Evidence for the Rapid Conversion of Stephacidin B into the Electrophilic Monomer

Avrainvillamide in Cell Culture

Jeremy E. Wulff, Seth B. Herzon, Romain Siegrist and Andrew G. Myers

J. Am. Chem. Soc.

Supporting Information

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A. Chemistry

A. Chemistry

General Experimental Procedures. All reactions were performed in single-neck, flame-dried, round-bottom flasks fitted with rubber septa under a positive pressure of argon, unless otherwise noted. Air- and moisture-sensitive liquids were transferred via syringe or stainless steel cannula. Where necessary (indicated in the text), solutions were deoxygenated by five successive freeze-pump-thaw cycles. Organic solutions were concentrated at ambient temperature by rotary evaporation at 40 Torr (house vacuum). Analytical thin-layer chromatography (TLC) was performed using glass plates pre-coated with silica gel (0.25 mm, 60 Å pore-size, 230-400 mesh, Merck KGA) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light, then were stained with iodine or by submersion in aqueous ceric ammonium molybdate (CAM), followed by brief heating on a hot plate. Flash-column chromatography was performed as described by Still et al.,[1] employing silica gel (60 Å, 32-63 μM, standard grade, Sorbent Technologies).

Materials. Commercial solvents and reagents were used as received with the following exceptions. Dichloromethane, benzene, tetrahydrofuran, and acetonitrile were purified by the method of Pangborn et al.[2] 2-Iodo-4,4,6,6-tetramethyl-cyclohexen-2-en-1-one (**14**),[3] and iodoarene **19**[4] were prepared as described previously.

Instrumentation. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at 400 or 500 MHz at 23 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale) downfield from tetramethylsilane, and are referenced to residual protium in the NMR solvent (CHCl$_3$, δ 7.26; C$_6$HD$_5$, δ 7.15; CHD$_2$OD, δ 3.30; (CHD$_2$)S(O)CD$_3$, δ 2.49). Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and/or multiple resonances, br = broad, app = apparent), integration, and coupling constant in Hertz. Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 100 or 125 MHz at 23 °C unless otherwise noted. Carbon chemical shifts are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent (CDCl$_3$, δ 77.0; C$_6$D$_6$, δ 128.0; CD$_2$Cl$_2$, δ 53.8; CD$_3$OD, δ 49.0; (CD$_3$)S(O)CD$_3$, δ 39.5). Infrared (IR) spectra were obtained using a Perkin-Elmer FT-IR spectrometer referenced to a polystyrene standard. Data are represented as follows: frequency of absorption (cm^{-1}), intensity of absorption (vs = very strong, s = strong, m = medium, w = weak, br = broad). Low- and high-resolution mass spectra were obtained at the Harvard University Mass Spectrometry Facility.

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[1] Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
[2] Pangborn, A. B.; Giardello, M. A.; Grubbs, R. H.; Rosen, R. K.; Timmers, F. J. *Organometallics* **1996**, *15*, 1518.
[3] Myers, A. G.; Herzon, S. B. *J. Am. Chem. Soc.* **2003**, *125*, 12080.
[4] Herzon, S. B.; Myers, A. G. *J. Am. Chem. Soc.* **2005**, *127*, 5342.

Synthetic Procedures.

*(For clarity, intermediates that have not been assigned numbers in the text are numbered sequentially in the supporting information, beginning with **7**).*

Scheme S1. Preparation of Activity-Based Probe **5**

8
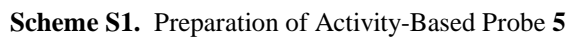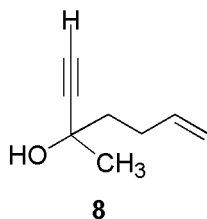
Tertiary Alcohol **8**.[5]   A solution of 5-hexen-2-one (14.9 mL, 129 mmol, 1 equiv) in tetrahydrofuran (55 mL) was added via cannula to a slurry of sodium acetylide (103 g, 18 wt. % in xylene/light mineral oil, 386 mmol, 3 equiv) at 0 °C. The reaction mixture was slowly warmed to room temperature (23 °C) and was stirred at that temperature for 2 d, before being poured onto ice. Saturated aqueous ammonium chloride was added and the resulting mixture was extracted with ethyl ether. The aqueous layer was acidified with concentrated hydrochloric acid and the acidified solution was further extracted with two portions of ethyl ether. The organic extracts were combined, the combined solution was dried over anhydrous magnesium sulfate, and the solids were removed by filtration. The filtrate was concentrated in vacuo. Fractional distillation (bp 57–58 °C at 10 Torr) afforded the product as a colorless oil (7.60 g, 48%).

R_f = 0.24 (hexanes-ethyl acetate 9:1). ^{1}H NMR (400 MHz, CDCl$_3$), δ 5.88 (ddt, 1H, J = 17.0, 10.6, 6.5 Hz), 5.09 (ddt, 1H, J = 17.0, 1.7, 1.5 Hz), 4.99 (dd, 1H, J = 10.4, 1.6 Hz), 2.46 (s, 1H), 2.40–2.22 (m, 2H), 1.96 (br s, 1H), 1.83–1.71 (m, 2H), 1.51 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 138.2, 114.9, 87.3, 71.6, 67.9, 42.3, 29.8, 29.0. IR (NaCl, thin film), cm^{-1} 3394 (br vs), 3302 (vs), 3079 (m), 2980 (s), 2933 (s), 2856 (m), 2111 (w), 1642 (s). HRMS-CI (m/z): [M + NH$_4$]$^+$ calcd for C$_8$H$_{16}$NO$^+$, 142.1232; found, 142.1238.


9
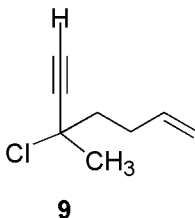
Propargyl Chloride **9**.[6] Calcium chloride dihydrate (4.28 g, 29 mmol, 0.5 equiv), copper chloride (2.28 g, 23 mmol, 0.4 equiv), and copper metal (240 mg, 3.8 mmol, 0.06 equiv) were added to concentrated hydrochloric acid (30.5 mL, 290 mmol, 5 equiv) at 0 °C. Alcohol **8** (7.23 g, 58.2 mmol, pre-cooled to 0 °C) was added over 5 min to the stirring acidic mixture at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, then was diluted with water, and the diluted solution was extracted with two portions of ethyl ether. The organic extracts were combined and the combined solution was washed successively with water and saturated aqueous sodium bicarbonate solution. The washed solution was dried over anhydrous magnesium

[5] Hashmi, A. S. K.; Ding, L.; Bats, J. W.; Fischer, P.; Frey, W. *Chem. Eur. J.* **2003**, 9, 4339.
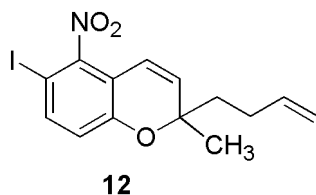[6] Hennion, G. F.; Boisselle, A. P. *J. Org. Chem.* **1961**, 26, 725.

sulfate, the solids were removed by filtration, and the filtrate was concentrated in vacuo. Fractional distillation (bp 40–50 °C at 10 Torr) afforded propargyl chloride **9** as a colorless, lachrymatory oil (6.50 g, 78%).

^{1}H NMR (400 MHz, CDCl$_3$), δ 5.84 (ddt, 1H, J = 17.0, 10.6, 6.6 Hz), 5.08 (ddt, 1H, J = 17.0, 1.8, 1.7 Hz), 5.00 (ddt, 1H, J = 10.0, 1.5, 1.4 Hz), 2.65 (s, 1H), 2.46–2.31 (m, 2H), 2.08–1.94 (m, 2H), 1.85 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 137.0, 115.2, 85.0, 73.4, 61.0, 44.8, 33.0, 29.8. IR (NaCl, thin film), cm^{-1} 3301 (vs), 3081 (m), 2980 (s), 2932 (s), 2119 (w), 1642 (s). HRMS-CI (*m/z*): [M + NH$_4$]$^+$ calcd for C$_9$H$_{15}$ClN$^+$, 160.0893; found, 160.0889.


11
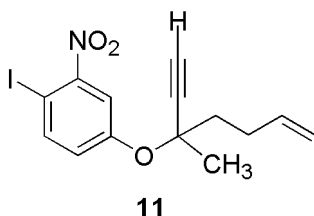
Aryl Ether **11**. Copper chloride (1.7 mg, 0.017 mmol, 0.01 equiv) was added in one portion to a vigorously stirred solution of propargyl chloride **9** (2.65 g, 18.6 mmol), 4-iodo-3-nitrophenol (**10**, 5.00 g, 18.9 mmol, 1 equiv), and triethylamine (2.9 mL, 21 mmol, 1.1 equiv) in acetonitrile (36 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and further at room temperature (23 °C) for 2 h, then was returned to the ice-water bath. A second portion of copper chloride (1.7 mg, 0.017 mmol, 0.01 equiv) was added and the mixture was slowly warmed to room temperature (23 °C) with stirring over 16 h. The reaction mixture was diluted with saturated aqueous ammonium chloride solution and the diluted solution was extracted with two portions of dichloromethane. The organic extracts were combined, the combined solution was dried over anhydrous sodium sulfate, and the solids were removed by filtration. The filtrate was adsorbed onto silica gel. This was transferred to the top of a column of silica gel, and the product was purified by flash-column chromatography (hexanes-ethyl acetate 20:1), affording aryl ether **11** as a yellow oil (3.76 g, 54%).

R$_f$ = 0.33 (hexanes-ethyl acetate 20:1). ^{1}H NMR (400 MHz, CDCl$_3$), δ 7.88 (d, 1H, J = 8.8 Hz), 7.77 (d, 1H, J = 2.8 Hz), 7.14 (dd, 1H, J = 8.8, 2.8 Hz), 5.85 (ddt, 1H, J = 17.0, 10.2, 6.6 Hz), 5.08 (dd, 1H, J = 17.0, 1.4 Hz), 5.01 (dd, 1H, J = 10.0, 1.4 Hz), 2.70 (s, 1H), 2.44–2.25 (m, 2H), 2.04 (ddd, 1H, J = 13.1, 11.7, 5.2 Hz), 1.93 (ddd, 1H, J = 13.5, 11.5, 5.4 Hz), 1.63 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 156.2, 153.0, 141.7, 137.4, 126.0, 117.8, 115.1, 83.2, 77.1, 77.0, 76.5, 41.6, 28.5, 26.6. IR (NaCl, thin film), cm^{-1} 3293 (m), 3079 (w), 2984 (m), 2934 (m), 2113 (w), 1534 (vs). HRMS-CI (*m/z*): [M + NH$_4$]$^+$ calcd for C$_{14}$H$_{18}$IN$_2$O$_3$$^+$, 389.0363; found, 389.0374.

12

Chromene **12**. A solution of aryl ether **11** (3.69 g, 9.94 mmol) and 2,6-di-*tert*-butyl-4-methylphenol (BHT, 40 mg, 0.2 mmol, 2 mol%) in *m*-xylene (100 mL) was heated to reflux for 6 h. The product mixture was concentrated in vacuo and the residue was subjected to flash-column chromatography (hexanes-ethyl acetate 20:1) to afford chromene **12** as a yellow oil (2.72 g, 74%).
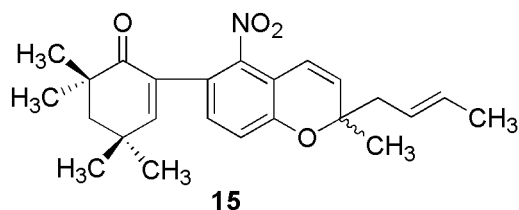
R_f = 0.53 (hexanes-ethyl acetate 20:1). ^{1}H NMR (500 MHz, CDCl$_3$), δ 7.53 (d, 1H, J = 8.8 Hz), 6.66 (d, 1H, J = 8.8 Hz), 6.22 (d, 1H, J = 10.3 Hz), 5.80 (ddt, 1H, J = 17.1, 10.3, 6.4 Hz), 5.74 (d, 1H, J = 10.3 Hz), 5.01 (ddt, 1H, J = 17.1, 2.0, 1.5 Hz), 4.96 (dd, 1H, J = 10.3, 1.5 Hz), 2.24–2.09 (m, 2 H), 1.85 (ddd, 1H, J = 13.9, 11.0, 5.6 Hz), 1.75 (ddd, 1H, J = 14.2, 11.2, 5.4 Hz), 1.4 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 153.9, 151.7, 139.3, 137.7, 133.5, 119.9, 116.4, 115.1, 114.9, 79.6, 72.9, 40.3, 28.1, 26.6. IR (NaCl, thin film), cm^{-1} 3087 (w), 2991 (m), 2957 (m), 1537 (vs). HRMS-CI (*m/z*): [M + NH$_4$]$^+$ calcd for C$_{14}$H$_{18}$IN$_2$O$_3$$^+$, 389.0363; found, 389.0357.

13
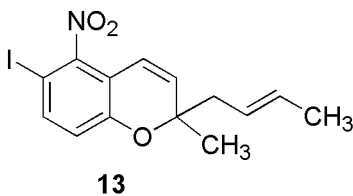
Chromene **13**. A solution of chromene **12** (2.55 g, 6.87 mmol, 1 equiv), allyloxy-*tert*-butyldimethylsilane[7] (4.2 g, 24 mmol, 3.5 equiv), and Grubbs' 2nd generation catalyst (230 mg, 0.27 mmol, 0.04 equiv) in dichloromethane (40 mL) was heated to reflux for 16 h. The reaction mixture was cooled to room temperature (23 °C) and then concentrated in vacuo. The residue was subjected to flash-column chromatography (hexanes-ethyl acetate 20:1) to afford the bond-migrated product **13** as a yellow oil (3.4:1 mixture of geometrical isomers, 2.47 g, 97%).

R_f = 0.53 (hexanes-ethyl acetate 20:1). ^{1}H NMR (500 MHz, CDCl$_3$, signals for the major isomer), δ 7.53 (d, 1H, J = 8.8 Hz), 6.66 (d, 1H, J = 8.8 Hz), 6.20 (d, 1H, J = 10.2 Hz), 5.75 (d, 1H, J = 10.2 Hz), 5.55–5.46 (m, 1 H), 5.46–5.37 (m, 1 H), 2.44–2.33 (m, 2H), 1.65 (d, 3H, J = 6.3 Hz), 1.40 (s, 3H). ^{13}C NMR (100 MHz, CDCl$_3$, signals for the major isomer), δ 153.9, 151.6, 139.2, 133.8, 130.0, 124.1, 120.0, 116.2, 115.5, 79.4, 72.9, 44.0, 25.8, 18.0. IR (NaCl, thin film), cm^{-1} 3026 (w), 2971 (m), 2931 (m), 1535 (vs). HRMS-CI (*m/z*): [M + NH$_4$]$^+$ calcd for C$_{14}$H$_{18}$IN$_2$O$_3$$^+$, 389.0363; found, 389.0366.

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[7] Van de Coevering, R.; Kuil, M.; Alfers, A. P.; Visser, T.; Lutz, M.; Spek, A. L.; Klein Gebbink, R. J. M.; van Koten, G. *Organometallics* **2005**, *24*, 6147.

15

Nitroketone **15**. A 25-mL 2-neck flask was charged with 2-iodo-4,4,6,6-tetramethyl-cyclohex-2-en-1-one[3] (**14**, 611 mg, 2.20 mmol. 1 equiv), copper powder (40 mesh, 700 mg, 11 mmol, 5 equiv), and tris(dibenzylideneacetone)dipalladium (200 mg, 0.22 mmol, 0.1 equiv). The flask was evacuated and purged with argon. A solution of iodochromene **13** (2.01 g, 5.42 mmol, 2.5 equiv) in methyl sulfoxide (8 mL, deoxygenated with 5 freeze-pump-thaw cycles) was added via cannula, and the reaction mixture was heated to 70 ℃ for 5 h. The reaction mixture was cooled to room temperature (23 ℃), then concentrated in vacuo. The residue was subjected to flash-column chromatography (1st column: hexanes-ethyl acetate 20:1; 2nd column: hexanes-dichloromethane 2:1 increasing to 100% dichloromethane) to afford the coupled product **15** as a yellow oil (3.4:1 mixture of geometrical isomers, 443 mg, 51%)

R_f = 0.22 (hexanes-ethyl acetate 20:1). ^{1}H NMR (500 MHz, CDCl$_3$, signals for the major isomer), δ 6.96 (d, 1H, J = 8.8 Hz), 6.90 (d, 1H, J = 8.8 Hz), 6.55 (s, 1H), 6.41 (d, 1H, J = 10.2 Hz), 5.76 (d, 1H, J = 10.2 Hz), 5.55–5.40 (m, 2H), 2.45–2.36 (m, 2H), 1.85 (s, 2H), 1.67 (dd, 3H, J = 5.9, 1.0 Hz), 1.41 (s, 3H), 1.25 (s, 6H), 1.21 (s, 6H). ^{13}C NMR (100 MHz, CDCl$_3$, signals for the major isomer), δ 201.7, 155.2, 153.3, 146.9, 133.0, 132.7, 131.2, 129.6, 124.5, 123.0, 118.7, 117.1, 114.3, 78.8, 48.9, 43.8, 41.2, 33.0, 30.5, 27.1, 25.7, 18.1. IR (NaCl, thin film), cm^{-1} 2959 (m), 2927 (m), 1683 (s), 1532 (vs). HRMS-CI (m/z): [M + NH$_4$]$^+$ calcd for C$_{24}$H$_{33}$N$_2$O$_4$$^+$, 413.2440; found, 413.2440.

16
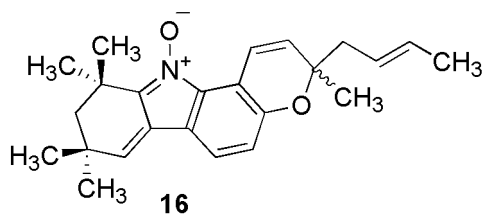
Nitrone **16**. (Note: nitrones such as **16** are light sensitive, and should be prepared and handled in the dark). A suspension of zinc powder (292 mg, 4.6 mmol, 4 equiv) in tetrahydrofuran (4 mL) was added portionwise (4 portions of 1 mL over 3 h) to a solution of nitroketone **15** (441 mg, 1.12 mmol, 1 equiv) in ethanol (12.9 mL), tetrahydrofuran (3.5 mL), and saturated aqueous ammonium chloride solution (2.6 mL) at room temperature (23 ℃). The yellow suspension was stirred at room temperature (23 ℃) for 1 h more, then was diluted with dichloromethane. The diluted suspension was filtered through a pad of Celite. The filtrate was concentrated in vacuo and the residue was subjected to flash-column chromatography (0% to 15% ethyl acetate in dichloromethane) to afford nitrone **16** as a bright yellow foaming oil (3.4:1 mixture of

geometrical isomers, 237 mg, 58%). A small amount of starting material (**15**, 58 mg, 13%) was also isolated in separate fractions.

R_f = 0.24 (100% dichloromethane). ^{1}H NMR (500 MHz, C_6D_6, signals for the major isomer), δ 8.62 (d, 1H, J = 9.9 Hz), 6.94 (d, 1H, J = 8.1 Hz), 6.85 (d, 1H, J = 8.1 Hz), 5.99 (s, 1H), 5.62–5.47 (m, 1H), 5.44 (d, 1H, J = 9.9 Hz), 5.39–5.29 (m, 1H), 2.34 (br d, 2H, J = 7.3 Hz), 1.58 (s, 6H), 1.52 (dd, 3H, J = 6.2, 1.5 Hz), 1.31 (s, 2H), 1.28 (s, 3H), 0.87 (s, 6 H). ^{13}C NMR (100 MHz, C_6D_6, signals for the major isomer), δ 155.0, 145.2, 140.7, 137.1, 131.1, 129.1, 125.6, 119.2, 118.9, 117.8, 115.5, 112.4, 78.7, 52.2, 44.3, 35.0, 32.9, 30.7, 25.8, 25.8, 18.2.[8] IR (NaCl, thin film), cm^{-1} 2958 (vs), 2919 (vs), 1633 (s). HRMS-ES (*m/z*): [M + H]$^+$ calcd for $C_{24}H_{29}NO_2^+$, 364.2276; found, 364.2284.

6
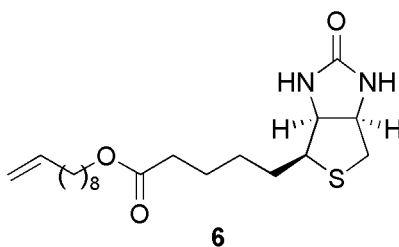
Biotin conjugate **6**. 9-Decen-1-ol (**17**, 1.10 mL, 6.17 mmol, 1.5 equiv) and triethylamine (5 mL) were added to a solution of (+)-biotin (**18**, 1.00 g, 4.09 mmol, 1 equiv), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl, 942 mg, 4.91 mmol, 1.2 equiv), and 1-hydroxybenzotriazole (HOBT, 664 mg, 4.91 mmol, 1.2 equiv) in *N,N*-dimethylformamide (DMF, 15 mL). The reaction mixture was stirred at room temperature (23 ℃) for 16 h, then was diluted with dichloromethane. The diluted solution was washed with saturated aqueous ammonium chloride solution. The aqueous layer was extracted with two portions of dichloromethane. The organic extracts were combined, the combined solution was dried over anhydrous magnesium sulfate, the solids were removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by flash-column chromatography (chloroform-methanol 9:1), affording ester **6** as a white solid (1.0 g, 64%).

R_f = 0.34 (chloroform-methanol 9:1). ^{1}H NMR (400 MHz, CD$_3$OD), δ 5.80 (ddt, 1H, J = 17.2, 10.2, 6.6 Hz), 4.98 (br d, 1H, J = 15.2 Hz), 4.91 (br d, 1H, J = 10.2 Hz), 4.48 (dd, 1H, J = 7.9, 4.5 Hz), 4.30 (dd, 1H, J = 8.05, 4.5 Hz), 4.06 (t, 2H, J = 6.6 Hz), 3.20 (dt, 1H, J = 8.1, 4.5 Hz), 2.92 (dd, 1H, J = 12.8, 4.9 Hz), 5.4 (d, 1H, J = 12.8 Hz), 2.34 (t, 2H, J = 7.5 Hz), 2.04 (dt, 2H, J = 7.3, 7.0 Hz), 1.79–1.53 (m, 6H), 1.50–1.28 (m, 12H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 175.4, 166.1, 140.1, 114.8, 65.6, 63.4, 61.6, 57.0, 41.1, 34.9, 30.5, 30.3, 30.1, 30.1, 29.8, 29.7, 29.5, 27.0, 26.0. IR (NaCl, thin film), cm^{-1} 3216 (br), 2927 (m), 2854 (m), 1703 (vs, br). HRMS-CI (*m/z*): [M + H]$^+$ calcd for $C_{20}H_{35}N_2O_3^+$, 383.2368; found, 383.2373.

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[8] An anticipated signal at 128 ppm was not distinguishable from the large solvent peak.

5
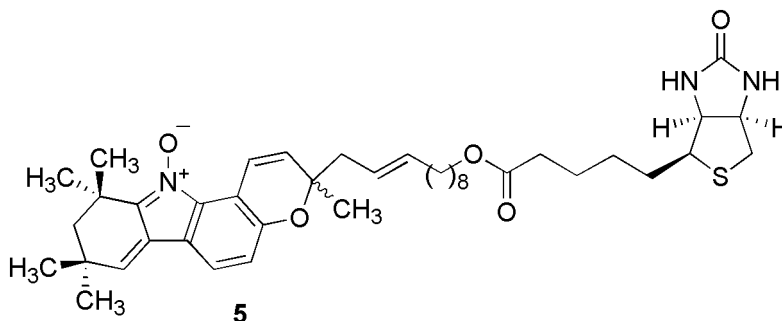
Nitrone-biotin conjugate **5**. A 25-mL flask was charged with olefin-biotin conjugate **6** (105 mg, 0.274 mmol, 5 equiv), nitrone **16** (20 mg, 0.055 mmol, 1 equiv), and Grubbs' 2nd generation catalyst (5 mg, 0.006 mmol, 0.1 equiv). A condensor was attached, and the flask/condensor was evacuated and purged three times with argon. Dichloromethane (20 mL) was added, and the resulting solution was heated to reflux for 14 h, then was cooled to room temperature (23 °C). The solvent was removed in vacuo, and the residue was subjected to flash-column chromatography (100% dichloromethane, followed by 1:1 acetonitrile:dichloromethane, followed by 1% methanol in 1:1 acetonitrile:dichloromethane). The semi-purified product was purified by HPLC (reverse phase, Beckman Coulter Ultrasphere ODS 5 µM, 30% to 100% acetonitrile in water) to afford **5** as a yellow film (5.5:1 mixture of geometrical isomers, 18.2 mg, 47%).

R_f = 0.54 (chloroform-acetonitrile-methanol 25:25:1). ^{1}H NMR (500 MHz, C$_6$D$_6$, signals for the major isomer), δ 8.63 (d, 1H, J = 10.3 Hz), 6.97 (d, 1H, J = 7.8 Hz), 6.88 (d, 1H, J = 8.3 Hz), 6.02 (s, 1H), 5.98 (br s, 1H), 5.58 (dt, 1H, J = 15.1, 7.3 Hz), 5.49 (d, 1H, J = 9.8 Hz), 5.44 (dt, 1H, J = 15.2, 6.8 Hz), 4.91 (br s, 1H), 4.07 (t, 2H, J = 6.6 Hz), 3.60–3.55 (m, 1H), 3.53–3.48 (m, 1H), 2.56–2.51 (m, 1H), 2.47–2.34 (m, 2H), 2.23 (dd, 1H, J = 12.7, 4.6 Hz), 2.20 (t, 2H, J = 7.6), 2.17 (d, 1H, J = 12.7 Hz), 2.02–1.94 (m, 2H), 1.60 (s, 3H), 1.60 (s, 3H), 1.62–1.41 (m, 6H), 1.36–1.16 (m, 17H), 0.89 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (100 MHz, CD$_2$Cl$_2$), δ 173.8, 163.2, 154.7, 146.4, 139.6, 139.6, 135.3, 131.5, 127.6, 124.2, 119.2, 118.8, 117.3, 115.5, 112.0, 78.8, 64.8, 62.1, 60.4, 55.7, 52.4, 44.2, 41.0, 35.5, 34.2, 33.1, 33.0, 30.9, 30.9, 29.7, 29.7, 29.6, 29.4, 29.0, 28.7, 28.6, 26.3, 25.8, 25.8, 25.8, 25.2. IR (NaCl, thin film), cm^{-1} 3215 (m, br), 2926 (s), 2850 (m), 1703 (vs, br). HRMS-CI (*m/z*): [M + H]$^+$ calcd for C$_{41}$H$_{58}$N$_3$O$_5$S$^+$, 704.4097; found, 704.4084.
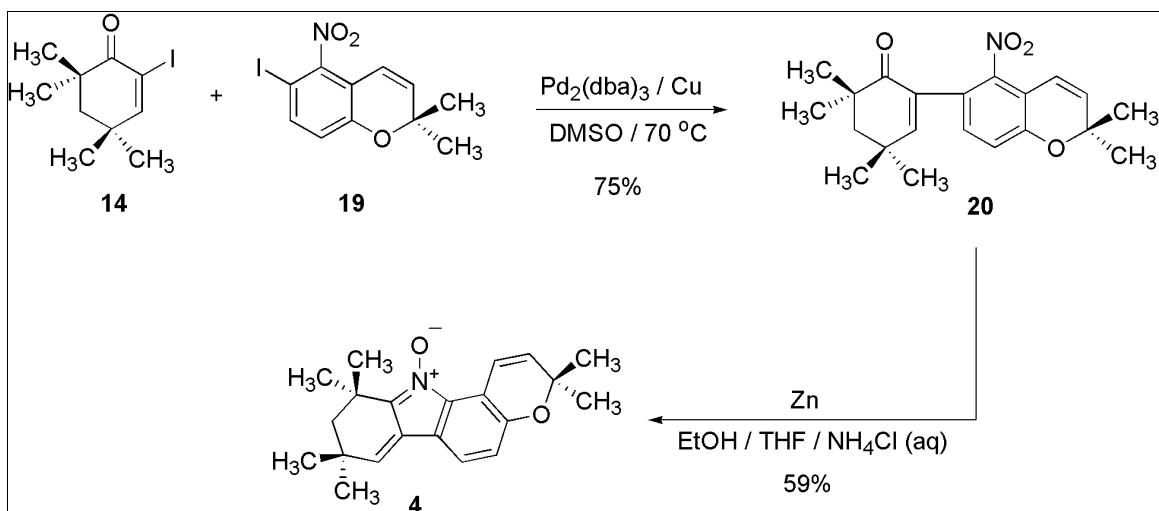
Scheme S2. Preparation of Nitrone **4**.

20
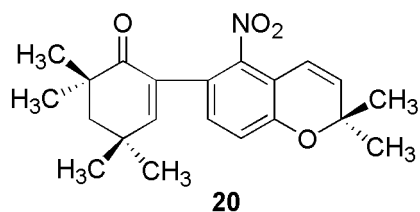
<u>Nitroketone 20</u>. 2-iodo-4,4,6,6-tetramethyl-cyclohex-2-en-1-one[3] (**14**, 556 mg, 2 mmol, 1 equiv), copper powder (636 mg, 10 mmol, 5 equiv), and tris(dibenzylideneacetone)dipalladium (183 mg, 0.2 mmol, 0.01 equiv) were added to a solution of iodoarene **19**[4] (1.66 g, 5 mmol, 2.5 equiv) in methyl sulfoxide (10 mL). The reaction mixture was stirred at 70 °C for 3 h, then was cooled to room temperature (23 °C) and diluted with dichloromethane. The diluted solution was washed with 30% aqueous ammonium chloride solution. The layers were separated and the aqueous phase was extracted with dichloromethane. The organic extracts were combined and the combined solution was washed successively with water and saturated aqueous sodium chloride solution. The washed solution was dried over anhydrous sodium sulfate, the solids were removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by flash-column chromatography (hexanes-dichloromethane 1:1 to 0:1), affording **20** as a yellow oil (532 mg, 75%).

R_f = 0.33 (hexanes-ethyl acetate 9:1). ^{1}H NMR (500 MHz, CDCl$_3$), δ 6.97 (d, 1H, J = 8.5 Hz), 6.91 (d, 1H, J = 8.5 Hz), 6.56 (s, 1H), 6.39 (d, 1H, J = 10.3 Hz), 5.80 (d, 1H, J = 10.3 Hz), 1.85 (s, 2H), 1.47 (s, 6H), 1.25 (s, 6H), 1.22 (s, 6H). ^{13}C NMR (100 MHz, CDCl$_3$), δ 201.9, 155.5, 153.5, 147.2, 134.1, 132.9, 131.5, 123.4, 119.1, 117.0, 114.4, 76.9, 49.2, 41.5, 33.3, 30.8, 28.0, 27.4. IR (thin film), cm^{-1} 2964 (m), 2927 (m), 1677 (s), 1530 (vs). HRMS-ESI (m/z): [M + H]$^{+}$ calcd for C$_{21}$H$_{26}$NO$_4^{+}$, 356.1856; found, 356.1848.
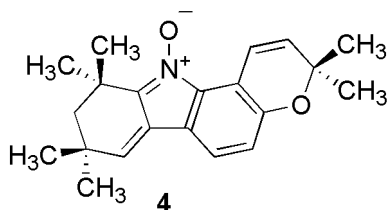
Nitrone **4**. A suspension of zinc powder (156 mg, 2.39 mmol, 5 equiv) in tetrahydrofuran (1 mL) was added in three portions (20 min between each addition) to a solution of nitroarene **20** (170 mg, 0.48 mmol, 1 equiv) in ethanol (5 mL) and 1M aqueous ammonium chloride solution (1.06 mL, 1.06 mmol, 2.2 equiv). The yellow suspension was stirred at room temperature (23 °C) for 2 h. The suspension was diluted with ethyl acetate and the diluted suspension was filtered over Celite. The filtrate was washed with saturated aqueous sodium chloride solution and the washed solution was dried over anhydrous sodium sulfate. The solids were removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (dichloromethane-ethyl acetate 100:5 to 100:20), yielding **4** as a yellow solid (92 mg, 59%).

R_f = 0.23 (dichloromethane-ethyl acetate 100:4). ^{1}H NMR (500 MHz, C_6D_6): δ 8.59 (d, 1H, J = 10.1 Hz), 6.94 (d, 1H, J = 8.2 Hz), 6.85 (d, 1H, J = 8.2 Hz), 5.99 (s, 1H), 5.37 (d, 1H, J = 10.1), 1.59 (s, 6H), 1.32 (s, 2H), 1.25 (s, 6H), 0.87 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$), δ 154.6, 146.9, 140.2, 140.0, 132.2, 127.5, 119.0, 118.4, 117.0, 115.7, 112.1, 76.7, 52.4, 35.5, 33.2, 31.1, 27.9, 26.1. IR (thin film), cm^{-1}: 2962 (m), 2921 (m), 1702 (w), 1636 (m). HRMS-ESI (*m/z*): [M + H]$^+$ calcd for $C_{21}H_{26}NO_2^+$, 324.1958; found, 324.1951.

B. Biology

General Experimental Procedures. All cell-culture work was conducted in a class II biological safety cabinet. All buffers were filter-sterilized (0.2 μm) prior to use. Antiproliferative assays and other operations requiring the handling of nitrone species were carried out in the dark to prevent the occurrence of photochemical rearrangement reactions. Compounds **1-6** were typically stored dry, in 50- or 100-μg portions, at –80 ℃, prior to analysis.

Materials. LNCaP, T-47D, βT-549 and MALME-3M cells were purchased from ATCC, and cultured in RPMI 1640 (ATCC or Mediatech) containing 10% fetal bovine serum (Hyclone), 10 mM HEPES, and 2 mM L-glutamine. Cells were grown in BD Falcon tissue culture flasks with vented caps. Bradford reagent was purchased from Sigma Aldrich. Antiproliferative assays were conducted in pre-sterilized 96-well flat-bottomed plates from BD Falcon. Solutions of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate (PMS) were purchased from Promega as the CellTiter 96 AQueous Non-Radioactive Cell Proliferation kit. Solutions of resazurin were purchased from Promega as the CellTiter-Blue Cell Viability Assay kit. Both kits were used according to the manufacturer's instructions. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast Novex tris-glycine mini gels (10% or 4-20% gradient, Invitrogen). Electrophoresis and semi-dry electroblotting equipment was purchased from Owl Separation Systems. Nitrocellulose membranes were purchased from Amersham Biosciences. Rabbit polyclonal antibodies to glutathione reductase and peroxiredoxin 1 were purchased from GeneTex (GR: GTX16801, PRX1: GTX15571). Rabbit polyclonal antibodies to heat shock protein 60 and exportin 1 were purchased from Santa Cruz Biotechnology (HSP60: sc-13966, XPO1: sc-5595). Antibody detection was performed using the SuperSignal West Pico Chemiluminscence kit (including a goat anti-rabbit–HRP conjugate) from Pierce. Western blots were visualized using CL-X Posure X-ray film from Pierce. Streptavidin-agarose was purchased from Sigma Aldrich. Protein bands were visualized using the Novex Colloidal Blue staining kit from Invitrogen, and were analyzed at the Taplin Biological Mass Spectrometry Facility (Harvard University).

Instrumentation. Absorbance and fluorescence measurements were made on Molecular Dynamics multiwell plate readers (absorbance: SPECTRAmax PLUS 384, fluorescence: SPECTRAmax GEMINI XS). Data was collected using SOFTmax PRO v. 4.3 (Molecular Dynamics), and was manipulated in Excel (Microsoft). The XLfit4 plugin (IDBS software) running in Excel was used for curve fitting. Analytical HPLC measurements were made on a Beckman Coulter System Gold HPLC, equipped with a reverse phase Beckman Coulter Ultrasphere ODS column (5 μM, 4.6 mm x 25 cm).

Antiproliferative Assays.

Method A (activity of **1**, **2**, *ent*-**1**, and *ent*-**2**, Table 1).[9] LNCaP, T-47D, βT-549, and MALME-3M cells were grown to approximately 80% confluence, then were trypsinized, collected, and pelleted by centrifugation (10 min at 183 x g). The supernatant was discarded, and the cell pellet was resuspended in fresh medium to achieve a concentration of approximately 1.0 to 1.5 x 10^6 cells/mL. A sample was diluted 10-fold in fresh medium, and the concentration of cells was determined using a hemacytometer.

The cell suspension was diluted to 6.0 x 10^4 cells/mL. A multichannel pipette was used to charge the wells of a 96-well plate with 50 μL per well of the diluted cell suspension. The plates were incubated for 24 h at 37 °C under an atmosphere of 5% CO_2.

The following day, 50-μg portions of **1**, **2**, *ent*-**1**, and *ent*-**2** were removed from the freezer, thawed, and dissolved in filter-sterilized DMSO to a concentration of 5 mM. A 6.5-μL aliquot of the nitrone solution was dissolved in 643.5 μL of medium to achieve a working concentration of 50 μM. Serial dilutions were employed to generate a range of different concentrations for analysis. Finally, 50-μL aliquots of this diluted nitrone solution were added to the wells containing adhered cells, giving final assay concentrations of up to 25 μM.

The treated cells were incubated for 48 h at 37 °C (5% CO_2). To each well was added 20 μL of complete CellTiter 96 AQueous Non-Radioactive Cell Proliferation kit (20:1 MTS:PMS), and the samples were returned to the incubator. Absorbance at 490 nm was recorded on a 96-well plate reader following 2.5 h- and 4.0 h-incubation periods (37 °C, 5% CO_2).

Percent growth inhibition was calculated for each well, based upon the following formula:

$$\text{Percent growth inhibition} = 100 \times (S - B_0) / (B_t - B_0)$$

where S is the sample reading, B_t is the average reading for a vehicle-treated population of cells at the completion of the assay, and B_0 is the average reading for an untreated population of cells at the beginning of the assay. All four compounds were run (in duplicate) at the same time, against all four cell lines. The experiment was repeated three times, over a three-week period. Averaged data was plotted as growth inhibition vs. sample concentration, and a GI_{50} value was calculated as the concentration required to cause 50% growth inhibition.

Interestingly, the values we obtained in our assay are rather higher (3-fold to 20-fold) than those determined for avrainvillamide (**2**) at the National Cancer Institute (NCI),[10] while our measured GI_{50} value

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[9] (–)-Stephacidin B (**1**) and (+)-avrainvillamide (**2**) were previously and independently screened against the colon cancer cell line HCT-116 (references 10 and 11). Although the reported activities were 10-fold different, these assays were conducted in different labs, using different protocols. No direct side-by-side comparison of **1** and **2** has been reported prior to this work, so far as we are aware.

[10] Fenical, W.; Jensen, P. R.; Cheng, X. C. Avrainvillamide, a Cytotoxic Marine Natural Product, and Derivatives Thereof. U.S. Patent 6,066,635, 2000.

for stephacidin B (**1**) in LNCaP cells is relatively close (within experimental error) to that obtained at Bristol-Myers Squibb (60 nM).[11,12] We attribute these differences predominantly to variations in assay conditions. For the purposes of high-throughput screening, the NCI assay relied upon sulforhodamine B-staining of residual cellular protein, following treatment of ~20,000 cells/well with 10-fold dilutions of compound. Our own assay, like that employed at Bristol-Myers Squibb,[11(b)] was based upon the metabolism of a tetrazolium salt, following treatment of ~3,000 cells/well with 1.5- to 3-fold dilutions of compound. Arguably, the former assay constitutes a predominantly physical measurement, while the latter conditions provide a more direct readout of cellular health, as well as a somewhat higher degree of precision. In any case, it is not surprising that somewhat different results emerge from such distinct sets of experimental conditions.

Method B (medium-throughput method for assaying the activities of structural analogs, including compounds **3 – 6**).[13] LNCaP and T-47D cells were grown to approximately 80% confluence, then were trypsinized, collected, and pelleted by centrifugation (10 min at 183 x g). The supernatant was discarded, and the cell pellet was resuspended in fresh medium to achieve a concentration of approximately 1.0 to 1.5 x 10^6 cells/mL. A sample was diluted 10-fold in fresh medium, and the concentration of cells was determined using a hemacytometer.

The cell suspension was diluted to 1.0 x 10^5 cells/mL. A multichannel pipette was used to charge the wells of a 96-well plate with 100 μL per well of the diluted cell suspension. The plates were incubated for 24 h at 37 °C under an atmosphere of 5% CO_2.

The following day, 100-μg portions of the nitrone samples were removed from the freezer, thawed, and dissolved in filter-sterilized DMSO to a concentration of 5mM. A 6.5-μL aliquot of the nitrone solution was dissolved in 643.5 μL of medium to achieve a working concentration of 50 μM. Serial dilutions were employed to generate a range of different concentrations for analysis. Finally, 100-μL aliquots of this diluted nitrone solution were added to the wells containing adhered cells, resulting in final assay concentrations of up to 25 μM.

The treated cells were incubated for 72 h at 37 °C (5% CO_2). To each well was added 20 μL of CellTiter-Blue reagent, and the samples were returned to the incubator. Fluorescence (560 nm excitation / 590 nm emission) was recorded on a 96-well plate reader following a 4.0 h incubation period (37 °C, 5% CO_2).

Percent growth inhibition was calculated for each well, based upon the following formula:

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[11] (a) Qian-Cutrone, J.; Krampitz, K. D.; Shu, Y.-Z.; Chang, L.-P.; Lowe, S. E. Stephacidin Antitumor Antibiotics. US Patent 6291461, 2001. (b) Qian-Cutrone, J.; Huang, S.; Shu, Y.-Z.; Vyas, D.; Fairchild, C.; Menendez, A.; Krampitz, K.; Dalterio, R.; Klohr, S. E.; Gao, Q. *J. Am. Chem. Soc.* **2002**, *124*, 14556–14557.

[12] The data in Table 1 were obtained following 48 h treatment of the cells with the compounds under study. In the Bristol-Myers Squibb assay, the incubation time was 72 h (see reference 11(b)). In separate experiments, we have observed increased activity for **2** following increased incubation times, a fact which would account for the somewhat greater activity reported for **1** by Bristol-Myers Squibb.

[13] In this assay, the GI_{50} for (+)-avrainvillamide (**2**) was 330 nM vs. LNCaP and 420 nM vs. T-47D.

$$\text{Percent growth inhibition} = 100 \text{ x } (S - B_0) / (B_t - B_0)$$

where S is the sample reading, B_t is the average reading for a vehicle-treated population of cells at the completion of the assay, and B_0 is the average reading for an untreated population of cells at the beginning of the assay.

Each analog was run a minimum of eight times, over a period of at least two weeks. For each compound, 14 separate concentrations were used in the assay, ranging from 25 μM to 8 nM. The average inhibition at each concentration was plotted against concentration, and a curve fit was generated. To eliminate positional effects (e.g., cell samples in the center of the plate routinely grew more slowly than those near the edge), the data was automatically scaled to ensure that the curves showed no inhibition at negligible concentrations of added compound. Such a precaution was found to generate more consistent data from week to week, without affecting the final results. Final GI_{50} values reflect the concentrations at which the resulting curves pass through 50 percent inhibition. In this assay, (+)-avrainvillamide (**2**) displayed GI_{50} values of 325 nM vs. LNCaP cells, and 422 nM vs. T-47D cells.

In Vitro Affinity-Isolation Experiments.

Preparation of LNCaP cell lysate. LNCaP cells were grown to approximately 90% confluence in 24 T-175 tissue culture flasks. The medium was discarded, and the cells were washed with phosphate-buffered saline (PBS, 10mL per flask). The cells were trypsinized (10 min, 37 °C, 8 mL per flask, 0.05% trypsin, 0.53 mM EDTA). RPMI-1640 (20 mL) was added to each flask, and the mixture was aspirated to detach any remaining cells. The resulting cell suspension was transferred to 50 mL centrifuge tubes, and the cells were pelletted by centrifugation (10 min at 1650 x g). The supernatant was discarded, and the cell pellets were resuspended in RPMI-1640, and transferred to two 15 mL centrifuge tubes. The cells were again pelletted by centrifugation (20 min at 1650 x g) and the supernatant was discarded, affording 6 mL of packed cells.

The cells were lysed at 4 °C, in 30 mL of mild lysis buffer (50 mM tris·HCl, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, pH 8.00)[14], using 50 strokes in a Dounce homogenizer. The mixture was centrifuged (15000 x g, 30 min, 4 °C), and the supernatant was course-filtered, briefly mixed, and partitioned into 500-μL and 1000-μL aliquots at 4 °C. These were flash-frozen in liquid N_2, and stored at –80 °C. Subsequent analysis (Bradford method)[15] showed the lysate to contain 8.1 mg/mL total protein.

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[14] The addition of protease inhibitors (190 mg/L phenylmethanesulfonyl fluoride, 10 mg/L aprotinin, 20 mg/L leupeptin trifluoroacetate, 20 mg/L pepstatin) did not appear to affect the pulldown efficiency, but was generally avoided, so as to leave any potential binding proteins in their native forms.

[15] Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.

LC-MS/MS identification of GR, PRX1, HSP60 and XPO1. A 1000-μL aliquot of LNCaP cell lysate was thawed at 4 ℃, then diluted in tris·HCl buffer (50 mM, pH 7.9, 3.0 mL). The resulting diluted lysate (containing 2 mg/mL protein) was partitioned into aliquots of 380 μL in 1.5-mL centrifuge tubes. To one aliquot was added (in the dark, on ice) conjugate **5** in DMSO (2 mM, 20 μL) to obtain a final concentration of 100 μM. To a second aliquot was added DMSO (20 μL) as a vehicle control.

The samples were mixed end-over-end, in the dark, for 1 h at 4 ℃, then well-suspended streptavidin-agarose resin (15 μL per sample of a buffered aqueous 2 mg/mL suspension) was added, and the samples were mixed for a further 16 h at 4 ℃. The samples were then centrifuged (12000 x g, 10 min, 4 ℃). The supernatant was discarded, and the collected resin was washed twice with tris·HCl buffer (500 μL for 10 min at 23 ℃, followed by centrifugation 10 min at 10000 x g). The resin was suspended in Laemmli loading buffer (Sigma, 2X concentration, 50 μL per sample), and the samples were heated to 95 ℃ for 6 min.

A tris-glycine mini gel (10%, 12-well) was loaded with 20 μL per well of the denatured protein mixture. The treated and untreated (vehicle control) samples were loaded in adjacent lanes. The protein samples were electroeluted (1 h, 23 ℃, 150 V), and the resulting gel was stained with Colloidal Blue to reveal the location of candidate proteins. Both the treated and untreated lanes showed a number of bands corresponding to nonspecific binding proteins. In addition, bands at approximately 58, 55, and 20 kDa were observed in the sample lane. These were excised and submitted for sequencing by LC-MS/MS. Due to the number of nonspecific binders observed near the 55 kDa band, a control sample (from the vehicle control lane) was also submitted.

Within the 58 kDa band, we observed good coverage (24 peptide matches) of pyruvate kinase isozymes M1/M2, an apparently nonspecific binding protein which was present in the 55 kDa control sample, and in several of our subsequent analyses. The next most abundant protein (in terms of peptide coverage) was heat shock protein 60 (HSP60, 21 peptide matches). Subsequent Western blot analysis (see Figure 2) confirmed this protein as a binding partner for avrainvillamide (**2**) and conjugate **5**.

Within the 55 kDa band, as anticipated, several proteins were observed by LC-MS/MS (9 proteins with ≥ 10 peptide matches). All but glutathtione reductase (GR) were observed with roughly equal coverage in the control sample. Subsequent Western blot analysis (see Figure 2) confirmed GR as a binding partner for avrainvillamide (**2**) and conjugate **5**.

Within the 22 kDa band, the protein with greatest coverage was peroxiredoxin 1 (PRX1, 15 peptide matches). Subsequent Western blot analysis (see Figure 2) confirmed PRX1 as a binding partner for avrainvillamide (**2**) and conjugate **5**.

In a closely related experiment, a sample containing 5 μM **5** was directly compared to one containing 5 μM **5** and 100 μM (+)-avrainvillamide (**2**). Following staining with Colloidal Blue, a band at 27 kDa was found to be particularly intense in the lane corresponding to treatment with **5** only. This was excised and submitted for analysis by LC-MS/MS. As a control, the 27 kDa region of the lane corresponding to treatment with both **5** and **2** was submitted. Once again, several proteins were found in

both samples. Of those which were only found in first sample, exportin 1 (XPO1)[16] was observed with the greatest coverage (5 peptide matches). Subsequent Western blot analysis (see Figure 2) confirmed XPO1 as a binding partner for avrainvillamide (**2**) and conjugate **5**.

LC-MS/MS identification of other protein binding partners. A 1000-µL aliquot of LNCaP cell lysate was thawed at 4 ℃, then diluted in tris·HCl buffer (50 mM, pH 7.8, 3.0 mL). The resulting diluted lysate (containing 2 mg/mL protein) was treated with well-suspended streptavidin-agarose resin (100 µL of buffered aqueous 1 mg/mL suspension) for 2 h at 4 ℃, mixing end-over-end. The sample was centrifuged (12000 x g, 10 min, 4 ℃), and the supernatant was transferred to a clean centrifuge tube and centrifuged again (1650 x g, 10 min, 4 ℃). The supernatant was partitioned (on ice) into 9 aliquots of 380 µL, in 1.5 mL centrifuge tubes. The remaining clarified lysate was saved for use as a positive control (see below). In the dark, on ice, was added DMSO and stocks of **2**, **3**, **5**, and **6** (in DMSO), as indicated:

sample:	volume lysate	volume DMSO	volume **2** 5000 µM	volume **3** 5000 µM	volume **5** 200 µM	volume **6** 2000 µM	final volume	% DMSO
1	380 µL	20 µL	x	x	x	x	400 µL	5%
2	380 µL	10 µL	x	x	10 µL (5 µM)	x	400 µL	5%
3	380 µL	2 µL	8 µL (100 µM)	x	10 µL (5 µM)	x	400 µL	5%
4	380 µL	10 µL	x	x	10 µL (5 µM)	x	400 µL	5%
5	380 µL	2 µL	x	8 µL (100 µM)	10 µL (5 µM)	x	400 µL	5%
6	380 µL	x	x	x	x	20 µL (100 µM)	400 µL	5%
7	380 µL	12 µL	8 µL (100 µM)	x	x	x	400 µL	5%
8	380 µL	12 µL	x	8 µL (100 µM)	x	x	400 µL	5%
9	380 µL	20 µL	x	x	x	x	400 µL	5%

The samples were mixed end-over-end in the dark for 4 h at 4 ℃, then tris·HCl buffer (50 mM, pH 7.8, 1 mL) was added to each sample, followed by well-suspended streptavidin-agarose resin (30 µL of buffered aqueous 1 mg/mL suspension). The samples were mixed end-over-end in the dark for 15.5 h at 4 ℃, then centrifuged (12000 x g, 10 min, 4 ℃). The supernatant was discarded, and the resin was washed

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[16] The molecular weight of XPO1 is 123 kDa. However, we regularly observed that protein (by both LC-MS/MS and Western blot) migrated to approximately 27 kDa on our gels – possibly due to a proteolytic cleavage event. The other protein binders discussed here (HSP60, GR, PRX1) were observed at their expected positions on the gels.

three times. Each wash consisted of 10 min mixing at 23 ºC, in 1 mL tris·HCl buffer, followed by centrifugation (10000 x g, 10 min, 23 ºC). The resin was suspended in Laemmli loading buffer (Sigma, 2X concentration, 90 µL per sample), and the samples were heated to 95 ºC for 6 min. As a positive control, a sample of unmodified clarified lysate (50 µL) was mixed with Laemmli loading buffer (Sigma, 2X concentration, 50 µL), and was likewise denatured at 95 ºC.

A tris-glycine mini gel (10%, 12-well) was loaded with 5 µL per well of the denatured protein mixture (samples 1 – 9). Other wells were loaded with molecular weight markers. The protein samples were electroeluted (1 h, 23 ºC, 150 V), and the resulting gel was stained with Silver Stain Plus (Bio-Rad) to reveal the presence of candidate proteins. Several such proteins were detected (see Figure S1), including some (defined here as type 1 binders) for which addition of **2** or **3** inhibited protein–**5** binding (as for XPO1 and HSP60 in Figure 2), and some (defined here as type 2 binders) for which addition of **2** or **3** enhanced protein–**5** binding (as for GR and PRX1 in Figure 2).
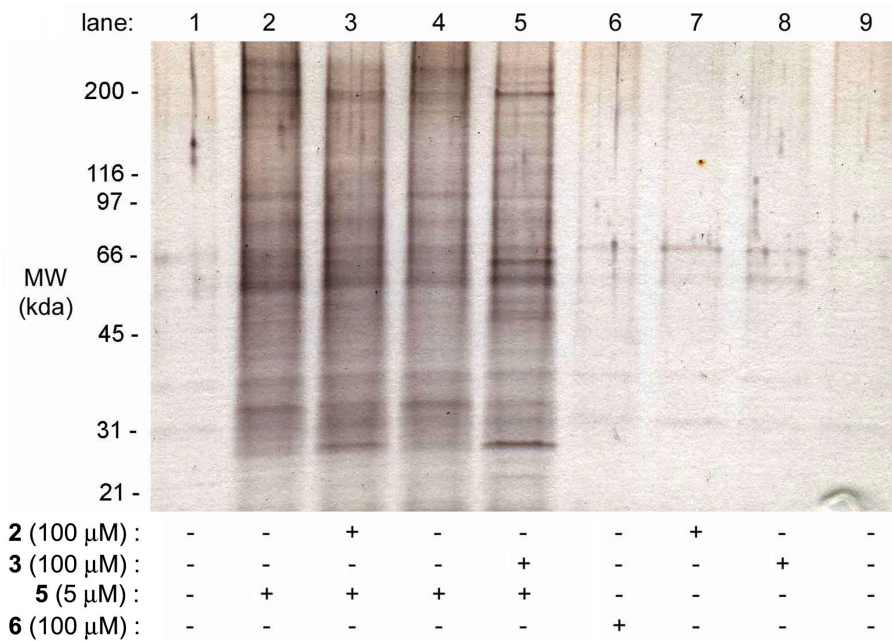
Figure S1. Silver stained gel, following in vitro affinity-isolation of potential binding proteins.

A second tris-glycine mini gel (10%, 12-well) was loaded with 20 µL per well of the denatured protein mixture (samples 1 – 9, as well as the positive control). One lane was loaded with 8 µL of Benchmark prestained protein ladder (Invitrogen). The protein samples were electroeluted (13 min, 23 ºC, 150 V) long enough to remove the colored protein standards from the well. The resulting gel was stained with Colloidal Blue. The entire lanes (approximately 1 cm) corresponding to the protein isolated from samples 1, 2, 3, 4 and 5, as well as the lane corresponding to the positive control, were submitted for protein sequencing by LC-MS/MS.

A total of 66 separate proteins were identified using this method (see Table S1), the majority of which were nonspecific binders. For our purposes, nonspecific binding proteins were defined as those which bound to the streptavidin-agarose resin (for example, pyruvate kinase isozymes M1/M2, discussed earlier), or those proteins for which binding to **5** was unaffected by the addition of **2** or **3**. Tubulin proteins (α-tubulin and β-tubulin) and the proteins associated with the ATP-synthase complex were particularly prominent among nonspecific binding proteins, as were other abundant cellular proteins such as heat shock protein 90 (HSP90) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Among type 1 binding proteins (those which were detected with greater coverage in samples 2 and 4 than in samples 3 and 5), we observed exportin 1 (XPO1, discussed earlier), as well as the structurally and functionally related proteins importin beta-1 subunit (IMB1) and importin alpha re-exporter (CSE1). With somewhat lower coverage, we observed exportin-t (XPOT), importin beta-3 (IMB3), exportin 7 (XPO7) and importin 7 (IPO7). Other type 1 binding proteins observed with good coverage included cullin-associated nedd8-dissociated protein 1 (CAND1) and DNA-dependent protein kinase catalytic subunit (PRKDC). See Figure S2 for a graphical display of abundant type 1 binding proteins.

Among type 2 binding proteins (those detected with greater coverage in samples 3 and 5 than in samples 2 and 4), we observed both peroxiredoxin 1 (PRDX1, discussed previously) and fatty acid synthase (FAS). See Figure S3 for a graphical display of abundant type 2 binding proteins.

Table S1. Proteins Identified by LC-MS/MS.

| protein	MW	lane 1	lane 2	lane 3	lane 4	lane 5	+ cntrl	description	assignment
				Peptide Matches					
XPO1	123	-	8	2	13	-	-	exportin 1	type 1
ATPB	57	1	11	11	12	6	13	atp synthase beta chain	nonspecific - ATP class
TBB2C	50	1	10	11	10	8	6	tubulin beta-2c chain	nonspecific - tubulin class
TBA1	50	-	7	8	10	5	4	tubulin alpha-1 chain	nonspecific - tubulin class
HS90A	85	1	8	11	10	8	14	heat shock protein hsp 90-alpha	nonspecific - abundant
IMB1	97	-	7	1	9	-	1	importin beta-1 subunit	type 1
KPYM	58	3	10	16	9	12	11	pyruvate kinase, isozymes m1/m2	nonspecific
ATPA	60	1	10	8	8	8	9	atp synthase alpha chain	nonspecific - ATP class
CAND1	136	-	5	-	8	-	1	cullin-associated nedd8-dissociated protein 1	type 1
CSE1	110	-	6	2	7	-	1	importin alpha re-exporter	type 1
CLH1	191	-	5	11	6	8	7	clathrin heavy chain 1	nonspecific
LDHA	37	-	4	5	5	5	12	l-lactate dehydrogenase a chain	nonspecific
HS90B	83	-	7	10	5	6	9	heat shock protein hsp 90-beta	nonspecific
PRKDC	469	-	4	-	5	-	1	DNA-dependent protein kinase catalytic subunit	type 1
SERA	57	-	6	9	5	4	5	d-3-phosphoglycerate dehydrogenase	probable nonspecific binder
PHB2	33	-	3	6	5	1	4	prohibitin 2	probable nonspecific binder
ADT1	33	-	4	3	5	1	-	adp/atp translocase 1	possible type 1
VDAC1	31	3	4	3	4	5	4	voltage-dependent anion-selective channel protein 1	probable nonspecific binder
GCN1L	293	-	9	4	4	-	2	gcn1-like protein 1	possible type 1
EF1A2	50	1	5	4	4	4	4	elongation factor 1-alpha-2	looks nonspecific here
XPOT	110	-	3	-	4	-	-	exportin t	type 1
AOFA	60	-	-	2	4	3	1	monoamine oxidase type a	probably nonspecific
IMB3	124	-	2	1	4	-	-	importin beta-3	possible type 1
TBB1	50	-	3	2	4	3	2	tubulin beta-1 chain	nonspecific - tubulin class
Q6PJ43	29	1	3	3	3	3	4	actg1 protein (fragment)	nonspecific
ACADV	70	-	5	2	3	-	4	acyl-coa dehydrogenase, very-long-chain specific	possible type 1
PHB	30	2	5	5	3	2	1	prohibitin	nonspecific
ADT2	33	-	3	2	3	1	2	adp/atp translocase 2	probable nonspecific binder
AL3A2	55	-	-	-	3	-	-	fatty aldehyde dehydrogenase	
XPO7	124	-	-	-	3	-	-	exportin 7	
PRDX1	22	-	3	5	3	9	4	poroxiredoxin-1	type 2
Q13707	37	2	3	6	3	4	5	acta2 protein (fragment)	nonspecific
TBB4	50	-	2	2	2	2	1	tubulin beta-4 chain	nonspecific - tubulin class
VDAC2	38	2	4	3	2	2	-	voltage-dependent anion-selective channel protein 2	probable nonspecific binder
ENPL	92	-	4	4	2	2	9	endoplasmin precursor	nonspecific
M2OM	34	-	2	2	2	-	-	mitochondrial 2-oxoglutarate/malate carrier protein	probable nonspecific binder
Q566M9	79	-	-	-	2	-	2	hypothetical protein (fragment)	
FAS	273	-	-	29	2	17	9	fatty acid synthase	type 2
BASI	42	-	1	-	2	-	-	basigin precursor (cd147 antigen)	possible type 1
ECH1	36	-	-	-	2	-	4	delta3,5-delta2,4-dienoyl-coa isomerase	
LPPRC	145	-	-	2	2	-	3	130 kda leucine-rich protein	
ATPO	23	-	3	2	2	1	-	atp synthase o subunit	nonspecific - ATP class
RDH11	35	-	2	3	2	2	1	retinol dehydrogenase 11	probable nonspecific binder
Q9UBV8	30	-	-	-	2	-	-	peflin (hypothetical protein flj10558)	
AT2A1	110	-	2	1	2	-	-	sarcoplasmic/endoplasmic reticulum calcium atpase	probable nonspecific binder
2AAA	65	-	2	3	2	-	2	serine/threonine protein phosphatase 2a	probable nonspecific binder
GAPDH	36	-	3	4	1	2	11	glyceraldehyde-3-phosphate dehydrogenase	nonspecific
AT1A1	113	-	3	-	1	-	2	Na/K-transporting atpase alpha-1 chain precursor	possible type 1
Q562L5	11	1	2	1	1	1	3	actin-like protein (fragment)	nonspecific
Q5TGV3	60	-	2	1	-	-	-	otthump00000016077 (fragment)	probable nonspecific binder
ACAD9	69	-	2	1	-	-	-	acyl-coa dehydrogenase family member 9	probable nonspecific binder
TRAP1	80	-	2	1	1	1	2	heat shock protein 75 kda	probable nonspecific binder
MPCP	40	-	2	-	1	-	2	phosphate carrier protein	possible type 1
IF5	49	-	2	-	1	-	-	eukaryotic translation initiation factor 5	possible type 1
ARF4	20	-	2	2	-	2	2	adp-ribosylation factor 4	probable nonspecific binder
IPO7	120	-	2	-	-	-	-	importin 7	
HNRPK	51	-	-	3	-	-	-	heterogeneous nuclear ribonucleoprotein k	
ODP2	66	-	-	3	-	-	-	pyruvate dehydrogenase complex e2 subunit	
VPS35	92	-	-	2	-	1	1	vacuolar protein sorting 35	
TCPZ	58	-	-	2	-	1	1	t-complex protein 1, zeta subunit	
COF1	18	-	-	2	-	1	5	cofilin 1	
IF4A1	46	-	-	2	-	-	4	eukaryotic initiation factor 4a-i	
K6PL	85	-	-	2	-	-	1	6-phosphofructokinase, liver type	
EFTU	50	-	-	2	-	1	-	elongation factor tu	
HNRPU	90	-	-	2	-	-	-	heterogenous nuclear ribonucleoprotein u	
RAB10	22	1	-	2	-	-	-	ras-related protein rab-10	
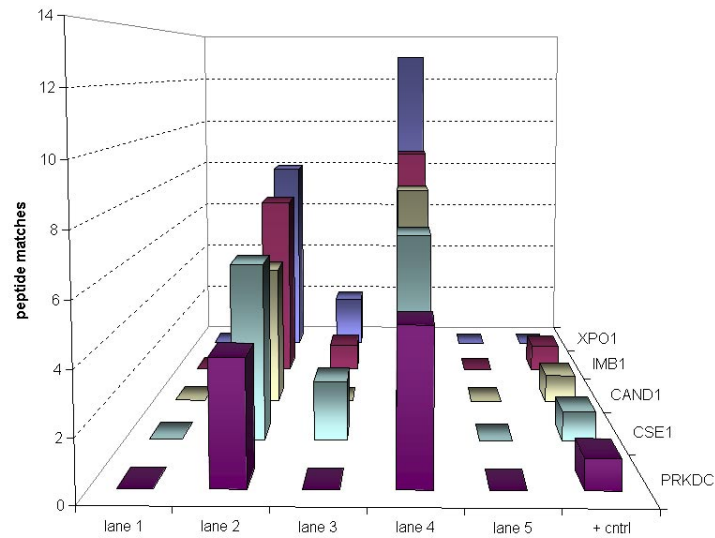
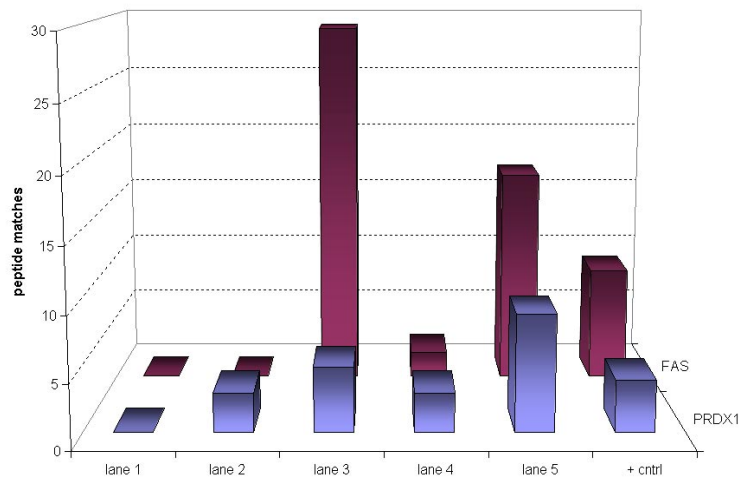**Figure S2.** Type 1 binding proteins observed with high coverage.

Figure S3. Type 2 binding proteins observed with high coverage.

Interestingly, although two of the four proteins (XPO1 and PRX1) identified in previous (excised band) experiments were found again in this broader search, the other two (GR, HSP60) were not, suggesting that complete coverage has not been reached.

Western Blot Observation of Protein Binding Partners.

Four tris-glycine mini gels (4 – 20%, 12-well) were loaded with 15 μL per lane of the denatured protein mixture described in the preceding section (samples 1 – 9, as well as the positive control). One lane was loaded with 8 μL of Benchmark prestained protein ladder (Invitrogen). The protein samples were

electroeluted (1 h, 23 ºC, 150 V), then transferred under semi-dry conditions to nitrocellulose membranes (100 mA, 23 ºC, 12 h).

The four membranes were each blocked for 1 h (40 mL 3% low fat milk in TBS buffer with 1% TWEEN-20), then rinsed (two ten min washes with TBS buffer containing 1% TWEEN-20), and treated 1 h with primary antibody solution (20 mL of 1% low fat milk in TBS buffer with 1% TWEEN-20, containing 10 – 20 µg of the appropriate rabbit polyclonal antibody). The membranes were rinsed again (two ten min washes with 40 mL TBS buffer containing 1% TWEEN-20) and treated with secondary antibody solution (20 mL of 1% low fat milk in TBS buffer with 1% TWEEN-20, containing 20 µg of goat anti-rabbit-HRP conjugate). The membranes were rinsed once more (three ten min washes with 40 mL TBS buffer containing 1% TWEEN-20) and treated with 6 mL of a 1:1 mixture of stabilized peroxide solution:enhanced luminol solution (Pierce; WestPico Chemiluminescent Substrate kit) for 3 min. Finally, the membranes were sealed in plastic wrap and exposed to X-ray film.

Competitive Binding Experiment with Iodoacetamide.

An identical procedure to that described in the preceding section was employed, except that in one sample (sample 6) iodoacetamide (10 mM) was added to the affinity-isolation experiment, as indicated:

sample:	volume lysate	volume DMSO	volume 2 5000 µM		volume 3 5000 µM		volume iodoacetamide 500 mM		volume 5 200 µM		volume 6 2000 µM		final volume	% DMSO
1	380 µL	20 µL	x		x		x		x		x		400 µL	5 %
2	380 µL	10 µL	x		x		x		10 µL	(5 µM)	x		400 µL	5 %
3	380 µL	2 µL	8 µL	(100 µM)	x		x		10 µL	(5 µM)	x		400 µL	5 %
4	380 µL	10 µL	x		x		x		10 µL	(5 µM)	x		400 µL	5 %
5	380 µL	2 µL	x		8 µL	(100 µM)	x		10 µL	(5 µM)	x		400 µL	5 %
6	380 µL	2 µL	x		x		8 µL	(10 mM)	10 µL	(5 µM)	x		400 µL	5 %
7	380 µL	x	x		x		x		x		20 µL	(100 µM)	400 µL	5 %
8	380 µL	12 µL	8 µL	(100 µM)	x		x		x		x		400 µL	5 %
9	380 µL	12 µL	x		8 µL	(100 µM)	x		x		x		400 µL	5 %

Following the sample workup described above, the protein binding partners HSP60, XPO1 and GR were detected by Western-blot analysis. The results (Figure S4) indicate that protein-**5** binding is reduced in the presence of iodoacetamide (compare lane 6 with lane 2 or 4).
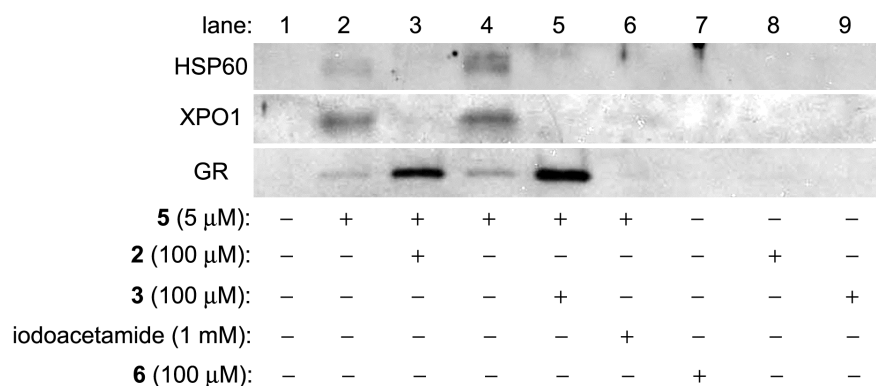
lane: 1 2 3 4 5 6 7 8 9

	1	2	3	4	5	6	7	8	9
HSP60									
XPO1									
GR									
5 (5 µM):	–	+	+	+	+	+	–	–	–
2 (100 µM):	–	–	+	–	–	–	–	+	–
3 (100 µM):	–	–	–	–	+	–	–	–	+
iodoacetamide (1 mM):	–	–	–	–	–	+	–	–	–
6 (100 µM):	–	–	–	–	–	–	+	–	–

Figure S4. Western blot detection following affinity-isolations of three protein binding partners with probe **5**, in the presence or absence of competitors (+)-avrainvillamide (**2**), structural analog **3** and iodoacetamide.

Enzyme Inhibition Assays.

General procedure. A sample of LNCaP cell lysate was thawed at 4 ºC. Glutathione reductase (GR) activity within the lysate (diluted to a final protein concentration of 0.1 mg/mL), in the absence or presence of several potential inhibitors, including **2**, *ent*-**2**, **3** and *S*-nitrosoglutathione (GSNO, as a positive control)[17] was assayed using the method of Smith, Vierheller and Thorne.[18] Inhibitors were added from DMSO stocks, and DMSO was assayed independently as a vehicle control.

Data. (+)-Avrainvillamide (**2**) was observed to be a more potent inhibitor of LNCaP GR than the simpler nitrone **3** (refer to Figure S5), but a much poorer inhibitor than GSNO.

In a second experiment, the diluted lysate was incubated with (+)-avrainvillamide (**2**) for varying periods of time, prior to the beginning of the assay (see Figure S6). The measured inhibition was not significantly affected by pre-incubation time, suggesting that (+)-avrainvillamide (**2**) is a reversible inhibitor of human glutathione reductase.[19]

[17] Becker, K.; Savvides, S. N. Keese, M.; Schirmer, R. H.; Karplus, P. A. *Nature Struct. Biol.* **1998**, *5*, 267.

[18] Smith, I. K.; Vierheller, T. L.; Thorne, C. A. *Anal. Biochem.* **1988**, *175*, 408.

[19] For a similar experiment with an irreversible inhibitor, see: Seefeldt, T.; Dwivedi, C.; Peitz, G.; Herman, J.; Carlson, L.; Zhang, Z.; Guan, X. *J. Med. Chem.* **2005**, *48*, 5224.
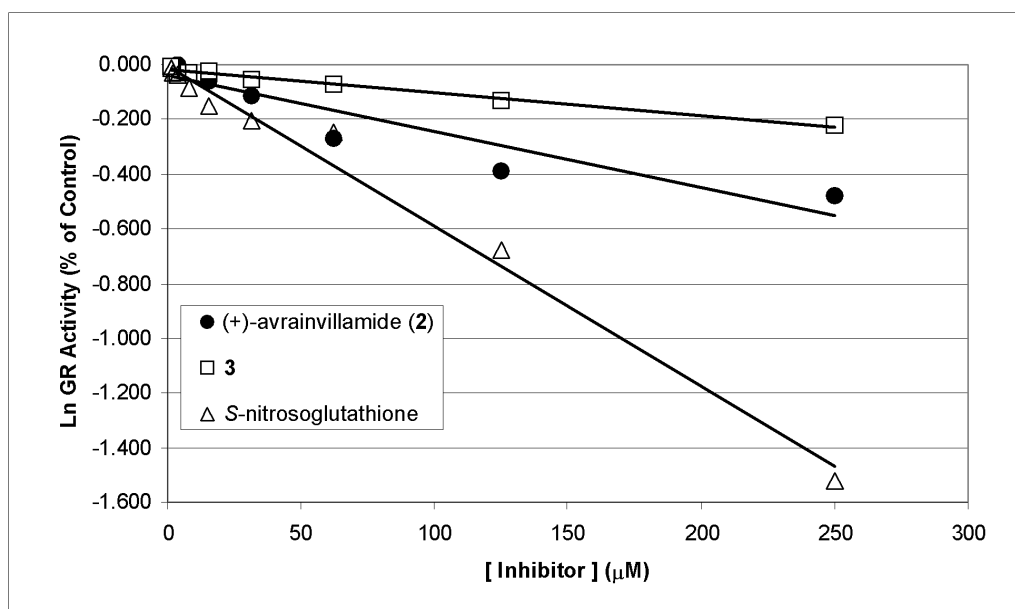
Figure S5. Inhibition of GR activity in an LNCaP cell lysate by **2** (filled circles), **3** (open squares) or GSNO (open triangles) in the presence of 850 µM glutathione disulfide.
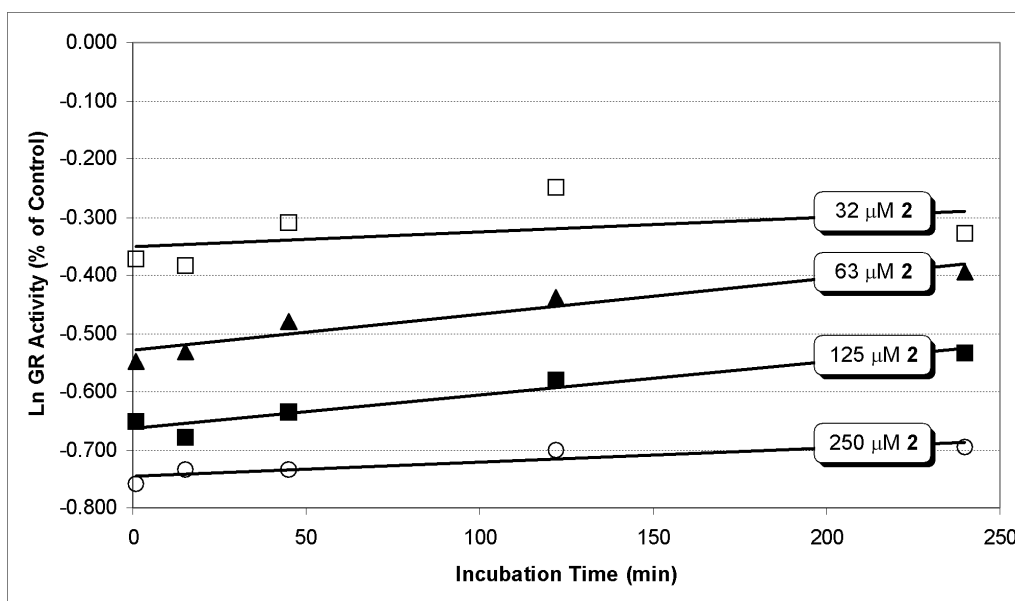

Figure S6. Inhibition of GR activity in an LNCaP cell lysate by **2** at 32 µM (open squares), 63 µM (filled triangles, 125 µM (filled squares) or 250 µM (open circles) with varying incubation times, in the presence of 570 µM glutathione disulfide.

In a third experiment, the inhibitory activities of (+)-avrainvillamide (**2**) and (-)-avrainvillamide (*ent*-**2**) were tested in the presence of varying concentrations of glutathione disulfide. The two compounds

were equally effective (within experimental error; see Figure S7) in inhibiting GR activity within our LNCaP lysate.
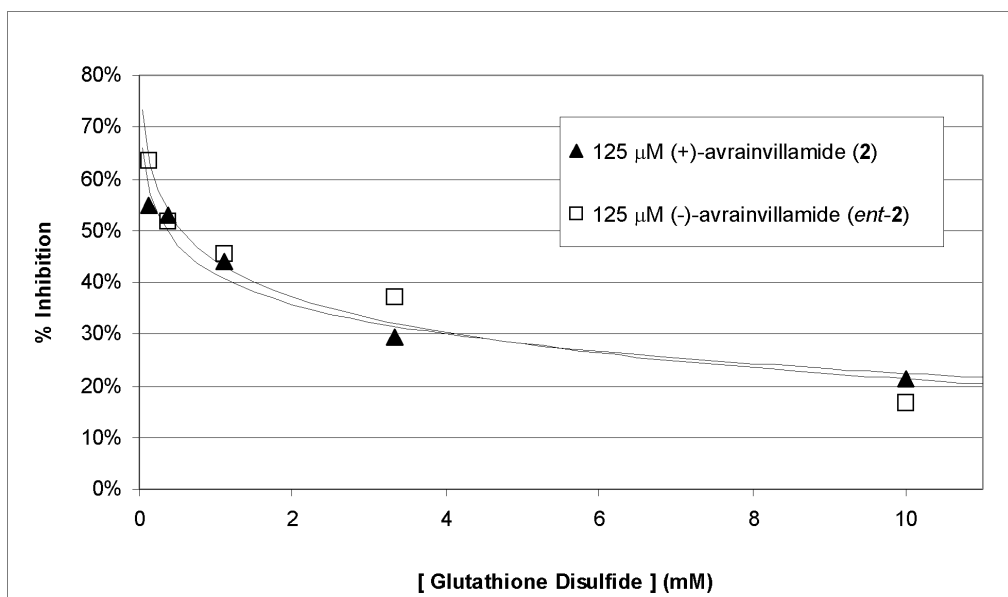

Figure S7. Inhibition of GR activity in an LNCaP cell lysate by 125 μM **2** (solid triangles) or 125 μM *ent*-**2** (open squares) in the presence of varying concentrations of GSSG.

The ability of **2** or *ent*-**2** to inhibit GR was dependant upon the concentration of glutathione disulfide (the natural substrate for the enzyme), which would be consistent with active-site inhibition. However, our use of a cell lysate that contained endogenous glutathione, glutathione disulfide, and other glutathione-processing enzymes as a source of GR precluded a more rigorous kinetic study.