Supporting Information

Regioisomeric family of novel fluorescent substrates for SHIP2

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EXPERIMENTAL PROCEDURES:

CHEMICAL SYNTHESIS:

Preparation of previously reported benzene polyphosphates. The phosphate- and hydroxyl-substituted benzenes were supplied by the Potter group, Department of Pharmacology, University of Oxford. The synthesis of compounds (1) to (8) was as described in previous reports [1, 2].

General: Chemicals were supplied by Alfa Aesar, Acros, Aldrich and Fluka. TLC, thin-layer chromatography was performed on Merck TLC aluminium sheets silica 60 F_{254}. Products were detected by dipping the TLC plate in a solution of phosphomolybdic acid in ethanol, then heating the plate until the compound develops. Organic solutions were dried over $MgSO_4$. It is assumed that *m*CPBA used in oxidation reactions is 100% for calculation purposes only and was always used in excess. Flash chromatography was carried out on Fisher Scientific Silica 60A (particle size 35–70 micron). Final compounds were judged by ^{1}H and ^{31}P NMR spectroscopic methods, purified by ion exchange chromatography, and were used in all biological evaluations as their triethylammonium salts and were greater than 95% pure by NMR. Ion exchange chromatography was performed on an LKB-Pharmacia Medium Pressure Ion Exchange Chromatograph using Q-Sepharose Fast Flow with gradients of triethylammonium bicarbonate (TEAB, 0→2.0 M) as eluent. Column fractions containing the benzene polyphosphates were identified by U.V. spectroscopy at 254 nm and quantified for total phosphate by a modification of the Briggs test[3]. NMR spectra (proton frequency 270 or 400 MHz) were referenced against $SiMe_4$, and HDO. The ^{31}P NMR shifts were measured in ppm relative to external 85% phosphoric acid. Melting points (uncorrected) were determined using a Reichert-Jung Thermo Galen Kofler block. Mass spectra were recorded using electrospray techniques.

Preparation of benzene polyphosphates (9) and (10).

1,2-Dihydroxybenzene 3,4-bisphosphate (9):

4,5-Dibenzyloxy-2-ethoxy-benzo[1,3]dioxole (13) A mixture of the aldehyde (**11**)[2] (3.53 g, 11.75 mmol) and *m*CPBA (2.42 g, 20 mmol) was stirred in dry dichloromethane (100 mL) for 20 h. TLC (hexane–ether, 2 : 1) revealed a new product R_f = 0.36 (same as the aldehyde) for the formate ester. The remaining solid was filtered off and washed with dichloromethane (100 mL). The organic layer was washed with 10% solution of sodium metabisulphite (2 × 100 mL), 10% aqueous solution of sodium hydrogen carbonate (100 mL) and dried over $MgSO_4$ to give the crude formate derivative (3.51 g). The formate ester derivative was dissolved in warm methanol (50 mL) and dry K_2CO_3 (2.0 g, 14.5 mmol) was added and the mixture was stirred for 1.5 h at 40 °C after which a new product had formed R_f = 0.32 for the phenol **(12)**. This intermediate was purified by flash chromatography (ether-hexane, 1:3) to give the phenol (2.78 g, 81 %). The colorless compound was dissolved in DMF (100mL) and K_2CO_3 (2.76 g, 20 mmol) was added followed by benzyl bromide (2.38 mL, 20 mmol) and the solution which was stirred overnight at 90 °C. After this time the mixture was cooled and the DMF was evaporated to give a red-brown oil which was partitioned between water and ether (100 mL of each). The solvent was evaporated and the crude product was purified by flash chromatography (ether-hexane, 1:3) to give the pure title compound (1**3**) as a colorless oil, (3.285 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) 1.26 (3 H, t, *J* 7.0 Hz, OCH_2CH_3), 3.56–3.72 (2 H, m, OCH_2CH_3), 5.05 (2 H, s, CH_2Ph), 5.30 (2 H, s, CH_2Ph), 6.44 (1 H, d, *J* 8.6 Hz, C*H*, Ar), 6.47 (1 H, d, *J* 8.6 Hz, C*H*, Ar), 6.86 (s, 1 H, C*H* methine), 7.25–7.49 (10 H, m, 2 × CH_2Ph); ^{13}C NMR (100 MHz, $CDCl_3$) 14.77 (q, OCH_2CH_3), 58.90 (t, OCH_2CH_3), 72.41 (t, CH_2Ph), 74.12 (t, CH_2Ph) 101.27 (d, methine *C*H), 107.98 (d, *C*H, Ar), 119.66 (d, *C*H, Ar), 127.80, 128.12, 128.27, 128.57, 128.65, 128.73, 128.93 (7 d, *C*H, CH_2Ph), 132.85, 137.20, 137.29, 137.51, 141.92, 146.83 (C_q, CH_2Ph and Ar); (HRMS,

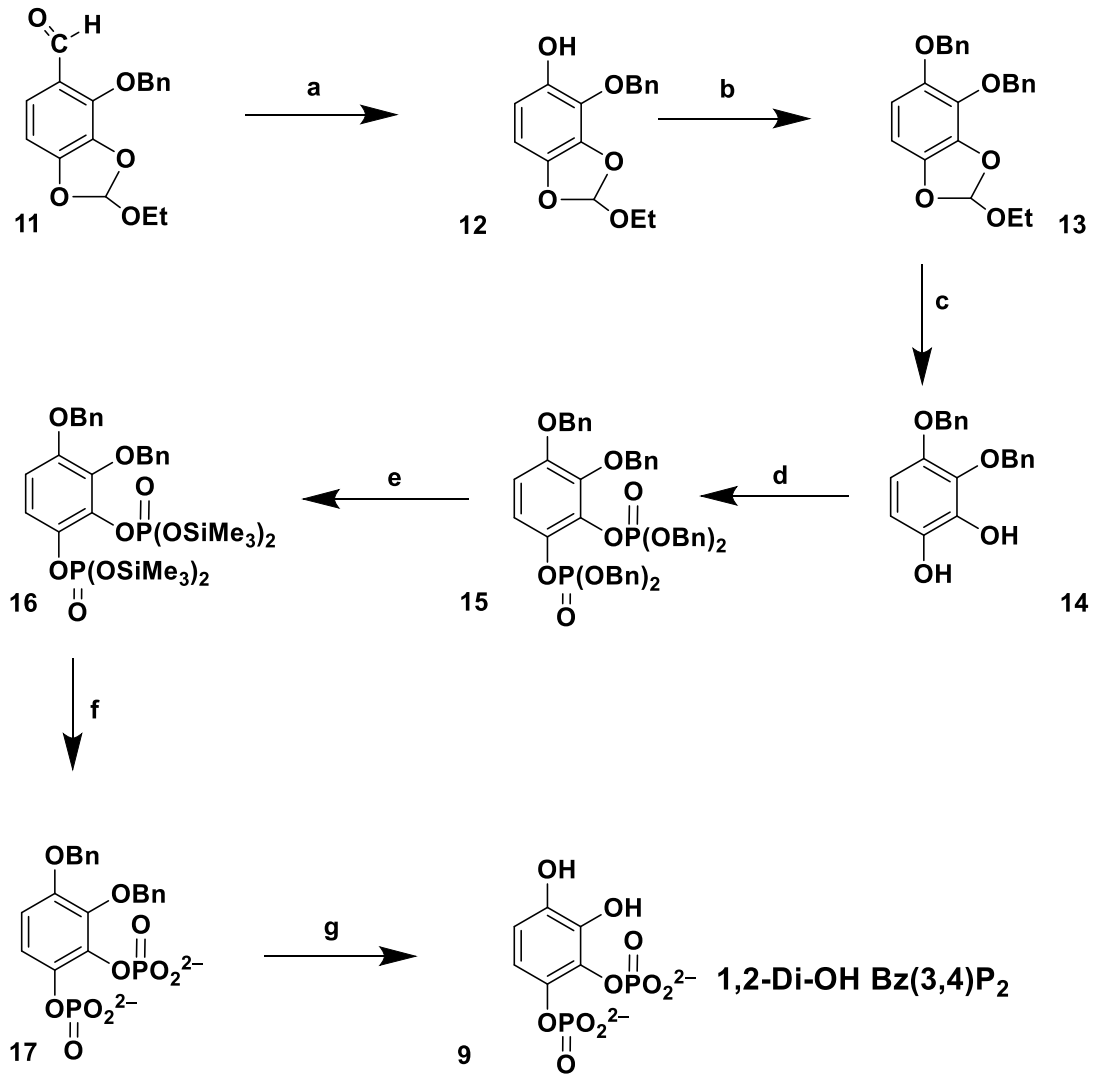
ES$^+$) *m/z* Calcd for $C_{23}H_{23}O_5$ $[M + H]^+$ 379.1540. Found 379.1537. Calcd for $C_{23}H_{22}O_5$ C, 73.00, H, 5.86. Found C, 73.0, H, 5.89.

1,2-Dibenzyloxy-3,4-dihydroxybenzene (14) 4,5-Dibenzyloxy-2-ethoxy-benzo[1,3]dioxole (**11**) (1.32 g, 3.49 mmol), was dissolved in MeOH (50 mL) and concentrated hydrochloric acid (5 drops) was added to give a colourless solution which was then stirred for 1.5 h after which TLC, (ether-hexane, 2:1) revealed a new product, R_f = 0.40. Triethylamine, (40 drops) were added until the acidic solution was neutralised. The solvents were evaporated and the remaining yellow residue was partitioned between ether and water (100 mL of each). The organic layer was evaporated to give a thick yellow syrup which was purified using ether-hexane (2:1) to give the pure diol (**14**) as a yellow syrup, (0.905 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) 5.09 (2 H, s, C*H*$_2$Ph), 5.12 (2 H, s, C*H*$_2$Ph), 4.80–5.80 (br, D_2O ex. 2 × Ar-O*H*), 6.47 (1 H, d, *J* 9.0 Hz, C*H*, Ar), 6.60 (1 H, d, *J* 9.0 Hz, C*H*, Ar), 7.25–7.47 (10 H, m, 2 × CH_2*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$) 71.60 (t, *C*H$_2$Ph), 75.50 (t, *C*H$_2$Ph), 105.67 (d, *C*H, Ar), 109.53 (d, *C*H, Ar), 127.51, 127.94, 128.51, 128.55, 128.61, 128.67 (6 d, *C*H, CH_2*Ph*), 135.55, 137.06, 138.76, 145.23 (C_q, CH_2*Ph* and Ar); (HRMS, ES$^+$) *m/z* Calcd for $C_{20}H_{19}O_4$ $[M + H]^+$ 323.1278. Found 323.1278. Calcd for $C_{20}H_{18}O_4$ C, 74.52, H, 5.63. Found C, 74.6, H, 5.72.

1,2-Dibenzyloxy-3,4-bis(dibenzyloxyphosphoryloxy)benzene (15) A mixture of carbon tetrachloride, (1.93 mL, 20 mmol), *N*,*N*-diisopropylethylamine (1.46 mL, 8.4 mmol), *N*,*N*-dimethylaminopyridine (49 mg, 0.4 mmol) and dibenzylphosphite (1.33 mL, 6.0 mmol) was stirred for 15 min at −10 °C in acetonitrile (20 mL) and the solution remained colourless[4]. 1,2-Dibenzyloxy-3,4-dihydroxybenzene (322 mg, 1 mmol), dissolved in acetonitrile, (8 mL) was then added dropwise to the phosphorylating mixture over 10 min at −10 °C (dry ice alone) and the mixture was stirred for a further 1 h. The solvents were evaporated and the remaining syrup was dissolved in dichloromethane (50 mL), washed with water (50 mL), dried ($MgSO_4$) and the title compound was purified by flash chromatography (ether−hexane 5:1), R_f = 0.21, to give the product as a colourless syrup (646 mg, 77 %). ^{1}H NMR (400 MHz, $CDCl_3$) 5.02–5.14 (12 H, m, 2 × ArOC*H*$_2$Ph and 4 × ArOP(O)OC*H*$_2$Ph), 6.73 (1 H, d, *J* = 9.4 Hz, C*H*, Ar), 7.12–7.42 (31 H, m, 2 × ArOCH$_2$*Ph*, 4 × ArOP(O)OCH$_2$*Ph*, C*H* Ar); ^{13}C NMR (100 MHz, $CDCl_3$) 69.82, 69.87, 69.98, 70.04, 71.44, 75.24 (6 t, 2 × ArO*C*H$_2$Ph and 4 × ArOP(O)O*C*H$_2$Ph), 110.18, 115.12 (2 d, 2 × *C*H, Ar), 127.53, 127.78, 127.98, 128.00, 128.14, 128.21, 128.23, 128.36, 128.50, 128.60, 128.62 (11 d, *C*H, ArOCH$_2$*Ph*, ArOP(O)O*C*H$_2$Ph), 135.38, 135.45, 135.68, 135.76, 136.38, 137.08, 141.60, 150.00 (C_q, ArOCH$_2$*Ph*, ArOP(O)OCH$_2$*Ph* and Ar); (HRMS, ES$^+$) *m/z* Calcd for $C_{48}H_{44}O_{10}P_2$ $[M + Na]^+$ 865.2302. Found 865.2284. Calcd for $C_{48}H_{44}O_{10}P_2$ C, 68.40, H, 5.26. Found C, 68.5, H, 5.32.

1,2-Dihydroxybenzene 3,4-bisphosphate (9) 1,2-Dibenzyloxy-3,4-bis(dibenzyloxy- phosphoryloxy) benzene (**5**) (183 mg, 217 μmol), was dissolved in dry $CDCl_3$ (5 mL), dry 2,4,6-collidine (0.39 mL, 3.0 mmol) was then added and the solution was stirred over an atmosphere of nitrogen. Bromotrimethylsilane (0.4 mL, 3 mmol) was also added and the solution was stirred for 2.5 h at room temperature to give intermediate (**16**). The solvents were evaporated and the reaction mixture was quenched using a mixed solvent of D_2O–2 M TEAB (2:1, 3 mL). The mixture was diluted with water (100 mL) and 1,2-dibenzyloxybenzene 3,4-bisphosphate (**17**) was purified by ion exchange chromatography using Q-Sepharose Fast Flow and a gradient of triethylammonium bicarbonate (TEAB) 0→2.0 M. The product eluted between 0.7 and 1.2 M TEAB buffer and isolated as the triethylammonium salt which was then used in the next step. The remaining 1,2-dibenzyloxybenzene 3,4-bisphosphate **(17)** was dissolved in water (10 mL) and stirred for 18 h in the presence of palladium hydroxide (300 mg) under an atmosphere of hydrogen. The solution was filtered through a PTFE syringe filter to remove the palladium hydroxide and the solvents

were evaporated to provide the title compound **(9)** (83 μmol) in 38% yield as quantified by the Briggs test[3]. ^{1}H NMR (400 MHz, D_2O/ 20 ºC) 6.46 (1 H, d, *J* = 9.0 Hz, Ar-*H*), 6.63 (1 H, d, *J* = 8.6 Hz, Ar-*H*). ^{31}P NMR (161 MHz, D_2O) 2.99 (1 P, s, Ar-O*P*O_3^{2-}), −1.38 (1 P, s, Ar-O*P*O_3^{2-}). (HRMS, ES^-) *m/z* Calcd for $C_6H_7O_{10}P_2$ $[M - H]^-$ 300.9520. found 300.9519.



Scheme 1

Reagents & Conditions: (a) *m*CPBA, CH_2Cl_2, purify, then K_2CO_3, MeOH, (81%); (b) K_2CO_3, DMF, BnBr, 90°C, (90%); (c) conc HCl (5 drops), MeOH, 1.5 h, (85%); (d) CCl_4, DMAP, DIPE, CH_3CN, −10°C, $(BnO)_2P(O)H$ (77%); (e & f) TMSBr, CH_2Cl_2, 2,4,6-collidine, 2.5 h, ion exchange; (g) $Pd(OH)_2$, H_2, 18 h, 38%.

1,3-Dihydroxy-2,4-bisphosphate (10):

2-Benzyloxy-3,4-dihydroxybenzaldehyde (18) A mixture of 4-Benzyloxy-2-ethoxy-benzo[1,3]dioxole-5-carbaldehyde (**11**)[2] (4.00 g, 14.06 mmol) was dissolved in MeOH (40 mL) and water (9 mL) and concentrated HCl (1 mL) was then added and the mixture was stirred for 19 h (overnight) at room temperature. The solvents were evaporated and the residue was partitioned between ether and water (100 mL of each). The yellow organic solution was dried and evaporated to give a thick yellow syrup, R_f = 0.29 (ether–hexane 2:1). The product was purified by flash chromatography (ether–hexane 1:1 → 2:1) and was isolated as an orange–yellow syrup (3.08 g, 90%). A small amount was then further purified by flash chromatography (CH_2Cl_2–CH_3CN, 100% to 2:1) to give a pale yellow syrup which crystallized from diisopropyl ether–hexane. (Note: overnight in the fridge a large single crystal appeared 8 × 8 × 3 mm). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.10 (2 H, s, ArOC*H*$_2$Ph), 5.92–6.72 (2 H, br, D_2O ex. 2 × Ar-O*H*), 6.78 (1 H, d, *J* 8.6 Hz, C*H*, Ar), 7.33–7.38 (6 H, m, CH_2*Ph*, C*H*, Ar), 9.94 (1 H, Ar-C*H*O); ^{13}C NMR (100 MHz, $CDCl_3$) 77.79 (t, ArO*C*H$_2$Ph), 111.73 (d, *C*-H, Ar), 122.44 (s, C_q, Ar), 123.21 (*C*-H, Ar) 128.70, 128.84, 128.96 (d, CH_2*Ph*), 135.88, 136.69 (s, C_q, CH_2*Ph*), 148.62, 151.32 (s, C_q, Ar), 189.56 (d, Ar-*C*HO). (HRMS, ES$^+$) *m/z* Calcd for $C_{14}H_{13}O_4$ $[M + H]^+$ 245.0808. found 245.0809.

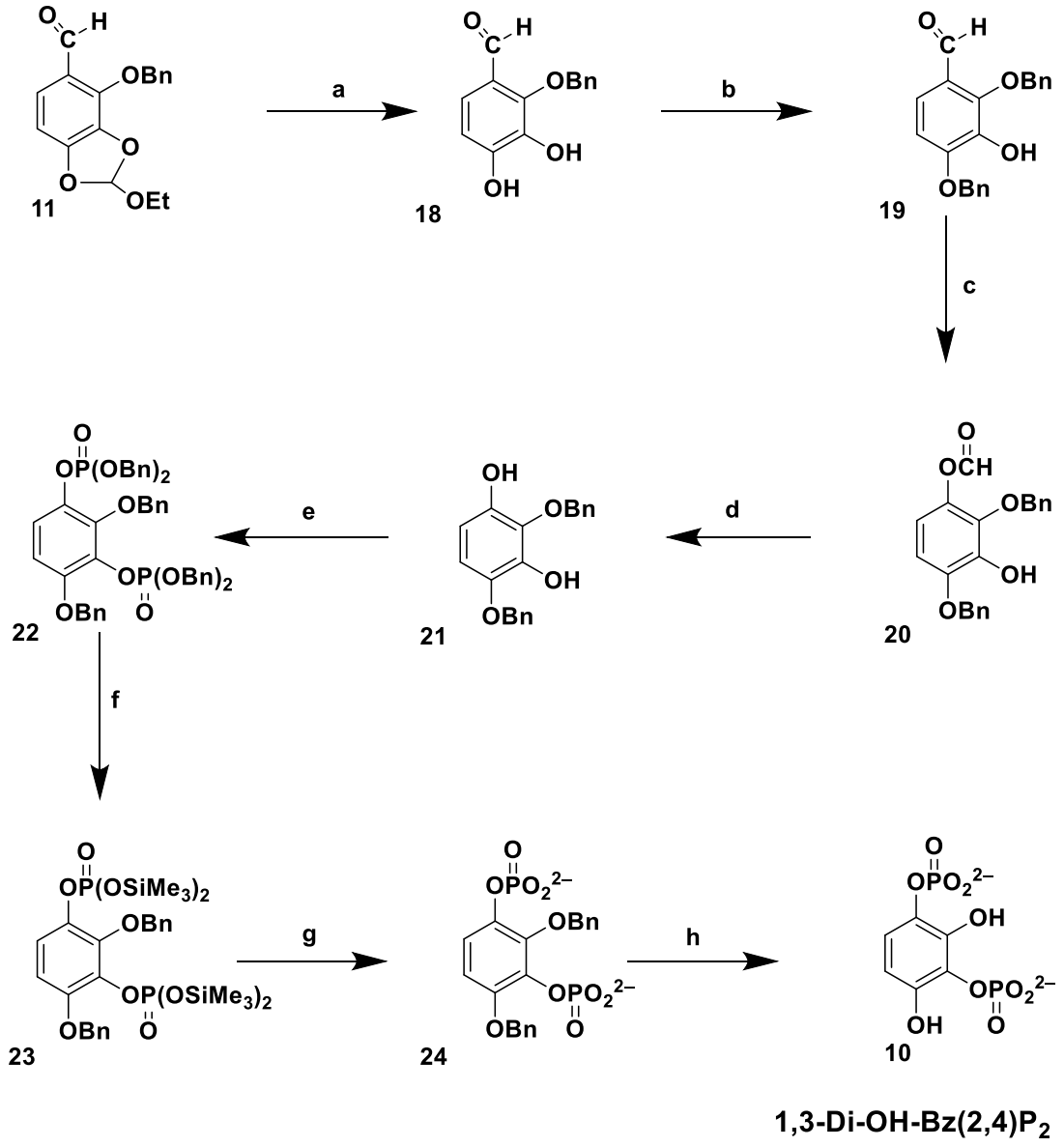
2,4-Bis(benzyloxy)-3-hydroxybenzaldehyde (19) A mixture of 2-benzyloxy-3,4-dihydroxybenzaldehyde **(18)** (2.016 g, 8.25 mmol), pre-dried $NaHCO_3$ (830 mg, 9.9 mmol)[5], benzyl bromide (1.08 mL, 9.075 mmol) and potassium iodide (137 mg, 0.825 mmol) were heated under reflux in acetonitrile (100 mL) for 17 h. The solvents were evaporated and CH_2Cl_2 and 1M aqueous hydrochloric acid (100 mL of each) were added to the residue. The organic layer was washed with water (100 mL) then dried and the solvent was evaporated. Flash chromatography (CH_2Cl_2) gave the desired product (1.247 g, 48 %) check (R_f =0.46 ether-hexane 2:1) (from ethyl acetate-hexane) together with the known tris-benzyloxy- derivative (851 mg, 24 %) (R_f =0.60 ether-hexane 2:1), (from hexane) and some starting material (not isolated) (R_f =0.29 ether-hexane 2:1). Mp = 100–101 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19, 5.24 (4 H, 2 s, 2 × ArOC*H*$_2$Ph), 5.78 (1 H, s, D_2O ex., Ar-O*H*), 6.79 (1 H, d, *J* 8.6 Hz, C*H*, Ar), 7.33–7.42 (11 H, m, 2 × CH_2*Ph*, C*H*, Ar), 10.12 (1 H, Ar-C*H*O); ^{13}C NMR (100 MHz, $CDCl_3$) 71.41, 76.08 (2 t, ArO*C*H$_2$Ph), 107.70, 119.96 (2 d, *C*-H, Ar), 123.93 (s, C_q, Ar), 127.85, 128.54, 128.60, 128.64, 128.71, 128.83 (d, CH_2*Ph*), 135.33, 136.40, 138.48 (s, C_q, CH_2*Ph*), 148.58, 151.80 (s, C_q, Ar), 189.13 (d, Ar-*C*HO); (HRMS, ES$^+$) *m/z* Calcd for $C_{21}H_{19}O_4$ $[M + H]^+$ 335.1278. found 335.1270.

1,3-Bis(benzyloxy)benzene-2,4-diol (**21**). *m*CPBA (1.21 g, 7.0 mmol) was added to a solution of 2,4-Bis(benzyloxy)-3-hydroxybenzaldehyde (**19**) (10 g, 3.29 mmol) in dry CH_2Cl_2 (50 mL) and the mixture was stirred for 21 h at room temperature. The yellow solution was washed with an aqueous solution of 10% sodium metabisulphite (2 × 50 mL), a saturated solution of sodium hydrogen carbonate (1 × 50 mL), acidified with 1 M aqueous HCl (150 mL) and finally washed with water (50 mL). The organic layer was dried and the solvent was evaporated to give the crude formate ester (R_f = 0.60, ether-hexane, 2:1) as a yellow solid which was purified by flash chromatography (ether–hexane, 2:1) to give the formate ester (**15**) as a colourless syrup. The formate ester was dissolved in a mixed solvent (MeOH 50 mL) and stirred for 16 h in the presence of Amberlyst (1.0 g). TLC indicated a new compound with a lower R_f = 0.46 (ether-hexane, 2:1, crude). The Amberlyst was filtered off over a bed of celite and the organic solution was concentrated. The title compound **21** was purified by flash chromatography using CH_2Cl_2 as eluent R_f = 0.40 (CH_2Cl_2) as a pale yellow solid (m.p. 61–62 ºC, not recrystallized) 824 mg (78 %). A small

portion was recrystallized from diisopropyl ether–hexane to give the title compound as an off white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.04, 5.16 (4 H, 2 s, 2 × ArOC*H*$_2$Ph), 5.30 (1 H, s, D_2O ex., Ar-O*H*), 5.72 (1 H, s, D_2O ex., Ar-O*H*), 6.38 (1 H, d, *J* 9.0 Hz, C*H*, Ar), 6.60 (1 H, d, *J* 9.0 Hz, C*H*, Ar), 7.35–7.44 (10 H, m, 2 × CH_2*Ph*); ^{13}C NMR (100 MHz, $CDCl_3$) 72.27, 75.06 (2 t, ArO*C*H$_2$Ph), 104.32, 108.38 (2 d, *C*-H, Ar), 127.90, 128.37, 128.55, 128.67, 128.69 (d, CH_2*Ph*), 133.17, 136.54, 137.05, 139.12, 140.20, 144.17 (s, C_q, CH_2*Ph*, Ar); (HRMS, ES$^+$) *m/z* Calcd for $C_{20}H_{19}O_4$ $[M + H]^+$ 323.1278. found 323.1265, calcd for $C_{20}H_{18}O_4$ C 74.52, H 5.63; found: C, 74.7, H, 5.68.

1,3-Bis(benzyloxy)benzene-2,4-bis(dibenzyloxyphosphoryloxy)benzene (22). A mixture of carbon tetrachloride, (1.93 mL, 20 mmol), *N*,*N*-diisopropylethylamine (1.46 mL, 8.4 mmol), *N*,*N*-dimethylaminopyridine (49 mg, 0.4 mmol) and 1,3-dibenzyloxy-2,4-dihydroxybenzene (**21**) (322 mg, 1 mmol) was stirred for 15 min at −10 °C in acetonitrile (20 mL) and the solution turned purple in colour. Dibenzylphosphite (1.33 mL, 6.0 mmol) was then added dropwise over 5 min at −10 °C (dry ice alone) and the mixture was stirred for a further 1 h under N_2. The solvents were evaporated and the remaining yellow syrup was dissolved in dichloromethane (50 mL), washed with water (50 mL), dried, and the title compound was purified by flash chromatography R_f = 0.24 (EtOAc−hexane, 1:1), to give the product as a colorless syrup (658 mg, 78 %) which was triturated with cold ether and hexane to give a white crystalline solid, m.p. 70–71 ºC. Any impurity below the R_f value can be removed using CH_2Cl_2−CH_3CN (5:1). Yield (658 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.90–5.12 (12 H, m, 2 × ArOC*H*$_2$Ph and 4 × ArOP(O)OC*H*$_2$Ph), 6.67 (1 H, d, J = 9.4 Hz, C*H*, Ar), 7.07–7.46 (31 H, m, 2 × ArOCH$_2$*Ph*, 4 × ArOP(O)OCH$_2$*Ph*, C*H*, Ar); ^{13}C NMR (100 MHz, $CDCl_3$) 69.56, 69.62, 69.93, 69.99 (4 t, × ArOP(O)O*C*H$_2$Ph), 71.42, 75.73 (2 t, 2 × ArO*C*H$_2$Ph), 108.08, 117.16 (2 d, 2 × *C*H, Ar), 127.61, 127.90, 127.97, 128.01, 128.12, 128.22, 128.25, 128.28, 128.35, 128.50, 128.57 (11 d, *C*H, ArOCH$_2$*Ph*, ArOP(O)OCH$_2$*Ph*), 134.92, 135.00, 135.34, 135.40, 135.72, 135.79, 135.98, 136.77, 138.27, 143.20, 148.84 (C_q, ArOCH$_2$*Ph*, ArOP(O)OCH$_2$*Ph* and Ar); ^{31}P NMR (100 MHz, $CDCl_3$) –5.74 (1 P, s, ArO*P*(O)OCH$_2$Ph), –5.91 (1 P, s, ArO*P*(O)OCH$_2$Ph); (HRMS, ES$^+$) *m/z* Calcd for $C_{48}H_{45}O_{10}P_2$ $[M + H]^+$ 843.2482. found 843.2501, calcd for $C_{48}H_{44}O_{10}P_2$ C 68.40, H 5.26; found: C, 68.6, H, 5.30.

1,3-Dihydroxybenzene-2,4-bisphosphate (10). 1,3-Bis(benzyloxy)benzene-2,4-bis(dibenzyloxyphosphoryloxy)benzene (**22**) (169 mg, 200 μmoles), was dissolved in dry $CDCl_3$ (5 mL), dry 2,4,6-collidine (0.39 mL, 3.0 mmol) was then added and the solution was stirred over an atmosphere of nitrogen. Bromotrimethylsilane (0.4 mL, 3 mmol) was also added and the solution was stirred for 3 h at room temperature to give **23**. The solvents were evaporated and the reaction mixture was quenched using a mixed solvent of D_2O–2 M TEAB (2:1, 3 mL). The mixture was diluted with water (100 mL) and 1,3-dibenzyloxybenzene 2,4-bisphosphate (**24**) was purified by ion exchange chromatography using Q-Sepharose Fast Flow and a gradient of triethylammonium bicarbonate (TEAB) 0→2.0 M. The product eluted between 1.1 and 1.4 M TEAB buffer and isolated as the triethylammonium salt which was then used to make the target compound. 1,3-Dibenzyloxybenzene 2,4-bisphosphate (**24**) was dissolved in water (20 mL) and stirred for 18 h in the presence of palladium on carbon (10%, 200 mg) under an atmosphere of hydrogen. The solution was filtered through a PTFE syringe filter to remove the palladium on carbon and the solvents were evaporated to provide the title compound (**10**) (151 μmol) in 75.5 % yield as quantified by the Briggs test[3]. ^{1}H NMR (400 MHz, D_2O/ 37 ºC) 6.42 (1 H, dd, *J* = 1.2, 9.0 Hz, Ar-*H*), 6.84 (1 H, dd, *J* = 1.2, 9.0 Hz, Ar-*H*); (400 MHz, D_2O/ 20 ºC) 6.06 (1 H, d, *J* = 9.0 Hz, Ar-*H*), 6.49 (1 H, d, 9.0 Hz, Ar-*H*); ^{31}P NMR (161 MHz, D_2O) 0.19 (1 P, s, Ar-O*P*O_3^{2-}), −2.68 (1 P, s, Ar-O*P*O_3^{2-}). (HRMS, ES$^-$) *m/z* Calcd for $C_6H_7O_{10}P_2$ $[M - H]^-$ 300.9520. found 300.9521.



Scheme 2

Reagents & Conditions: (a) MeOH, H_2O, conc HCl (40:9:1), 19 h, 90%; (b) BnBr, (1.1 eq.), KI, (0.1 eq), CH_3CN, reflux, $NaHCO_3$, 1 h, 48%; (c) *m*CPBA, CH_2Cl_2, 21 h, R.T. purify; (d) MeOH, Amberlyst, 78%; (e) CCl_4, DMAP, CH_3CN, −10°C, (H)P(O)$(OBn)_2$, 78%; (f & g) $CDCl_3$, 2,4,6-collidine, TMSBr, 3 h, then purify by ion exchange; (h) H_2O, Pd/C, H_2, 18 h, 75.5%.

PROTEIN PURIFICATION:

Preparation of SHIP2:

An expression clone containing the catalytic domain of SHIP2 residues 419-832 (SHIP-cd) with an N-terminal 6xHis-tag followed by a TEV protease cleavage site sub-cloned into the vector pNIC-MBP was purchased via the Structural Genomics Consortium (SGC) from Source BioScience (Clone accession TC124029) and was used to transform *E. coli* Rosetta2 (DE3) cells (Novagen). Cultures were grown in LB medium supplemented with 25 μg ml^{-1} kanamycin at 37°C with shaking until OD_{600} reached 0.8. Target expression was induced by addition of 0.5mM IPTG and incubation at 23°C with shaking overnight. Cells were harvested by centrifugation (5,000 × g, 30 min, 4°C) and the pellet re-suspended in lysis buffer (100 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 8.0 plus completec EDTA-free protease inhibitor (Roche)). Cells were disrupted by French Press (three passes at 17,000 psi) and cell debris removed by centrifugation (42,000 × g, 60 min, 4°C. The filtered lysate was loaded onto a Ni-charged HiTrap chelating HP column (GE Healthcare). The column was washed with buffer A (20 mM HEPES, 500 mM NaCl, 1% glycerol, 500 mM imidazole, 0.5 mM TCEP, pH 7.5) and eluted with an elution gradient 20 mM to 500 mM imidazole over 50 ml, finishing with buffer B (20 mM HEPES, 500 mM NaCl, 1% glycerol, 20 mM imidazole, 0.5 mM TCEP, pH 7.5). The SHIP2-containing fractions were concentrated to 5 ml and loaded onto a 16/60 Superdex 75 gel filtration column, equilibrated and eluted with gel filtration buffer (20 mM HEPES, 300 mM NaCl, 1% glycerol, 20 mM, 0.5 mM TCEP, pH 7.5). The SHIP2-containing fractions were pooled and the N-terminal histidine tag was proteolytically removed by incubating with His-tagged TEV protease (Invitrogen) in a molar ratio of 30:1 at 4°C overnight. SHIP2 was purified from tag and protease by passing the reaction mixture over a buffer A equilibrated Ni-charged HiTrap chelating column and eluted with buffer A. The SHIP2 with His tag removed was concentrated and exchanged into gel filtration buffer using an Amicon concentrator with a 10 KDa MWCO membrane. For aqueous phase experiments, the protein was diluted into experiment buffer; 20 mM HEPES, 1 mM $MgCl_2$, pH 7.3.

ANALYTICAL TECHNIQUES:

IC_{50} determination by displacement of 2-FAM $InsP_5$:

Fluorescence polarization was determined as previously described[6] using a BMG ClarioStar plate reader with excitation wavelength 485 nm and emission wavelength 520 nm.384 well plates were used with a volume of 20 μl in each well. For EC_{50} determination, varying amounts of SHIP2 (in 20 mM HEPES, 1 mM $MgCl_2$, pH7.3) were added to 2 nM 2-FAM-IP_5. Displacement experiments used 2 nM 2-FAM-$InsP_5$ and 1 μM SHIP2 with additions of displacing ligand/substrate ranging from nM to mM.

Phosphate release assay:

SHIP2 was diluted into 200 mM HEPES, 2 mM $MgCl_2$, pH 7.3 and incubated with the individual compounds for a period of 10 mins at 30°C. The mixtures were cooled on ice. The phosphate detection reagent was freshly prepared; 4 parts 12.14 M ammonium molybdate in 1M sulphuric acid to 1 part 388 mM ferrous sulphate[7]. Using 384 well plates, 10 μl each reaction mixture was aliquoted into a well followed by 10 μl phosphate detection reagent. These mixtures were incubated for 10minutes at room temperature before using a Hidex Sense plate reader to determine the absorbance at 700 nm. A calibration curve was constructed, using KH_2PO_4 standards ranging from 0 to 100 μM, prepared in the same buffer and treated in the same way as the samples.

HPLC:
Reaction products were analyzed on a 3 mm × 250 mm CarboPac PA200 column (Dionex, UK) fitted with a 3 mm × 50 mm guard column of the same material. Samples (20µl) were injected and compounds were eluted with a gradient derived by mixing water (A) and 0.6 M methanesulfonic acid (B) according to the following schedule: time (min), % B; 0, 0; 25, 100; 38, 100. The eluent flow rate was 0.4 mL min^{-1}. Compounds were detected by fluorescence, excitation at 280 nm, emission at 330 nm, on a Jasco FP-4250 Fluorescence detector.

Fluorescence Spectroscopy:
Excitation and emission spectra of compounds were recorded using a Jasco FP8500 spectrometer in the fluorescence mode and polarisers were used to reduce spectral background arising from scattering of the excitation wavelength.

TD-DFT calculations:
All calculations were performed using the Gaussian 09 set of programs (Revision C. Gaussian, Inc. Wallingford) using the long-range corrected CAM-B3LYP[8] functional with the 6-31+G** basis set without symmetry constraints. Solvent effects treated using the polarizable continuum (PCM) model[9] with all calculations performed in acetonitrile. The geometries of ground and excited states were confirmed as minima by frequency calculation. Excitation and emission wavelengths and oscillator strengths were calculated using state-specific solvation method[10].

FIGURE S1:

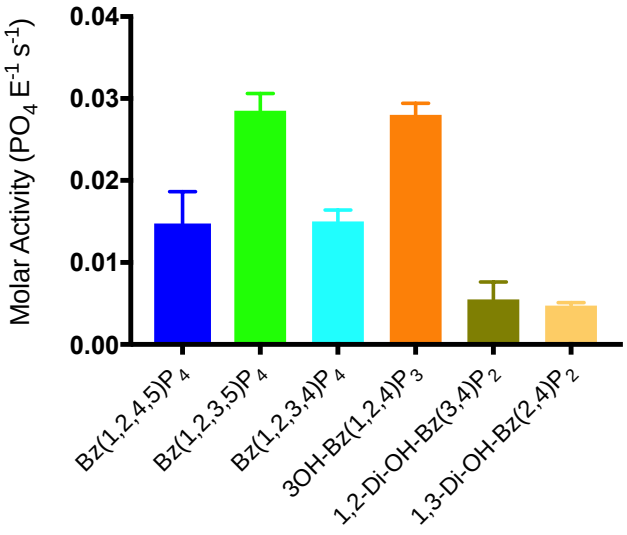


Figure S1. Benzene phosphates and hydroxybenzene phosphates as substrates of SHIP2. Substrates (100µM) were incubated with 4µM. SHIP2 for 10min at 30°C. Means and s.d. of triplicate determinations are shown.

FIGURE S2:

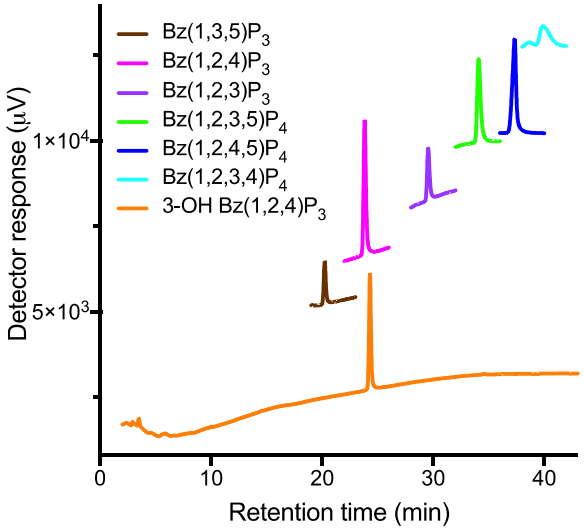


Figure S2. HPLC separation of benzene phosphates. Equal amounts of BzPs were resolved on a CarboPac PA-200 column eluted with methanesulfonic acid. Traces were offset in y direction to aid visualization and detector response (Abs_{280}) scaled down by a factor of 10 for $Bz(1,2,4,5)P_4$.

FIGURE S3:

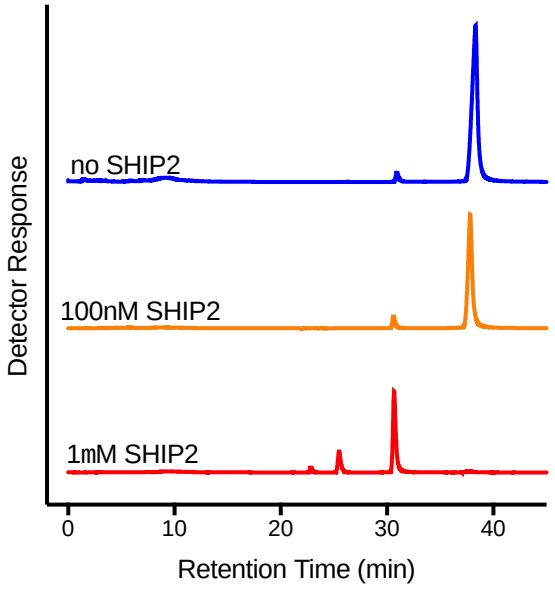


Figure S3. HPLC assay of SHIP2 action on compound (6) $Bz(1,2,4,5)P_4$. Substrate (100µM) was incubated with varying amounts of SHIP2 for 2h at 16°C. Substrate and products were detected by fluorescence (excitation at 280 nm, emission at 330 nm)

FIGURE S4:

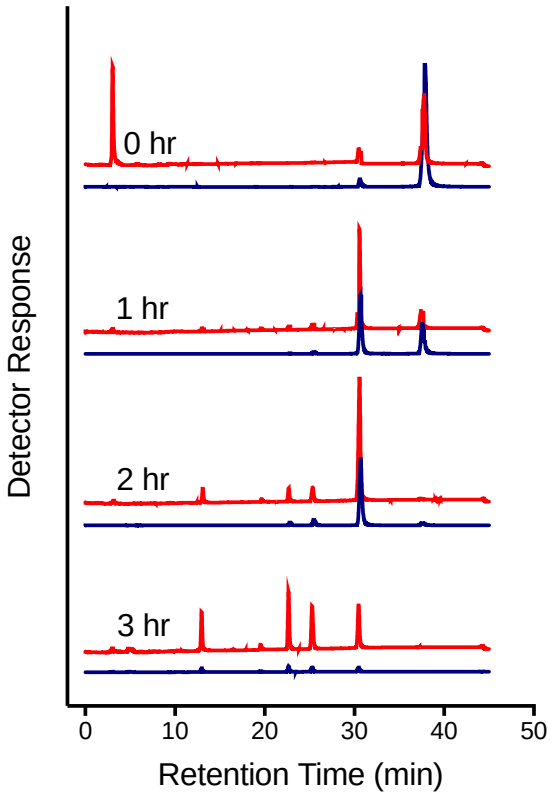
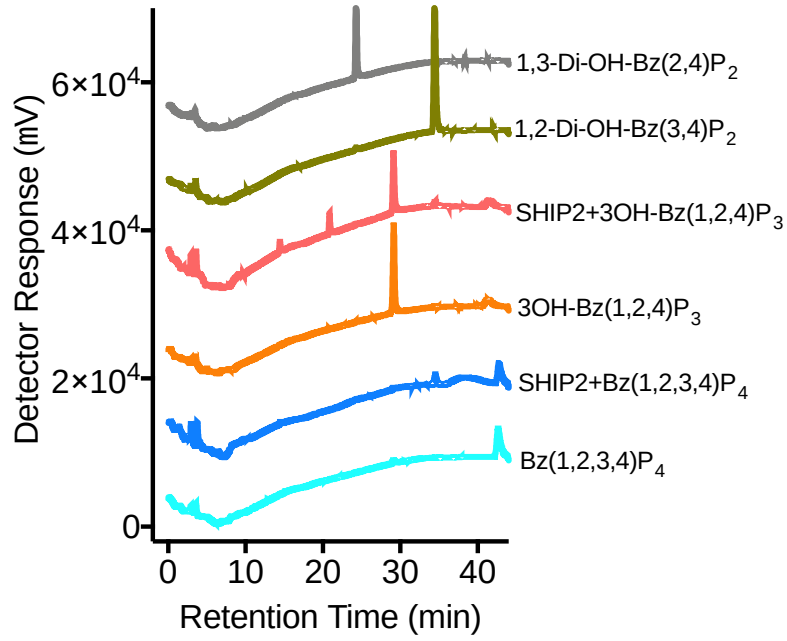


Figure S4. HPLC assay of SHIP2 action on compound (6) Bz(1,2,4,5)P_4. Substrate (100μM) was incubated with 1μM SHIP2 at 16°C. Substrate and products detected by tandem measurement of absorbance at 280nm (red line) and fluorescence (excitation at 280 nm, emission at 330 nm) (blue line)

FIGURE S5:



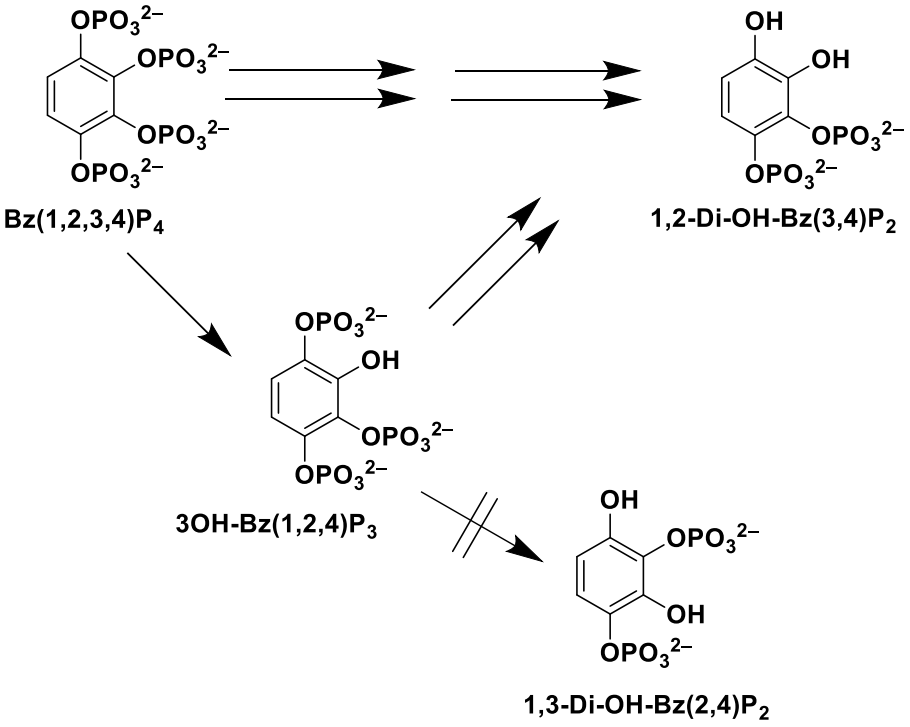


Figure S5. HPLC separation of compound (4) Bz(1,2,3,4)P_4, compound (7) 3-OH-Bz(1,2,3)P_3, compound (9) 1,2-Di-OH-Bz(3,4)P_2 and compound (10) 1,3-Di-OH-Bz(2,4)P_2 and products formed by the action of 1µM SHIP2 on 100µM compound (4) Bz(1,2,3,4)P_4 and compound (7) 3-OH-Bz(1,2,4)P_3, 2 hours at 16°C. Compounds detected by absorbance measurement at 280nm. Schematic of reactions catalyzed by SHIP2, double arrows indicate reactions confirmed; single arrows possible reactions.

TD-DFT

TD-DFT DISCUSSION OF RESULTS:

TD-DFT analysis of weakly fluorescent compound (2) Bz(1,3,5)P_3 and compound (5) Bz(1,2,3,5)P_4 reveals that excitation from the ground state to the first excited state exhibits highly mixed character. Significant contributions from HOMO→LUMO, HOMO-1→LUMO+1 transitions were noted, and in the case of compound (2) Bz(1,3,5)P_3, additional HOMO-1→LUMO transitions were observed, reflected in the weakest oscillator strength (Tables SA and SC). For compound (3) Bz(1,2,4)P_3 and compound (6) Bz(1,2,4,5)P_4, the transitions from the ground state to first excited state are predominantly of HOMO→LUMO character, but also with notable contributions from HOMO-1→LUMO+2 (Tables SE and SG), resulting in intermediate oscillator strengths. In contrast, compound (8) 5-OH-Bz(1,2,4)P_3 and tryptophan excitations are dominated by the HOMO→LUMO transition with only minor contributions from other orbitals (Tables SI and SK). Electronic transitions for the emission processes are predominantly LUMO→HOMO for benzene phosphates and tryptophan (Tables SB, SD, SF, SH, SJ, SL) and, for the respective classes of molecule, exhibit the same order of oscillator strengths as for the excitation process (Tables S1 and S2). The measured fluorescence of compound (6) Bz(1,2,4,5)P_4, was 30 times more intense than that of the next most fluorescent benzene tetrakisphosphate, compound (5) Bz(1,2,3,5)P_4, and approximately 10% of that of tryptophan (Figure 4). These observations match the trends in oscillator strengths predicted by TD-DFT (Tables S1 and S2).

Our TD-DFT analysis systematically underestimates excitation wavelengths for both benzene phosphates and tryptophan compared with experimental results (Table S1 and S2). A similar observation has been noted for TD-DFT analysis using CAM-B3LYP/6-31+G* level of theory[10]. The predicted emission wavelengths also demonstrate an underestimate of up to 40 nm for the benzene phosphates, possibly as the quality of geometry optimization for molecules in excited states is known to be less accurate than for ground states. As the errors in the TD-DFT predictions are systematic, fluorescent properties of different compounds can reasonably be compared in terms of wavelength shifts.

TD-DFT TABLES AND FIGURES:

Table S1. Comparison and assignment of ground state to first excited state absorption transitions with TD-DFT. * Fig. 5B

Compound	Absorption Wavelength (nm)		Predicted Oscillator Strength	Transition	Contribution (%)
	Experiment	Predicted			
Tryptophan	279	259	0.0858	HOMO →LUMO	87.6
5-OH Bz(1,2,4)P_3*	283 *(Bz(1,2,4,5)P_4+7)*	243 *(Bz(1,2,4,5)P_4+6)*	0.0728	HOMO →LUMO	73.8
Bz(1,2,4,5)P_4	276	237	0.0390	HOMO →LUMO	73.4
				HOMO-1 →LUMO+2	22.5
Bz(1,2,4)P_3	274	231	0.0135	HOMO →LUMO	62.9
				HOMO-1 →LUMO+2	20.6
Bz(1,2,3,5)P_4	276	237	0.0034	HOMO →LUMO	58.6
				HOMO-1 →LUMO+1	38.3
Bz(1,3,5)P_3	270	231	0.0002	HOMO-1 →LUMO	24.1
				HOMO →LUMO	23.5
				HOMO-1 →LUMO+1	19.2

Table S2. Comparison and assignment of ground state to first excited state emission transitions with TD-DFT. * Fig. 5C

Compound	Emission Wavelength (nm)		Predicted Oscillator Strength	Transition	Contribution (%)
	Experiment	Predicted			
Tryptophan	332	321	0.1087	HOMO →LUMO	95.5
5-OH Bz(1,2,4)P_3*	349 *(Bz(1,2,4,5)P_4+25)*	306 *(Bz(1,2,4,5)P_4+25)*	0.1052	HOMO →LUMO	91.6
Bz(1,2,4,5)P_4	324	281	0.0750	HOMO →LUMO	87.8
Bz(1,2,4)P_3	310	264	0.0591	HOMO →LUMO	82.0
Bz(1,2,3,5)P_4	330	284	0.0495	HOMO →LUMO	86.2
Bz(1,3,5)P_3	317	273	0.0329	HOMO →LUMO	79.7

Compound (2) Bz(1,3,5)P_3

Table SA. Contributions to the ground state to first excited state absorption for compound (2) Bz(1,3,5)P_3 calculated using TD-DFT.

MO	Contribution (%)
92 -> 94	24.10
92 -> 95	19.21
92 -> 96	4.34
93 -> 94	23.50
93 -> 95	12.70
93 -> 96	10.78

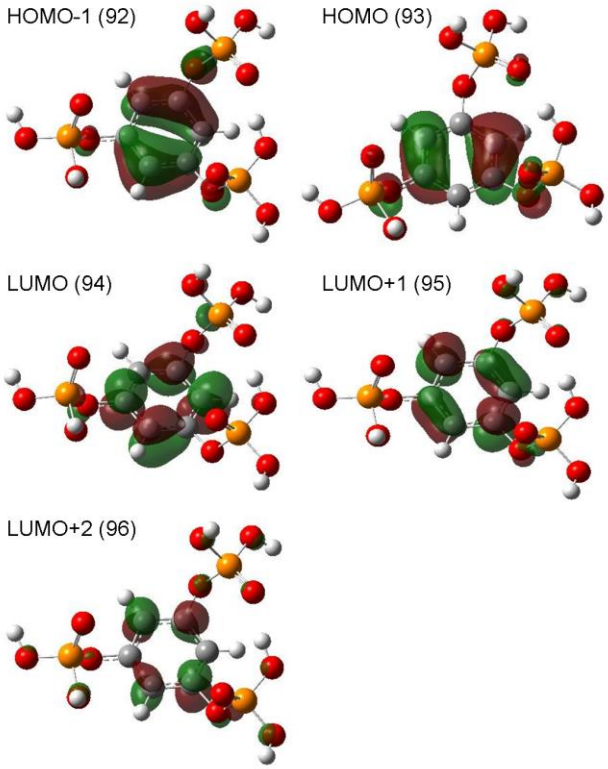


Figure SA. Ground state molecular orbitals involved in significant components of the excitation process for compound (2) Bz(1,3,5)P_3.

Table SB. Contributions to excited state to ground state emission for compound (2) Bz(1,3,5)P_3 calculated using TD-DFT.

MO	Contribution (%)
92 -> 94	3.13
92 -> 96	12.20
93 -> 94	79.68

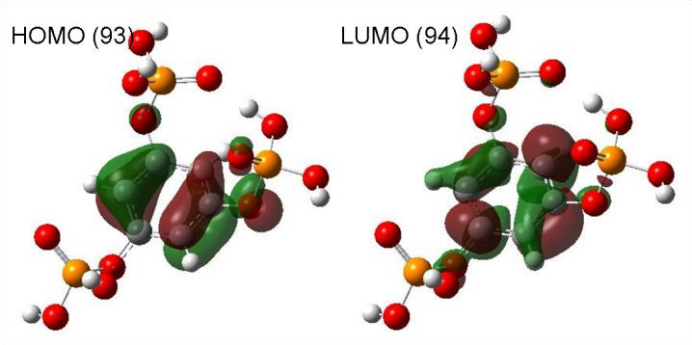


Figure SB. Excited state molecular orbitals involved in significant components of the emission process for compound (2) Bz(1,3,5)P_3.

Compound (5) Bz(1,2,3,5)P_4

Table SC. Contributions to the ground state to first excited state absorption for compound (5) Bz(1,2,3,5)P_4 calculated using TD-DFT.

MO	Contribution (%)
116 ->119	38.33
117 ->118	58.56

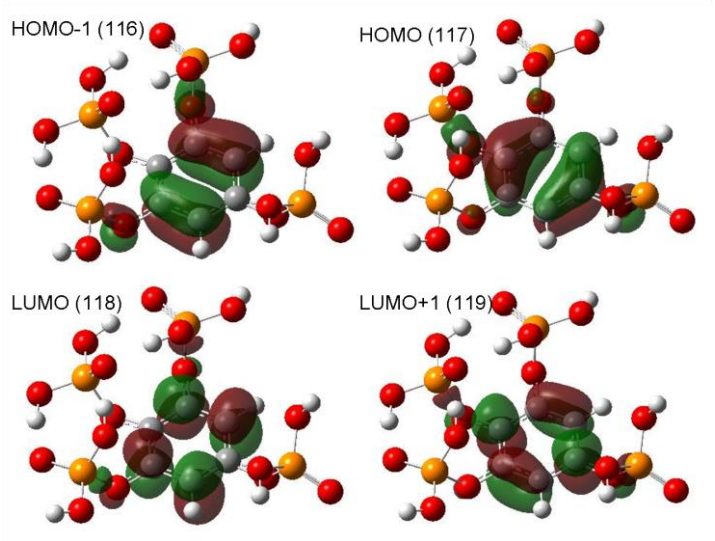


Figure SC. Ground state molecular orbitals involved in significant components of the excitation process for compound (5) Bz(1,2,3,5)P_4.

Table SD. Contributions to excited state to ground state emission for compound (5) Bz(1,2,3,5)P_4 calculated using TD-DFT.

MO	Contribution (%)
116 ->119	9.25
117 ->118	86.21

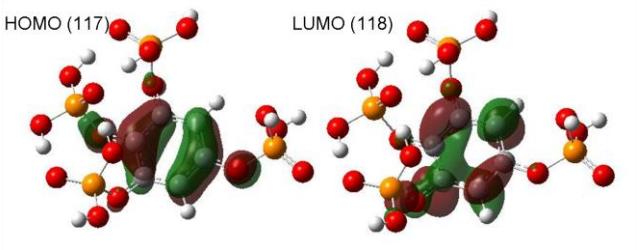


Figure SD. Excited state molecular orbitals involved in significant components of the emission process for compound (5) Bz(1,2,3,5)P_4.

Compound (3) Bz(1,2,4)P_3

Table SE. Contributions to the ground state to first excited state absorption for compound (3) Bz(1,2,4)P_3 calculated using TD-DFT.

MO	Contribution (%)
92 -> 95	8.56
92 -> 96	20.59
93 -> 94	61.95
93 -> 95	5.72

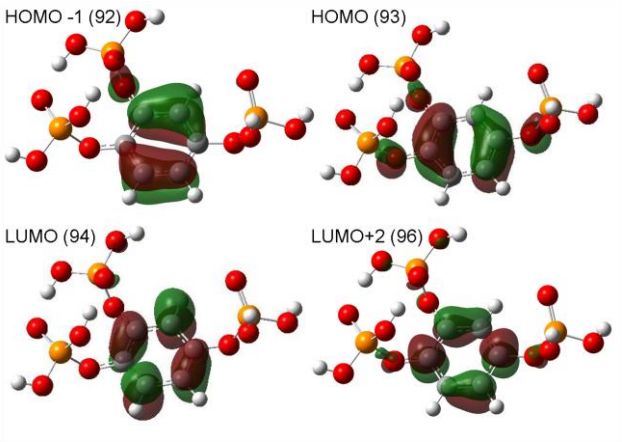


Figure SE. Ground state molecular orbitals involved in significant components of the excitation process for compound (3) Bz(1,2,4)P_3.

Table SF. Contributions to excited state to ground state emission for compound (3) Bz(1,2,4)P_3 calculated using TD-DFT.

MO	Contribution (%)
92 -> 96	7.30
92 -> 97	4.23
93 -> 94	81.98
93 -> 95	3.31

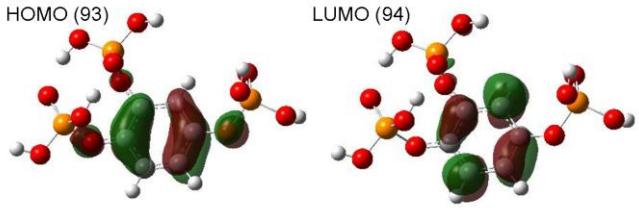


Figure SF. Excited state molecular orbitals involved in significant components of the emission process for compound (3) Bz(1,2,4)P_3.

Compound (6) Bz(1,2,4,5)P_4

Table SG. Contributions to the ground state to first excited state absorption for compound (6) Bz(1,2,4,5)P_4 calculated using TD-DFT.

MO	Contribution (%)
116 ->120	22.53
117 ->118	73.39
117 ->119	2.65

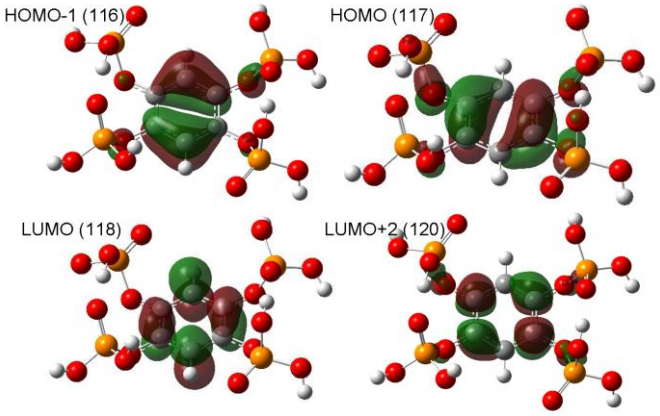


Figure SG. Ground state molecular orbitals involved in significant components of the excitation process for compound (6) Bz(1,2,4,5)P_4.

Table SH. Contributions to excited state to ground state emission for compound (6) Bz(1,2,4,5)P_4 calculated using TD-DFT.

MO	Contribution (%)
116 ->121	8.80
117 ->118	87.84

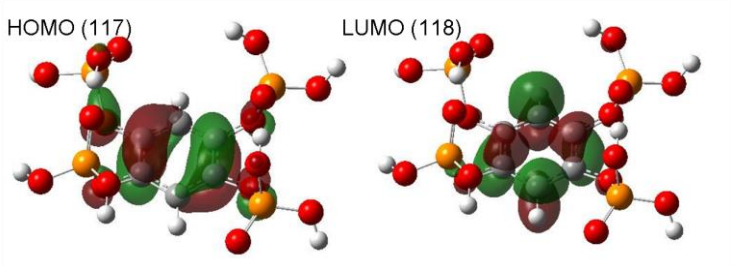


Figure SH. Excited state molecular orbitals involved in significant components of the emission process for compound (6) Bz(1,2,4,5)P_4.

Compound (8) Bz (1,2,4)P_3 (5-OH)

Table SI. Contributions to the ground state to first excited state absorption for compound (8) Bz(1,2,4)P_3(5-OH) calculated using TD-DFT.

MO	Contribution (%)
96 -> 101	12.01
96 -> 102	4.14
97 -> 98	73.76
97 -> 99	8.02

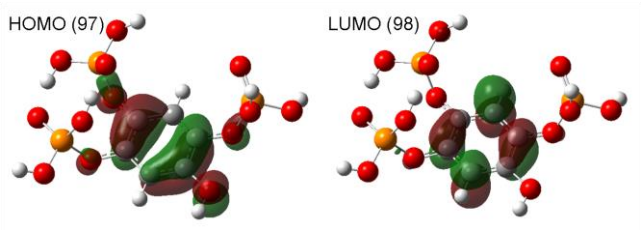


Figure SI. Ground state molecular orbitals involved in significant components of the excitation process for compound (8) Bz(1,2,4)P_3(5-OH).

Table SJ. Contributions to excited state to ground state emission for compound (8) Bz(1,2,4)P_3(5-OH) calculated using TD-DFT.

MO	Contribution (%)
96 -> 102	4.30
97 -> 98	91.59
97 -> 99	2.74

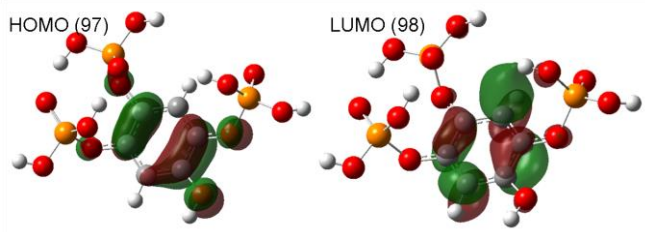


Figure SJ. Excited state molecular orbitals involved in significant components of the emission process for compound (8) Bz(1,2,4)P_3(5-OH).

Tryptophan

Table SK. Contributions to the ground state to first excited state absorption for tryptophan calculated using TD-DFT.

MO	Contribution (%)
53 > 55	2.71
53 > 62	3.71
54 > 55	87.57

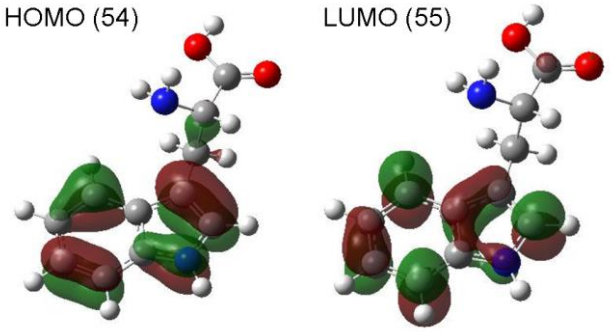


Figure SK. Ground state molecular orbitals involved in significant components of the excitation process for tryptophan.

Table SL. Contributions to excited state to ground state emission for tryptophan and calculated using TD-DFT.

MO	Contribution (%)
54 > 55	95.45

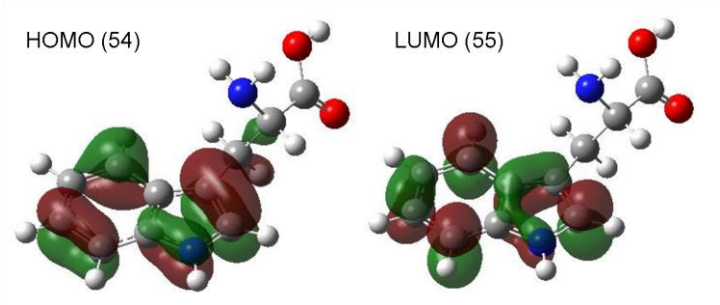


Figure SL. Excited state molecular orbitals involved in significant components of the emission process for tryptophan

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