone was then added in one portion to the brown suspension to give a bright yellow suspension (gas was evolved at this point). The reaction mixture was then cooled to 0° C in an ice bath and unsaturated phosphonate 122 (0.30 g / 1.39 mmol) was added in one portion. The mixture was stirred at 0° C for one hour before dilution with EtOAc (20 mL), the mixture was then filtered over Celite followed by washing of the solution with brine (1 x 5 mL). The organic phase was dried with MgSO4 and the solvent removed under reduced pressure to give the crude product as a
yellow oil. Purification by flash chromatography (3 : 1 EtOAc : Hex) gave the product as a colorless oil (0.11 g / 34% yield). \( ^1H \) NMR (CDCl\(_3\)) \( \delta \) 1.25 (t, 6H, \( J = 7.1 \) Hz), 2.92 (m, 2H), 4.05 (m, 4H), 5.25 (m, 1H). \(^{31}P\) NMR: (CDCl\(_3\)) \( \delta \) 17.82 (d, \( J = 74.47 \) Hz). \(^{19}F\) NMR: (CDCl\(_3\)) \( \delta \) -203.45 (m).

A round bottom flask was charged with unsaturated phosphonate 122 (0.10 g / 0.48 mmol) and then placed under vacuum for four hours. The flask was then sealed with a rubber septa and placed under Argon and TMSBr (218 \( \mu \)L / 1.61 mmol) was added dropwise to the neat phosphonate at room temperature. The rubber septa was removed and quickly replaced with a yellow Caplugs stopper and the reaction was stirred at room temperature overnight. Volatile materials were then removed (first on the rotovap, then on high vacuum for four hours) and water (1.5 mL) was added to the resulting oil and the solution stirred overnight at room temperature. (The solution instantly became turbid before slowly clearing overnight). Water was then removed by freeze drying to yield the phosphonic acid product as a colorless, viscous oil which was used without further purification in the next step (0.07 g / 95% yield). \(^1H\) NMR: (D\(_2\)O) \( \delta \) 2.25 (m, 2H), 4.60 (m, 1H), 4.91 (m, 2H), 5.62 (m, 1H). \(^{13}C\) NMR: \( \delta \) (D\(_2\)O) 40.15, 62.45 (m), 112.76, 132.04. \(^{31}P\) NMR: (D\(_2\)O) \( \delta \) 16.34 (d, \( J = 74.49 \) Hz). \(^{19}F\) NMR: (D\(_2\)O) \( \delta \) -200.48 (m).
This compound was prepared in the same way as 123 in 25% yield. $^1$H NMR: (D$_2$O) $\delta$ 2.45 (m, 2H), 4.62 (m, 1H). $^{13}$C NMR: $\delta$ (D$_2$O) 39.90 (d, $J = 21.7$ Hz), 91.05 (dd, $J = 169.7$, 153.1 Hz), 180.11. $^{31}$P NMR: (D$_2$O) $\delta$ 14.55 (d, $J = 70.11$ Hz). $^{19}$F NMR: (D$_2$O) $\delta$ -204.75 (m).

Protected D-mannitol was cleaved as described for the synthesis of ester 95. The resulting aldehyde (5.16 g / 39.7 mmol) was dissolved in EtOH (45 mL) and the solution cooled to 0°C before the slow addition of NaBH$_4$ over a period of five minutes. The solution was then allowed to warm to room temperature and stirred at room temperature for two hours. The reaction was then quenched by the addition of 3 M HCl until the pH was neutral. EtOH was then removed under reduced pressure and the aqueous phase was extracted with CH$_2$Cl$_2$ (4 x 40 mL). The combined organic phases were dried with MgSO$_4$ and the solvent removed under reduced pressure to give the crude product as a colorless oil. The crude product was purified by flash chromatography (10 : 1 Hex : EtOAc) to give the pure product as a colorless oil (4.0 g / 76% yield). $^1$H NMR (CDCl$_3$) $\delta$ 1.37 (s, 3H) 1.42 (s, 3H), 3.58 (m, 1H), 3.74 (m, 1H), 4.05 (m, 1H), 4.24 (m, 1H). $^{13}$C NMR $\delta$ (CDCl$_3$) 25.6, 26.8, 62.5, 65.0, 77.1, 108.4.
A solution of alcohol 127 (750 µL / 6.30 mmol) and pyridine (627 µL / 7.75 mmol) in CH₂Cl₂ (15 mL) was cooled to -60° C before the addition of triflic anhydride at the same temperature. The resulting solution was stirred at -60° C for 30 minutes before allowing to warm to room temperature over one hour. The organic layer was washed with brine (1 x 6 mL), dried with MgSO₄, and concentrated under reduced pressure to give a dark brown / purple oil which was used immediately in the next step without further purification.⁷⁷

A round bottom flask equipped with a pressure equalizing dropping funnel was flame dried while purging with Argon before charging with n-butyllithium (2.71 mL / 10.6 mmol of a 2.0 M solution in hexanes) in THF (15 mL). Dibromofluorophosphonate 40 (469 µL / 2.45 mmol) and TMSCl (2.45 mmol / 310 µL) in THF (10 mL) were added to the dropping funnel. The flask was cooled to -78° C before the dropwise addition of the contents of the dropping funnel at the same temperature. The resulting dark yellow / brown solution was stirred at -78° C for 15 minutes before the addition of triflate 128 (0.65 g / 2.45 mmol) in THF (8 mL) at the same temperature. This solution was stirred at -78° C for one hour before warming to 0° C followed by the dropwise addition of a solution of lithium ethoxide (prepared by adding 0.2 g of lithium wire to 12 mL of ethanol) and the resulting solution allowed to stir at 0° C for one hour before the solution was poured into 7 mL of saturated NH₄Cl. THF was removed under reduced pressure and the aqueous layer extracted with EtOAc (3 x 20 mL). The combined organics were dried with MgSO₄ and the solvent removed under reduced pressure to give the crude product which was purified by flash chromatography (8 : 1 Hex : EtOAc to 2 : 1 Hex : EtOAc) to give the pure
product as a slightly yellow oil (0.49 g / 70%). 1H NMR: (CDCl₃) δ 1.25 (m, 12H), 2.10 (m, 2H), 3.48 (m, 1H), 4.10 (m, 6H), 4.80 (m, 1H). 13C NMR: δ (CDCl₃) 16.5 (d, J = 3.2 Hz), 25.6, 26.8, 27.0, 33.8 (d, J = 19.75 Hz), 34.95, 62.9, 63.1 (d, J = 6.60 Hz), 63.5 (m), 68.8, 69.4, 71.7 (d, J = 14.89 Hz), 72.5 (d, J = 11.74 Hz), 83.8 (d, J = 17.75 Hz), 86.1 (dd, J = 7.73, 18.32 Hz), 88.4 (d, J = 17.46 Hz), 109.26. 31P NMR: (CDCl₃) δ 18.67 (d, J = 74.07 Hz), 18.35 (d, J = 75.30 Hz). 19F NMR (CDCl₃): δ -207.9 (m), -212.3 (m).

A round bottom flask charged with protected phosphonate 129 (100 μL / 0.39 mmol) was dried under high vacuum for four hours before sealing with a rubber septa followed by the dropwise addition of TMSBr (302 μL / 2.30 mmol) to the neat compound at room temperature. The rubber septa was immediately replaced with a yellow caplug stopper, and the resulting solution stirred at room temperature for four hours. Volatile materials were then removed; first on the rotovap and then on high vacuum for three hours. Water (1 mL) was then added, and the resulting solution was stirred overnight at room temperature. The resulting solution was then loaded onto a column of Dowex IX-8 resin (HCO₃⁻ form, prepared by washing the resin sequentially with di H₂O, 1 M NaHCO₃ and diH₂O until the pH was neutral) and eluted with 100 mM NH₄HCO₃. The eluant was then freeze dried to give the ammonium salt as a white powder. The free phosphonic acid was obtained by several cycles of freeze drying. 1H NMR: (D₂O) δ 1.65 (m, 2H), 3.25 (m, 2H), 3.65 (m, 1H), 4.65 (m, 1H). 13C NMR: (D₂O) 34.35 (d, J = 19.5 Hz), 64.74, 65.60, 68.10 (dd, J = 2.9, 11.2 Hz), 69.80 (dd, J = 2.3, 11.5 Hz), 89.45 (dd, J =
169.8, 133.45 Hz), 91.23 (dd, J = 169.5, 154.3 Hz). $^{31}$P NMR: (D$_2$O) $\delta$ 16.8 ($d$, J = 71.4 Hz), 17.0 ($d$, J = 70.11 Hz). $^{19}$F NMR: (D$_2$O) $\delta$ -203.75 (m), -207.8 (m).

A solution of diol (32 $\mu$mol / 6 mg), NAD$^+$ (50 mg / 80 $\mu$mol), glycerol-1-phosphate dehydrogenase (80 U), and oxidase from L. Sansfrancinsens (80 U) in deuterated KP$_i$ was gently stirred for 24 hours at room temperature. The reaction was monitored by $^{19}$F NMR. After 24 hours, integration between the two diastereomers changed from 0.8 : 1 to 0.4 : 1 (Figure 3-45).
Figure 3-1. Phosphate triester, phosphonate and α-fluorinated phosphonates

![Chemical structures of phosphate triester, phosphonate, α-fluorophosphonate, and α-diFluorophosphonate](image)

25. Phosphate Triester
26. Phosphonate
27. α-Fluorophosphonate
28. α-DiFluorophosphonate

Figure 3-2. Phosphate and phosphonic acid $pK_a$ values

![Chemical structures of phosphates and phosphonates with $pK_a$ values](image)

29. $pK_a = 6.5$
30. $pK_a = 7.5 - 8$
31. $pK_a = 6.5$
32. $pK_a = 5.5 - 6$

Figure 3-3. Dihedral angle comparison for phosphate and phosphonic acids

![Chemical structures with dihedral angles](image)

33
34
35
36

Figure 3-4. Bisphosphonate route to α-fluorophosphonates

![Chemical reaction diagram](image)

37 → 1) LDA / THF / -78°C → 2) Benzaldehyde → 46% → 38

84
Figure 3-5. Savignac’s routes to α-fluorophosphonates
Figure 3-6. Fluorination via nucleophilic and electrophilic sources
Figure 3-7. Methods for asymmetric $\alpha$-fluorophosphonate synthesis
Figure 3-8. Glycerol-3-phosphate analogues synthesized by O’Hagan

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
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<tr>
<td>61 X = O</td>
<td>0.20 mM</td>
</tr>
<tr>
<td>62 X = CH$_2$</td>
<td>0.18 mM</td>
</tr>
<tr>
<td>63 X = CHF</td>
<td>0.17 mM</td>
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<tr>
<td>64 X = CF$_2$</td>
<td>0.73 mM</td>
</tr>
</tbody>
</table>

Figure 3-9. O’Hagan’s Glycerol-1-phosphate dehydrogenase assay

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Figure 3-33. RuO₄ catalyzed alkene oxidation and phosphonate deprotection

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Figure 3-40. Future route to optically pure α-fluorophosphonic acids
Figure 3-41. Future synthetic methods
Figure 3-42. Proposed synthesis of optically pure $\alpha$-fluorophosphonate 63b

Figure 3-43. Proposed synthesis of $\alpha$-fluorophosphonic acid 63a
Figure 3-44. Proposed synthesis of α-fluorophosphonic acid carbohydrates
Figure 3-45. Decrease in starting material over a 24 hour period
Table 3-1. Fluorination conditions of carbonate

<table>
<thead>
<tr>
<th>Base / Solvent / Temperature</th>
<th>F⁺ Agent / Solvent / Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH / THF / 0° C</td>
<td>Selectfluor / THF / 0° C</td>
</tr>
<tr>
<td>NaH / THF / 0° C</td>
<td>Selectfluor / CH₃CN / 0° C</td>
</tr>
<tr>
<td>NaH / CH₃CN / 0° C</td>
<td>Selectfluor / CH₃CN / 0° C</td>
</tr>
<tr>
<td>NaH / CH₃CN / -10° C</td>
<td>2 equiv. Selectfluor / CH₃CN / -10° C</td>
</tr>
<tr>
<td>LDA / THF / -78° C to -10° C</td>
<td>Selectfluor / THF / -10° C</td>
</tr>
<tr>
<td>LDA / THF / -78° C to -10° C</td>
<td>Selectfluor / CH₃CN / -10° C</td>
</tr>
<tr>
<td>KH / THF / -10° C</td>
<td>Selectfluor / CH₃CN / -10° C</td>
</tr>
<tr>
<td>NaH / THF / -10° C</td>
<td>NFSI / THF / -10° C</td>
</tr>
<tr>
<td>LDA / THF / -78° C to -10° C</td>
<td>NFSI / THF / -10° C</td>
</tr>
<tr>
<td>KH / THF / -10° C</td>
<td>NFSI / THF / -10° C</td>
</tr>
</tbody>
</table>
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BIOGRAPHICAL SKETCH

Neil Stowe was born in Atlanta, GA. He went to Delta State University in Cleveland, MS where he received his B.S. in Chemistry in 2003. He came to the University of Florida in Fall, 2003. Upon completion of his Ph. D. he plans to work at the Scripps Research Institute in San Diego, California.