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## Design, synthesis, and activity of 2,3-diphosphoglycerate analogs as allosteric modulators of hemoglobin O<sub>2</sub> affinity

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### Abstract

Four phosphonate derivatives of 2,3-diphosphoglycerate (2,3-DPG), in which the phosphate group is replaced by a methylene or difluoromethylene, were successfully synthesized for use as allosteric modulators of hemoglobin (Hb) O<sub>2</sub> affinity. The syntheses were accomplished in four steps and the reagents were converted to their potassium salts to allow for effective binding with Hb in aqueous media. O<sub>2</sub> equilibrium measurements of the chemically modified Hbs exhibited P<sub>50</sub> values in the range 8.9–12.8 with Hill coefficients in the range of 1.5–2.4.

**Keywords:** Allosteric effector, bioisosterism, blood substitutes, hemoglobin-based oxygen carriers, synthesis, stroma-free

### Introduction

Development of a suitable red blood cell (RBC) substitute, which can be stored at ambient temperature and be infused safely regardless of recipient blood type, has been of significant interest to the scientific community. Efforts to find a safe and effective replacement for RBCs date back to the 17th century (Winslow 1995, Palmer et al. 2009). In recent years, the use of artificial O<sub>2</sub> carriers has received significant attention due to public concerns over the safety of blood products, complications from blood transfusion, and post-partum treatment of hemorrhage. Progress in the field was accelerated by the emergence of the human immunodeficiency virus (HIV) which is epidemic in the 1980s and the realization that infectivity was transmissible via blood, since the use of blood replacement fluids avoids many of the adverse consequences of blood transfusion (Schreiber et al. 1996).

Perfluorocarbons were the first class of materials to advance to human trials and receive FDA approval, while an alternate approach was based on cell-free hemoglobin (Hb). The use of cell-free Hb as a blood substitute is associated with two major problems. First, it suffers from a very short circulation lifetime in the blood stream, mainly due to its breakdown from a large tetrameric protein,  $\alpha_2\beta_2$ , into two

smaller  $\alpha\beta$  dimeric units, consequently facilitating its rapid renal elimination and afflicting considerable renal toxicity. Second, cell-free Hb exhibits a P<sub>50</sub> value of 12–14 mm Hg (partial pressure of O<sub>2</sub> at which half of the O<sub>2</sub> binding sites on the Hb molecule are saturated). This is significantly lower than that of whole blood which exhibits a P<sub>50</sub> of 27 mm Hg. With such a strong affinity for O<sub>2</sub>, Hb's ability to release oxygen to surrounding tissues is greatly reduced thus limiting its ability to act as an effective blood substitute (Bakker et al. 1992, Gould et al. 1992, Spahn 2000). The drawbacks of stroma-free Hb have been attributed to the loss of the natural allosteric effector, 2,3-disphosphoglycerate (2,3-DPG), upon its isolation from the red blood cell (Horton 1993, Astrup and Rorth 1971). This work seeks to develop novel analogs of 2,3-DPG that will maintain the integrity of the tetrameric protein and mimic the P<sub>50</sub> of whole blood.

Recently, phosphonates have been identified as important mimics of phosphates. This is particularly so in the field of phosphate chemistry, owing to the great importance of phosphate-containing molecules in biological processes (Thatcher and Campbell 1993). Unlike a phosphate group, the phosphonate linkage is not readily hydrolyzed in aqueous solution, and this unique property has made these compounds attractive as phosphate analogs in numerous applications (Engel 1977, Tozer and Herpin 1996, Blackburn 1981). The structural correspondence of C–C–P bonds with C–O–P bonds, although significantly dissimilar in their chemical characteristics, has provided an avenue to systematic chemical and biomedical studies of phosphonic acids and their derivatives (Wiemer 1997, Waschbüsch et al. 1997, Moonen et al. 2004, Palacios et al. 2005, Caplan et al. 1998, Stremmer and Poulter 1987, Chambers et al. 1990). The driving force for the development of analogs of the phosphate moiety is the unwanted lability of the ester P–O bond. There are numerous examples of enzyme inhibitors in the literature incorporating phosphonates as phosphate mimics (Burke et al. 1993). Furthermore, phosphonates have been used successfully to mimic monophosphate and triphosphate nucleotides (Blackburn and Kent 1981).

In this study, a series of analogs of 2,3-DPG (Figure 1) containing phosphate isosters were synthesized and characterized for use as site-directed affinity reagents for human Hb.

## Methods: experimental procedure

### Materials and methods

Reagents and chemicals were purchased from Fischer Scientific and Sigma-Aldrich. Commercial reagents and chemicals were used without further purification. Solvents were dried over sodium metal prior to use. Reagents used for preparation of buffers were analytical grade. Human Hb was obtained from human RBCs via tangential flow filtration (a).  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{31}\text{P}$  NMR spectra were obtained on a Bruker Avance 400 MHz Ultra Shield<sup>TM</sup>. Chemical shifts for  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz) were referenced to  $\text{CDCl}_3$  or  $\text{D}_2\text{O}$  and reported in ppm. FT-IR spectra were collected using a Nicolet Avatar-IR 370 or a Perkin Elmer series 100 spectrometer. Mass spectra were obtained using an Agilent Technologies 5973 inert mass selective detector. MALDI-TOF analyses were carried out using a Perkin Elmer Voyager-DE STR Biospectrometry workstation equipped with a two-stage acceleration source. Reaction progress was monitored using thin-layer chromatography (TLC) using Silica Gel 60 F254 glass-coated plates. Products were purified with column chromatography using 63–200  $\mu\text{m}$  mesh silica gel or fractional distillation under reduced pressure or recrystallization with acetonitrile.

### Diallyl phosphonate (1)

In a 500 mL two-neck round bottom flask fitted with an addition funnel, 17.45 mL (200 mmol) of phosphorus trichloride was dissolved in 150 mL of dry benzene at  $-10^\circ\text{C}$ . A mixture of 27.27 mL (400 mmol) of allyl alcohol and 55.64 mL (400 mmol) of triethyl amine was added slowly over a period of 1 hrs under vigorous stirring. After allowing the resulting mixture to warm to room temperature, additional 14.13 mL (208 mmol) of allyl alcohol was added. Then the reaction

was allowed to stir overnight at room temperature, the triethylamine hydrochloride precipitate was removed by filtration, and washed with benzene, with the washings being added to the liquid reaction product. The organic layer was washed with 100 mL of water, and then dried over anhydrous sodium sulfate. Benzene and other low-boiling impurities were removed under reduced pressure at a temperature less than  $50^\circ\text{C}$ . The diallyl phosphonate produced was recovered by reduced-pressure distillation at  $68^\circ\text{C}$ – $72^\circ\text{C}$  in 87% yield. Spectroscopic analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 4.5–4.7 (m, 4H) [ $\text{CH}_2\text{OP}$ ], 5.28–5.39 (dd, 2H) [ $\text{CH}_2=$ ], 5.41–5.59 (dd, 2H) [ $\text{CH}_2=$ ], 5.91–6.12 (m, 2H) [ $=\text{CH}$ ], 7.8 (d, 1H) [ $\text{PH}$ ];  $^{13}\text{C}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 63.3 [ $\text{CH}_2\text{O}$ ], 119.1 [ $\text{CH}_2=$ ], 130.1 [ $=\text{CH}$ ]; MS ( $m/z$ , %): 121 ( $\text{M}^+$ -41( $\text{CH}_2=\text{CH}-\text{CH}_2$ )), 105 ( $\text{M}^+$ -57( $\text{CH}_2=\text{CH}-\text{CH}_2\text{O}$ )), 79 (18), 80 (17), 57 ( $\text{M}^+$ -105 (2( $\text{CH}_2=\text{CH}-\text{CH}_2\text{O}$ ))), 41 ( $\text{M}^+$ -121( $\text{HPO}_3\text{C}_3\text{H}_5$ )).

### Tetraallyl 1,4-bisphosphonate butane (2)

Sodium hexamethyl disilazane (10.35 mL 2M in THF, 20 mmol) was added to a stirred solution of diallyl phosphonate (1), (3 g in 5 mL THF, 19.0 mmol) at room temperature. The reaction mixture was stirred for 2 hrs before dropwise addition of 1,4-dibromobutane (0.9 mL 7.5 mmol). The reaction was stirred overnight at room temperature affording the sodium bromide precipitate which was removed by filtration through Celite and washed with THF. The washings were added to the liquid reaction product. The solvent was evaporated at reduced pressure and purified via column chromatography (50/50, ethyl acetate:hexane) to afford brown oil with 78%. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 1.8–1.9 (dd, 4H) [ $\text{CH}_2\text{CH}_2\text{P}$ ], 1.95–2.1 (m, 4H) [ $\text{CH}_2\text{P}$ ], 4.5–4.7 (m, 8H) [ $\text{CH}_2\text{OP}$ ], 5.28–5.39 (dd, 4H) [ $\text{CH}_2=$ ], 5.41–5.59 (dd, 4H) [ $\text{CH}_2=$ ], 5.91–6.12 (m, 4H) [ $=\text{CH}$ ];  $^{13}\text{C}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 32.1 [ $\text{CH}_2\text{CHP}$ ], 35.1 [ $\text{CH}_2\text{P}$ ], 65.3 [ $\text{CH}_2\text{O}$ ], 119.1 [ $\text{CH}_2=$ ], 133.1 [ $=\text{CH}$ ]; MALDI M + 1 379.7379.

### Tetrapotassium 1,4-bisphosphonate butane (3)

To Compound 2 (1.5 g, 4.0 mmol) in 10 mL dichloromethane, potassium 2-ethylhexanoate (4.37 g, 24.0 mmol), tetrakis(triphenylphosphine)palladium(0) (0.36 g, 0.32 mmol), and triphenylphosphine (0.36 g, 1.52 mmol) were added and the mixture was stirred at  $20$ – $25^\circ\text{C}$  in the dark until all starting materials had reacted as determined by TLC. The reaction was then allowed to stir overnight at room temperature in the dark. The mixture was then diluted with 20 mL of ethyl acetate, and the solvent was evaporated. The residue was washed with ether, and dried in vacuo to afford the crude product. Trituration with ethyl acetate/methanol (95/5) gave the pure 3 in 42% yield as a water soluble hygroscopic white powder. Spectroscopic Analysis:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 1.2–1.3 (d, 2H) [ $\text{CH}_2\text{CH}_2\text{P}$ ], 1.3–1.4 (d, 2H) [ $\text{CH}_2\text{P}$ ];  $^{31}\text{P}$  NMR (100 MHz,  $\text{PPh}_3$ ,  $\delta$ ): 20; ESI-MS:  $m/z$  371.1017 ( $[\text{M} + \text{H}]^+$  calcd 369.8344).

### Diallyl ( $\alpha,\alpha$ -Difluoromethyl)phosphonate (4)

To a solution of diallyl phosphonate (1) (7 g, 43.21 mmol) in 10 mL THF at room temperature was added sodium bis(trimethyl)amide (23.9 mL of a 2 M solution in heptanes/ethylbenzene). The reaction mixture was allowed to stir for

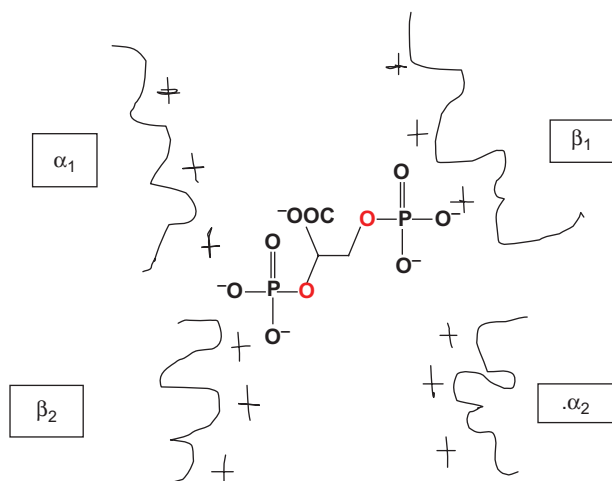


Figure 1. Electrostatic interaction between the anionic charges of DPG and the cationic charges of amino acids present on the  $\beta$  subunits of Hb.

40 min. Chlorodifluoromethane (Freon 22) was bubbled into the solution for 40 min, and the reaction mixture was stirred for additional 12 hrs under balloon atmosphere of chlorodifluoromethane. Precipitated NaCl was removed by filtration through Celite and the solvent was evaporated. The resulting liquid was distilled to yield **4** as a pale yellow liquid. Spectroscopic analysis:  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 4.71 (m, 4H) [ $\text{CH}_2\text{O}$ ], 5.28–5.39 (dd, 2H) [ $\text{CH}_2=$ ], 5.41–5.89 (br dd, 2H) [ $\text{CH}_2=$ ], 5.90 (dd, 1H) [ $\text{CF}_2\text{H}$ ], 5.91–6.12 (m, 2H) [=CH];  $^{13}\text{C}$  NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 68.3 [ $\text{CH}_2\text{O}$ ], 69.9 [ $\text{CF}_2$ ], 119.9 [ $\text{CH}_2=$ ], 140.1 [=CH]; MALDI:  $\text{M} + 1$  216.1101.

#### Tetraallyl 1,1,6,6-tetrafluorohexane-1, 6-bisphosphonate (**5**)

A solution of **4** (2 g, 9.4 mmol) in THF (10 mL) was cooled in a dry ice-acetone bath, and treated via syringe with a solution of lithium diisopropylamide (4.7 mL, 2 M in THF/*n*-heptane/ethylbenzene) and 1.63 mL hexamethylphosphoramide. After stirring for 2 hrs, solution was cannulated into a cold solution ( $-78^\circ\text{C}$ ) of 1,4-dibromobutane (2 g, 9.4 mmol) in THF (10 mL). The reaction mixture was allowed to warm to room temperature, concentrated, and quenched with 0.5 N HCl, and extracted with EtOAc ( $2 \times 100$  mL). The organic layer was washed with 1 N HCl ( $4 \times 10$  mL), saturated  $\text{NaHCO}_3$  (20 mL), and saturated NaCl (20 mL). The EtOAc solution was dried over  $\text{Na}_2\text{SO}_4$ , filtered, and evaporated. The oily residue was chromatographed on silica gel (90%  $\text{CH}_2\text{Cl}_2$ :10% MeOH as eluent) to give **5** as a yellow oil in 81% yield. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 1.36–1.48 (m, 4H) [ $\text{CH}_2\text{CH}_2\text{CF}_2$ ], 1.78–2.00 (m, 4H) [ $\text{CH}_2\text{CF}_2$ ], 4.50–4.71 (m, 4H) [ $\text{CH}_2\text{O}$ ], 5.28–5.41 (dd, 2H) [ $\text{CH}_2=$ ], 5.42–5.60 (br dd, 2H) [ $\text{CH}_2=$ ], 5.93–6.13 (m, 1H) [=CH];  $^{13}\text{C}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 32.10 [ $\text{CH}_2$ ], 35.10 [ $\text{CH}_2\text{CF}_2$ ], 68.3 [ $\text{CH}_2\text{O}$ ], 71.1(d) [ $\text{CF}_2$ ], 119.0 [ $\text{CH}_2=$ ], 133.1 [=CH].

#### Tetrapotassium 1,1,6,6-tetrafluorohexane-1, 6-bisphosphonate (**6**)

To a solution of **5** (3.0 g, 6.45 mmol) in dichloromethane (10 mL) was added potassium 2-ethylhexanoate (6.29 g, 34.0 mmol), tetrakis(triphenylphosphine)palladium(0) (1.0 g, 0.882 mmol), and triphenylphosphine (1.05 g, 3.8 mmol). The mixture was stirred at  $20$ – $25^\circ\text{C}$  in the dark until all starting materials had reacted as determined by TLC. The reaction was then allowed to stir overnight at room temperature in the dark. The mixture was diluted with 20 mL of ethyl acetate, and the solvent was evaporated. The residue was washed with ether, and dried in vacuo to afford the crude product. Triturating with ethyl acetate/methanol (95/5) gave **6** in 41% yield as a water soluble hygroscopic white powder. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{D}_2\text{O}$ ,  $\delta$ ): 1.20–1.27 (m, 4H) [ $\text{CH}_2\text{CH}_2\text{CF}_2$ ], 1.38–1.51 (m, 4H) [ $\text{CH}_2\text{CF}_2$ ];  $^{31}\text{P}$ -NMR (100 MHz,  $\text{PPh}_3$ ,  $\delta$ ): 5.5 (t,  $J = 99.8$  Hz); ESI-MS:  $m/z$  470.8910 [ $\text{M} + 1$ ] calcd 469.8280.

#### Tetraallyl $\alpha,\alpha$ -diphosphonate-*p*-xylene (**7**)

To a clean dry 250 mL round-bottomed flask was added diallyl phosphonate (**1**) (4.5 g, 28.5 mmol) and 10 mL dry THF. The resulting mixture was stirred for 10 min. Sodium hexamethyldisilazane (15.6 mL, 30 mmol) in THF was added

slowly and the mixture was stirred for an additional 2 hrs at room temperature. To the reaction mixture was added  $\alpha,\alpha$ -dibromobenzene (2.97 g, 11.25 mmol), the reaction mixture was stirred at room temperature for 16 hrs. Precipitated NaBr was removed by filtration through Celite, and the solvent was evaporated. The resulting red yellow oil was purified by column chromatography on silicagel and eluted with ethyl acetate/hexane (60% ethyl acetate: 40% hexane) to obtain **7** as orange oil with 80% yield. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 3.50–3.45 (d, 4H) [ $\text{CH}_2\text{P}$ ], 4.5–4.7 (m, 8H) [ $\text{CH}_2\text{OP}$ ], 5.28–5.39 (dd, 4H) [ $\text{CH}_2=$ ], 5.41–5.59 (dd, 4H) [ $\text{CH}_2=$ ], 5.91–6.12 (m, 4H) [=CH], 7.25–7.32 (dd, 4H);  $^{13}\text{C}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 31.0 [ $\text{CH}_2\text{P}$ ], 32.1 [ $\text{CH}_2\text{P}$ ], 65.3 [ $\text{CH}_2\text{O}$ ], 119.1 [ $\text{CH}_2=$ ], 128.0 [ $\text{CH}_2=$ , Ar], 131.0 [ $\text{CH}_2=$ , Ar], 133.1 [=CH]; MS ( $m/z$ ): ( $\text{M}-1$ ) $^+$  425.

#### Tetrapotassium $\alpha,\alpha$ -diphosphonate-*p*-xylene (**8**)

To compound **7** (6.0 g, 14.0 mmol) in dichloromethane (20 mL) was added potassium 2-ethylhexanoate (7.28 g, 39.92 mmol), tetrakis(triphenylphosphine)palladium(0) (1.2 g, 1 mmol), and triphenylphosphine (1.2 g, 4.5 mmol). The mixture was stirred at  $20$ – $25^\circ\text{C}$  in the dark until all starting materials had reacted as determined by TLC. The reaction was allowed to stir overnight at room temperature in the dark. The mixture was next diluted with 20 mL of ethyl acetate, and the solvent was evaporated. The residue was washed with ether and dried in vacuo to afford the crude product. Trituration with ethyl acetate/methanol (95/5) gave the pure **8** in 38% yield as a water soluble hygroscopic white powder. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 3.50–3.45 (d, 4H) [ $\text{CH}_2\text{P}$ ], 7.25–7.32 (dd, 4H); ESI-MS:  $m/z$  422.2236 [ $\text{M} + 1$ ] calcd 417.8344.

#### 3-bromo methylpropionate (**9**)

A solution of 3-bromopropionic acid (10 g, 0.065 mmol) in methanol (125 mL) was cooled to  $0^\circ\text{C}$  in a dried round bottom flask. Sulfuric acid (5 mL) was added to the cold solution. The reaction mixture was refluxed for 6 hrs and the solvent was evaporated. The residue was dissolved in  $\text{CH}_2\text{Cl}_2$  and washed with water, saturated sodium bicarbonate and sodium chloride. The combined organic layers were dried over anhydrous sodium sulfate. The organic solution was concentrated in vacuo to give brown oil. The resulting oil was purified by column chromatography on silica gel ( $\text{CH}_2\text{Cl}_2$ /hexane; 95:5) to give red oil with 63% yield. Spectroscopic Analysis:  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 2.85–2.91 (t, 2H) [ $\text{CH}_2\text{C}(\text{O})$ ], 3.5–3.59 (t, 2H) [ $\text{CH}_2\text{Br}$ ], 3.69–3.71 (s, 3H) [ $\text{OCH}_3$ ];  $^{13}\text{C}$ -NMR (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 27 [ $\text{CH}_2\text{C}(\text{O})$ ], 38 [ $\text{CH}_2\text{Br}$ ], 53 [ $\text{CH}_3\text{O}$ ], 171 [ $\text{C}=\text{O}$ ]; IR:  $1734\text{ cm}^{-1}$  [ $\text{C}=\text{O}$ ]; 166 [ $\text{M}-1$ ] $^+$ , 135 [ $\text{CH}_3\text{O}$ ] $^+$ , 87 [ $\text{M}-\text{Br}$ ].

#### Diallyl 3-phosphonate methylpropionate (**10**)

To a clean dry 250 mL round-bottomed flask was added diallyl phosphonate (**1**) (4.0 g, 24.6 mmol) and dry THF (10 mL). The resulting mixture was stirred for 10 min. Sodium hexamethyldisilazane in THF (12.9 mL, 25.8 mmol) was added dropwise and stirring was continued for additional 2 hrs at room temperature. Then 3-bromo methylpropionate (3.25 g, 19.5 mmol) was added slowly and the reaction mixture was



stirred at room temperature for 16 hrs. Precipitated NaBr was removed by filtration through Celite, and the solvent was evaporated. The resulting reddish yellow oil was purified by column chromatography on silicagel and eluted with ethyl acetate/hexane (50/50) to obtain **10** as brown red oil with 49% yield. Spectroscopic Analysis:  $^1\text{H-NMR}$  (400MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 2.85–2.91 (t, 2H) [ $\text{CH}_2\text{C}(\text{O})$ ], 3.5–3.59 (t, 2H) [ $\text{CH}_2\text{P}$ ], 3.69–3.71 (s, 3H) [ $\text{OCH}_3$ ], 4.5–4.7 (m, 8H) [ $\text{CH}_2\text{OP}$ ], 5.28–5.39 (dd, 4H) [ $\text{CH}_2=$ ], 5.41–5.59 (dd, 4H) [ $\text{CH}_2=$ ], 5.91–6.12 (m, 4H) [ $=\text{CH}$ ];  $^{13}\text{C-NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ) 27 [ $\text{CH}_2\text{C}(\text{O})$ ], 28 [ $\text{CH}_2\text{P}$ ], 53 [ $\text{CH}_3\text{O}$ ], 65.3 [ $\text{CH}_2\text{O}$ ], 119.1 [ $\text{CH}_2=$ ], 133.1 [ $=\text{CH}$ ], 171 [ $\text{C}=\text{O}$ ]; MS ( $m/z$ ): 249 ( $\text{M} + 1$ ) $^+$ .

### Dipotassium 3-phosphonate methylpropionate (**11**)

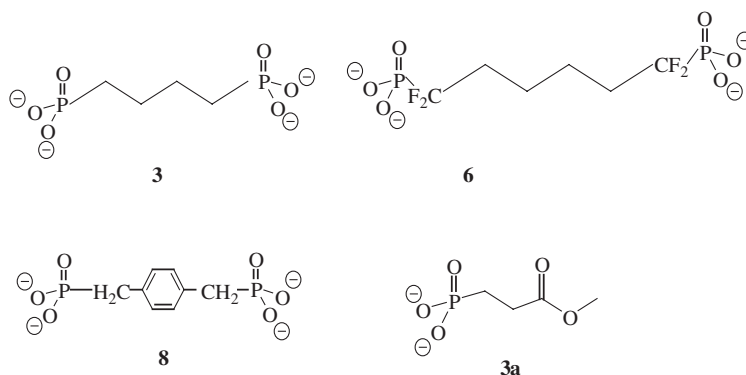
To a solution of the ester **10** (3.0 g, 12.1 mmol) in dichloromethane (10 mL) was added potassium-2-ethylhexanoate (6.29 g, 34.5 mmol), tetrakis(triphenylphosphine) palladium(0) (1.025 g, 0.882 mmol), and triphenylphosphine (1.05 g, 4 mmol). The mixture was stirred at 20–25°C in the dark until all starting materials had reacted as determined by TLC. The reaction was then allowed to stir overnight at room temperature in the dark. The mixture was then diluted with 20 mL of ethyl acetate, and the solvent was evaporated. The residue was washed with ether, and dried in vacuo to afford the crude product. Trituration with ethyl acetate/methanol (95/5) gave the pure **11** in 48% yield as a water soluble hygroscopic white powder. Spectroscopic Analysis:  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 1.61–1.69 (t, 2H) [ $\text{CH}_2\text{C}(\text{O})$ ], 2.39–2.42 (t, 2H), 3.60 (s, 3H);  $^{13}\text{C-NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 23 [ $\text{CH}_2\text{P}$ ], 24 [ $\text{CH}_2\text{C}(\text{O})$ ], 50 [ $\text{CH}_3\text{O}$ ], 174 [ $\text{C}=\text{O}$ ];  $^{31}\text{P}$  NMR

(100 MHz,  $\text{PPh}_3$ ,  $\delta$ ): 21; ESI-MS:  $m/z$  244.9383 ( $[\text{M} + 1]$  calcd. 243.9305).

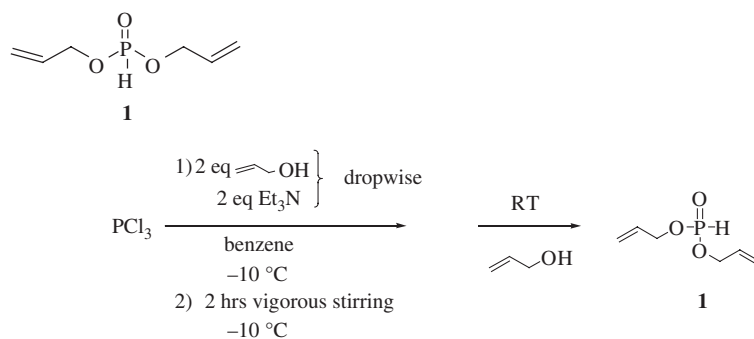
### $\text{O}_2$ Affinity and Cooperativity of Hb

The equilibrium  $\text{O}_2$  binding curve of Hb exposed to the 2,3-DPG analogs was measured using a Hemox-Analyzer (TCS Scientific Corporation, New Hope, PA) at 37°C. Samples were prepared by thoroughly mixing 50–100  $\mu\text{L}$  of Hb (> 300 mg/mL) with 5 mL of Hemox buffer (pH 7.4), at a 40:1 molar ratio of 2,3-DPG mimic to Hb, 20  $\mu\text{L}$  of Additive-A, 10  $\mu\text{L}$  of Additive-B and 10  $\mu\text{L}$  of anti-foaming agent, all purchased from TCS Scientific Corporation. The samples were allowed to saturate to a  $p\text{O}_2$  (i.e. partial pressure of  $\text{O}_2$ ) of  $145 \pm 2$  mm Hg using compressed air for about 45 mins. After giving the sample enough time to equilibrate, the gas stream was switched to pure  $\text{N}_2$  to deoxygenate the sample. The absorbance of oxygenated and deoxygenated Hb in solution was recorded as a function of  $p\text{O}_2$  via dual wavelength spectroscopy.  $\text{O}_2$ -Hb equilibrium curves were fit to a four-parameter ( $A_0$ ,  $A_\infty$ ,  $P_{50}$ ,  $n$ ) Hill model (Eq. 1), where  $Y$  is the fractional saturation of Hb ( $Y$  has no units and varies from 0–1, a value of 0 corresponds to fully deoxygenated Hb while a value of 1 corresponds to fully oxygenated Hb), while  $A_0$  and  $A_\infty$  represent the absorbance at 0 mm Hg and full saturation, respectively.  $P_{50}$  represents the  $p\text{O}_2$  at which the heme-binding sites in the Hb sample are 50% saturated with  $\text{O}_2$ , while  $n$  is the Hill coefficient, a measurement of cooperativity in  $\text{O}_2$  binding.

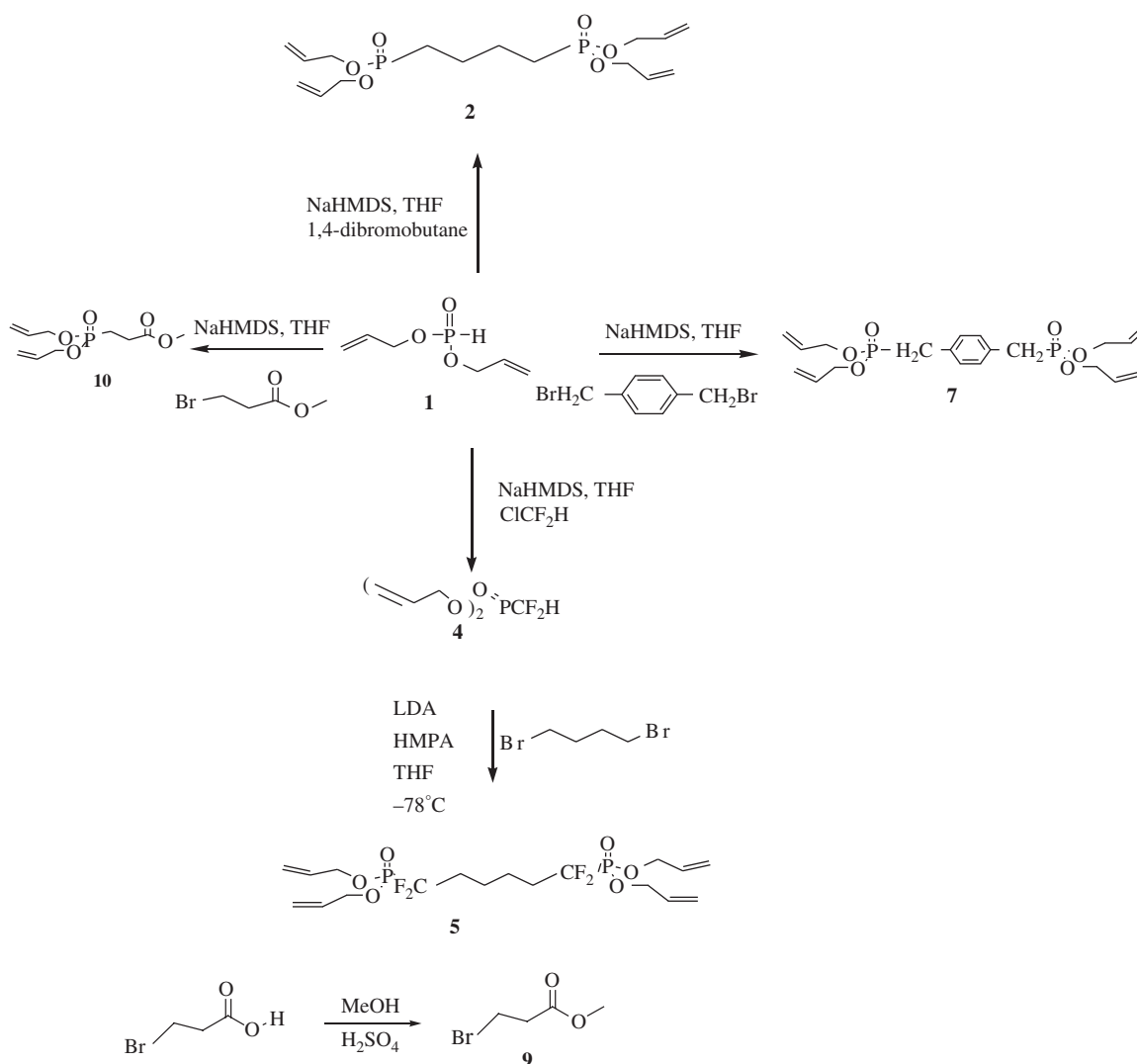
$$Y = \frac{Abs - A_0}{A_\infty - A_0} = \frac{p\text{O}_2^n}{p\text{O}_2^n + P_{50}^n} \quad (1)$$



Synthesized phosphonate analogs of 2,3-diphosphoglycerate.



Scheme 1. Synthesis of diallyl phosphonate.



Scheme 2. Synthesis of alkyl phosphonates.

The goodness of fit was verified by comparing the experimental data ( $Y = \frac{Abs - A_0}{A_\infty - A_0}$  versus  $pO_2$ ) with the curve fitted data ( $Y = \frac{pO_2^n}{pO_2^n + P_{50}^n}$  versus  $pO_2$ ).

## Results and discussion

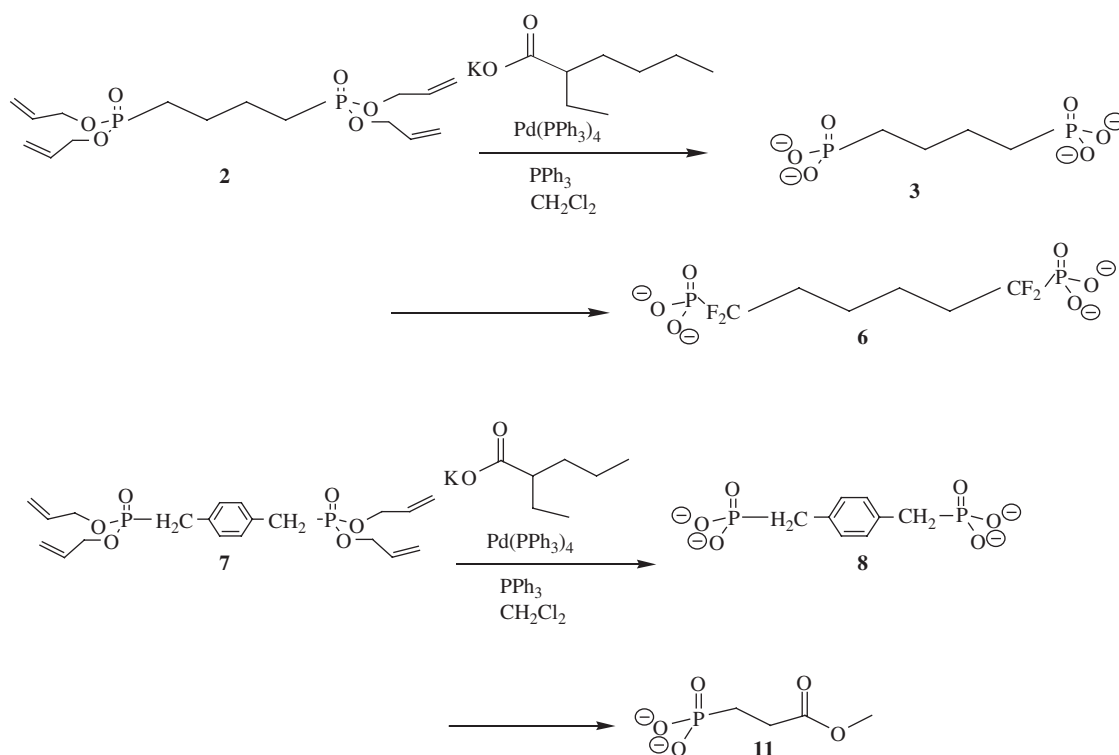
### Synthesis of Hb allosteric effectors

Initial attempts at synthesizing diallyl phosphonate from allyl alcohol and phosphorous trichloride via Kennedy's method resulted in low yields (Kennedy 1957). This prompted us to vary the reaction parameters which included solvent, temperature, base, order of addition, and reaction time. After several experiments, we obtained optimum results by carrying out the reaction at temperatures that ranged from  $-10$  to  $-20^\circ\text{C}$ . Furthermore, we found it necessary to stir the reaction mixture for approximately 2 hrs before addition of the third equivalent of allyl alcohol. The diallyl phosphonate was purified via reduced-pressure

distillation and isolated in yields ranging from 85% to 90% (Scheme 1).

Replacement of the P-O bond of 2,3-DPG was achieved by reaction of diallyl phosphonate with chlorodifluoromethane in the presence of sodiumhexamethyldisilazane to afford,  $\alpha$ -(diallyl difluoromethyl)phosphonate **4**. Subsequent deprotonation of **4** with LDA afforded the lithium salt which was reacted with 1,4-dibromobutane to provide the difluorinated alkyl phosphonate **5**. Deprotection of the allyl group gave the tetrapotassium salt **6** which was evaluated as an allosteric effector of Hb. The non-fluorinated phosphonate salts **3** and **8** were also synthesized by reacting two equivalents of diallyl phosphonate with the appropriate dihalide via the Michaelis-Becker reaction (Scheme 2) (Berkowitz and Sloss 1995). Deprotection of the allyl group gave the desired reagents in good yields.

Synthesis of the dipotassium 3-phosphonate methylpropionate allosteric effector **11** was also prepared from diallyl phosphonate. The diallyl phosphonate was reacted with NaHMDS and methyl 3-bromopropionate in a 1:1 molar



Scheme 3. Deprotection of the allyl group.

ratio. Deprotection gave the dipotassium salt in 90% yield (Scheme 3) (Kamber and Just 1985).

### Evaluation of Hb allosteric effectors

The ideal allosteric effector of Hb is a reagent that closely mimics the chemical structure of 2,3-DPG, while specifically targeting the  $\beta$ -cleft of Hb and causing the least conformational change in the native structure of Hb upon subsequent cross-linking. 2,3-DPG forms a salt bridge with the positively charged amino acid residues on the  $\beta$  globins and controls Hb  $O_2$  affinity and stabilizes the tetramer. These novel reagents carry two or more anions, which should guide them towards the  $\beta$  cavity to form a stable tetramer with tunable  $O_2$  affinity. Compounds **3**, **6** and **8** each consist of four anionic charges and are structurally comparable to DPG. Compound **11** encompasses an ester functional group at one end, in order to make a covalent bond with an amine group on one of the 2  $\beta$  globin chains.

Structural comparison shows that the four oxygen anions on **3**, **6** and **8** are at similar positions to each other as on 2,3-DPG. Although there are only four anionic charges on **3**, **6** and **8** compared to 2,3-DPG which has five anionic charges, the anions should direct these phosphonate reagents to the  $\beta$  cleft site to form a salt bridge with the positively charged amino acid residues in this region.

### Activities of phosphonate analogs of 2,3-DPG on Hb

The effect of phosphonate analogs of 2,3-DPG on the  $O_2$  affinity of human Hb was measured using a Hemox Analyzer. The activity of these phosphonate compounds, **3**, **6**, **8**, and **11**, as regulators of Hb  $O_2$  affinity was evaluated

on highly purified human Hb. The  $O_2$  affinities of these Hbs ranged from somewhat greater than to lower than that of unmodified, human Hb (control 12.1 torr). As shown in Table I, the  $P_{50}$  values range from 8.9 mm Hg for **3** to 12.8 mm Hg for **8**. Values for the Hill coefficient, which represents the cooperative effect of  $O_2$  binding to the four globin subunits in the Hb tetramer, are in the range of 1.5–2.6 compared to the control (cell-free Hb) with a value of 2.5. As seen in Figure 2 and represented in Table I, these compounds maintained the cooperativity of tetrameric Hb. The most significant and potentially useful results for  $O_2$  affinity and cooperativity were obtained using compound **11**, which combines covalent and electrostatic interactions to cross-link the  $\beta$  chains.

Hb modified with compound **3** exhibited an  $O_2$  affinity higher than that of unmodified Hb, but it is important to note that it is still considered to be in a range capable of delivering  $O_2$  to tissues (Macdonald and Winslow 1992). A new generation of chemically modified Hbs with even lower  $P_{50}$ s (i.e. high  $O_2$  affinity) and decreased cooperativity are

Table I.  $O_2$  Binding properties of the Hb exposed to 2,3-DPG analogs.

Compound	$P_{50}$ (mm Hg)	$n$
Control (cell-free Hb)	11.4	2.4
<b>3</b>	8.9	1.5
<b>6</b>	12.7	2.6
<b>8</b>	12.8	1.8
<b>11</b>	12.6	2.4

Conditions for  $P_{50}$  and  $n$  determination: 30  $\mu$ l of 400 mg/mL of Hb was added to 5 ml of Hemox solution (pH 7.4), 10  $\mu$ l of anti-forming solution, 10  $\mu$ l of additive B, 20  $\mu$ l of BSA (TCS Scientific) with the 2,3-DPG analog at an analog:Hb molar ratio of 40:1 at 37°C for 45 min.

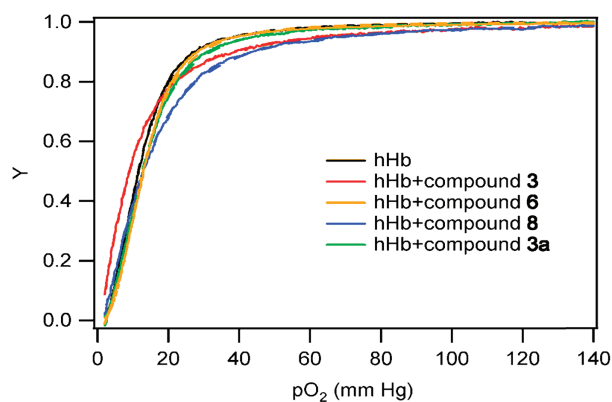


Figure 2. Oxygenation curve of Hb in the presence of **3**, **6**, **8**, and **11**.

under development as O<sub>2</sub> therapeutics based on the idea that they may enhance targeted delivery of O<sub>2</sub> to the tissues that need it most (Tsai et al. 2003, Wettstein et al. 2003, Winslow 2003). Compound **6** exhibited an O<sub>2</sub> affinity and cooperativity similar to the control of cell-free Hb. The *n* value of the Hb modified with compound **8**, is lower than that of unmodified Hb. One possible explanation for this may be by the presence of the aromatic rigid structure which might reduce movement of the protein that is vital for cooperativity of the subunits.

## Conclusion

In general, Hb modified with these phosphonate anions maintained cooperativity of the subunits. With the exception of compound **3**, all of them have exhibited low O<sub>2</sub> affinity compared to the Hb control, which is paramount for O<sub>2</sub> transport. Compound **11** yielded the most favorable result, since it maintained cooperativity while decreasing O<sub>2</sub> affinity. Based on this result and other literature references we deduced that electrostatic interactions alone may not be enough to give the desired interaction. It may be necessary to establish a covalent interaction between hemoglobin and the allosteric effector. In conclusion, these results show that the phosphonate analogs investigated in this study represent compounds that can regulate the O<sub>2</sub> affinity of Hb.

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## Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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## References

- Astrup P, Rorth M. 1971. Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status. Academic Press: New York.
- Bakker JC, Berbers GA, Bleeker WK, den Boer PJ, Biessels PT. 1992. Preparation and characterization of cross linked and polymerized hemoglobin solutions. *Biomater Artif Cells Immobilization Biotechnol.* 20:233–241.
- Berkowitz DB, Sloss DG. 1995. *J Org Chem.* 60:7047–7050.
- Blackburn GM, Kent DE. 1981. A novel synthesis of  $\alpha$ - and  $\gamma$ - fluoroalkylphosphonates. *J Chem Soc Chem Commun.* 11:511–513.
- Blackburn GM. 1981. *Chem Ind (London).* 5:134–138.
- Burke TR Jr., Smyth MS, Nomizu M, Otaka A, Roller PP. 1993. *J Org Chem.* 58:1336–1340.
- Caplan NA, Pogson CI, Hayes DJ, Blackburn GM. 1998. *Bioorg Med Chem Lett.* 8:515–520.
- Chambers RD, O'Hagan D, Lamont RB, Jain SC. 1990. The difluoromethylene phosphonate moiety as a phosphate mimic: X-ray structure of 2-amino-1,1-difluoroethylphosphonic acid. *Chem Commun.* 15:1053–1054.
- Engel R. 1977. *Chem Rev.* 77:349–367.
- Gould SA, Sehgal LR, Sehgal HL, Moss GS. 1992. Artificial blood: current status of hemoglobin solutions. *Crit Care Clin.* 8:293–309.
- Horton H, Moran L, Ochs R, Rawl J, Scrimgeour K. 1993. *Principles of Biochemistry.* New Jersey: Neil Patterson.
- Kamber M, Just G. 1985. *Can J Chem.* 63:825.
- Kennedy J. 1957. Patent Brit 778,077. *Chem Abstr.* 51:17980b.
- Macdonald VW, Winslow RM. 1992. Oxygen Delivery and myocardial function in rabbit hearts perfused with cell-free hemoglobin. *J Appl Physiol* 72:476–483.
- Moonen K, Laureyn I, Stevens CV. 2004. *Chem Rev.* 104:6177.
- Palacios F, Alonso C, de los Santos JM. 2005. *Chem Rev.* 105:899.
- Palmer AF, Sun G, Harris DR. 2009. Tangential flow filtration of hemoglobin. *Biotechnol Prog.* 25:189–199.
- Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. 1996. The risk of transfusion-transmitted viral infections. The retrovirus epidemiology donor study. *N Engl J Med.* 334:1685–1690.
- Spahn DR. 2000. Current status of artificial oxygen carriers. *Adv Drug Deliv Rev.* 40:143–151.
- Stremmel KE, Poulter CDJ. 1987. *Am Chem Soc.* 109:5542–5544 (geranyl diphosphate analogs as FDP synthase pseudo-substrates).
- Thatcher GRJ, Campbell AS. 1993. *J Org Chem.* 58:2272–2281.
- Tozer MJ, Herpin TF. 1996. *Tetrahedron.* 52:8619–8683.
- Tsai A, Vandergriff KD, Intalietta M, Winslow RM. 2003. Targeted O<sub>2</sub> delivery by low-P50 hemoglobin: a new basis for O<sub>2</sub> therapeutics. *Am J Physiol Heart Circ Physiol.* 285:H1411–H1419.
- Waschbüsch R, Carran J, Marinetti A, Savignac P. 1997. *Chem Rev.* 97:3401.
- Wettstein R, Tsai AG, Winslow RM. 2003. Resuscitation with poly(ethylene glycol)-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters. *Crit Care Med.* 31:1824–1830.
- Wiemer DF. 1997. *Tetrahedron.* 53:16609.
- Winslow RM. 1995. Blood substitutes, a moving target. *Nat Med.* 1:1212–1215.
- Winslow RM. 2003. Current status of blood substitute research towards a new paradigm. *J Intern Med.* 253:508–517.