Electronic Supplementary Information

Stabilized Wittig Olefination for Bioconjugation

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I. General Information—Chemistry

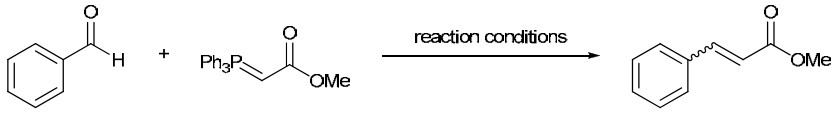
Reagents and solvents were purchased at the highest commercial quality and used without further purification. Moisture-sensitive reactions were performed under dry nitrogen or argon, using anhydrous solvents obtained by passing commercially available pre-dried, oxygen-free formulations through activated alumina columns. Yields refer to chromatographically and spectroscopically homogeneous materials. Thin-layer chromatography (TLC) was carried out on 0.25 mm E. Merck silica gel plates (60F-254). Chromatograms were visualized with UV light and developed with iodine vapor, ninhydrin solution, or cerium ammonium molybdate solution (CAM). E. Merck silica gel (60, particle size 0.040 – 0.063 mm) was used for flash column chromatography. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-600 instrument and referenced using residual undeuterated solvent. ^{31}P NMR spectra were recorded on a Bruker Avance III 400 MHz instrument (equipped with 5-mm PABBO probe) and referenced by spiking samples with triphenylphosphine oxide (29.1 ppm in $CDCl_3$). The following abbreviations were used to indicate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. IR spectra were recorded on a Perkin-Elmer Spectrum One FTIR spectrometer with diamond ATR accessory. Data are represented as follows: frequency of absorption (cm^{-1}), intensity of absorption (s = strong, m = medium, w = weak, br = broad). High-resolution mass spectra (HRMS) were recorded on an Agilent ESI-TOF mass spectrometer at 3500 V emitter voltage. Exact m/z values are reported in Daltons.

II. Preliminary Studies with a Model Phosphoranylidene Ester

General procedure for Wittig olefination in aqueous media

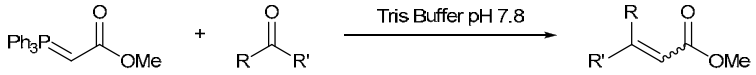
To a solution of methyl 2-(triphenylphosphoranylidene)acetate (10.0 mg, 0.030 mmol, 1.0 equiv) in reaction solvent (2 mL) was added benzaldehyde (6.4 uL, 0.060 mmol, 2.0 equiv) at the required temperature. The mixture was stirred at 4 °C for 12 h then extracted with CH_2Cl_2 (5 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the filtrate was removed concentrated in vacuo. The product was purified using flash column chromatography to afford the corresponding cinnamate ester.

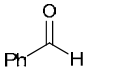
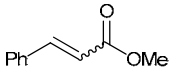
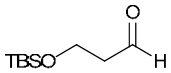
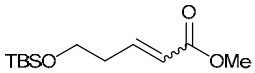
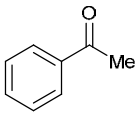
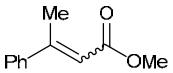
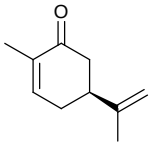
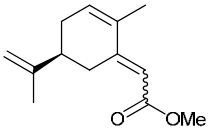
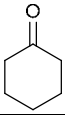
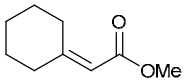
Table 1. Screening of Stabilized Wittig Olefination



Entry	Medium	Temperature (°C)	Time (h)	Yield (%)
1	Distilled Water	25	1	88
2	Distilled Water	4	1	83
3	Tris buffer pH 7.8	25	1	79
4	Tris buffer pH 7.8	4	1	68
5	Tris buffer pH 7.8	4	12	71
6	Tris buffer pH 7.8	4	1	69
7	PBS pH 7.6	4	1	75
8	RIPA buffer pH 7.8	4	1	65
9	RPMI1640 (10% FBS)	25	1	58
10	RPMI1640 (10% FBS)	4	12	49

Table 2. Screening of Carbonyl Compounds with Model Phosphoranylidene in Tris Buffer



Entry	Carbonyl Compound	Wittig Product	Yield (%)
1			71
2			42
3			no reaction
4			no reaction
5			no reaction

III. Rate Studies of Stabilized Wittig Olefination

The rate constants of the reaction between methyl 2-(triphenylphosphoranylidene)acetate and nonyl-biotin probe **3** with benzaldehyde were determined using the method of initial rates. Pentamethylbenzene was used as an internal standard for ^{1}H NMR analysis. Reactions were performed with excess benzaldehyde, under pseudo-first order conditions and allowed to proceed for 300 s at 4°C before quenching with 5 N HCl (20 µL). The concentration of the product formed after this time was determined by ^{1}H NMR using the integration ratio of the product olefin doublet (CD_3CN or $CDCl_3$: 6.51 ppm, J = 16.0 Hz) to the aryl singlet of the ISTD (CD_3CN: 6.51 ppm; $CDCl_3$: 6.84 ppm). The pseudo-first order rate constant (k_{obs}) was calculated from the slopes obtained by plotting the initial rate of product formation ($M \bullet s^{-1}$) versus the concentration of phosphoranylidene (M).

A) Reaction of methyl 2-(triphenylphosphoranylidene)acetate with benzaldehyde in CD_3CN. Reactions were initiated by adding 15 µL of benzaldehyde to a 500 µL solution of the ylide (5 – 25 mM in CD_3CN) at 4 °C and mixed by vortexing. The resulting initial concentration of benzaldehyde was 300 mM, which was in excess to ensure pseudo-first order conditions. After quenching with 5 N HCl solution, the reaction mixture was directly analyzed by ^{1}H NMR. The second-order rate constant was determined by dividing k_{obs} by the initial concentration of benzaldehyde.

B) Reaction of methyl 2-(triphenylphosphoranylidene)acetate with benzaldehyde in saturated Tris buffer. An equal volume of Tris buffer (50 mM, pH 7.8) and benzaldehyde were mixed, allowed to stand at 4 °C for 2 h, and an aliquot withdrawn from the cloudy top layer to obtain benzaldehyde-saturated Tris buffer. Reactions were initiated by adding 400 µL of benzaldehyde-saturated Tris buffer to a suspension (in 20 µL DMSO) of the ylide and ISTD at 4 °C, with the resulting initial concentration of the ylide ranging between 10 – 40 mM. After quenching with 5 N HCl solution, the reaction mixture was extracted once with 0.6 mL of $CDCl_3$, filtered through a pad of anhydrous $MgSO_4$, and the filtrate analyzed by ^{1}H NMR. A lower-bound estimate of the second-order rate constant was determined by dividing k_{obs} by the aqueous solubility of benzaldehyde at 25 °C reported in the literature.[1]

C) Reaction of nonyl-biotin probe 3 with benzaldehyde in Tris buffer. Reactions were initiated by adding 5 µL of benzaldehyde to a solution of the probe (5 – 25 mM in Tris buffer) at 4 °C. After quenching with 5 N HCl solution, the reaction mixture was extracted once with 0.5 mL of $CDCl_3$, filtered through a pad of anhydrous $MgSO_4$, and the filtrate analyzed by ^{1}H NMR. The second-order rate constant was estimated by dividing k_{obs} by the aqueous solubility of benzaldehyde reported by Carless and Swarbrick.[1]

[1] J. E. Carless and J. Swarbrick, *Pharm. Pharmacol.*, 1964, **16**, 632.

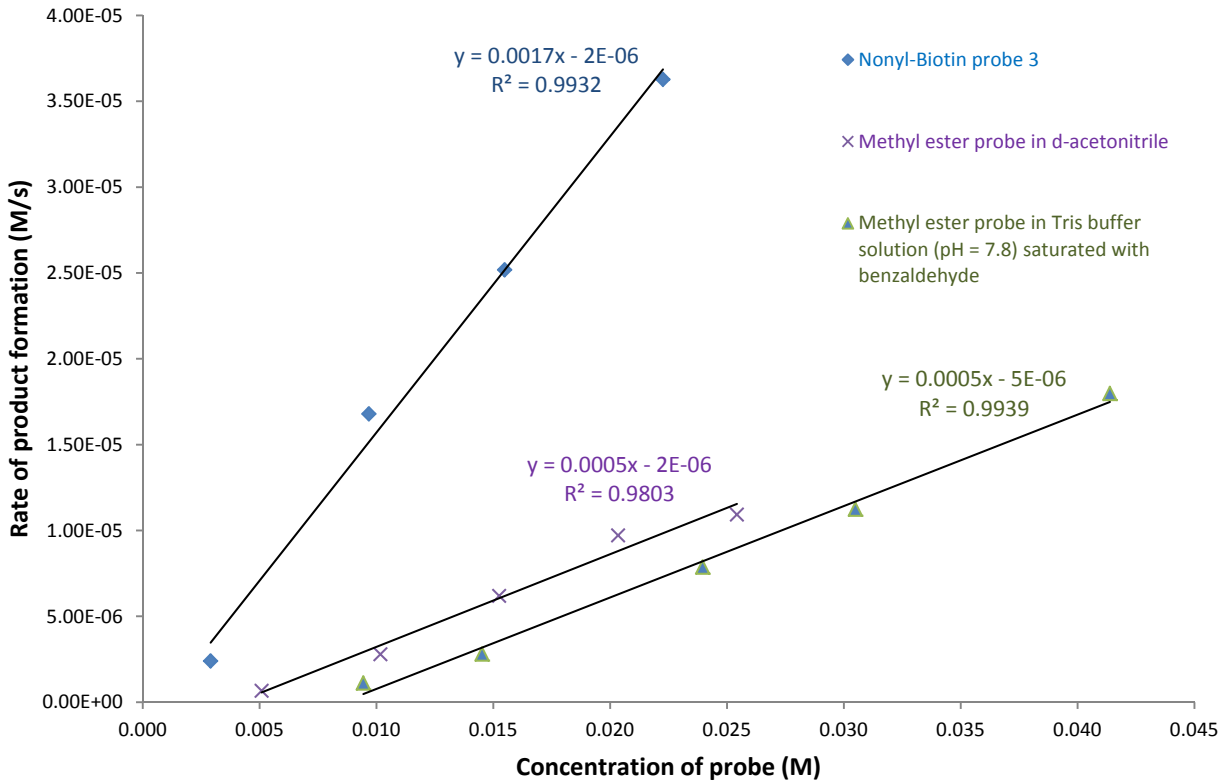


Figure 1. Kinetics plots of rate of product formation (Ms^{-1}) against phosphoranylidene probe concentration (M) in the determination of rate constants for the Wittig olefination in aqueous media.

Table 3. Comparison of Rate Constant in Wittig Olefination Against Other Reactions Used for Bioconjugation

Reaction	Rate constant, k ($M^{-1}s^{-1}$)	Conditions	Temperature (°C)
Wittig olefination*	0.28×10^{-2}	Tris buffer	4
Staudinger ligation[2]	0.25×10^{-2}	5% H_2O/CH_3CN	25
Strain-promoted azide-alkyne cycloaddition[3]	7.6×10^{-2}	CH_3CN	25
Inverse electron demand tetrazine- norbornene cycloaddition[4]	160×10^{-2}	Fetal bovine serum	25

* The rate constant (k) for Wittig olefination in buffered media was obtained by dividing k_{obs} (gradient of plot of nonyl-biotin probe) by the aqueous solubility of benzaldehyde, which was taken as 0.06 M.

[2] J. M. Baskin , J. A. Prescher, S.T Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.*, 2007, **104**, 16793.
[3] F. L. Lin, H. M. Hoyt, H.Van Halbeek, R. G. Bergman and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2005, **127**, 2686.
[4] N. K. Devaraj, R. Weissleder and S. A. Hilderbrand, *Bioconjug. Chem.* 2008, **19**, 2297.

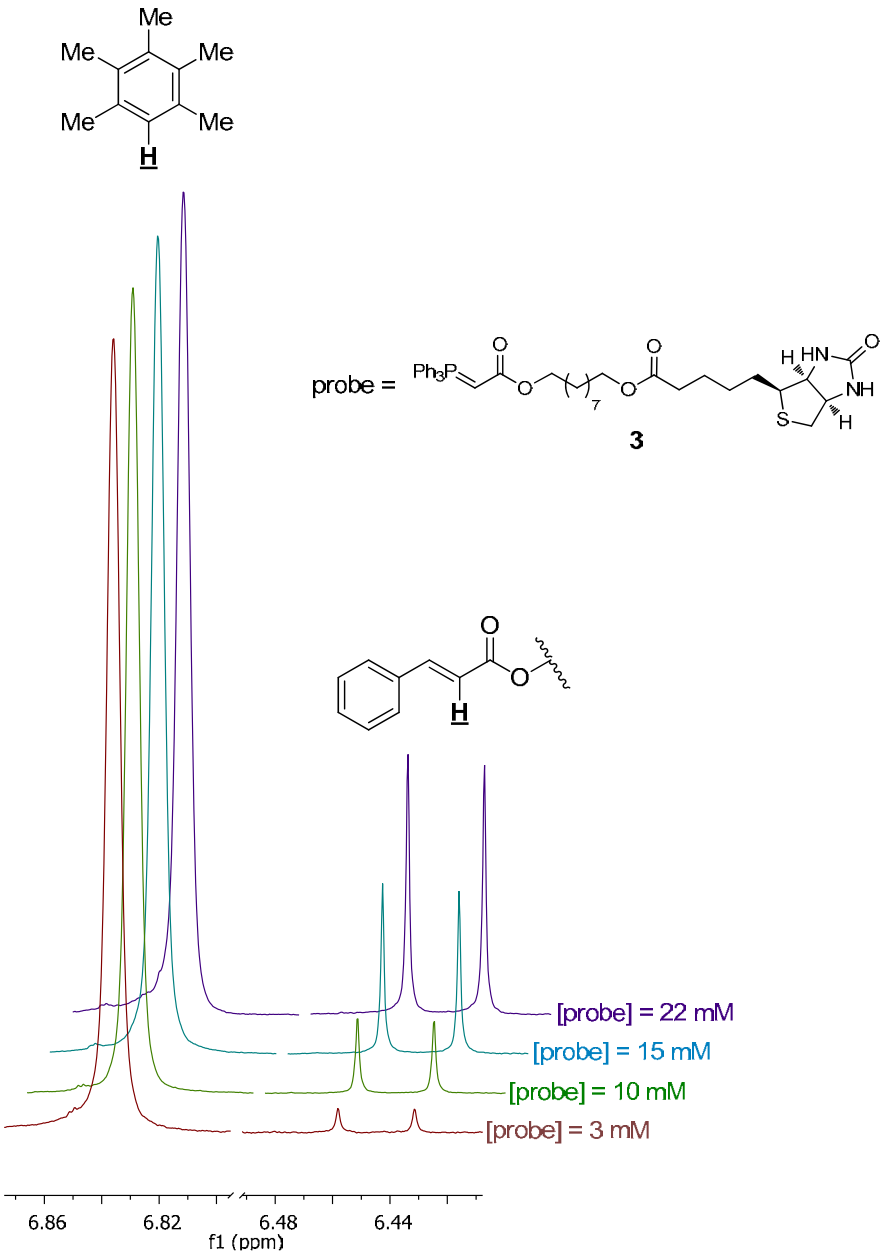


Figure 2. Representative overlayed NMR spectra of reaction mixtures showing the signals of the internal standard (pentamethylbenzene) and the olefinic proton of the product with increasing phosphoranylidene probe concentration.

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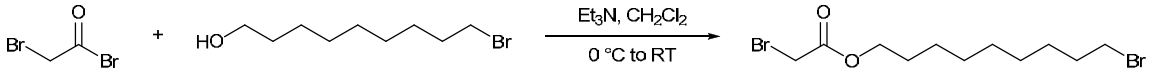
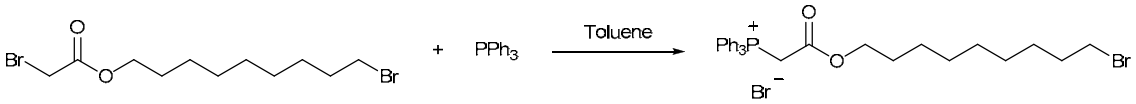
IV. Chemical Synthesis



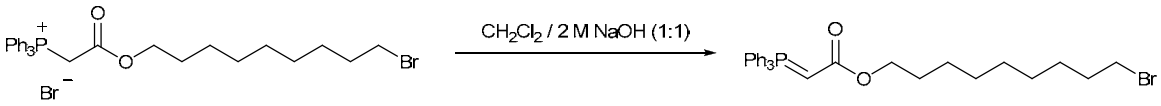
9-bromononyl 2-bromoacetate (1). To a solution of 9-bromononanol (48.1 mg, 0.216 mmol, 1 equiv) and triethylamine (0.05 mL, 0.356 mmol, 1.6 equiv) in CH_2Cl_2 (3 mL) was added bromoacetyl bromide (0.03 mL, 0.344 mmol, 1.6 equiv) at 0 °C over a period of 1 minute, in the dark. After 5 min, the resulting solution was allowed to warm up to room temperature and was stirred for another 6 h. The pale yellow solution was slowly quenched with an equivalent volume of water and the resulting mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with saturated Na_2CO_3 solution (50 mL), water (50 mL), brine (50 mL), and dried over anhydrous Na_2SO_4. The dried solution was filtered, concentrated in vacuo, and purified by flash column chromatography (5% EtOAc–hexane) to provide 62.4 mg of 9-bromononyl 2-bromoacetate **6** as a pale yellow oil (89%).

TLC: (10% EtOAc–hexane) R_f = 0.57 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 4.15 (t, J = 6.7 Hz, 2H), 3.81 (s, 2H), 3.39 (t, J = 6.8 Hz, 2H), 1.87 – 1.79 (m, 2H), 1.68 – 1.61 (m, 2H), 1.44 – 1.37 (m, 2H), 1.36 – 1.32 (m, 2H), 1.32 – 1.27 (m, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 167.4, 66.5, 34.1, 32.9, 29.3, 29.1, 28.7, 28.5, 28.2, 26.0, 25.8. FTIR (neat), cm^{-1}: 2928 (s), 2855 (s), 1735 (s), 1464 (m), 1279 (s), 1163 (s), 1110 (s), 995 (m). HRMS (ESI): calcd for $(C_{11}H_{20}Br_2O_2 + Na)^+$ 364.9722, found 374.9717.



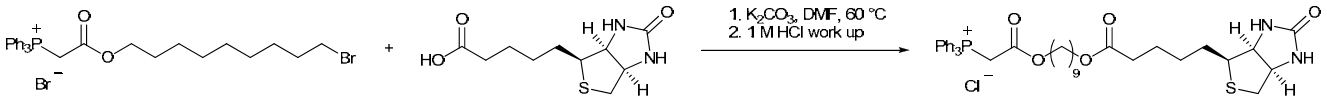
(2-((9-bromononyl)oxy)-2-oxoethyl)triphenylphosphonium bromide (2a). 9-bromononyl 2-bromoacetate **1** (54.5 mg, 0.16 mmol, 1 equiv) and triphenylphosphine (48.0 mg, 0.17 mmol, 1.1 equiv) were dissolved in dry toluene (1.0 mL). The mixture was allowed to stir for 18 h and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (5% MeOH–CH_2Cl_2) to provide 86.7 mg of the desired product as a thick yellow gel (91%).

TLC: (10% MeOH–EtOAc) R_f = 0.37 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.89 (m, 6H), 7.77 (m, 3H), 7.65 (m, 6H), 5.58 (d, J = 13.7 Hz, 2H), 3.94 (t, J = 6.8 Hz, 2H), 3.38 (t, J = 6.8 Hz, 2H), 1.82 (m, 2H), 1.44 – 1.34 (m, 4H), 1.30 – 1.24 (m, 2H), 1.24 – 1.18 (m, 4H), 1.17 – 1.12 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 164.7, 135.2, 134.1 (d, J = 10.7 Hz), 130.3 (d, J = 13.2 Hz), 118.2 (d, J = 89.0 Hz), 67.0, 34.2, 33.4 (d, J = 56.3 Hz), 32.8, 29.3, 29.0, 28.7, 28.2, 28.1, 25.7. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 20.9. FTIR (neat) cm^{-1}: 2931 (s), 2854 (m), 1728 (s), 1438 (s), 1306 (m), 1110 (s), 997 (m). HRMS (ESI): calcd for $(C_{29}H_{35}BrO_2P + H)^+$ 525.1558, found 525.1580.



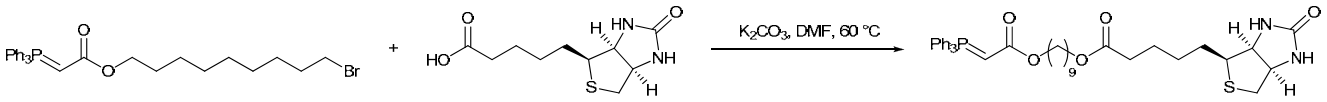
9-bromononyl 2-(triphenylphosphoranylidene)acetate (2). The crude nonylphosphonium bromide salt **2a** from a separate reaction beginning with 9-bromononyl 2-bromoacetate (645.6 mg, 1.88 mmol, 1 equiv) was dissolved in a 1:1 suspension of CH_2Cl_2 / NaOH (8 mL). The biphasic mixture was allowed to stir for 2 h at room temperature before dilution with water (10 mL). The mixture was extracted with CH_2Cl_2 (5 × 10 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified using flash column chromatography to give 740.9 mg of the desired product as a thick yellow oil (75%).

TLC: (10% MeOH–EtOAc) R_f = 0.19 (I_2) ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.82 – 7.71 (m, 5H), 7.69 – 7.62 (m, 4H), 7.59 – 7.50 (m, 6H), 3.90 (t, J = 6.6 Hz, 2H), 3.49 (t, J = 6.7 Hz, 2H), 2.01 (br s, 1H), 1.75 – 1.69 (m, 2H), 1.39 – 1.33 (m, 4H), 1.25 – 1.20 (m, 2H), 1.19 – 1.13 (m, 4H), 1.12 – 1.07 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.63, 133.13 (d, J = 10.0 Hz), 132.01 (d, J = 2.6 Hz), 128.82 (d, J = 12.2 Hz), 128.21 (d, J = 91.7 Hz), 62.36, 34.21, 32.97, 30.18 (d, J = 124.9 Hz), 29.45, 29.39, 28.82, 28.30, 26.16. ^{31}P NMR (162 MHz, $CDCl_3$) δ 17.5. FTIR (neat), cm^{-1}: 2927 (s), 2854 (m), 1615 (s), 1437 (s), 1334 (s), 1186 (w), 1105 (s), 891 (m). HRMS (ESI): calcd for $(C_{29}H_{35}BrO_2P)^+$ 525.1553, found 525.1580.



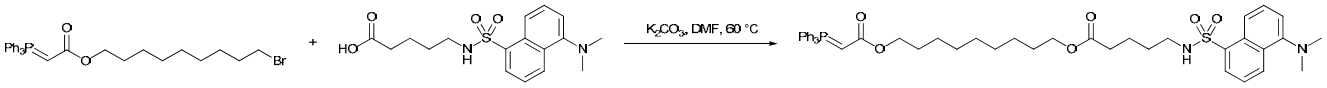
(2-((9-biotinyloxynonyl)oxy)-2-oxoethyl)triphenylphosphonium chloride (3a). Phosphonium bromide **2a** (44.8 mg, 0.0739 mmol, 1.0 equiv) and *D*-(+)-biotin (18.1 mg, 0.0741 mmol, 1.0 equiv) were dissolved in DMF (0.5 mL) and potassium carbonate (45.6.1 mg, 0.330 mmol, 4.6 equiv) was added in one portion. The mixture was stirred at 60 °C for 18 h before diluting with 1 N HCl (5 mL). The aqueous solution was extracted with CH_2Cl_2 (5 × 10 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified by flash column chromatography (0-10% MeOH–CH_2Cl_2) to give 18.5 mg of product as a pale oil (64%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.20 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.92 – 7.84 (m, 6H), 7.78 – 7.70 (m, 3H), 7.68 – 7.59 (m, 6H), 6.08 – 5.48 (m, 2H), 5.47 (s, 1H), 5.20 (s, 1H), 4.57 – 4.49 (m, 1H), 4.34 – 4.25 (m, 1H), 4.06 (t, *J* = 6.5 Hz, 2H), 3.95 (t, *J* = 6.7 Hz, 2H), 3.19 – 3.10 (m, 1H), 2.91 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.79 (d, *J* = 12.8 Hz, 1H), 2.32 (t, *J* = 7.2 Hz, 2H), 1.78 – 1.55 (m, 6H), 1.54 – 1.38 (m, 4H), 1.37 – 1.08 (m, 10H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.85, 165.30 (d, *J* = 3.1 Hz), 163.49, 135.09 (d, *J* = 3.0 Hz), 134.15 (d, *J* = 10.8 Hz), 130.32 (d, *J* = 13.2 Hz), 118.52 (d, *J* = 89.0 Hz), 67.00, 64.57, 61.99, 60.37, 55.51, 40.74, 34.21, 32.51 (d, *J* = 57.5 Hz), 29.82, 29.32, 29.15, 29.09, 28.70, 28.52, 28.45, 28.26, 25.99, 25.70, 25.01. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 21.2 (s). FTIR (neat), cm^{-1}: 3249 (br, s), 2928 (s), 2855 (m), 1726 (s), 1694 (s), 1458 (m), 1439 (s), 1264 (m), 1182 (m), 1111 (s), 997 (w), 865 (w), 721 (m). HRMS (ESI): calcd for $(C_{39}H_{50}N_2O_5PS)^+$ 689.3173, found 689.3184.



9-biotinyloxynonyl 2-(triphenylphosphoranylidene)acetate (3). Phosphoranylidene bromide **2** (150 mg, 0.28 mmol, 1.0 equiv) and *D*-(+)-biotin (75 mg, 0.31 mmol, 1.1 equiv) were dissolved in DMF (1 mL). To this potassium carbonate (116 mg, 0.84 mmol, 3.0 equiv) was added in one portion. The mixture was stirred at 60 °C for 18 h, and the solvent was removed under reduced pressure. The residue was dissolved in water (5 mL) and CH_2Cl_2 (5 mL) and the layers were separated. The aqueous solution was extracted with CH_2Cl_2 (5 × 10 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified by flash column chromatography (0-10% MeOH–CH_2Cl_2) to give 102.0 mg of product as a pale oil (51%).

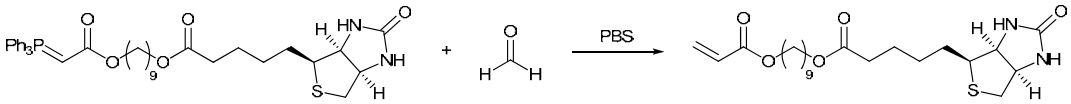
TLC: (5% MeOH–EtOAc) R_f = 0.16 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.92 – 7.84 (m, 6H), 7.78 – 7.70 (m, 3H), 7.68 – 7.59 (m, 6H), 5.47 (s, 1H), 5.20 (s, 1H), 4.57 – 4.49 (m, 1H), 4.34 – 4.25 (m, 1H), 4.06 (t, *J* = 6.5 Hz, 2H), 3.95 (t, *J* = 6.7 Hz, 2H), 3.19 – 3.10 (m, 1H), 2.91 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.79 (d, *J* = 12.8 Hz, 1H), 2.32 (t, *J* = 7.2 Hz, 2H), 1.78 – 1.55 (m, 6H), 1.54 – 1.38 (m, 4H), 1.37 – 1.08 (m, 12H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 173.2, 171.2, 163.2, 133.1 (d, *J* = 10.0 Hz), 132.2 (br s), 132.1 (d, *J* = 30.7 Hz), 128.8 (d, *J* = 12.0 Hz), 64.6, 61.9, 60.1, 55.3, 40.5, 33.9, 30.2 (d, *J* = 120.7 Hz), 29.8, 29.7, 29.4, 29.3, 29.1, 28.6, 28.3, 28.3, 26.0, 25.9, 24.8. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 17.5. FTIR (neat), cm^{-1}: 3249 (br, s), 2928 (s), 2855 (s), 1726 (s), 1694 (s), 1439 (s), 1308 (m), 1264 (m), 1182 (m), 1111 (s), 997 (w), 721 (m), 690 (s). HRMS (ESI): calcd for $(C_{39}H_{49}N_2O_5PS + H)^+$ 689.3173, found 689.3180.



9-(2-(triphenylphosphoranylidene)acetoxy)nonyl 6-(5-(dimethylamino)naphthalene-1-sulfonamido)hexanoate (4). Phosphoranylidene bromide **2** (40.5 mg, 0.077 mmol, 1.0 equiv) and *N*-dansyl-6-aminohexanoic acid (28.0 mg, 0.077 mmol, 1.0 equiv) were dissolved in DMF (0.5 mL). Potassium carbonate (48.9 mg, 0.35 mmol, 4.6 equiv) was added in one portion. The mixture was stirred at 60 °C for 18 h, and the DMF was removed under vacuum. The residue was dissolved in CH_2Cl_2 (10 mL) and the organic layer was washed with water (2 × 5 mL). The organic extract was dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified by flash column chromatography (0-10% MeOH–CH_2Cl_2) to give 53 mg of the desired product as a yellow solid (62%).

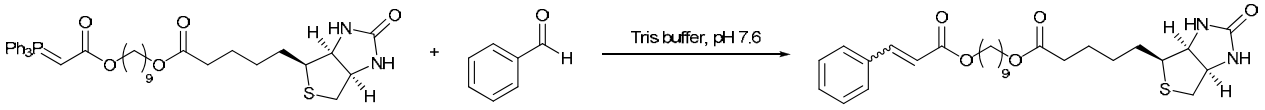
TLC: (10% MeOH–EtOAc) R_f = 0.18 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.53 (d, *J* = 8.5 Hz, 1H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.23 (dd, *J* = 7.3, 1.1 Hz, 1H), 7.71 – 7.61 (m, 6H), 7.58 – 7.50 (m, 4H), 7.45 (qd, *J* = 7.8, 2.8 Hz, 7H), 7.18 (d, *J* = 7.4 Hz, 1H), 4.85 (br s, 1H), 4.12 – 4.09 (m, 1H), 4.06 – 4.02 (m, 1H), 4.01 (t, *J* = 6.7 Hz, 2H), 3.88 (t, *J* = 6.5 Hz, 2H), 3.28 – 3.21 (m, 1H), 2.88 (s, 6H), 2.87 – 2.84 (m, 2H), 2.16 – 2.11 (m, 2H), 1.87 (dd, *J* = 6.9, 1.7 Hz, 1H), 1.76 – 1.67 (m, 2H),

1.59 – 1.54 (m, 2H), 1.45 – 1.42 (m, 2H), 1.40 – 1.36 (m, 2H), 1.31 – 1.27 (m, 2H), 1.27 – 1.24 (m, 3H), 1.20 – 1.16 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 173.6, 171.2, 152.0, 134.8, 133.0 (d, J = 10.0 Hz), 132.0 (br s), 131.9, 130.4, 129.8 (d, J = 39.9 Hz), 129.7, 129.6, 128.7 (d, J = 12.1 Hz), 128.4, 123.2, 118.8, 115.2, 64.6, 60.4, 45.4, 45.4, 43.0, 34.0, 29.5 (d, J = 55.4 Hz), 29.3, 29.2, 29.2, 29.2, 29.1, 28.6, 26.0, 25.9, 25.9, 24.2. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 17.2. FTIR (neat), cm^{-1}: 3158 (br), 2928 (s), 2855 (m), 2784 (w), 1730 (s), 1612 (s), 1574 (m), 1437 (s), 1326 (s), 1142 (s), 1103 (s), 887 (m), 791 (m). HRMS (ESI): calcd for $(C_{47}H_{57}N_2O_6PS + H)^+$ 809.3748, found 809.3733.



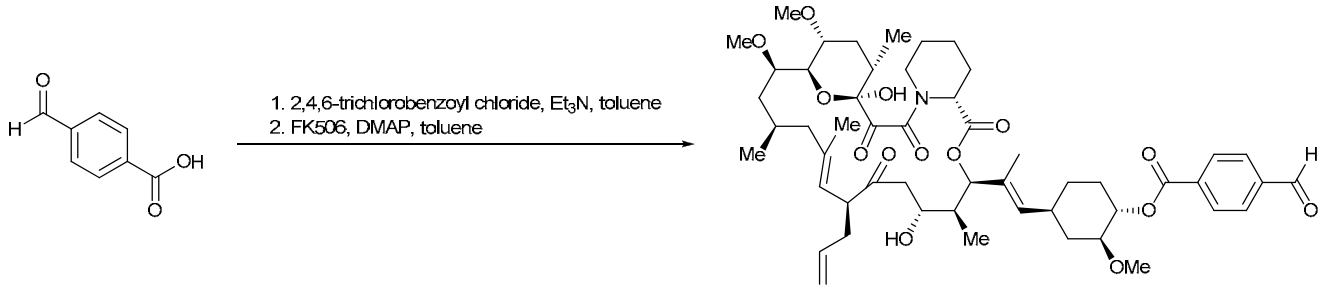
9-biotinyloxynonyl acrylate (5). A solution of 2% formaldehyde solution in phosphate-buffered saline (1 mL) was added to biotinylated phosphoranylidene ester **3** (8 mg, 0.012 mmol, 1 equiv) at 4 °C. The mixture was stirred at 4 °C for 12 h and the aqueous solution was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified by flash column chromatography (5% MeOH–EtOAc) to give 4.3 mg of the desired product (78%).

TLC: (5% MeOH–EtOAc) R_f = 0.38 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 6.42 (dd, J = 17.3, 1.4 Hz, 1H), 6.15 (dd, J = 17.3, 10.4 Hz, 1H), 5.84 (dd, J = 10.4, 1.4 Hz, 1H), 4.75 (s, 1H), 4.60 (s, 1H), 4.55 (dd, J = 7.6, 5.2 Hz, 1H), 4.36 (ddd, J = 7.5, 4.6, 1.2 Hz, 1H), 4.18 (t, J = 6.8 Hz, 2H), 4.09 (t, J = 6.6 Hz, 2H), 3.23 – 3.18 (m, 1H), 2.97 (dd, J = 12.8, 5.0 Hz, 1H), 2.76 (d, J = 12.9 Hz, 1H), 2.35 (t, J = 7.3 Hz, 2H), 1.74 – 1.67 (m, 3H), 1.66 – 1.63 (m, 3H), 1.53 – 1.44 (m, 3H), 1.39 – 1.32 (m, 7H), 0.93 – 0.85 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 173.6, 166.4, 162.6, 130.5, 128.6, 64.7, 64.6, 64.6, 61.8, 55.1, 40.5, 33.9, 31.9, 29.7, 29.3, 29.2, 28.6, 28.6, 28.3, 25.9, 25.9, 24.8. FTIR (neat), cm^{-1}: 3236 (br, m), 2927 (s), 2853 (m), 1734 (s), 1698 (s), 1462 (m), 1258 (m), 1193 (m), 1034 (m), 869 (w). HRMS (ESI): calcd for $(C_{22}H_{36}N_2O_5S + H)^+$ 441.2418, found 441.2405.



9-biotinyloxynonyl cinnamate (6). To a solution of biotinylated phosphoranylidene ester **3** (8 mg, 0.012 mmol, 1 equiv) in Tris buffered solution (pH 7.6, 1 mL) was added benzaldehyde (6.4 µL, 0.06 mmol, 5.0 equiv) at 4 °C. The mixture was stirred at 4 °C for 12 h and the aqueous solution was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified using flash column chromatography to give 4.5 mg of the desired product (94%).

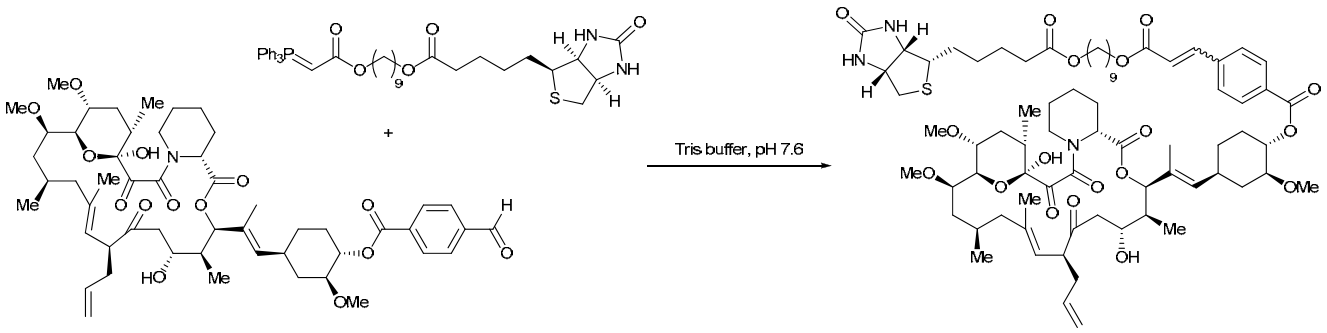
TLC: (5% MeOH–EtOAc) R_f = 0.32 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.68 (d, J = 16.0 Hz, 1H), 7.54 – 7.52 (m, 2H), 7.40 – 7.37 (m, 3H), 6.44 (d, J = 16.0 Hz, 1H), 4.60 (s, 1H), 4.54 – 4.50 (m, 1H), 4.46 (s, 1H), 4.32 (m, 1H), 4.20 (t, J = 6.8 Hz, 2H), 4.06 (t, J = 6.8 Hz, 2H), 3.17 (ddd, J = 8.6, 6.3, 4.7 Hz, 1H), 2.94 (dd, J = 12.8, 5.1 Hz, 1H), 2.73 (d, J = 12.8 Hz, 1H), 2.32 (t, J = 7.3 Hz, 2H), 1.73 – 1.65 (m, 6H), 1.64 – 1.60 (m, 3H), 1.50 – 1.45 (m, 2H), 1.43 – 1.38 (m, 2H), 1.37 – 1.30 (m, 7H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 173.7, 167.3, 162.6, 144.8, 134.6, 130.4, 129.0, 128.2, 118.4, 64.85, 64.7, 61.9, 60.2, 55.2, 40.7, 34.0, 29.5, 29.3, 29.3, 28.9, 28.8, 28.4, 28.4, 26.1, 26.0, 24.9. FTIR (neat), cm^{-1}: 3255 (br, m), 2924 (s), 2853 (m), 1705 (s), 1463 (m), 1312 (m), 1206 (m), 1174 (m), 1106 (w), 765 (m). HRMS (ESI): calcd for $(C_{28}H_{40}N_2O_5S + Na)^+$ 539.2550, found 539.2554.



FK506 4-formylbenzoate (7). To a solution of 4-formylbenzoic acid (3.3 mg, 0.022 mmol, 2.0 equiv) in toluene (1 mL) was added triethylamine (4.6 µL, 0.033 mmol, 3.0 equiv) and 2,4,6-trichlorobenzoyl chloride (3.8 µL, 0.024 mmol, 2.2 equiv) at room temperature. The reaction was allowed to stir for 3 h at room temperature. A solution of FK506 (9.8 mg,

0.011 mmol, 1.0 equiv) and DMAP (2.7 mg, 0.022 mmol, 2.0 equiv) in toluene (0.5 mL) was added to the reaction mixture and was stirred for 12 h at room temperature. The reaction was quenched with saturated ammonium chloride solution (3 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic extracts were washed with water (5 mL) and dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The crude residue was purified using flash column chromatography to give the 4.3 mg of the desired product (42%).

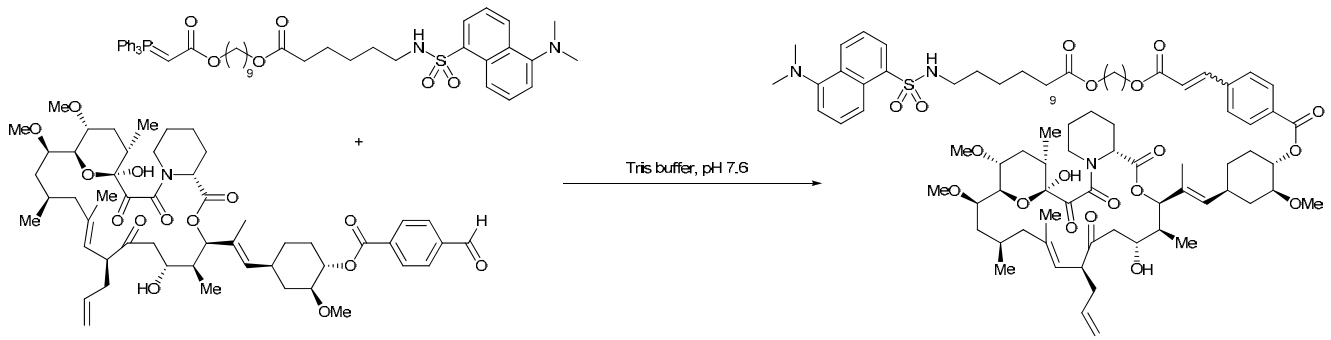
TLC: (40% EtOAc–hexane) R_f = 0.21 (CAM); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 10.11 (s, 1H), 8.20 (dd, *J* = 8.3, 1.9 Hz, 2H), 7.96 (dd, *J* = 8.4, 1.8 Hz, 2H), 5.76-5.67 (m, 1 H), 5.34 and 5.20 (rotamers, d, *J* = 1.5 Hz, 1 H), 5.10 (br d, *J* = 9.0 Hz, 1H), 5.07 – 5.04 (m, 1H), 5.01 (br d, *J* = 10.1 Hz, 1H), 4.95 (m, 1H), 4.86 and 4.24 (rotamers, br s, 1 H), 4.63 (br d, *J* = 5.0 Hz, 1H), 4.44 and 3.75 (rotamers, br d, *J* = 12.0 Hz, 1H), 3.96 and 3.92 (rotamers, m, 1 H), 3.88 and 3.68 (rotamers, dd, *J* = 9.5, 2.4 Hz, 1H), 3.61-3.55 (m, 1H), 3.50-3.44 (m, 1H), 3.402, 3.398, 3.395, 3.380, 3.340, 3.308, (rotamers of 3 methoxyls, s, total of 9 H), 3.02 (td, *J* = 13.4, 2.9 Hz, 1H), 2.79 and 2.73 (rotamers, dd, *J* = 16.0, 2.6, 1 H), 2.49 and 2.44 (rotamers, dt, *J* = 14.2, 7.0 Hz, 1H), 2.39-2.26 (m, 3 H), 2.21-2.14 (m, 3 H), 2.12 – 2.07 (m, 2H), 2.05 – 2.01 (m, 2H), 1.97 (ddd, *J* = 13.8, 10.1, 5.7 Hz, 1H), 1.93-1.88 (m, 1H), 1.84-1.73 (m, 3H), 1.63 – 1.32 (m, 9H), 1.68 and 1.67 (rotamers, d, *J* = 0.8 Hz, 3H), 1.63 and 1.60 (rotamers, br s, 3 H), 1.23 – 1.16 (m, 2H), 1.08 – 1.03 (m, 2 H), 1.00, 0.97, 0.94, 0.92, 0.87, 0.83 (rotamers of 3 methyls, d, *J* = 6.3, 6.6, 6.5, 7.2, 7.2, 6.5 Hz, total of 9 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 212.9 and 212.8 (rotamers), 196.2, 192.5 and 191.7 (rotamers), 171.2, 169.0 and 168.7 (rotamers), 165.9 and 165.1 (rotamers), 164.7, 139.9 and 139.1 (rotamers), 139.1 and 139.0 (rotamers), 135.7 and 135.6 (rotamers), 135.3, 132.9 and 132.2 (rotamers), 130.2, 129.5, 128.9 and 128.7 (rotamers), 122.6 and 122.3 (rotamers), 116.7 (rotamers), 98.7 and 97.0 (rotamers), 80.9, 77.2, 75.2, 73.7 and 73.6 (rotamers), 72.8, 72.1, 70.1, 69.0, 60.4, 57.8, 57.7, 57.6, 57.0, 56.6, 56.4, 56.2, 52.9 and 52.7 (rotamers), 48.5 and 48.4 (rotamers), 43.9 and 42.8 (rotamers), 40.2 and 39.6 (rotamers), 39.3, 36.5 and 36.4 (rotamers), 35.7, 35.5, 35.1, 34.7, 34.6 (rotamers), 33.6, 32.8, 32.7 and 32.6 (rotamers), 30.6, 29.8 and 29.7 (rotamers), 27.7, 26.3 and 26.2 (rotamers), 26.0, 24.6, 24.5, 22.7, 21.2, 21.0, 20.9, 20.5, 19.4, 16.3, 16.1, 15.8, 14.4 (rotamers), 14.2, 9.8, 9.4. FTIR (neat), cm^{-1}: 3650 (br, m), 2936 (s), 2860 (m), 1706 (s)2828 (m), 2953 (m), 1649 (s), 1451 (m), 1384 (m), 1322 m), 1275 (s), 1201 (m), 1173 (m), 1103 (s), 1038 (m), 1015 (m), 989 (m), 914 (w), 734 (s). HRMS (ESI): calcd for $(C_{52}H_{73}NO_{14} + Na)^+$ 959.4957, found 959.4974.



FK506 (*E*)-4-(3-((9-biotinyloxynonyl)oxy)-3-oxoprop-1-en-1-yl)benzoate (8). To a solution of biotinylated phosphoranylidene ester **3** (5 mg, 0.0096 mmol, 3.0 equiv) in Tris buffered solution (pH 7.6, 1 mL) was added FK506 4-formylbenzoate **7** (3 mg, 0.0032 mmol, 1.0 equiv) at 4 °C. The mixture was stirred at 4 °C for 12 h and the aqueous solution was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified using flash column chromatography to give 4.0 mg of the desired biotinylated FK506 adduct (73%).

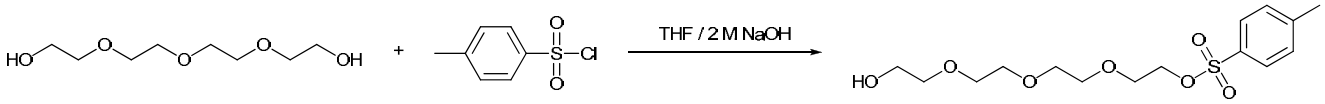
TLC: (5% MeOH–EtOAc) R_f = 0.24 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.06 (dd, *J* = 8.4, 1.7 Hz, 2H), 7.70 (d, *J* = 16.0 Hz, 1H), 7.59 (d, *J* = 8.3 Hz, 2H), 6.52 (d, *J* = 16.0 Hz, 1H), 5.76 – 5.66 (m, 1H), 5.34 and 5.20 (rotamers, d, *J* = 1.5 Hz, 1 H), 5.10 (br d, *J* = 9.0 Hz, 1H), 5.07 – 5.04 (m, 1H), 5.01 (br d, *J* = 10.1 Hz, 1H), 4.98 – 4.92 (m, 1H), 4.62 (d, *J* = 5.5 Hz, 1H), 4.54 (dd, *J* = 7.0, 5.1 Hz, 1H), 4.44 (d, *J* = 12.2 Hz, 1H), 4.34 (dd, *J* = 7.4, 4.6 Hz, 1H), 4.21 (t, *J* = 6.7 Hz, 2H), 4.06 (t, *J* = 6.7 Hz, 2H), 3.96 (s, 1H), 3.92 (ddd, *J* = 9.6, 3.9, 2.9 Hz, 1H), 3.88 and 3.68 (dd, J = 9.5, 2.4 Hz, 1H), 3.60 – 3.55 (m, 1H), 3.49 – 3.44 (m, 1H), 3.406, 3.401, 3.396, 3.382, 3.342, 3.308, (rotamers of 3 methoxyls, s, total of 9 H), 3.20 – 3.16 (m, 1H), 3.02 (td, *J* = 13.4, 2.9 Hz, 1H), 2.93 (dd, *J* = 12.9, 5.0 Hz, 1H), 2.79 and 2.73 (rotamers, dd, *J* = 16.0, 2.6, 1 H), 2.49 and 2.44 (rotamers, dt, *J* = 14.2, 7.0 Hz, 1H), 2.39 – 2.28 (m, 4H), 2.21 – 2.13 (m, 3H), 2.12 – 2.06 (m, 2H), 2.00 – 1.89 (m, 4H), 1.88 – 1.74 (m, 8H), 1.73 – 1.65 (m, 8H), 1.64 – 1.59 (m, 4H), 1.52 – 1.45 (m, 3H), 1.43 – 1.37 (m, 4H), 1.35 – 1.31 (m, 5H), 1.28 – 1.24 (m, 10H), 1.23 – 1.16 (m, 2H), 1.08 – 1.03 (m, 2 H), 1.00, 0.97, 0.94, 0.92, 0.87, 0.83 (rotamers of 3 methyls, d, *J* = 6.3, 6.6, 6.5, 7.2, 7.2, 6.5 Hz, total of 9 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 213.0 and 212.0 (rotamers), 196.2, 175.5, 173.6 and 173.5 (rotamers), 169.0 and 168.7 (rotamers), 166.7, 165.5, 164.7, 143.2, 139.0, 138.6, 135.6, 135.3, 132.9 and 132.2 (rotamers), 131.9 and 131.8 (rotamers), 103.9, 130.2, 130.0, 129.8, 129.3, 128.9 and 128.8 (rotamers), 127.9, 122.6 and 122.3 (rotamers), 120.6, 116.8 and 116.7 (rotamers), 98.7 and 98.4 (rotamers), 97.0, 80.9,

77.5, 75.2, 73.7 and 73.6 (rotamers), 72.8, 72.1, 70.6 and 70.1 (rotamers), 69.1, 68.2, 64.9, 64.7 and 64.6 (rotamers), 61.8, 60.1, 58.1, 57.9, 57.8 (rotamers), 57.7, 57.6, 57.0, 56.7, 56.4, 56.2, 55.1, 52.9 and 52.7 (rotamers), 48.5 and 48.4 (rotamers), 43.9 and 43.6 (rotamers), 42.8, 40.5, 40.2 and 39.6 (rotamers), 39.3 and 38.8 (rotamers), 36.5 and 36.4 (rotamers), 37.4, 37.1, 36.6 and 36.5 (rotamers), 36.0, 35.9, 35.7, 35.5, 35.4, 35.1, 34.7, 34.7 and 34.6 (rotamers), 33.8, 33.7, 33.6, 33.5, 33.4, 32.8, 32.7 and 32.6 (rotamers), 31.9 (rotamers), 30.5, 30.2, 30.0, 29.8 (rotamers), 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.3, 29.3 and 29.2 (rotamers), 29.2, 29.2 and 29.1 (rotamers), 29.1 (rotamers), 28.7, 28.6 (rotamers), 28.3, 28.3, 27.7, 27.2, 27.2, 27.1, 26.3 and 26.2 (rotamers), 26.0, 25.9, 25.9, 25.5, 25.2, 24.8 and 24.7 (rotamers), 24.5, 22.7, 21.2, 20.5, 19.4, 16.3, 16.1, 16.1, 15.8, 14.4 (rotamers), 14.1, 9.8, 9.4. FTIR (neat), cm^{-1}: 3333 (br, m), 2926 (s), 2855 (m), 1712 (s), 1652 (s), 1453 (m), 1274 (s), 1173 (s), 1103 (s), 1016 (m), 782 (s). HRMS (ESI): calcd for $(C_{73}H_{107}N_3O_{18}S + H)^+$ 1346.7343, found 1346.7349.



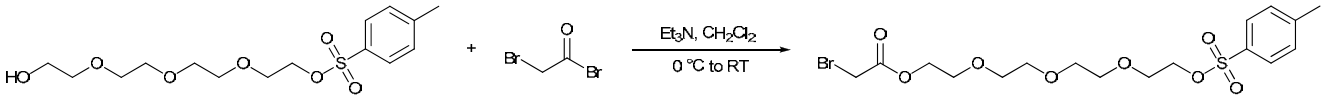
FK506 (*E*)-4-(3-((9-((*N*-dansyl-6-aminohexanoyl)oxy)nonyl)oxy)-3-oxoprop-1-en-1-yl)benzoate (9). A solution of dansyl phosphoranylidene ester **4** (7.8 mg, 0.0096 mmol, 3.0 equiv) in Tris buffered solution (pH 7.6, 1 mL) was added FK506 4-formylbenzoate **7** (3 mg, 0.0032 mmol, 1.0 equiv) at 4 °C. The mixture was stirred at 4 °C for 12 h and the aqueous solution was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was removed under reduced pressure. The product was purified using flash column chromatography to give 4.0 mg of the desired dansylated FK506 adduct (76%).

TLC: (5% MeOH–EtOAc) R_f = 0.27 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.56 (d, *J* = 8.5 Hz, 1H), 8.29 (d, *J* = 8.6 Hz, 1H), 8.26 (dd, *J* = 7.3, 1.2 Hz, 1H), 8.07 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.72 (d, *J* = 16.0 Hz, 1H), 7.60 (dd, *J* = 8.5, 1.9 Hz, 1H), 7.59 (dd, *J* = 8.9, 1.3 Hz, 1H), 7.57 – 7.52 (m, 1H), 7.20 (d, *J* = 7.5 Hz, 1H), 6.54 (dd, *J* = 16.0, 1.2 Hz, 1H), 5.77 – 5.69 (m, 1H), 5.36 and 5.22 (rotamers, br s, 1 H), 5.10 (br d, *J* = 8.9 Hz, 1H), 5.09 – 5.06 (m, 1H), 5.05 – 5.00 (m, 2H), 4.99 – 4.94 (m, 1H), 4.86 and 4.24 (rotamers, br s, 1 H), 4.65 – 4.62 (m, 1H), 4.46 and 3.78 (rotamers, br d, *J* = 13.6 Hz, 1H), 4.23 and 4.22 (rotamers, br s, 1H), 4.11 and 4.04 (t, *J* = 6.8 Hz, 1H), 3.97 and 3.93 (rotamers, m, 1 H), 3.90 (dd, *J* = 9.5, 2.4 Hz, 1H), 3.70 (d, *J* = 9.5 Hz, 1H), 3.60 (dd, *J* = 11.5, 2.6 Hz, 1H), 3.50-3.46 (m, 1H), 3.423, 3.418, 3.414, 3.400, 3.359, 3.325 (rotamers of 3 methoxyls, s, total of 9 H), 3.40 – 3.37 (m, 1H), 3.16 (d, *J* = 2.4 Hz, 1H), 3.04 (dd, *J* = 13.2, 2.4 Hz, 1H), 2.91 (s, 6H), 2.81 and 2.75 (rotamers, dd, *J* = 16.1, 2.6 Hz, 1H), 2.51 and 2.45 (rotamers, dt, *J* = 14.2, 7.0 Hz, 1H), 2.41 – 2.33 (m, 2H), 2.33 – 2.27 (m, 1H), 2.23 – 2.18 (m, 2H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.14 – 2.07 (m, 2H), 2.07 – 2.04 (m, 1H). 2.01 and 1.99 (dd, *J* = 5.3, 3.4 Hz, 1H), 1.94-1.89 (m, 1H), 1.84-1.73 (m, 3H), 1.74 – 1.70 (m, 3H), 1.68 and 1.65 (rotamers, br s, 3H), 1.66 – 1.53 (m, 15H), 1.61 and 1.60 (rotamers, br s, 3 H), 1.51 – 1.45 (m, 3H), 1.43 – 1.38 (m, 3H), 1.36 – 1.32 (m, 4H), 1.29 – 1.26 (m, 4H), 1.25 – 1.19 (m, 3H), 1.10 – 1.04 (m, 1H), 1.02, 0.99, 0.96, 0.94, 0.89, 0.84 (rotamers of 3 methyls, d, *J* = 6.3, 6.6, 6.4, 6.9, 7.2, 6.5 Hz, total of 9 H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 212.9 and 212.8 (rotamers), 196.2, 192.4, 173.5, 169.0 and 168.7 (rotamers), 166.7, 165.9 and 165.5 (rotamers), 164.7, 152.1, 143.2, 139.9 and 139.1 (rotamers), 135.6 and 135.5 (rotamers), 134.7, 132.9 and 132.2 (rotamers), 131.9 and 131.8 (rotamers), 130.4, 130.1 and 129.2 (rotamers), 129.7 and 129.6 (rotamers), 129.4, 129.3, 128.9 and 128.8 (rotamers), 128.4, 127.9, 123.2, 122.6 and 122.3 (rotamers), 120.6 (rotamers), 118.6, 116.7 (rotamers), 115.2, 98.7 and 97.0 (rotamers), 80.9, 77.2, 75.2, 73.7 and 73.6 (rotamers), 72.8, 72.1, 70.1, 69.1, 64.9 and 64.7 (rotamers), 64.5, 57.9 and 57.8 (rotamers), 57.7, 57.0, 56.7, 56.4, 56.2, 52.9 and 52.7 (rotamers), 48.5 and 48.4 (rotamers), 45.4, 43.9 and 42.6 (rotamers), 43.1, 42.8, 40.2 and 39.6 (rotamers), 39.3, 36.7 and 36.5 (rotamers), 35.7 and 35.5 (rotamers), 35.1, 34.7, 34.6, 33.9, 33.6 and 32.8 (rotamers), 32.7 and 32.6 (rotamers), 31.9, 30.5, 29.8, 29.7 (rotamers), 29.4, 29.3, 29.2, 29.1, 28.7, 28.6, 27.7, 26.3 and 26.2 (rotamers), 26.0, 25.9, 25.9, 25.9, 24.6, 24.2, 22.7, 21.5 and 20.9 (rotamers), 20.6 and 19.4 (rotamers), 16.3, 16.1 and 15.8 (rotamers), 14.5 and 14.4 (rotamers), 14.1, 9.8 and 9.4 (rotamers). FTIR (neat), cm^{-1}: 3484 (br, m), 3316 (br, m), 2930 (s), 2855 (m), 1715 (s), 1647 (s), 1452 (m), 1321 (m), 1273 (s), 1201 (m), 1172 (s), 1101 (s), 1015 (m), 789 (s). HRMS (ESI): calcd for $(C_{80}H_{113}N_3O_{19}S + Na)^+$ 1474.7581, found 1474.7583.



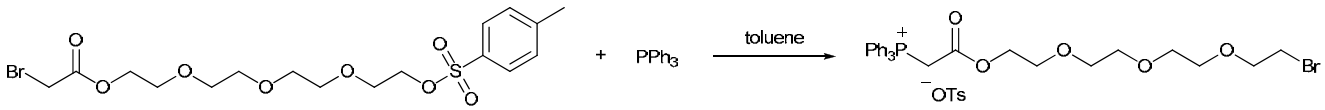
Tetraethylene glycol tosylate (10). To a solution of tetraethylene glycol (2.22 g, 11.4 mmol, 3.8 equiv) in THF at 0 °C was added 2.5 M NaOH (2 mL, 5.0 mmol, 1.7 equiv) and stirred for 30 min. Tosyl chloride (565.3 mg, 3.0 mmol, 1 equiv) was added in small portions and the mixture stirred for 5 h. The solvent was removed under reduced pressure, residue taken up in CH_2Cl_2 (15 mL) and washed with water (3 × 10 mL). The aqueous wash was further extracted with CH_2Cl_2 (3 × 10 mL) and the combined organic fractions were washed with brine and dried over anhydrous Na_2SO_4. The dried solution was filtered and the filtrate concentrated under reduced pressure to give a 900.8 mg of a pale yellow oil (87%). The product was sufficiently pure to be used without further purification.

TLC: (60% EtOAc–hexane) R_f = 0.12 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.80 (d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.5, 2H), 4.17 – 4.15 (m, 2H), 3.73 – 3.67 (m, 4H), 3.67 – 3.61 (m, 4H), 3.61 – 3.58 (m, 6H), 2.44 (s, 3H), 2.40 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 144.9, 133.1, 130.0, 128.1, 72.6, 70.9, 70.8, 70.6, 70.5, 69.4, 68.9, 61.9, 21.8. FTIR (neat), cm^{-1}: 3444 (br, s), 2873 (s), 1598 (m), 1453 (m), 1353 (s), 1176 (s), 1096 (s), 922 (s), 818 (m), 776 (m). HRMS (ESI): calcd for $(C_{15}H_{24}O_7S + Na)^+$ 349.1316, found 349.1310.



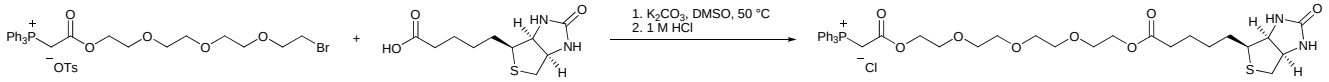
Tetraethylene glycol 2-bromoacetate tosylate (11). To a solution of tetraethylene glycol tosylate (1.07 g, 3.1 mmol, 1.0 equiv) and triethylamine (0.60 mL, 4.3 mmol, 1.4 equiv) in CH_2Cl_2 (20 mL) was added bromoacetyl bromide (0.35 mL, 3.6 mmol, 1.2 equiv) at 0 °C over a period of 1 minute, in the dark. After 5 min, the resulting solution was allowed to warm up to room temperature and stirred for 6 h. The pale yellow solution was slowly quenched with an equivalent volume of water and the resulting mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were washed with saturated sodium bicarbonate solution (20 mL) and dried over anhydrous Na_2SO_4. The dried solution was filtered, concentrated in vacuo and purified by flash column chromatography (10% EtOAc–hexane) to provide 1.27 g of the desired product as a yellow oil (88%).

TLC: (50% EtOAc–hexane) R_f = 0.36 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.80 (d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.1 Hz, 2H), 4.34 – 4.31 (m, 2H), 4.17 – 4.15 (m, 2H), 3.87 (s, 2H), 3.73 – 3.71 (m, 2H), 3.70 – 3.68 (m, 2H), 3.66 – 3.61 (m, 4H), 3.60 (s, 4H), 2.45 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 167.4, 145.0, 133.2, 130.0, 128.1, 70.9, 70.8, 70.8, 70.7, 69.4, 68.9, 68.9, 65.5, 26.0, 21.8. FTIR (neat), cm^{-1}: 2877 (s), 1738 (s), 1598 (m), 1451 (m), 1354 (s), 1284 (s), 1175 (s), 1098 (s), 1018 (s), 921 (s). HRMS (ESI): calcd for $(C_{17}H_{25}BrO_8S + Na)^+$ 491.0346, found 491.0328.



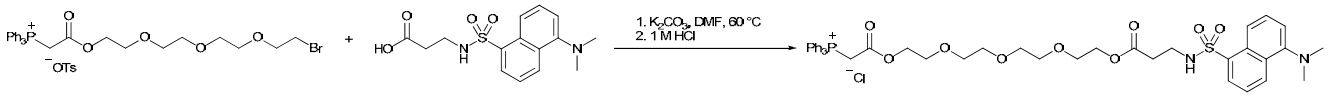
(14-bromo-2-oxo-3,6,9,12-tetraoxatetradecyl)triphenylphosphonium tosylate (12). To a solution of bromide **11** (450.3 mg, 0.959 mmol, 1 equiv) in toluene (5 mL) was added triphenylphosphine (0.31 g, 1.2 mmol, 1.2 equiv) in one portion. The mixture was allowed to stir for 72 h, after which the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (10% MeOH–CH_2Cl_2) to give 627.5 mg of the desired product as a sticky off-white solid (89%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.4 (ninhydrin); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.87 – 7.81 (m, 6H), 7.78 – 7.72 (m, 3H), 7.69 (d, J = 8.1 Hz, 2H), 7.63 (td, J = 7.9, 3.6 Hz, 6H), 7.05 (d, J = 8.2 Hz, 2H), 5.35 (d, J = 13.5 Hz, 2H), 4.15 – 4.11 (m, 2H), 3.76 (t, J = 6.3 Hz, 2H), 3.64 – 3.59 (m, 4H), 3.58 – 3.55 (m, 2H), 3.54 – 3.49 (m, 4H), 3.43 (t, J = 6.3 Hz, 2H), 2.30 (s, 3H).^{13}C NMR (151 MHz, CDCl3) δ: 164.9 (d, J = 3.3 Hz), 144.1, 138.8, 135.1 (d, J = 3.0 Hz), 134.1 (d, J = 10.7 Hz), 130.3 (d, J = 13.2 Hz), 128.4, 126.2, 118.2 (d, J = 89.1 Hz), 71.2, 70.5, 70.5, 70.5, 68.4, 65.8, 31.8 (d, J = 57.9 Hz), 30.6, 21.4. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 21.0 (s). FTIR (neat), cm^{-1}: 3440 (br, s), 2874 (s), 1739 (s), 1439 (s) 1194 (s), 1112 (s), 1034 (s), 1011 (s), 816 (m), 722 (m). HRMS (ESI): calcd for $(C_{28}H_{33}BrO_5P)^+$ 559.1243, found 559.1264.



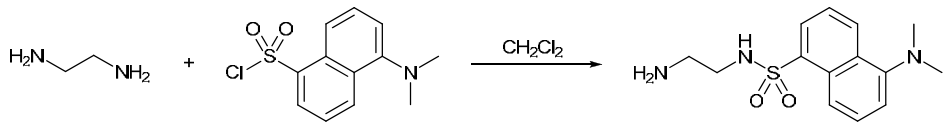
(14-biotinyloxy-2-oxo-3,6,9,12-tetraoxatetradecyl)triphenylphosphonium chloride (13). The phosphonium salt **12** (25.9 mg, 0.035 mmol), *D*-(+)-biotin (10.4 mg, 0.0425 mmol, 1.2 equiv), and potassium carbonate (9.8 mg, 0.708 mmol, 2 equiv) were dissolved in dimethylsulfoxide (0.5 mL). The mixture was heated at 50 °C for 2 h. An equivalent volume of 1 M HCl was then added and the mixture stirred for an additional 2 h at room temperature. Direct purification of the mixture by reverse phase HPLC (acetonitrile/water) gave 10.8 mg of the desired product as a pale waxy oil (40%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.48 (I_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.92 (dd, *J* = 13.4, 7.5 Hz, 6H), 7.77 (dd, *J* = 8.2, 6.6 Hz, 3H), 7.67 (td, *J* = 7.8, 3.6 Hz, 6H), 5.83 (dd, *J* = 13.5, 8.3 Hz, 2H), 5.33 (s, 1H), 5.30 (s, 1H), 4.58 – 4.50 (m, 1H), 4.32 (dd, *J* = 7.4, 4.5 Hz, 1H), 4.21 (dd, *J* = 8.1, 4.1 Hz, 2H), 4.15 (dd, *J* = 5.3, 2.8 Hz, 2H), 3.69 – 3.65 (m, 2H), 3.64 – 3.62 (m, 3H), 3.57 (ddd, *J* = 11.0, 5.7, 3.2 Hz, 6H), 3.16 (dd, *J* = 11.7, 7.1 Hz, 1H), 2.92 (dd, *J* = 12.7, 4.8 Hz, 1H), 2.81 (d, *J* = 12.8 Hz, 1H), 2.34 (t, *J* = 7.3 Hz, 2H), 1.79 – 1.56 (m, 4H), 1.50 – 1.39 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 173.7, 165.3, 165.3, 135.1 (d, *J* = 2.9 Hz), 134.3 (d, *J* = 10.8 Hz), 130.3 (d, *J* = 13.2 Hz), 118.5 (d, *J* = 89.1 Hz), 70.7, 70.7, 70.7, 70.6, 69.3, 68.5, 65.8, 63.5, 62.0, 60.4, 55.5, 40.69, 34.0, 32.5 (d, *J* = 57.5 Hz), 28.4, 24.9. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 21.0. FTIR (neat), cm^{-1}: 3348 (br, m), 2921 (s), 2854 (m), 1703 (s), 1616 (m), 1457 (m), 1438 (m), 1275 (s), 1260 (s), 1113 (s), 763 (s). HRMS (ESI): calcd for $(C_{38}H_{47}N_2O_8PS + H)^+$ 723.2864, found 723.2870.



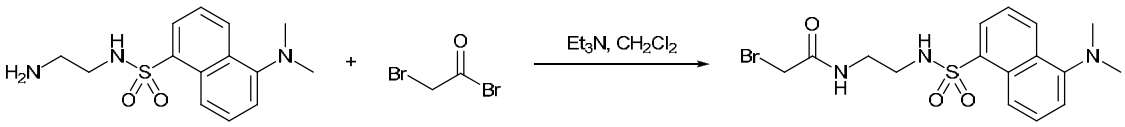
***N*-dansyl-(18-amino-2,16-dioxo-3,6,9,12,15-pentaoxaoctadecyl)triphenylphosphonium (14)**. The phosphonium salt **12** (97.3 mg, 0.0958 mmol, 1 equiv), *N*-dansyl *β*-alanine (62.9 mg, 0.195 mmol, 1.5 equiv), and potassium carbonate (72.4 mg, 0.524 mmol, 4.0 equiv) were dissolved in DMF (2 mL). The mixture was heated at 60 °C for 6 h. An equivalent volume of 1 M HCl was then added and the mixture stirred for an additional 30 min at room temperature. The mixture was concentrated to dryness in vacuo, partitioned between 1 M HCl (10 mL) and CH_2Cl_2 (5 mL each), and the aqueous layer was further extracted with CH_2Cl_2 (4 × 5 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated in vacuo, and the residue purified by flash column chromatography (10% MeOH–CH_2Cl_2) to provide 68.0 mg of the desired product as a bright yellow-green wax (61%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.2 (UV); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.56 (d, *J* = 8.6 Hz, 1H), 8.32 (d, *J* = 8.6 Hz, 1H), 8.25 (dd, *J* = 7.1, 1.3 Hz, 1H), 7.98 – 7.90 (m, 6H), 7.81 – 7.76 (m, 3H), 7.72 – 7.65 (m, 6H), 7.60 (t, *J* = 8.2 Hz, 1H), 7.54 (t, *J* = 7.8 Hz, 1H), 7.22 – 7.18 (m, 1H), 6.09 (s br, 1H), 5.71 (d, *J* = 13.5 Hz, 2H), 4.20 – 4.18 (m, 2H), 4.18 – 4.15 (m, 2H), 3.70 – 3.55 (m, 12H), 3.20 (q, *J* = 6.4 Hz, 2H), 2.91 (s, 6H), 2.53 (t, *J* = 6.3 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 171.8, 165.3, 151.9, 135.3, 135.0 (d, *J* = 3.1 Hz), 134.1 (d, *J* = 10.7 Hz), 130.3 (d, *J* = 13.2 Hz), 130.0, 129.7, 129.3, 128.4, 126.2, 123.3, 119.2, 118.6 (d, *J* = 89.1 Hz), 115.3, 71.4, 71.2, 70.7, 70.7, 70.6, 70.6, 70.5, 70.5, 70.5, 68.9, 68.5, 65.7, 65.5, 63.7, 45.5, 45.5, 42.9, 38.9, 34.5, 31.9 (d, *J* = 57.5 Hz), 30.6. ^{31}P NMR (162 MHz, $CDCl_3$) δ: 20.9. FTIR (neat), cm^{-1}: 2922 (s), 2867 (s), 1732 (s), 1454 (m), 1438 (s), 1314 (s), 1182 (m), 1142 (s), 1110 (s), 948 (m). HRMS (ESI): calcd for $(C_{43}H_{50}N_2O_9PS)^+$ 801.2969, found 801.3026.



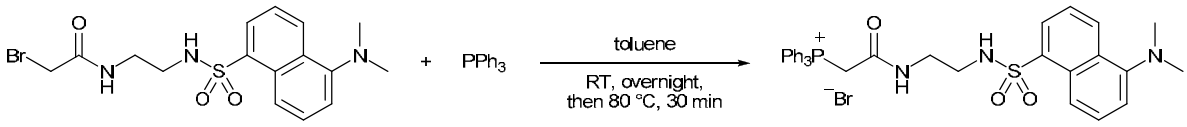
***N*-dansyl-1,2-diaminoethane (15).** A solution of dansyl chloride (110.1 mg, 0.408 mmol, 1 equiv) in CH_2Cl_2 (4 mL) was added dropwise to a stirring solution of ethylenediamine (1.0 mL, 18.5 mmol, 45 equiv) in CH_2Cl_2. After 4 h, the reaction was diluted with CH_2Cl_2 (10 mL). The reaction mixture was extracted with 1 M HCl (4 × 10 mL) and the combined aqueous extracts basified with 6 M NaOH (10 mL). The aqueous phase was then extracted with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated in vacuo, to give a 111.0 mg of pure product as a yellow foam (93%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.1 (UV); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.51 (dt, *J* = 8.6, 1.1 Hz, 1H), 8.30 (d, *J* = 8.6 Hz, 1H), 8.22 (dd, *J* = 7.2, 1.3 Hz, 1H), 7.53 (dd, *J* = 8.6, 7.5 Hz, 1H), 7.50 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.15 (dd, *J* = 7.7, 0.9 Hz, 1H), 2.93 – 2.89 (m, 2H), 2.86 (s, 6H), 2.72 – 2.65 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 152.1, 134.7, 130.5, 129.9, 129.7, 129.7, 128.5, 123.3, 118.8, 115.3, 45.5, 45.5, 40.9, 29.8. FTIR (neat), cm^{-1}: 3290 (br, m), 2941 (m), 2786 (m), 1574 (m), 1455 (m), 1315 (br, s), 1160 (s), 1143 (s), 944 (m), 791 (s). HRMS (ESI): calcd for $(C_{14}H_{19}N_3O_2S + H)^+$ 294.1271, found 294.1283.



2-bromo-*N*-(2-dansylaminoethyl)acetamide (16). To a stirring solution of amine **15** (28.9 mg, 0.0985 mmol, 1 equiv) and triethylamine (30.3 mg, 0.300 mmol, 3.0 equiv) in CH_2Cl_2 (1 mL) at 0 °C was added bromoacetyl bromide (30.3 mg, 0.15 mmol, 1.5 equiv) in one portion. After 6 h, the reaction was diluted with EtOAc (10 mL) and filtered through a pad of Celite. The filtrate was washed with saturated $NaHCO_3$ solution (20 mL) then extraced with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated in vacuo, and the residue purified by flash column chromatography (50% EtOAc–Hexane) to give a 16.4 mg of product as a waxy bright-yellow solid (40%).

TLC: (50% EtOAc–Hexane) R_f = 0.2 (UV); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.56 (d, *J* = 8.5 Hz, 1H), 8.26 – 8.24 (m, 2H), 7.59 (dd, *J* = 8.6, 7.6 Hz, 1H), 7.53 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.20 (dd, *J* = 7.5, 0.6 Hz, 1H), 6.67 (br s, 1H), 5.27 (t, *J* = 6.0 Hz, 1H), 3.68 (s, 2H), 3.34 (dt, *J* = 5.7 Hz, 2H), 3.08 (dt, *J* = 5.7 Hz, 2H), 2.90 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 166.5, 152.2, 134.2, 130.8, 130.0, 129.8, 129.4, 128.7, 123.2, 118.5, 115.4, 45.4, 45.4, 43.0, 39.9, 28.7. FTIR (neat), cm^{-1}: 3290 (br, m), 2926 (m), 2867 (m), 1655 (s), 1573 (m), 1539 (m), 1455 (m), 1317 (s), 1143 (s), 1093 (m), 946 (m), 790 (s). HRMS (ESI): calcd for $(C_{16}H_{20}BrN_3O_3S+H)^+$ 414.0482, found 414.0487.



(2-((2-(N-dansyl)aminoethyl)amino)-2-oxoethyl)triphenylphosphonium bromide (17). To a solution of bromide **16** (8.0 mg, 0.0194 mmol, 1 equiv) in toluene (1 mL) was added triphenylphosphine (10.6 mg, 0.0404 mmol, 2 equiv) in one portion. The mixture was allowed to stir overnight, but a faint trace of starting material was visible by TLC. The reaction mixture was thus heated at 80 °C for 30 min, allowed to cool, and the solvent removed under reduced pressure. The residue was purified by flash column chromatography (5% MeOH–CH_2Cl_2) to give 10.9 mg of the desired product as a waxy bright-yellow solid (83%).

TLC: (10% MeOH–CH_2Cl_2) R_f = 0.5 (UV/ninhydrin); ^{1}H NMR (600 MHz, $CDCl_3$) δ: 9.61 (br s, 1H), 8.50 (d, *J* = 8.5 Hz, 1H), 8.40 (d, *J* = 8.7 Hz, 1H), 8.19 (dd, *J* = 7.3, 1.3 Hz, 1H), 7.82 – 7.77 (m, 9H), 7.69-7.67 (m, 6H), 7.58 (dd, *J* = 8.6, 7.6 Hz, 1H), 7.48 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.15 (dd, *J* = 7.6, 0.7 Hz, 1H), 6.64 (br t, *J* = 5.7 Hz, 1H), 4.76 (d, *J* = 14.3 Hz, 2H), 3.14 (dt, *J* = 5.1 Hz, 2H), 2.97 (dt, *J* = 5.4 Hz, 2H), 2.86 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 162.9 (d, *J* = 5.0 Hz), 135.7, 135.4 (d, *J* = 3.0 Hz), 134.2 (d, *J* = 10.6 Hz), 130.4 (d, *J* = 13.1 Hz), 130.1, 129.9 (d, *J* = 40.9 Hz), 129.1, 128.6, 123.3, 120.0, 118.5, 117.9, 115.4, 45.6, 45.6, 42.1, 41.3, 32.6 (d, *J* = 55.4 Hz). ^{31}P NMR (162 MHz, $CDCl_3$) δ: 22.0
FTIR (neat), cm^{-1}: 3198 (br, m), 3057 (m), 2941 (m), 2869 (m), 2830 (m), 1672 (s), 1587 (m), 1571 (m), 1438 (s), 1321 (s), 1143 (s), 1110 (s), 996 (m), 793 (s), 730 (s), 688 (s). HRMS (ESI): calcd for $(C_{34}H_{35}N_3O_3PS)^+$ 596.2131, found 596.2127.

V. General Information—Biology

A. Cell Biology

All cell-culture work was conducted in a Class II biological safety cabinet. Buffers were filter-sterilized (0.2 μm) prior to use. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. Synthetic compounds used in biological experiments were stored in the dark at –20 °C as 10 mM stock solutions in DMSO.

B. Materials

Chinese Hamster Ovary (CHO) and Jurkat cells were purchased from ECACC Cat. No. #85050302 and #90112119 respectively. HeLa cells were purchased from NCI. CHO cells were cultured in Ham's F-12 (Gibco) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine and 1% Penicillin-Streptomycin (Gibco). Jurkat and HeLa cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine and 1% penicillin-streptomycin (Gibco). Cells were grown in BD Falcon tissue culture flasks with vented caps. RIPA buffer with protease and phosphatase inhibitors for whole cell lysate preparation was purchased from Thermo Scientific (#89900 and #78444). Streptavidin-agarose was purchased from Thermo Scientific (#0020359). Bradford reagent was purchased from Bio-Rad (#500-0006) and Laemmli loading buffer (2X concentration) were purchased from Sigma Aldrich (#S3401). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast Mini-PROTEAN TGX (4-20%) precast gels from Bio-Rad (#456-8123). Precision Plus Dual Colour standard from Bio-Rad (#161-0374) was used as a protein molecular marker. Electrophoresis and semi-dry electroblotting was carried out with Bio-Rad equipment (Mini-PROTEAN® Tetra Cell System). Nitrocellulose membranes were purchased from PALL Life Sciences (BioTrace NT). A mouse monoclonal antibody to FKBP12 (clone 1E5-A12) was purchased from Sigma Aldrich (#WH0002280M1). Western-blot detection was performed using the SuperSignal West Pico Chemiluminscence kit (including a goat anti-rabbit-HRP or goat anti-mouse-HRP conjugate) from Pierce (#34080). Western blots were visualized using Amersham HyperFilm ECL (#28-9068-40). Imaging dishes 35 mm μ-Dish were purchased from ibidi.

C. Instrumentation

Absorbance measurements were acquired on a Tecan Infinite® M1000 Pro microplate reader. Fluorescence microscopy work was performed on an Olympus IX-3 wide-field inverted microscope equipped with a Photometrics CoolSnapHQ^2 camera and a set of ET Sedat Quad 89000 filters from Chroma Technologies. Dansyl excitation was filtered at 350 nm (AT350/50x) and emission at 579-631 nm (ET605/52m). Images were acquired using Metamorph software and processed using FIJI.[5]

VI. Representative Procedures—Biology

A. Preparation of Solutions

Wash buffer:
50 mM Tris•HCl, pH 7.6
75 mM NaCl
0.5 mM EDTA
0.5% Triton X-100
0.5% Sodium deoxycholate
0.05% SDS

Tris buffer:
50 mM Tris•HCl, pH 7.8

TBST buffer
0.1% TWEEN 20 in Tris buffer

[5] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods.*, 2012, **9**, 676.

B. Preparation of Cell Lysates

Cell lysates were freshly prepared for each experiment. Cells were grown to approximately 90% confluence in 4×T-150 tissue culture flasks. For suspension cells (Jurkat), the cell-containing media was directly transfered into 50 mL-BD Falcon tubes on ice; for adherent cells (CHO, HeLa), the cell layer was first trypsinized (0.25% trypsin, 3 min, 37 °C) and diluted 4X with media. Samples were centrifuged (1000 x g, 5 min, 4 °C) and the supernatant was discarded. The cell pellets were resuspended in PBS (1 mL) and transferred to a 1.5-mL centrifuge tube. Cells were pelletted by centrifugation (6000 x g, 5 min, 4 °C) and the supernatant was aspirated. Ice-cold RIPA buffer (300 µL / cell pellet) was added to the pellets, and the mixture was rotated end-over-end for 1 h at 4 °C to lyse cells. The cell suspension in RIPA was centrifuged (12000 x g, 10 min, 4 °C), and the supernatant was transferred to a clean centrifuge tube. A 50-µL aliquot of washed, well-suspended, two-fold diluted streptavidin-agarose resin was added, and the resulting slurry was mixed (2 h, 4 °C), and then centrifuged (12000 x g, 10 min, 4 °C) to remove endogenous biotin. Protein concentration was determined using the Bradford method. A 1-mL aliquot of biotin-free lysate was diluted with Tris buffer to afford a protein concentration of 1.5 mg / mL. This was partitioned into 1.5-mL centrifuge tubes for subsequent dosing with compounds.

C. Dosing of Compounds in Lysates

Solutions of the bait or probes were prepared by serial dilution from a stock (10 mM in DMSO). Lysates were dosed with the appropriate concentration of bait at 4 °C (or the relevant control), mixing end-over-end in the dark for 8 h at 4 °C. After which, each sample was dosed with phosphoranylidene probe (or the relevant control) at 4 °C and mixed end-over-end for 12 h. Refer to the respective dosing tables in Part VII for the various experiments.

D. Preparation of Streptavidin Resins

A 500-µL aliquot of streptavidin-agarose suspension was transferred to a centrifuge tube. Wash buffer (1 mL) was added, and the resulting suspension was mixed end-over-end (5 min, 4 °C). The mixture was centrifuged (12000 x g, 2 min, 4 °C), and the supernatant was discarded. The resin was washed with wash buffer (2 × 1 mL, 10 min at 4 °C, followed by centrifugation at 12000 x g, 2 min, 4 °C) then thoroughly resuspended in Tris buffer (1 mL).

E. Streptavidin Pulldown Assay

Each sample was treated with a 50-µL aliquot of washed, well-suspended, two-fold diluted streptavidin-agarose resin. The resulting slurry was mixed end-over-end (15 h, 4 °C) then centrifuged (12000 x g, 10 min, 4 °C). The supernatant was removed and the collected resins washed with wash buffer (3×1 mL) then Tris buffer (2×1 mL). Each wash consisted of 10 min of mixing (4 °C), 10 min of centrifugation (4 °C) and then removal of the supernatant. Laemmli loading buffer was added to the resin (50 µL, 2X concentration) and the samples were heated to 95 °C for 6 min. The denatured protein mixture was then analyzed by SDS-PAGE and Western Blotting.

F. SDS-PAGE and Western Blot

A Tris-glycine mini gel (4-20%, 10-well) was loaded with the denatured protein mixture (20 µL / lane). The protein samples were electroeluted (150 V, 23°C, 45 min) then wet transferred to a nitrocellulose membrane (300 mA, 23°C, 1.5 h). The membrane was treated with blocking solution (40 mL, 5% low-fat milk in TBST, 1 h), primary antibody solution (20 mL of 1% low-fat milk in TBST containing 10 µg antibody, 15 h at 4°C), secondary antibody solution (20 mL of 1% low-fat milk in TBST containing 20 µg of HRP conjugate, 1 h), and finally 1:1 solution of stabilized peroxide and enhanced luminol (6 mL, 1 min); Between each of these treatments, the membrane was washed thoroughly (3×40 mL TBST). The membrane was sealed in a transparent plastic wrap and exposed to X-ray film to provide the Western blot.

G. Cellular Pulldown Assay

HeLa cells were grown to approximately 80% confluence and harvested as described above. The concentration of cells in suspension was determined using a hemocytometer. The suspension was diluted to 4.5 x 10^5 cells / mL and partitioned (8 mL each) into 75-mL cell culture flasks. Cells were incubated overnight. Before the experiment, spent media was aspirated, the cells were washed with DPBS (10 mL), and fresh media (8 mL) was added to each flask. Dosing solutions were prepared by diluting the respective DMSO stocks of compounds with culture media to a final volume of 250 µL (refer to dosing table in Part VII–D). An aliquot (200 µL) of this solution was dosed into the media in the flasks. Cells were incubated with bait **7** (or the relevant control) for 8 h, after which, the media was changed and cells carefully washed as previously described. Cells were then incubated with the appropriate probe (or control) for 15 h and subsquently harvested

for lysates.

H. Live Cell Fluorescence-Labelling

HeLa cells were grown until confluent and harvested as described above. Th cell suspension was partioned into 35-mm imaging dishes (Greiner #657160). Cells were seeded in wells at a density of 3×10^5 cells / mL in 2 mL of media. The cells were dosed with bait and probe in a similar manner to the pulldown assay. The culture media was changed as previously described before imaging. Within the same experiment, all images were acquired using exactly the same settings.

VII. Affinity Isolation of FKBP12

A. Probe Titration Experiment with Jurkat Lysates

Table 4. Preparation of Dosing Solutions for Probe Titration

		Preparation of Bait Solution*			Preparation of Probe Solution								
Sample	Vol. of lysate (µL)	Vol. of DMSO (µL)	Vol. of **7** from 10 mM stock (µL)	Vol. of Tris (µL)	Vol. of **3** from 5 µM stock (µL)	Vol. of **3** from 10 µM stock (µL)	Vol. of **3** from 50 µM stock (µL)	Vol. of **3** from 100 µM stock (µL)	Vol. of **3** from 500 µM stock (µL)	Vol. of Tris (µL)	Final Conc. of Probe **3** (µM)	Final Vol. of lysate (µL)	Components
1	200	5	x	47.5	X	X	X	X	X	47.5	X	300	DMSO
2	200	2.5	2.5	47.5	X	X	X	X	X	47.5	0.05	300	**3**
3	200	2.5	x	47.5	2.5	X	X	X	X	47.5	X	300	**7**
4	200	X	2.5	47.5	2.5	X	X	X	X	47.5	0.05	300	**7** + **3**
5	200	X	2.5	47.5	X	2.5	X	X	X	47.5	0.1	300	**7** + **3**
6	200	X	2.5	47.5	X	X	2.5	X	X	47.5	0.5	300	**7** + **3**
7	200	X	2.5	47.5	X	X	X	2.5	X	47.5	1	300	**7** + **3**
8	200	X	2.5	47.5	X	X	X	X	2.5	47.5	5	300	**7** + **3**
9	200	X	X	47.5	X	X	X	X	5	45.0	10	300	**7** + **3**

* Final bait concentration is consistent at 0.10 µM for all samples. DMSO concentration is consistent for all samples at 2.6%.

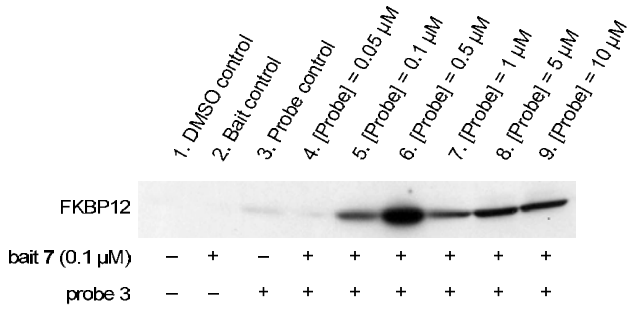


Figure 3. Western blot for the titration of probe **3** in Jurkat cell lysates. Lanes 1 –3: controls for DMSO, bait and probe **3** (5 µM) showing absence of FKBP12. Lanes 4 – 9 are dosed with increasing concentration of probe **3**.

B. Bait Titration Experiment with CHO Lysates

Table 5. Preparation of Dosing Solutions for Bait Titration

			Preparation of Bait Solution							Preparation of Probe Solution*			
Sample	Vol. of lysate (µL)	Vol. of DMSO (µL)	Vol. of **7** from 5 µM stock (µL)	Vol. of **7** from 10 µM stock (µL)	Vol. of **7** from 50 µM stock (µL)	Vol. of **7** from 100 µM stock (µL)	Vol. of **7** from 500 µM stock (µL)	Vol. of Tris (µL)	Final Conc. of Bait **7** (µM)	Vol. of **3** from 10 mM stock (µL)	Vol. of Tris (µL)	Final Vol. of lysate (µL)	Components
1	200	6.5	X	X	X	X	X	47.5	X	X	46.0	300	DMSO
2	200	4	2.5	X	X	X	X	47.5	0.05	X	46.0	300	**3**
3	200	4	X	X	X	X	X	47.5	X	4	46.0	300	**7**
4	200	4	2.5	X	X	X	X	47.5	0.05	4	46.0	300	**7** + **3**
5	200	4	X	2.5	X	X	X	47.5	0.1	4	46.0	300	**7** + **3**
6	200	4	X	X	2.5	X	X	47.5	0.5	4	46.0	300	**7** + **3**
7	200	4	X	X	X	2.5	X	47.5	1	4	46.0	300	**7** + **3**
8	200	4	X	X	X	X	2.5	47.5	5	4	46.0	300	**7** + **3**
9	200	6.5	X	X	X	X	X	47.5	X	X	46.0	300	Lysate control (20X Dilution)

* Final probe concentration is consistent at 5 µM. DMSO concentration is consistent for all samples at 2.6%.

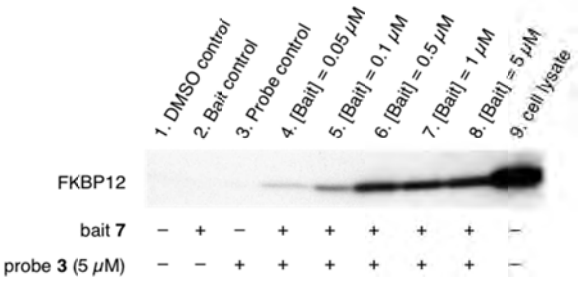


Figure 4. Western blot for the titration of bait **7** in CHO cell lysates. Lanes 1–3: controls for DMSO, bait (0.1 µM) and probe **3** showing absence of FKBP12. Lanes 4 – 8 are dosed with increasing concentration of bait **7**. Lane 9: CHO whole cell lysate control to indicate relative abundance of FKBP12.

C. Comparison Between Phosphoranylidene and Phosphonium Probe

Table 6. Preparation of Dosing Solutions for FK506, 7, 3 and 3a

	Preparation of Bait Solution						Preparation of Probe Solution						
Sample	Vol. of lysate (µL)	Vol. of DMSO* (µL)	Vol. of FK506 from 10 mM stock (µL)	Vol. of **7** from 10 mM stock (µL)	Vol. of Tris (µL)	Final Conc. of FK506 or Bait **7** (µM)	Vol. DMSO (µL)	Vol. of **3** from 10 mM stock (µL)	Vol. of **3a** from 10 mM stock (µL)	Vol. of Tris (µL)	Final Conc. of Probe **3** or **3a** (µM)	Final Vol. of lysate (µL)	Components
1	200	2.5	X	X	77.5	X	3	X	X	77	X	300	DMSO
2	200	X	2.5	X	77.5	0.1	3	X	X	77	X	300	FK506
3	200	X	X	2.5	77.5	0.1	3	X	X	77	X	300	**7**
4	200	2.5	X	X	77.5	X	3	3	X	77	0.1	300	**3**
5	200	X	X	X	77.5	X	3	X	3	77	0.1	300	**3**a
6	200	X	2.5	X	77.5	0.1	X	3	X	77	0.1	300	**7** + **3**
7	200	X	2.5	X	77.5	0.1	X	X	3	77	0.1	300	**7** + **3a**
8	200	X	X	2.5	77.5	0.1	X	3	X	77	0.1	300	FK506 + **3**
9	200	X	X	2.5	77.5	0.1	X	X	3	77	0.1	300	FK506 + **3a**

* DMSO concentration is consistent for all samples at 2.6%.

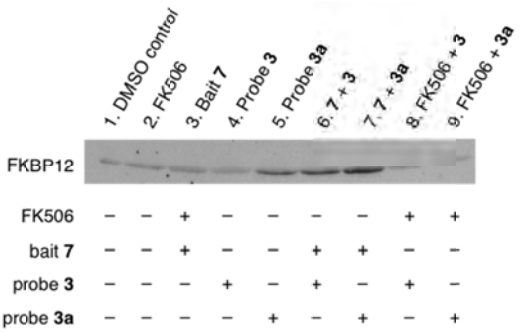


Figure 5. Western blot for the comparison of probes **3** and **3a**. Lanes 1–5: controls for DMSO, FK506, bait **7**, probes **3** and **3a** respectively. Lanes 6–9: lysates dosed with interacting pairs of reagents to show reactivity and chemoselectivity of both **3** and **3a** against FK506 and **7**.

D. Live Cell Affinity Isolation of FKBP12

Table 7. Preparation of Dosing Solutions for Affinity Isolation of FKBP12 in Live HeLa Cells

		Preparation of Bait Dosing Solution					Preparation of Probe Dosing Solution						
Sample	Vol. of DMSO* (μL)	Vol. of **7** from 1 mM stock (μL)	Vol. of **8** from 1 mM stock (μL)	Vol. of Media (μL)	Conc. of Bait Dosing Sol. (μM)	Final Conc. of Bait (μM)	Vol. of DMSO (μL)	Vol. of **3a** from 10 mM stock (μL)	Vol. of **13** from 10 mM stock (μL)	Vol. of Media (μL)	Conc. of Probe Dosing Sol. (μM)	Final Conc. of Probe (μM)	Components
1	10	X	X	190	40	1	5	X	X	195	200	X	DMSO
2	X	10	X	190	40	1	5	X	X	195	200	X	**7**
3	10	X	X	190	40	1	X	5	X	195	200	5	**3a**
4	10	X	X	190	40	1	X	X	5	195	200	5	**13**
5	X	10	X	190	40	1	X	5	X	195	200	5	**7** + **3a**
6	X	10	X	190	40	1	X	10	5	195	200	5	**7** + **13**
7	X	X	10	190	40	1	X	X	X	X	X	X	**8**

* DMSO concentration is consistent at 0.1% in the first dose (bait dosing) and 0.05% in the second (probe dosing).

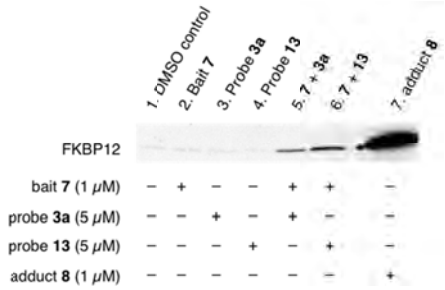


Figure 6. Western blot for affinity isolation of FKBP12 in live HeLa cells. Lanes 1–4: controls for DMSO, bait **7**, probes **3a** and **13** respectively. Lanes 5–6: bands corresponding to FKBP12 as a result of Wittig olefination between the probes (**3a** and **13**) and bait **7** in live cells. Lane 7: treatment of live cells with preconjugated adduct **8** as a positive control.

VIII. Fluorescence Labeling of FKBP12

Table 8. Preparation of Dosing Solutions for Fluorescence Labeling of FKBP12 in Live HeLa Cells

	Dosing of Bait				Dosing of Fluorescent Probes				
Sample	Vol. of DMSO* (μL)	Vol. of **7** from 1 mM stock (μL)	Vol. of Media (mL)	Conc. of Bait (μM)	Vol. of DMSO* (μL)	Vol. of Probe **(4, 14, or 17)** from 10 mM stock (μL)	Vol. of Media (mL)	Conc. of Probe (μM)	Components
1	1	X	2	1	1	X	2	X	DMSO
2	1	X	2	1	1	X	2	X	**7**
3	1	X	2	1	X	1	2	5	**4**
4	1	X	2	1	X	1	2	5	**14**
5	1	X	2	1	X	1	2	5	**17**
6	X	1	2	1	X	1	2	5	**7** + **4**
7	X	1	2	1	X	1	2	5	**7** + **14**
8	X	1	2	1	X	1	2	5	**7** + **17**

*DMSO concentration is consistent at 0.05% in the first dose and 0.25% in the second.

Two replicates of conditions 5 and 8 were subsequently performed.

A. Comparison of Dansyl Probes Bearing Different Linkers

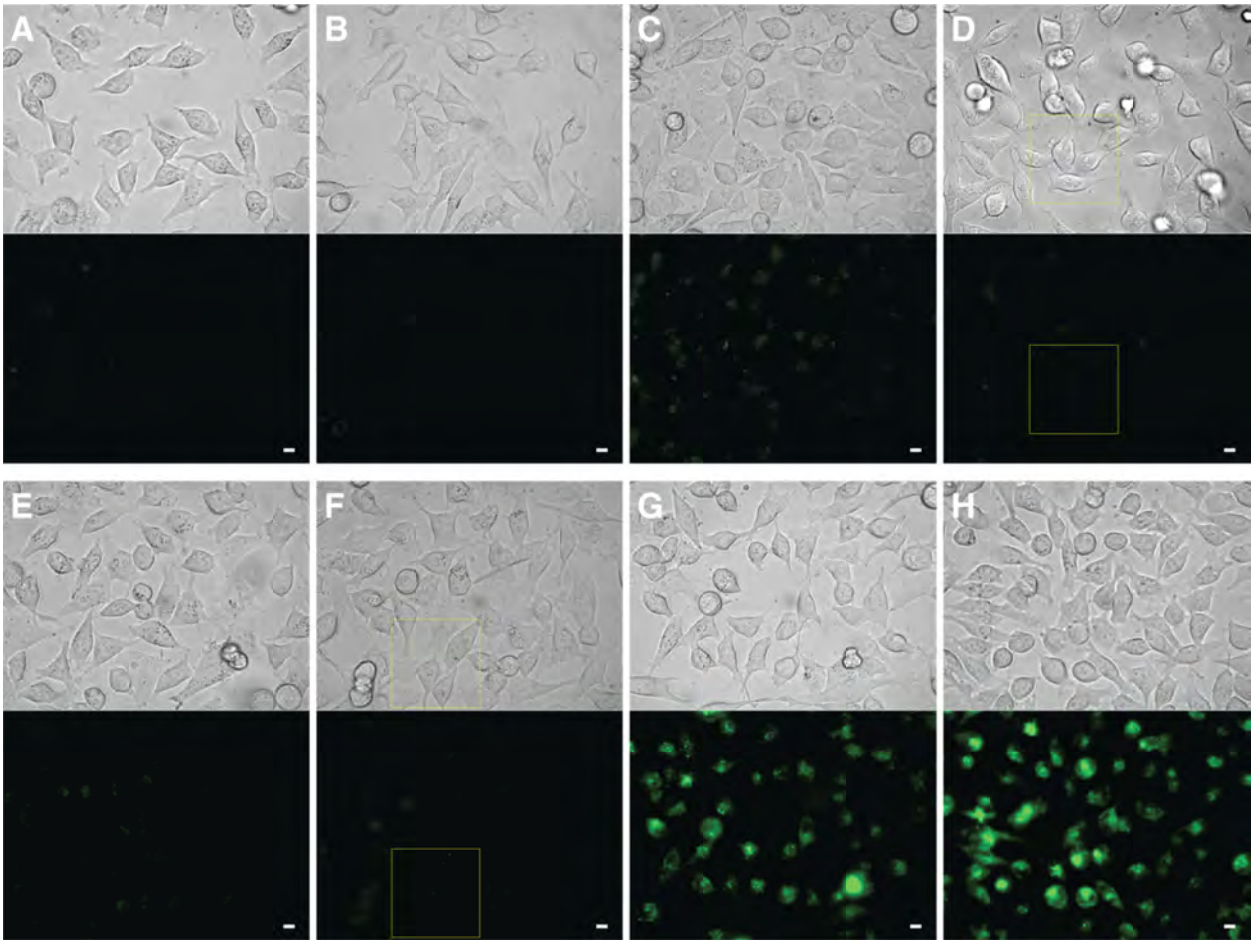


Figure 7. Comparison of all the dansyl probes (**4, 14** and **17**) in a single fluorescence microscopy experiment. DIC and fluorescence images of cells treated with (A) DMSO only, (B) bait **7** only, (C) probe **4** only, (D) bait **7** and probe **4**, (E) probe **14** only, (F) bait **7** and probe **14**, (G) probe **17** only, and (H) bait **7** and probe **17**. Cells treated with probe **17** show marked difference in fluorescence intensity compared to those with either probe **4** or **14**. The yellow boxes indicate the regions displayed in the main text. Scale bars represent 25 μm.

B. Comparison of Cells Treated With Bait and Probe Versus Bait Alone

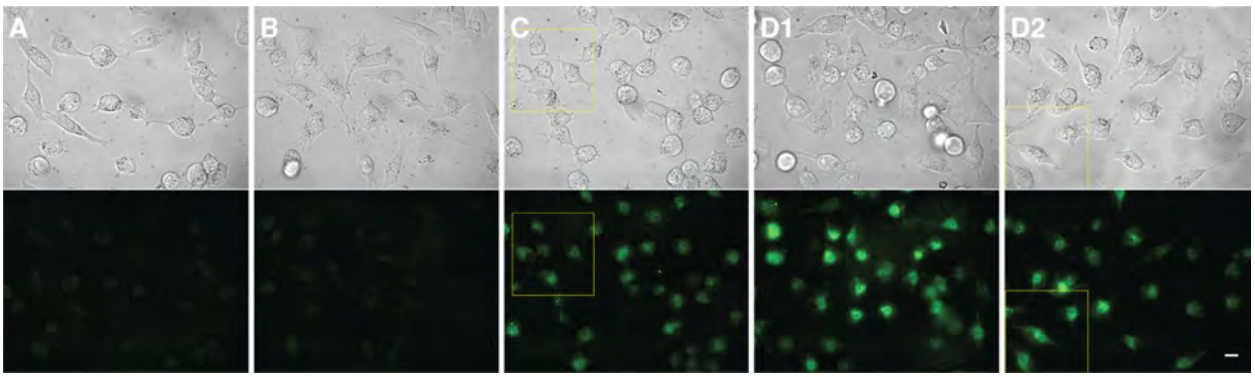


Figure 8. Live cell labeling experiment using bait **7** and probe **17**. Representative DIC and fluorescence images of cells treated with (A) DMSO, (B) bait **7** only, (C) probe **17** only, (D1–2) bait **7** and probe **17** taken from the same microscope sample at different region. The yellow boxes indicate the regions displayed in the main text. The scale bar represents 25 μm.