Supporting Information

Affinity-purification probes of potential use to investigate the endogenous Hsp70 interactome in cancer

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General Considerations

NMR spectra were recorded on a Bruker AV-III-500 MHz NMR spectrometer. Chemical shifts are reported in δ values in ppm downfield from TMS as the internal standard. ^{1}H data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constant (Hz), integration. ^{13}C chemical shifts are reported in δ values in ppm downfield from TMS as the internal standard. High resolution mass spectra were recorded on a Waters LCT Premier system. Low resolution mass spectra were obtained on Waters Acquity Ultra Performance LC with electrospray ionization and SQ detector. Analytical HPLC was performed on a Waters Autopurification system with PDA, MicroMass ZQ and ELSD detector. Analytical thin layer chromatography was performed on 250 μM silica gel F_{254} plates. Preparative thin layer chromatography was performed on 1000 μM silica gel F_{254} plates. Flash column chromatography was performed employing 230-400 mesh silica gel. Solvents were HPLC grade. All reagents were purchased from either Aldrich or Acros Organics and used without purification. All reactions were performed under argon protection. The synthesis of **1**-**4** and **11** is reported elsewhere.[1]

Synthesis

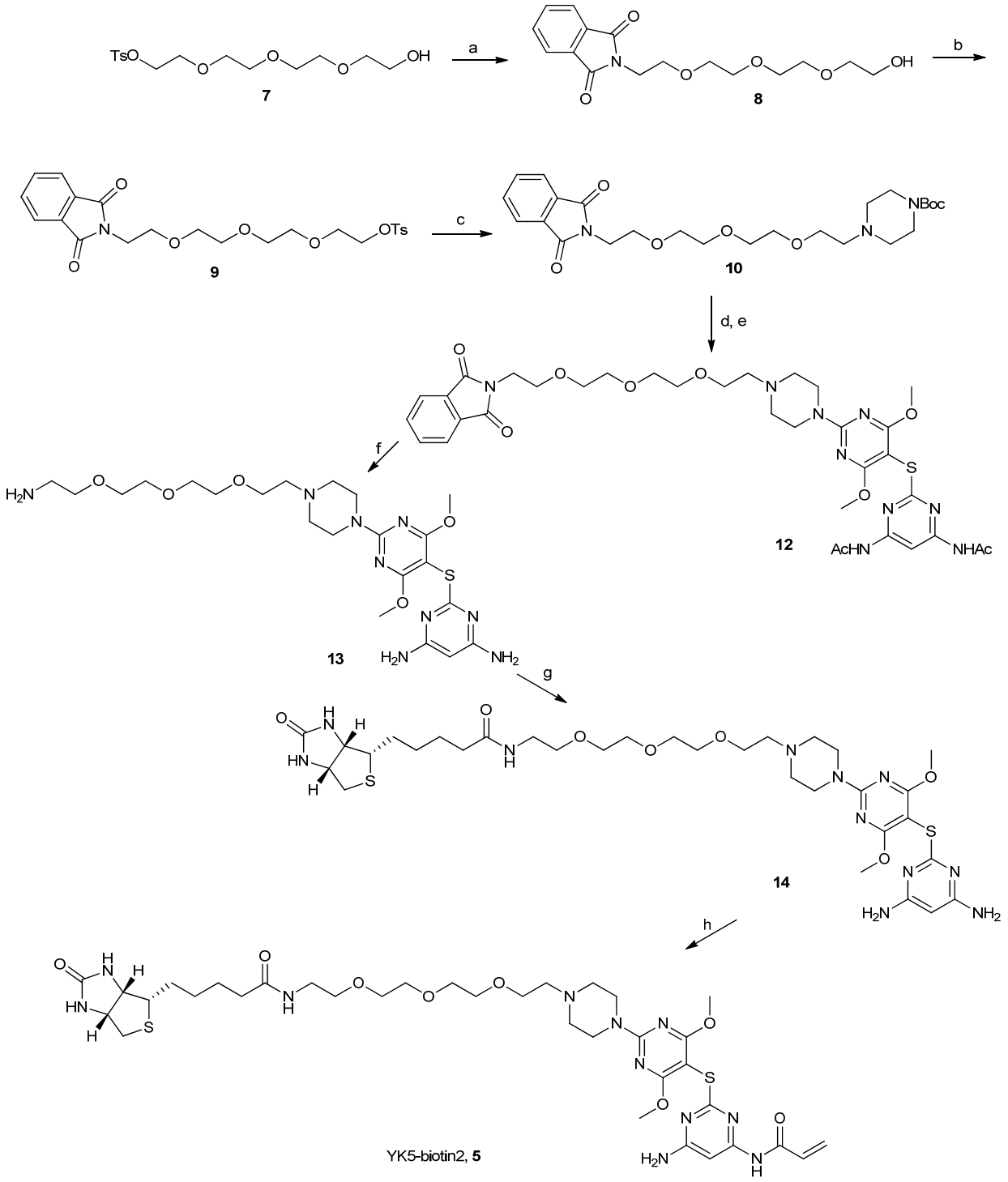
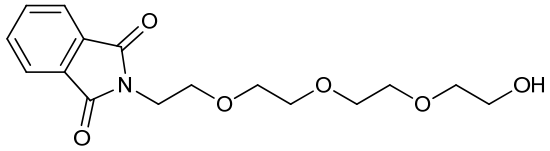
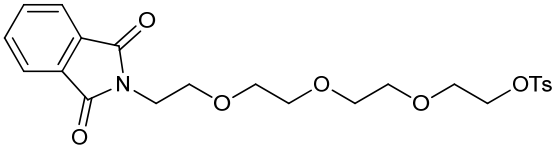


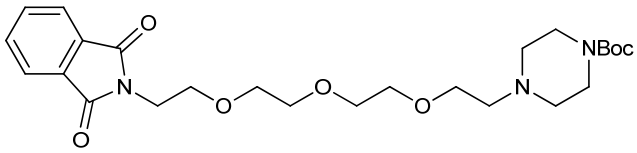
Figure S1. Synthesis of YK5-biotin2 (**5**).
Reagents and conditions: a. potassium phthalimide, DMF, 110 °C,18 h, 84%; b. tosyl chloride, Et_3N, DMAP, CH_2Cl_2, 5 °C to rt, 24 h, 87%; c. 1-Boc-piperazine, K_2CO_3, dioxane, 80 °C, 22 h, 72%; d. TFA:CH_2Cl_2 (1:4), rt, 1 h; e. N,N'-(2-((2-fluoro-4,6-dimethoxypyrimidin-5-yl)thio)pyrimidine-4,6-diyl)diacetamide (**11**), K_2CO_3, DMF, 90 °C, 1.5 h, 65%; f. NH_2NH_2, MeOH, rt, 2 h then 1M NaOH (*aq.*), 50 °C, 1.5 h, 93%; g. D-biotin, EDCI, DMAP, CH_2Cl_2, sonicate, 2 h, 55%; h. acryloyl chloride, Et_3N, CH_2Cl_2, 0 °C, 37%.



2-(2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl)isoindoline-1,3-dione (8).[2] **7** (1.22 g, 3.5 mmol) and potassium phthalimide (0.713 g, 3.85 mmol) were suspended in anhydrous DMF (10 mL) and heated at 110 °C for 18 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in CH_2Cl_2 (50 mL) and washed with 1M HCl (2 x 20 mL), brine (2 x 20 mL), dried over $MgSO_4$, and filtered. Solvent was removed under reduced pressure to give an oil which was purified by column chromatography (EtOAc) to afford 0.95 g (84%) of **8**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (dd, *J* = 3.1, 5.4 Hz, 2H), 7.72 (dd, *J* = 3.0, 5.5 Hz, 2H), 3.91 (t, *J* = 5.9 Hz, 2H), 3.75 (t, *J* = 5.8 Hz, 2H), 3.55-3.73 (m, 12H); MS (*m*/*z*): $[M+Na]^+$ 346.1.

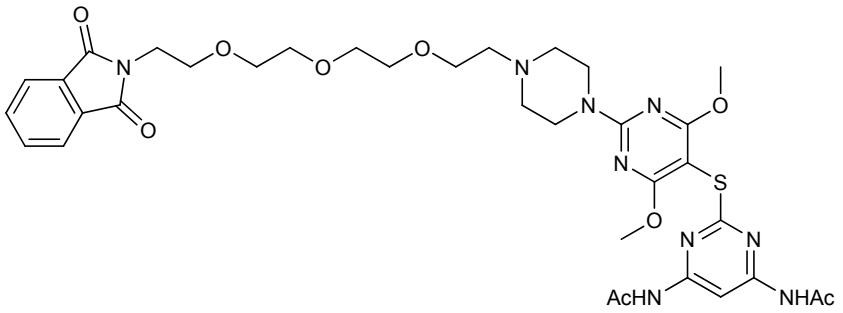


2-(2-(2-(2-(1,3-Dioxoisoindolin-2-yl)ethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (9).(*2*) A solution of **8** (0.95 g, 2.7 mmol), Et_3N (395 µL, 0.287 g, 2.8 mmol) and DMAP (33 mg, 0.27 mmol) in CH_2Cl_2 (30 mL) was cooled to 5°C with ice-bath. *p*-Toluenesulfonyl chloride (0.515 g, 2.7 mmol) was added in portions at 5 °C and after 30 minutes the ice-bath was removed and stirring continued at rt for 24 h. The reaction mixture was added to a seperatory funnel and washed with 1N HCl (2 x 25 mL), water (25 mL), and brine (2 x 25 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated to give an oil which was purified by column chromatography (hexane:EtOAc, 6:4 to 4:6) to give 1.12 g (87%) of **9**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.84 (dd, *J* = 3.1, 5.4 Hz, 2H), 7.79 (d, *J* = 8.3 Hz, 2H), 7.72 (dd, *J* = 3.0, 5.5 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 4.14 (t, *J* = 4.8 Hz, 2H), 3.89 (t, *J* = 5.9 Hz, 2H), 3.73 (t, *J* = 5.8 Hz, 2H), 3.48-3.68 (m, 10H), 2.44 (s, 3H); MS (*m*/*z*): $[M+Na]^+$ 500.0.

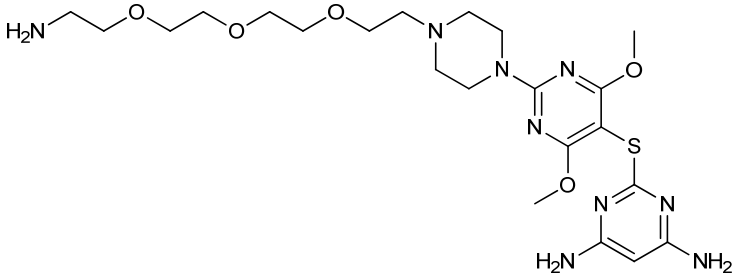


***tert*-Butyl 4-(2-(2-(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethoxy)ethoxy)ethyl)piperazine-1-carboxylate (10).** To a solution of **9** (1.10 g, 0.0023 mol) in dioxane (25 mL) was added 1-Boc-piperazine (1.07 g, 0.0058 mol) and K_2CO_3 (1.37 g, 0.0099 mol) and heated at 80 °C for 22 h. Solvent was removed under reduced pressure and the residue was taken up into CH_2Cl_2 (100 mL) and washed with water (2 x 50 mL) and brine (2 x 50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated to an oil which was purified by column chromatography (CH_2Cl_2:MeOH-NH_3 (7N), 1:0 to 30:1) to give 0.819 g (72%) of **10**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.84 (dd, *J* = 3.1, 5.4 Hz, 2H), 7.71 (dd, *J* = 3.0, 5.5 Hz, 2H), 3.90 (t, *J* = 5.9 Hz, 2H), 3.74 (t, *J* =

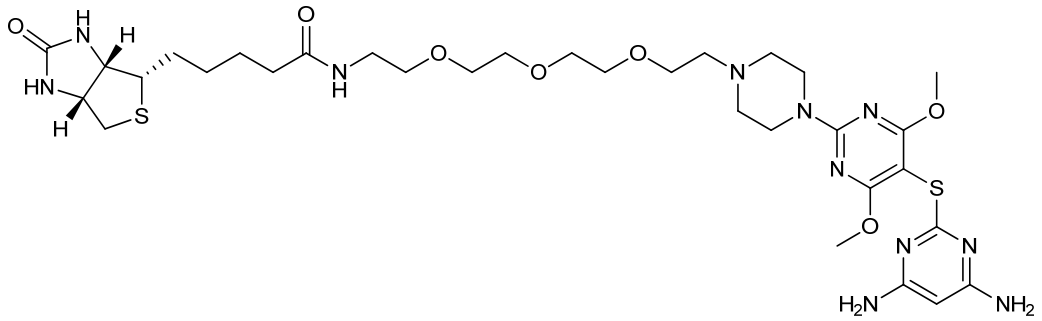
5.9 Hz, 2H), 3.52-3.67 (m, 10H), 3.43 (m, 4H), 2.57 (t, *J* = 6.0 Hz, 2H), 2.43 (m, 4H), 1.45 (m, 9H); MS (*m*/*z*): $[M+H]^+$ 492.1.



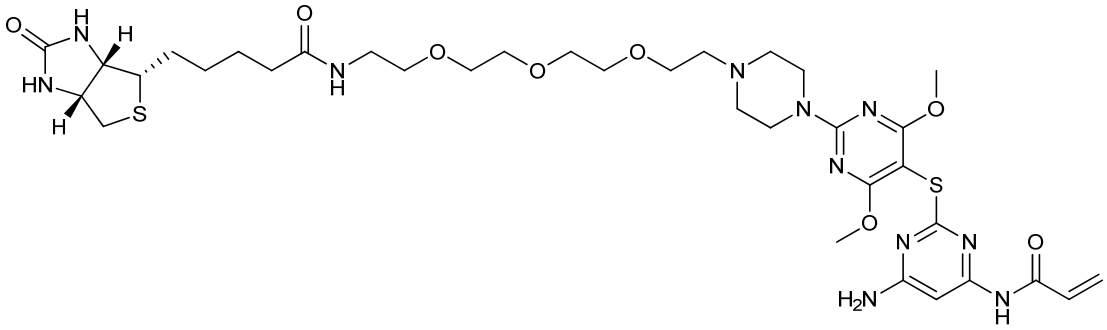
N,N'-(2-(2-(4-(2-(2-(2-(2-(1,3-Dioxoisoindolin-2-yl)ethoxy)ethoxy)ethoxy)ethyl)piperazin-1-yl)-4,6-dimethoxypyrimidin-5-ylthio)pyrimidine-4,6-diyl)diacetamide (12). To **10** (542 mg, 1.10 mmol) in CH_2Cl_2 (28 mL) was added TFA (7 mL) dropwise and stirred at rt for 1 h. The reaction mixture was concentrated under reduced pressure and TFA removed by co-evaporating with MeOH several times and drying under high vacuum overnight. To this was added K_2CO_3 (381 mg, 2.76 mmol) and DMF (20 mL) and the resulting suspension was stirred at rt for 10 min. Then **11** (421 mg, 1.1 mmol) was added and the mixture was heated at 90 °C for 90 min. The solvent was removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2:MeOH, 100:1 to 25:1) to give 0.537 g (65%) of **12**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35 (s, 1H), 7.84 (dd, *J* = 3.1, 5.5 Hz, 2H), 7.81 (bs, 2H), 7.71 (dd, *J* = 3.1, 5.5 Hz, 2H), 3.83-3.92 (m, 12H), 3.74 (t, *J* = 5.8 Hz, 2H), 3.55-3.67 (m, 10H), 2.64 (t, *J* = 5.7 Hz, 2H), 2.56 (m, 4H), 2.15 (s, 6H); MS (*m*/*z*): $[M+H]^+$ 754.2.



2-(2-(4-(2-(2-(2-(2-Aminoethoxy)ethoxy)ethoxy)ethyl)piperazin-1-yl)-4,6-dimethoxypyrimidin-5-ylthio)pyrimidine-4,6-diamine (13). 12 (300 mg, 0.398 mmol) in MeOH (9 mL) was added hydrazine hydrate (580 µL, 598 mg, 11.9 mmol) and stirred at rt for 2 h. Then 4.5 mL of 1M NaOH (*aq.*) was added and the reaction mixture was heated at 50 °C for 1.5 h. The reaction mixture was concentrated to dryness and the residue purified by column chromatography (CH_2Cl_2:MeOH, 60:1 to 10:1) to give 0.214 g (93%) of **13**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.16 (s, 1H), 4.56 (s, 4H), 3.84-3.91 (m, 10H), 3.61-3.69 (m, 10H), 3.51 (t, *J* = 5.2 Hz, 2H), 2.86 (t, *J* = 5.2 Hz, 2H), 2.66 (t, *J* = 5.7 Hz, 2H), 2.57 (m, 4H); MS (*m*/*z*): $[M+H]^+$ 540.1.



N-(2-(2-(2-(2-(4-(5-((4,6-Diaminopyrimidin-2-yl)thio)-4,6-dimethoxypyrimidin-2-yl)piperazin-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (14). 20.0 mg (0.0371 mmol) of **13**, 18.1 mg (0.0741 mmol) of D-(+)-biotin, DMAP (cat.), 14.2 mg (0.0741 mmol) of EDCI in 1 ml of CH_2Cl_2 was sonicated for 2 h in a sealed tube. The reaction mixture was evaporated to dryness and the residue was purified by preparatory TLC (CH_2Cl_2:MeOH-NH_3 (7N), 10:1) to give 15.6 mg (55%) of **14**. ^{1}H NMR (500 MHz, $CDCl_3$/MeOH-d_4): δ 5.23 (s, 1H), 4.49 (m, 1H), 4.30 (m, 1H), 3.86-3.91 (m, 10H), 3.60-3.72 (m, 12H), 3.40 (m, 2H), 3.12-3.18 (m, 1H), 2.91 (dd, *J* = 5.0, 12.9 Hz, 1H), 2.72 (d, *J* = 12.9 Hz, 1H), 2.68 (t, *J* = 5.6 Hz, 2H), 2.60 (m, 4H), 2.19 (dd, *J* = 2.1, 7.7 Hz, 2H), 1.38-1.76 (m, 6H); MS (*m*/*z*): $[M+H]^+$ 766.25.



N-(2-(2-(2-(2-(4-(5-((4-Acrylamido-6-aminopyrimidin-2-yl)thio)-4,6-dimethoxypyrimidin-2-yl)piperazin-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (YK5-biotin2; 5). To 15 mg (0.020 mmol) of **14** in 2 ml of CH_2Cl_2 at 0 °C was added 83 µl (60 mg, 0.6 mmol) of Et_3N. Then 3.3 µl (3.6 mg, 0.04 mmol) of acryloyl chloride in CH_2Cl_2 (0.5 mL) was added dropwise at 0°C. After 1 h, an additional 3.3 µl of acryloyl chloride in CH_2Cl_2 (0.5 mL) was added dropwise. This was repeated four more times at 30 min. intervals for a total reaction time of 3.5 hours (total acryloyl chloride, 19.8 µl, 21.6 mg, 0.24 mmol). The reaction mixture was concentrated under reduced pressure and the residue purified by preparatory TLC (CH_2Cl_2:MeOH-NH_3 (7N), 10:1) to yield 6.0 mg (37%) of **5**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.57 (s, 1H), 7.13 (s, 1H), 6.94 (br s, 1H), 6.53 (br s, 1H), 6.32-6.45 (m, 2H), 5.72 (dd, *J* = 2.4, 9.0 Hz, 1H), 5.58 (br s, 2H), 5.19 (br s, 1H), 4.48 (m, 1H), 4.34 (m, 1H), 3.82-3.93 (m, 10H), 3.55-3.77 (m, 12H), 3.39 (m, 2H), 3.08-3.15 (m, 1H), 2.89 (dd, *J* = 5.1, 12.9 Hz, 1H), 2.81 (m, 2H), 2.72 (d, *J* = 12.9 Hz, 1H), 2.70 (m, 4H), 2.19 (m, 2H), 1.38-1.76 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.6, 171.0, 170.3, 165.1, 164.8, 163.8, 159.8, 157.1, 130.9, 128.8, 88.7, 79.5, 71.0, 70.4, 70.3, 70.2, 69.5, 62.3, 59.9, 57.3, 56.0, 54.2, 53.4, 52.5, 43.6, 40.7, 39.2, 35.4, 28.5, 28.0, 25.7; MS (*m*/*z*): $[M+H]^+$ 820.3.

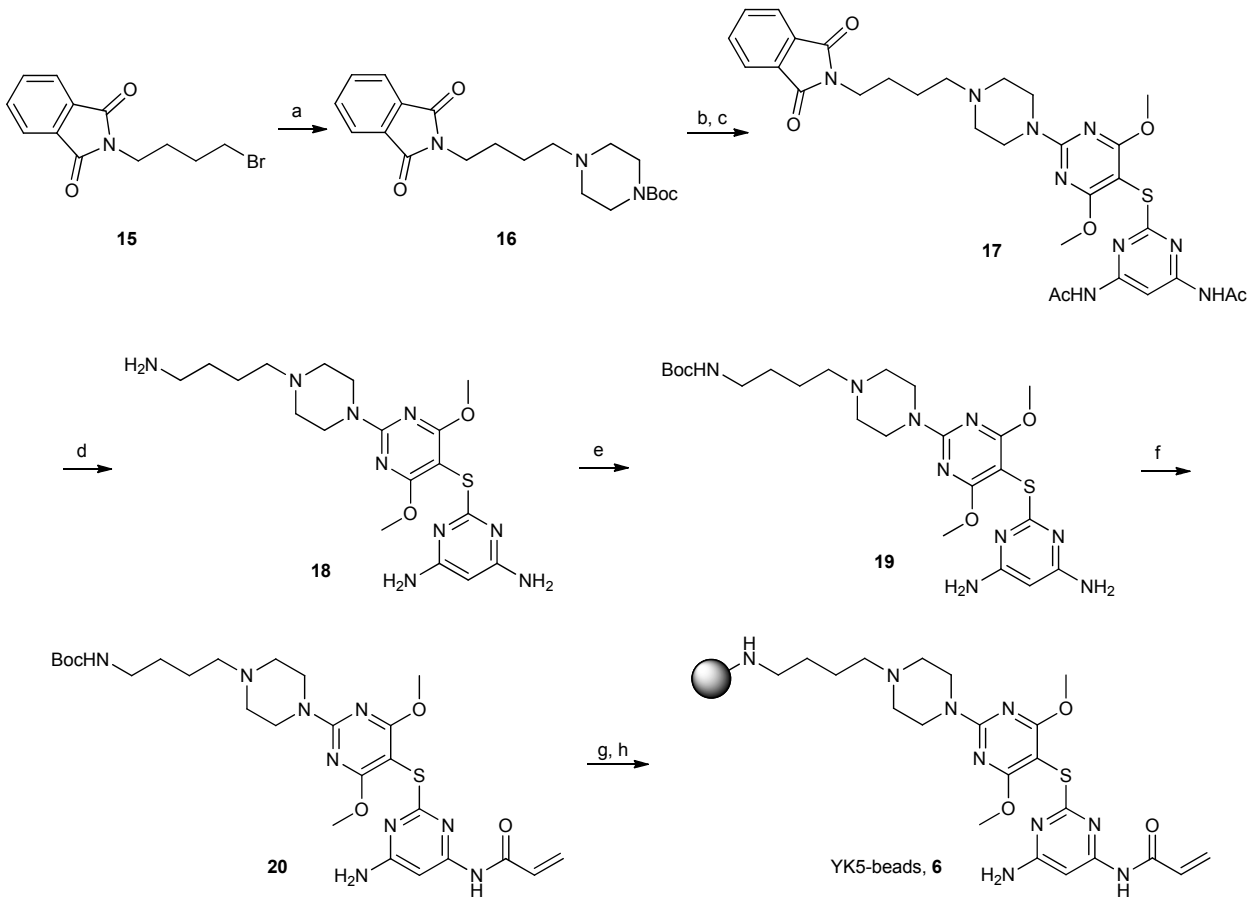
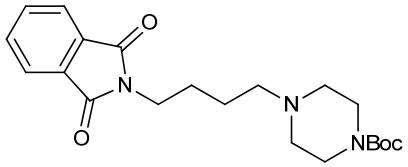
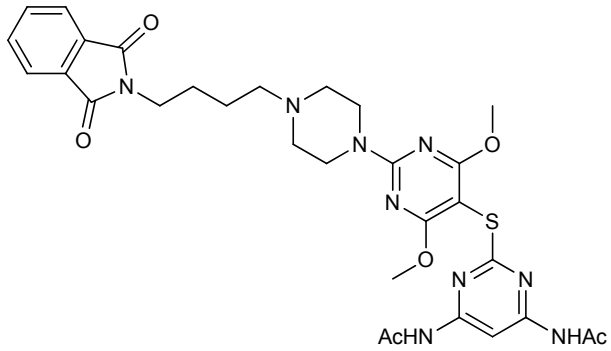


Figure S2. Synthesis of YK5-beads (**6**).
Reagents and conditions: a. 1-Boc-piperazine, NaI, K_2CO_3, acetone, reflux, 22 h; b. CH_2Cl_2:TFA (4:1), rt, 1 h; c. **11**, K_2CO_3, 90 °C, 1.5 h; d. hydrazine hydrate, MeOH, rt, 2 h, then 1M NaOH, 55 °C, 2 h; e. di-*t*-butyldicarbonate, Et_3N, CH_2Cl_2, rt, 20 h; f. acryloyl chloride, Et_3N, CH_2Cl_2, 0 °C, 2 h; g. CH_2Cl_2:TFA (4:1), rt, 45 min.; h. Affi-Gel® 10 beads, DIEA, DMAP, DMF, 3 h.

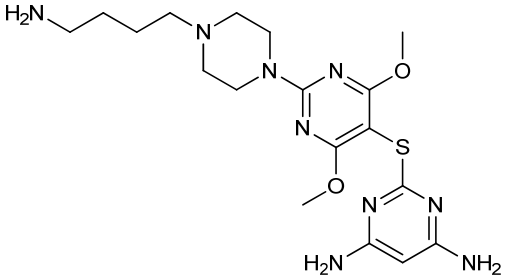


***tert*-Butyl 4-(4-(1,3-dioxoisoindolin-2-yl)butyl)piperazine-1-carboxylate (16).** N-(4-bromobutyl)phthalimide (**15**; 1.95 g, 6.89 mmol) and sodium iodide (81 mg, 0.537 mmol) were added to a suspension of K_2CO_3 (1.64 g, 11.88 mmol) and 1-Boc-piperazine (1.00 g, 5.37 mmol) in acetone (25 mL) and refluxed for 22 h. The reaction mixture was filtered and the solid was washed with acetone (3 x 50 mL). The filtrate was concentrated and the residue purified by column chromatography (hexane:EtOAc, 7:3 to 0:1) to afford 2.08 g (100%) of **16**. ^{1}H NMR (500

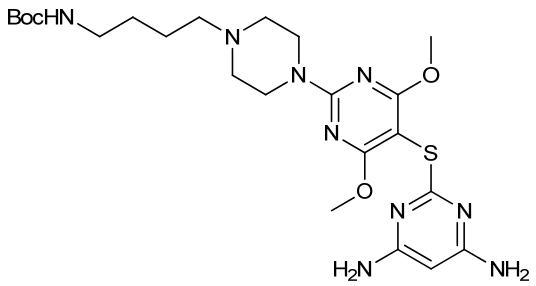
MHz, $CDCl_3$): δ 7.84 (dd, *J* = 3.0, 5.4 Hz, 2H), 7.71 (dd, *J* = 3.0, 5.4 Hz, 2H), 3.71 (t, *J* = 7.1 Hz, 2H), 3.38-3.43 (m, 4H), 2.32-2.40 (m, 6H), 1.70 (m, 2H), 1.53 (m, 2H), 1.45 (s, 9H); MS (*m*/*z*): $[M+H]^+$ 388.4.



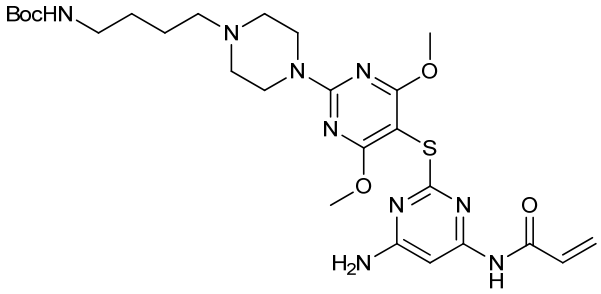
N,N'-(2-(2-(4-(4-(1,3-dioxoisoindolin-2-yl)butyl)piperazin-1-yl)-4,6-dimethoxypyrimidin-5-ylthio)pyrimidine-4,6-diyl)diacetamide (17). To **16** (429.7 mg, 1.11 mmol) in CH_2Cl_2 (12 mL) was added TFA (3 mL) dropwise and stirred at rt for 1 h. The reaction mixture was concentrated under reduced pressure and TFA removed by co-evaporating with MeOH several times and drying under high vacuum overnight. To this was added K_2CO_3 (384 mg, 2.78 mmol) and DMF (21 mL) and the resulting suspension was stirred at rt for 10 min. Then **11** (424 mg, 1.11 mmol) was added and the suspension was heated at 90 °C for 90 min. Solvent was removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2:MeOH, 100:1 to 40:1) to give 0.355 g (49%) of **17**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35 (br s, 1H), 7.82-7.88 (m, 4H), 7.72 (dd, *J* = 3.1, 5.5 Hz, 2H), 3.88 (s, 6H), 3.84 (m, 4H), 3.74 (t, *J* = 7.2 Hz, 2H), 2.48 (m, 4H), 2.43 (t, *J* = 7.4 Hz, 2H), 2.16 (s, 6H), 1.74 (m, 2H), 1.59 (m, 2H); MS (*m*/*z*): $[M+H]^+$ 650.5.



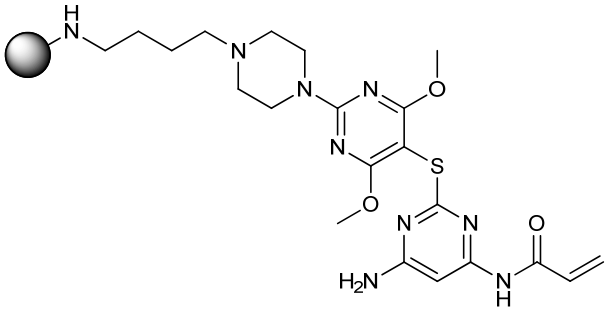
2-(2-(4-(4-aminobutyl)piperazin-1-yl)-4,6-dimethoxypyrimidin-5-ylthio)pyrimidine-4,6-diamine (18). 17 (0.355 g, 0.546 mmol) in MeOH (10 mL) was added hydrazine hydrate (797 µL, 0.820 g, 16.4 mmol) and stirred at rt for 2 h. Then 5 mL of 1M NaOH was added and the reaction mixture was heated at 55°C for 2 h. The reaction mixture was concentrated under reduced pressure and the residue purified by column chromatography (CH_2Cl_2:MeOH-NH_3 (7N), 20:1 to 5:1) to give 0.228 g (96%) of **18**. ^{1}H NMR (500 MHz, $CDCl_3$/MeOH-d_4): δ 5.22 (s, 1H), 3.88 (s, 10H), 2.72 (t, *J* = 7.1 Hz, 2H), 2.53 (m, 4H), 2.42 (t, *J* = 7.2 Hz, 2H), 1.46-1.62 (m, 4H); MS (*m*/*z*): $[M+H]^+$ 436.4.



***tert*-butyl 4-(4-(5-(4,6-diaminopyrimidin-2-ylthio)-4,6-dimethoxypyrimidin-2-yl)piperazin-1-yl)butylcarbamate (19). 18** (0.221 g, 0.507 mmol) in CH_2Cl_2 (6 mL) was added Et_3N (107 µL, 77 mg, 0.761 mmol) and di-*t*-butyldicarbonate (0.133 g, 0.611 mmol) and stirred at rt for 20 h. The reaction mixture was concentrated under reduced pressure and the residue purified by column chromatography (CH_2Cl_2:MeOH-NH_3 (7N), 100:1 to 30:1) to give 0.254 g (93%) of **19**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.19 (s, 1H), 4.41 (s, 4H), 3.89 (m, 10H), 3.14 (m, 2H), 2.50 (m, 4H), 2.40 (t, *J* = 6.8 Hz, 2H), 1.54-1.63 (m, 4H), 1.44 (s, 9H); MS (*m*/*z*): $[M+H]^+$ 536.5.



***tert*-butyl 4-(4-(5-(4-acrylamido-6-aminopyrimidin-2-ylthio)-4,6-dimethoxypyrimidin-2-yl)piperazin-1-yl)butylcarbamate (20).** To 80 mg (0.149 mmol) of **19** in 8 ml of CH_2Cl_2 at 0 °C was added 414 µl (298 mg, 2.98 mmol) of Et_3N. Then 14.5 µl (16.2 mg, 0.179 mmol) of acryloyl chloride was added. After 30 min. an additional 14.5 µl of acryloyl chloride was added at 0 °C. This was repeated two more times for a total reaction time of 2 hours (total acryloyl chloride, 58 µl, 64.8 mg, 0.716 mmol). The reaction was quenched by the addition of 1 mL MeOH and then concentrated under reduced pressure. The residue was purified by preparatory TLC (CH_2Cl_2:MeOH-NH_3 (7N), 15:1) to yield 42.5 mg (48%) of **20**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.78 (s, 1H), 7.05 (s, 1H), 6.42 (d, *J* = 16.9 Hz, 1H), 6.18 (dd, *J* = 10.4, 16.9 Hz, 1H), 5.80 (d, *J* = 10.2, 1H), 5.15 (br s, 1H), 4.80 (br s, 2H), 3.89 (s, 10H), 3.14 (m, 2H), 2.54 (m, 6H), 1.58 (m, 4H), 1.44 (s, 9H); MS (*m*/*z*): $[M+H]^+$ 590.5.



YK5-beads (6). A solution of **20** (45 mg, 0.076 mmol) in 3 ml of CH_2Cl_2 was added 0.75 mL of TFA dropwise at rt. After stirring for 45 min., the reaction mixture was concentrated under reduced pressure. TFA was removed by co-evaporating with MeOH several times and drying

under high vacuum overnight to yield a residue which was dissolved in DMF (2 mL) and added to 4.2 mL (0.0636 mmol) of Affi-Gel® 10 beads (prewashed, 3 x 6 mL DMF) in a solid phase peptide synthesis vessel. 100 µL of N,N-diisopropylethylamine and several crystals of DMAP were added and this was shaken at rt for 3 h. Then the solvent was removed and the beads washed for 10 minutes each time with CH_2Cl_2 (4 x 10 mL), DMF (4 x 10 mL), and *i*-PrOH (3 x 10 mL).

Storage

The YK5 beads (**6**) were stored in *i*-PrOH at -80 °C. YK5-biotin1 (**4**) and YK5-biotin2 (**5**) working stocks of 100mM in DMSO were stored at -20°C. The DMSO stock of YK5-biotin1 was stable for approximately 2-3 months, whereas YK5-biotin2 had the advantage of greater stability upon long term storage. This compound can be stored for at least two years at -20°C with no evidence of decomposition.

Additional References

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2. Lankshear, M., Dudley, I., Chan, K.-M., Cowley, A., Santos, S., Felix, V., and Beer, P. (2008) Cooperative and Ion-Pair Recognition by Heteroditopic Calix[4]diquinone Receptors, *Chem. Eur. J. 14*, 2248–2263.