Electronic Supplementary Material (ESI) for Chemical Communications

Coordinate-bond-dependent solid-phase organic synthesis of biotinylated desferrioxamine B: A new route for metal-specific probes

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Supporting Information

1. General experimental details

Unless otherwise stated, all reactions were conducted under an inert atmosphere of nitrogen or argon. Tetrahydrofuran (99%) and *N*,*N*-dimethylformamide (99.8%) were from Sigma-Aldrich. Tetrahydrofuran was distilled from sodium prior to use. Methanol was from Lomb Scientific (99.8%) and was dried over 4 Å molecular sieves. ^{1}H NMR spectra were obtained using a Varian INOVA instrument operating at 400 MHz. All spectral data are reported in ppm (δ) relative to the residual solvent peaks for CD_3OD (3.31 ppm, 4.87 ppm). Coupling constants (*J*) are reported in Hz. Electrospray ionization mass spectrometry (ESI-MS) was carried out using a Finnigan LCQ mass spectrometer (San Jose, CA, USA) in positive ion mode. Samples (1 mg) were dissolved in 50 µL of DMF or DMSO. The mobile phase was methanol, with a flow rate = 0.20 mL min^{-1}; cone voltage was 25 V and the injection volume was 20 µL. The electronic absorption spectra of solid samples of dried resin (approximately 30 mg each) were acquired from 370-800 nm as ground powders smeared onto a filter paper using a Cary 1E UV-visible spectrophotometer in diffuse reflectance mode. Analytical-scale HPLC was conducted on an Agilent 1200 HPLC system with an Agilent Eclipse XDB-C18 column (particle size 5 µm, column dimension 4.6 × 150 mm) under the following conditions. Mobile phase, A: H_2O/TFA 100:0.1; B: ACN:TFA 100:0.1; gradient, 5 to 100% B over 30 min; flow rate, 0.5 mL min^{-1}; UV detection, 220 nm.

2. Assay procedures

2.1. Iron(III) addition. An aliquot of $Fe(ClO_4)_3$ in 0.2 M $HClO_4$ (100 µL, 10 mM) was added to a 200-µL subsample of a fraction in a 96-well plate and the absorbance value of the solution was measured after 10 min at λ = 450 nm using an SpectraMax M5 microplate reader.[2] A standard curve for DFOB·mesylate (0, 10, 20, 30, 40 or 50 µg) was generated as follows. Aliquots (0, 10, 20, 30, 40 or 50 µL) from a 1 mg mL^{-1} stock solution of DFOB·mesylate in HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) were made to 200 µL with HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) and an aliquot (100 µL) of Fe(III) solution was added and the absorbance value of the solution was read at λ = 450 nm. A separate standard curve was generated for biotin-DFOB (0, 10, 20, 30, 40 or 50 µg). As stipulated throughout the

Supporting Information, fractions of different volume sizes were collected during different procedures: 0.5 mL, 1.0 mL, 1.5 mL or 8 mL. The estimated μmol concentration of DFOB or biotin-DFOB in a 200-μL fraction subsample was corrected using the respective multiplier (2.5, 5, 7.5, 40) to determine the μmol concentration in the original fraction.

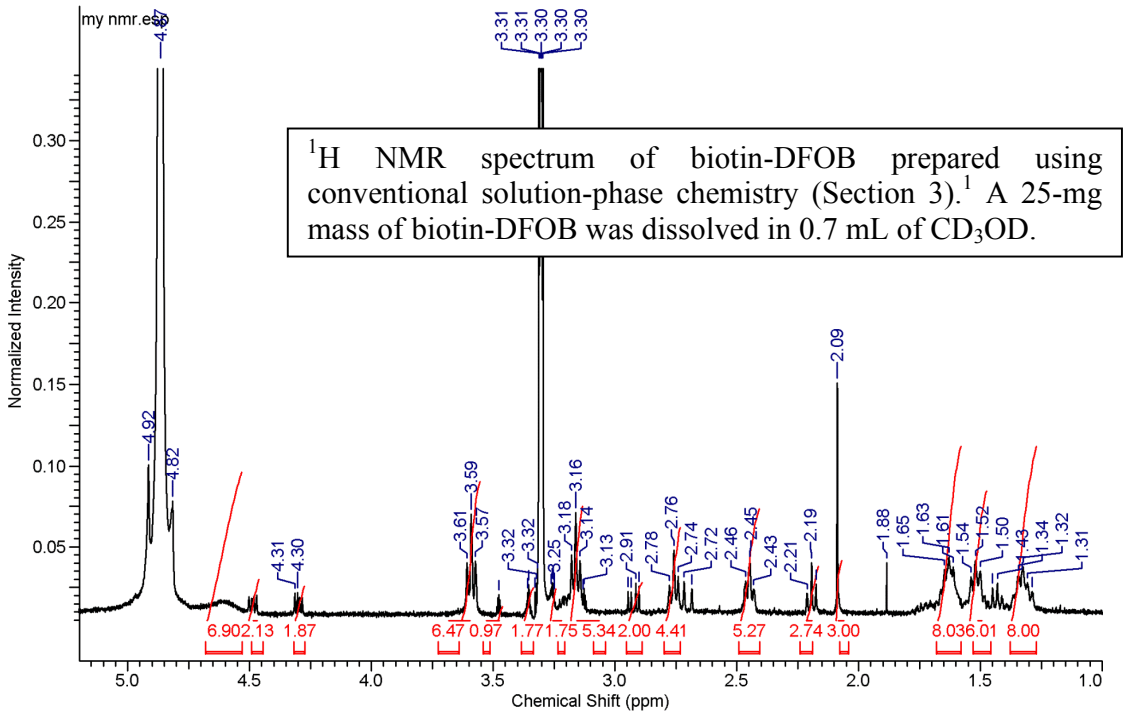
2.2. Reactive amine group. A 1% (w/v) solution of 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution was prepared in DMF. An approximate 5-mg mass of the test resin was transferred into a micro test tube and a 1-mL aliquot of ethanol was added to the tube. One drop of TNBS reagent was added to the tube and the slurry was briefly vortexed and the tube was incubated at room temperature for 30 min with the resin settling to the bottom of the tube. A 5-mg solid sample of DFOB·mesylate was dissolved in 1-mL ethanol and treated as above. A red-orange color indicated the presence of reactive amine groups.[3]

3. Preparation of biotin-DFOB using conventional solution-phase synthesis

3.1. Preparation of NHS-biotin. *N*-Hydroxysuccinimide-biotin was prepared based upon literature.[4] *N*-Hydroxysuccinimide (NHS) (0.214 g, 1.8 mmol) and *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) (0.388 g, 2 mmol) were added to a solution of biotin (0.42 g, 1.7 mmol) in 8 mL of DMF. The solution was stirred overnight at room temperature. The volume of the solution was reduced via rotary evaporation (external bath temperature 55 °C) to approximately 4 mL upon which a white precipitate appeared. The vessel was left at –22 °C overnight and the solid was removed via filtration. Yield, 0.41 g (70%). The product was used in the subsequent steps without further characterization.

3.2. Preparation of biotin-DFOB. Biotin-DFOB was prepared based upon literature.[1] Desferrioxamine B·mesylate (0.031 g, 0.047 mmol) and NaOH (0.0021 g, 0.0053 mmol) were dissolved in methanol (7 mL) and NHS-biotin (0.032 g, 0.094 mmol) was added to the solution. The mixture was heated under reflux at 60 °C overnight. The product was filtered off and washed with small amounts of methanol. Yield, 20.3 mg (55%). ^{1}H NMR (400 MHz, CD_3OD): δ 1.32 (8H, *m*, $C\underline{H}_2C\underline{H}_2C\underline{H}_2$ of DFOB, $C\underline{H}_2$ of biotin), 1.43 (2H, *m*, $C\underline{H}_2$ of biotin), 1.52 (6H, *m*,

C<u>H</u>$_2$ of DFOB), 1.63 (8H, *m*, 3 × C<u>H</u>$_2$ of DFOB, C<u>H</u>$_2$CHS of biotin), 2.09 (3H, *s*, C<u>H</u>$_3$ of DFOB), 2.19 (2H, *t*, *J*=8.01, COC<u>H</u>$_2$ of biotin), 2.45 (4H, *t*, *J*=8.01, 2 × NHCOC<u>H</u>$_2$CH$_2$CONOH), 2.72 (1H, *d*, *J*=12.0, SC<u>H</u>$_2$), 2.76 (4H, *d*, *J*=8.0, NHCOCH$_2$C<u>H</u>$_2$CONOH), 2.92 (1H, *dd*, *J*=4.0, 12.0, SC<u>H</u>$_2$), 3.16 (6H, *t*, *J*=8.0, NHC<u>H</u>$_2$(C<u>H</u>$_2$)C<u>H</u>$_2$NOH), 3.26 (1H, *m*, C<u>H</u>SCH$_2$), 3.35 (1H, *m*, N<u>H</u>CONH), 3.48 (1H, *m*, NHCON<u>H</u>), 3.59 (6H, *t*, *J*=8.0, 3 × CONOHC<u>H</u>$_2$), 4.3 (1H, *dd*, *J*=4.0, 8.0, C<u>H</u>CHSCH$_2$CH), 4.49 (1H, *dd*, *J*=4.0, 8.0 CHCHSCH$_2$C<u>H</u>), 4.6 (6H, *brs*, 3 × N<u>H</u>CO and 3 × NO<u>H</u>CH$_2$). ESI-MS calcd for $C_{35}H_{62}N_8O_{10}S$ $[M + Na^+]^+$ 809.42, found 809.61. The compound was analyzed by RP-HPLC (**Fig. 2a**, LHS) and positive ion ESI-MS (**Fig. 2a**, RHS) and shown to be >90% pure.



4. Preparation of biotin-DFOB using solid-phase organic synthesis with immobilized metal affinity resin as a solid support

4.1. Preparation of DFOB solution. A 50 mM stock solution of DFOB·mesylate was prepared by dissolving 0.06568 g of DFOB·mesylate (Sigma, 92.5%) in 2 mL of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9). An aliquot (0.3 mL) of the DFOB·mesylate stock

solution (15 μmol) was made to 5 mL with HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9).

4.2. Preparation of Ni(II)-based resin for ligand binding. Immobilized metal affinity resin pre-loaded with Ni(II) (GE Healthcare, Ni(II) Sepharose 6 Fast Flow) was used as a free flowing resin. An approximate 7-8-mL aliquot of resin slurry was loaded into a 1.5 cm-i.d. column with a base frit (GE Healthcare) and the resin bed was settled with gravity to a final bed volume of 5 mL. To remove non-specifically bound Ni(II), the resin was washed using gravity flow with 2 × CV of imidazole buffer (0.5 M imidazole, 20 mM Na_2HPO_4/NaH_2PO_4, 0.5 M NaCl, pH 8), 5 × CV of phosphate buffer (20 mM Na_2HPO_4/NaH_2PO_4, 0.5 M NaCl, pH 7) and 5 × CV of water. In preparation for ligand binding, the resin was pre-equilibrated with 10 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9). A sample that was similarly prepared was washed with THF and taken to dryness for analysis by solid-state electronic absorption spectroscopy (30 mg, **Fig. 1b**, LHS) or for the presence of reactive amine groups (5 mg) (**Fig. 1b**, RHS).

4.3. Solvent leaching. A column bed volume of 0.5 mL of Ni(II)-loaded resin was washed according to the procedure detailed in Section 4.2 to remove adventitious Ni(II) and was then equilibrated with 10 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) before being loaded with 30 μL of a 50 mM solution of DFOB·mesylate (loading = 1.5 μmol or 3.0 μmol/mL resin) in HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9). The column was washed with 14 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9); and 26 × CV of THF (**Fig. S1a**), MeOH (**Fig. S1b**) or DMF (**Fig. S1c**). The experiments were conducted at room temperature. Fractions of 0.5 mL were analyzed for μmol DFOB using the Fe(III) addition assay.

4.4. Preparation of DFOB-loaded Ni(II)-based resin for solid-phase organic synthesis. A 5-mL aliquot of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) containing 15 μmol DFOB·mesylate (Section 4.1) was loaded onto a bed volume of 5-mL resin that had been prepared for ligand binding according to Section 4.2. The concentration of DFOB loaded was in slight excess of capacity (loading = 15 μmol or 3.0 μmol/mL wet resin). Excess DFOB was eluted from the resin by washing with ~4 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M

NaCl, pH 9) and 1-mL fractions were collected. In preparation for the solid-phase organic synthesis (SPOS) procedure, the resin was washed with ~5 × CV of THF and 1-mL fractions were collected. The aqueous- and THF-based fractions were analyzed for DFOB using the Fe(III) addition assay which showed that approximately 0.55 μmol (3.7%) of the 15 μmol DFOB loaded onto the resin was eluted during these washes, leaving 14.45 μmol DFOB bound to the resin (**Fig. S2a**). The THF-equilibrated resin was taken to dryness via rotary evaporation to yield approximately 0.235 g of solid DFOB-Ni(II)-immobilized resin ([DFOB] = 62 μmol g^{-1} dry resin). Two subsamples of resin were removed for analysis either by solid-state electronic absorption spectroscopy (30 mg) (**Fig. 1c**, LHS) or for the presence of reactive amine groups (5 mg) (**Fig. 1c**, RHS). The remaining portion of resin (0.2 g) was used to undertake the SPOS procedure. The 0.2 g of dry resin used in the SPOS procedure contained 12.3 μmol of bound DFOB.

4.5. Solid-phase organic synthesis. A 0.2 g portion of dry resin containing 12.3 μmol bound DFOB (Section 4.4) was suspended in 8 mL of THF in a 100-mL round-bottomed flask. An aliquot (100 μL) of THF that was made to pH 9.1 by the addition of a sub-mg-quantity of solid NaOH was added to the suspension and the slurry was stirred for 15 min at 40 °C. An aliquot of a 0.35 M solution of NHS-biotin in DMSO (0.78 mL, 273 μmol) was added to the slurry and the mixture was stirred at 40 °C overnight. After 24 h, the slurry was transferred from the round-bottomed flask to a 1.5 cm-i.d. column with a base frit (GE Healthcare) and the filtrate from the slurry (~8 mL) was retained. This 8-mL volume solution was analyzed by the Fe(III) addition assay and by RP-HPLC and ESI-MS and was shown to contain biotin-DFOB in a yield of 0.067 μmol, which was leached from the resin during the overnight reaction procedure. The resin was washed with a further 6 mL of THF at 40 °C and four fractions of 1.5 mL were collected and analyzed using the Fe(III) addition assay which showed the presence of a further 0.063 μmol biotin-DFOB (**Fig. S2b**). The fourth THF-based fraction analyzed as negative in the Fe(III) addition assay (**Fig. S2b**, data point at cumulative volume, 49 mL). The THF-suspended resin was taken to dryness via rotary evaporation to yield approximately 0.2 g of resin. Two subsamples of resin were removed for analysis either by solid-state electronic absorption spectroscopy (30 mg) (**Fig. 1e**, LHS) or for the presence of reactive amine groups (5 mg) (**Fig. 1e**, RHS).

A 50-mg subsample of the resin was processed to elute bound components as follows. The 50-mg sample of resin was suspended in 1.5 mL of THF and the slurry was transferred to a 1.5 cm-i.d. column with a base frit (GE Healthcare). The THF-based eluent was negative in the Fe(III) addition assay (**Fig. S2c**, data point at cumulative volume 50 mL). The resin, which swelled to a bed volume of approximately 1.5 mL, was washed with 26 × CV of water at pH 6 and the 1.5-mL fractions collected were analyzed using the Fe(III) addition assay (**Fig. S2c**). The water fractions were combined and the volume was reduced from 39 mL to 5 mL via rotary evaporation and this concentrated, aqueous solution was analyzed by RP-HPLC (**Fig. 2b**, LHS) and positive-ion ESI-MS (**Fig. 2b**, RHS).

Additional comment 1. In this first example of a coordinate-bond-dependent SPOS procedure, due attention was given to verifying using solid-state electronic spectroscopy and tests for reactive amine groups the components bound to the IDA-based resin in solid form. This required that the resins were prepared under aqueous conditions and equilibrated with THF before removing the solvent to furnish solid resin. Having established in this work the integrity of the THF-equilibrated DFOB-bound Ni(II)-loaded resin is maintained in the solid-state, it would be tractable to use the THF-washed resin in wet form directly in a THF-based SPOS-based procedure.

Additional comment 2. Preliminary one-pot amide coupling reactions between DFOB-bound Ni(II)-loaded IDA resin using 1-hydroxy-benzotriazole (HOBt)-based, *N*,*N*′-dicyclohexylcarbodiimide (DCC)-activation of biotin gave more complex mixtures, which prompted the use of milder reaction conditions and the use of NHS-biotin.

4.6. Preparation of biotin-DFOB-loaded Ni(II)-based resin with standard biotin-DFOB. A 50 mM solution of biotin-DFOB as synthesized using conventional procedures (Section 3.2) was prepared in HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9). An aliquot (9 μL) was sorbed onto a 0.75 mL volume of Ni(II)-loaded resin that had been equilibrated with HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) according to Section 4.2. The resin was washed with 4 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) followed by 4 × CV of THF. The solvent was removed via rotary evaporation to yield about 35 mg of dry resin.

Two subsamples of resin were analyzed either by solid-state electronic absorption spectroscopy (30 mg) (**Fig. 1d**, LHS) or for the presence of reactive amine groups (5 mg) (**Fig. 1d**, RHS).

5. Reaction using the solid-phase organic synthesis format with metal free resin

5.1. Removing Ni(II) from resin. An aliquot of Ni(II) Sepharose 6 Fast Flow resin (2.5 mL) in a 1.5 cm-i.d. column with a base frit (GE Healthcare) was washed with 5 × CV of buffer containing 20 mM Na_2HPO_4/NaH_2PO_4, 100 mM EDTA, 0.3 M NaCl, pH 8; 5 × CV of 20 mM Na_2HPO_4/NaH_2PO_4, 0.2 M NaCl, pH 8; and 5 × CV water. The color of the resin changed from green to white. The slurry was taken into a round-bottomed flask and the solvent was removed via rotary evaporation to yield approximately 100 mg of Ni(II)-free resin. Two subsamples of the Ni(II)-free resin were removed for analysis either by solid-state electronic absorption spectroscopy (30 mg) (**Fig. 1a**, LHS) or for the presence of reactive amine groups (5 mg) (**Fig. 1a**, RHS).

5.2. Solid-phase organic synthesis format using metal-free resin. DFOB·mesylate (0.015 g, 0.023 mmol) and NaOH (1 mg, 0.025 mmol) were dissolved in THF (3.5 mL) in a round-bottomed flask. A 10-mg mass of dry Ni(II)-free resin (Section 5.1) was suspended in 1 mL of THF and added to the reaction vessel followed by the addition of a solid sample of NHS-biotin (0.032 g, 0.094 mmol). The suspension was heated under reflux at 40 °C overnight. The slurry was transferred to a 1.5 cm-i.d. column with a base frit (GE Healthcare) and the filtrate from the slurry (~3.5 mL) was retained and analyzed by RP-HPLC (**Fig. 2c**, LHS) and positive-ion ESI-MS (**Fig. 2c**, RHS) revealing the presence of multiple species including a significant concentration of DFOB (>50%) and a low concentration (~10%) of biotin-DFOB.

6. References

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(4) Chan, E. W. S.; Chattopadhaya, S.; Panicker, R. C.; Huang, X.; Yao, S. Q. *J. Am. Chem. Soc.* **2004**, *126*, 14435-14446.

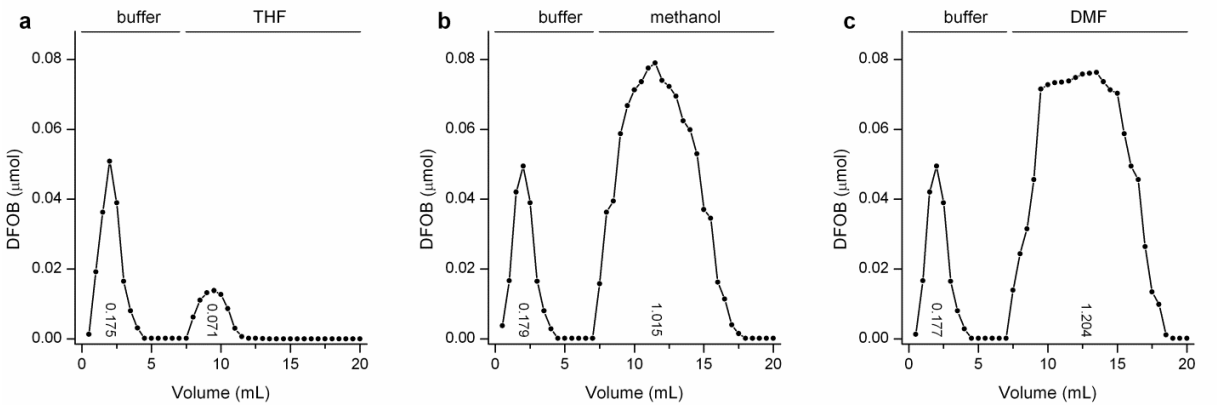

Figure S1. Room-temperature binding/leaching behavior of 1.5 μmol of DFOB loaded onto 0.5 mL of Ni(II)-loaded resin upon washing with 14 × CV of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) and 26 × CV of (**a**) THF; (**b**) methanol; or (**c**) DMF. In all experiments, about 0.177 μmol of DFOB was eluted from the column in the buffer wash, reflecting a loading in slight excess of the binding capacity of the resin. Of the DFOB that remained bound to the resin (1.323 μmol), the amount of DFOB leached upon washing with THF, methanol or DMF was 0.071 μmol (5%), 1.015 μmol (77%), or 1.204 μmol (91%), respectively.

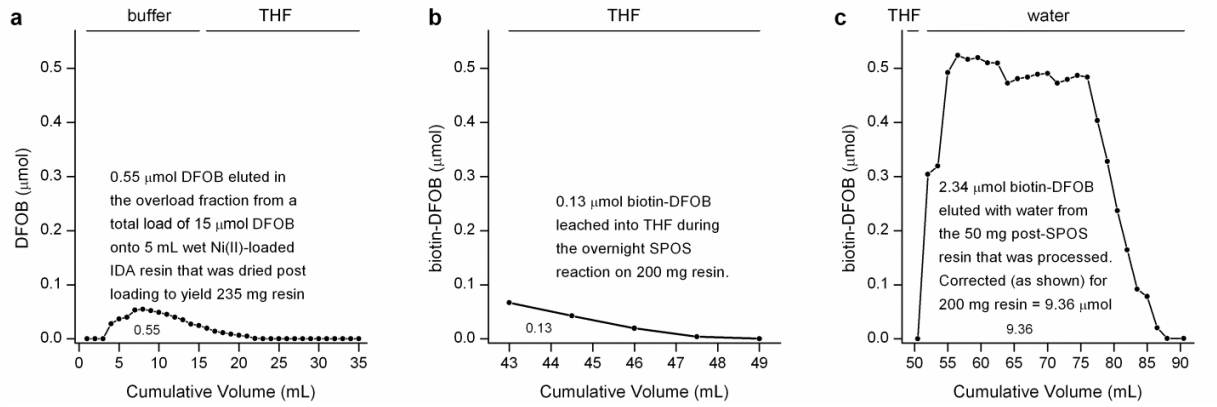

Figure S2. The pathway used for the preparation of biotin-DFOB using solid-phase organic synthesis with immobilized metal affinity resin as a solid support. In (**a**), 5 mL of wet Ni(II)-loaded resin that had been processed according to Section 4.2, was loaded with 15 μmol DFOB and the fraction (0.55 μmol) that was present in excess of the binding capacity of the resin was eluted by washing with 15 mL of HEPES buffer (25 mM HEPES-NaOH, 0.5 M NaCl, pH 9) and 21 mL of THF. The THF-washed resin, which contained 14.45 μmol bound DFOB, was taken to dryness to yield 235 mg solid resin. A sample (200 mg, containing 12.3 μmol bound DFOB) was subject to the solid-phase organic synthesis procedure described in Section 4.5. After the reaction (**b**), the reaction solvent was collected (~8 mL THF) and the resin was washed with a further 6 mL of THF until there was no positive result in the Fe(III) addition assay and the THF-based resin slurry was taken to dryness to yield 200 mg resin. The combined THF washings in (**b**) were shown to contain 0.13 μmol of biotin-DFOB that had leached from the resin during the overnight reaction. In (**c**), a 50-mg subsample of the resin from (**b**) was suspended in 1.5 mL of THF and then the resin bed (1.5 mL) was washed with 26 × CV of water, pH 6.0, to yield a corrected (50 mg to 200 mg) amount of biotin-DFOB of 9.4 μmol. The overall yield of biotin-DFOB was 76% (9.4 μmol/12.3 μmol).