Supplementary Methods

A small molecule that binds Hedgehog and blocks its signaling in human cells

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Contents:

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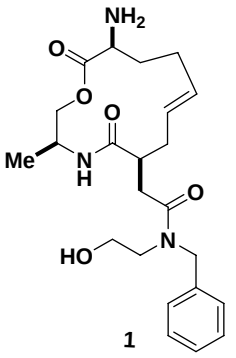
I. Supplementary figures



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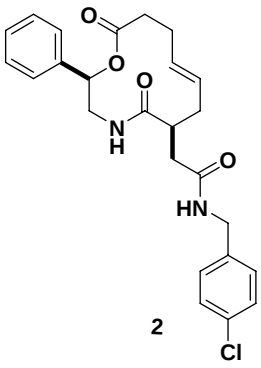

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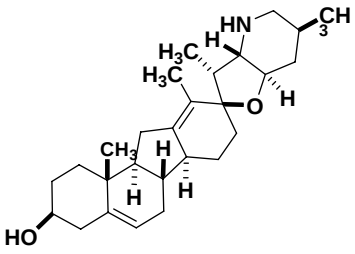

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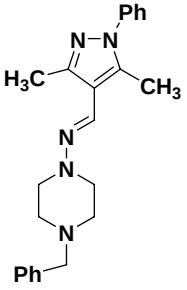

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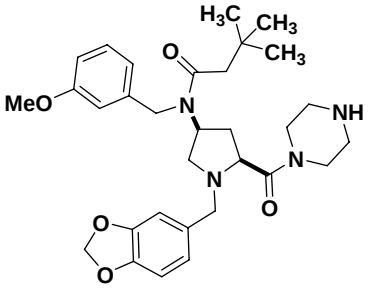

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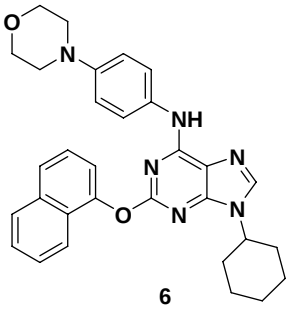

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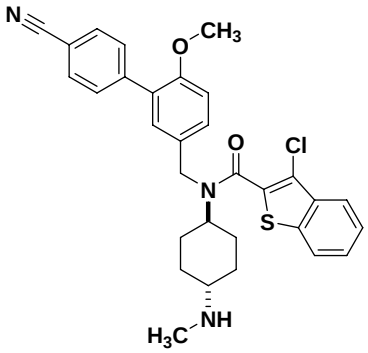

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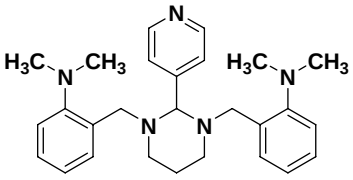

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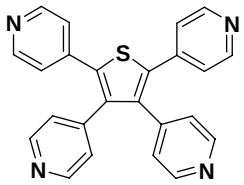

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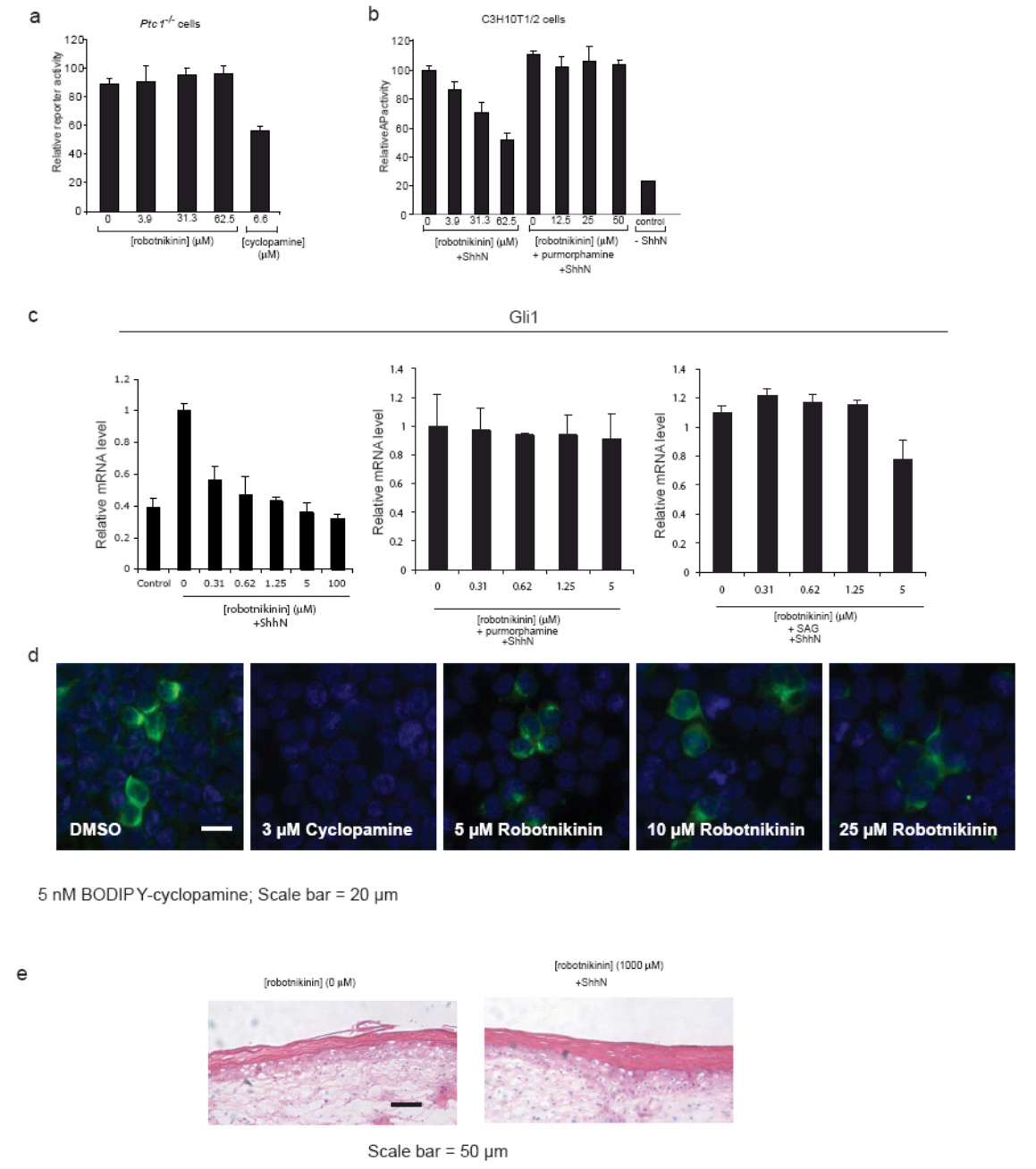
Supplementary Fig. 1 Structures of Shh pathway modulators. Shh Small Molecule Microarray screening hit (**1**), Robotnikinin (**2**), cyclopamine (**3**),

SANT1 (**4**), Cur61414 (**5**), purmorphamine (**6**), Hh-Ag1.2 (SAG; **7**), GANT61 (**8**), GANT58 (**9**).



Supplementary Fig. 2 (a) When a *Ptc1*$^{-/-}$ MEF cell line was treated with robotnikinin at the indicated concentrations, pathway inhibition was not observed, in contrast to the results observed in (Ptc1-containing) Shh-LIGHT2 cells. Each datum point represents the average of five experiments, and error bars represent standard deviations. All data

were normalized for cell titer. **(b)** C3H10T1/2 cells showed repressed osteogenic potency, indicated by alkaline phosphatase (AP) induction, in the presence of robotnikinin, but the effect was reversed in the presence of 3.6 μM purmorphamine **(c)** primary human keratinocytes displayed repression of Gli1 nRNA by qPCR **(d)** robotnikinin did not compete with BODIPY-cyclopamine for Smo binding to the surface of HEK293 cells overexpressing Smo **(e)** there was not a marked change in the histology of synthetic human skin, which was derived from dehydrated collagen populated with primary human keratinocytes, in the presence of robotnikinin.

II. Materials and methods

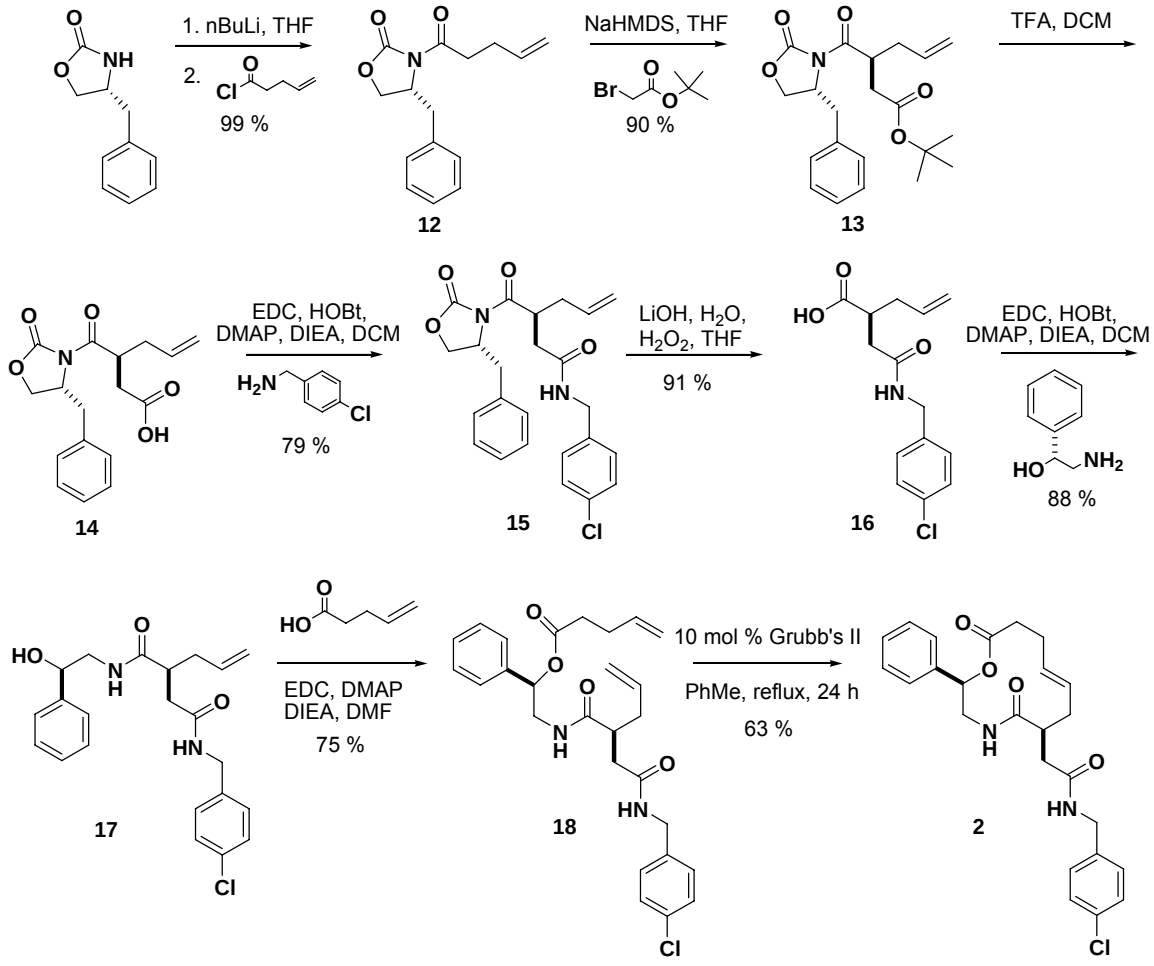
Commercially available reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI), Fluka Chemical Corp. (Milwaukee, WI), TCI America (Portland, OR), and Toronto Research Chemicals Inc. (ON, Canada) and used as received unless otherwise noted. All solvents for reactions, were dispensed from a solvent purification system that passes solvents through packed columns (THF, CH_3CN, and CH_2Cl_2: dry neutral alumina; DMF: activated molecular sieves). Water was double distilled. Reactions were monitored by analytical thin-layer chromatography using Merck silica gel 60 F254 plates. Compounds were visualized with a UV lamp (λ 254) and staining with I_2/SiO_2.

Purification and analysis

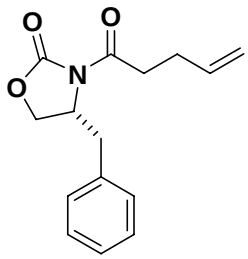
Flash chromatography was performed using a CombiFlash Companion system (Teledyne, ISCO, Inc.) with prepacked FLASH silica columns (Biotage, Inc.). ^1H NMR spectra were recorded at 23 °C on a Varian Mercury400 (400 MHz), a and Varian Unity/Inova500 (500 MHz) Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and referenced to residual protium in the NMR solvent ($CDCl_3$, δ = 7.26). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (J) in Hertz (Hz), and integration. ^{13}C NMR spectra were recorded at 23 °C on a Varian Mercury400 (400 MHz) and a Varian Unity/Inova500 (500 MHz) spectrometer. Chemical shifts (δ) are reported in parts per million (ppm)

downfield from tetramethylsilane and referenced to carbon resonances in the NMR solvent ($CDCl_3$, δ = 77.23, center line). High-resolution mass spectra (HRMS) were obtained at the mass spectrometry facility at Harvard University using a mass resolution of 10.000.

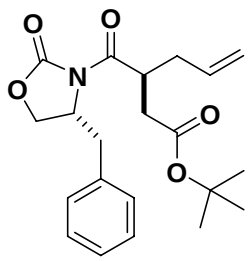
III. Synthetic procedures



Scheme S1. Synthesis of robotnikinin (**2**)**.**

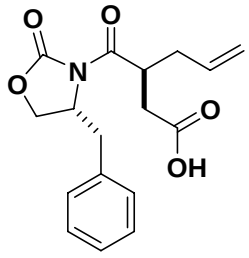


(R)-4-benzyl-3-pent-4-enoyloxazolidin-2-one (12). A solution of (*R*)-4-benzyl-2-oxazolidinone (2 g, 0.011 mmol) in dry THF (33 mL) was cooled to -78°C. To this solution n-butyllithium (1.6 M in hexanes, 6.87 mL, 0.011 mmol) was added over a ten-minute period. Following this addition, 4-pentenoyl chloride (1.32 mL, 0.012 mmol) was added in a single portion. The reaction mixture was stirred under argon at -78°C for 30 minutes and then warmed to room temperature. The reaction progress was monitored by TLC (~ 1 h). The reaction mixture was quenched with saturated aqueous ammonium chloride (60 mL) and the product was extracted with dichloromethane (2 x 80 mL). The combined organic layers were washed successively with 1N sodium hydroxide and brine, dried over Na_2SO_4, and filtered. The solution was concentrated *in vacuo* and the residue was cooled at 4°C overnight. The resulting solid was triturated using cold hexanes and dried to give a white solid, **12** (2.838 g, 99%). ^{1}H NMR ($CDCl_3$, 500MHz): δ 7.33 - 7.37 (m, 1 H), 7.28 - 7.32 (m, 1 H), 7.20 - 7.24 (m, 1 H), 5.84 - 5.96 (m, 1 H), 5.13 (dd, *J*=17.1, 1.5 Hz, 1 H), 5.05 (dd, *J*=10.3, 1.0 Hz, 1 H), 4.69 (ddd, 1 H), 4.15 - 4.25 (m, 1 H), 3.31 (dd, *J*=13.2, 3.4 Hz, 1 H), 2.99 - 3.16 (m, 1 H), 2.77 (dd, *J*=13.2, 9.8 Hz, 1 H), 2.44 - 2.51 (m, 1 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.8, 136.9, 135.5, 129.6, 129.2, 127.6, 116.0, 110.0, 66.4, 55.4, 38.2, 35.0, 28.4. HRMS calcd for $C_{15}H_{17}NO_3$ (M + H) *m/z* 260.1286, found 260.1277.



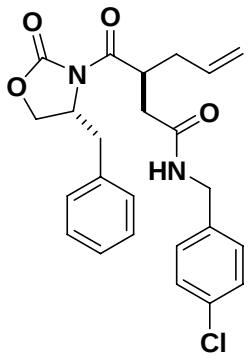
(S)-tert-butyl 3-((R)-4-benzyl-2-oxooxazolidine-3-carbonyl)hex-5-enoate (13). A dry round bottom flask was charged with **12** (3.2 g, 12.34 mmol) and dry THF (123 mL) and then cooled to -78°C. To this solution NaHMDS (1M in THF, 13.57 mL, 13.57 mmol) was added over a period of 10 minutes. To ensure complete enolization, the reaction mixture was stirred for an

additional 20 minutes at -78°C. A solution of tert-butyl bromoacetate (2.74 mL, 18.51 mmol) in THF (6 mL) was then introduced to the reaction flask. The solution was stirred for 1 hour at -78°C and then warmed to -48°C while still stirring. The reaction was monitored by TLC (~3 h) and quenched with saturated aqueous NH_4Cl. The reaction mixture was concentrated by the removing the THF under reduced pressure. The residue was then diluted with dichloromethane. This solution was washed successively with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure to give white crystals of **13** (4.27 g, 93%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 - 7.37 (m, 2 H) 7.27 - 7.30 (m, 3 H) 5.74 - 5.85 (m, 1 H) 5.05 - 5.13 (m, 2 H) 4.63 - 4.70 (m, 1 H) 4.25 - 4.32 (m, 1 H) 4.16 (d, *J*=4.9 Hz, 2 H) 3.34 (dd, *J*=13.4, 2.7 Hz, 1 H) 2.74 - 2.87 (m, 2 H) 2.39 - 2.52 (m, 2 H) 2.19 - 2.26 (m, 1 H) 1.44 (s, 9 H); ^{13}C NMR (126 MHz, $CDCl_3$) *δ* 175.0, 171.1, 153.0, 135.6, 134.4, 129.4, 128.8, 127.1, 117.7, 80.6, 65.8, 55.4, 39.0, 37.5, 36.6, 36.1, 28.0. HRMS calcd for $C_{21}H_{27}NO_5$ (M + H) *m/z* 374.1967, found 374.1971.

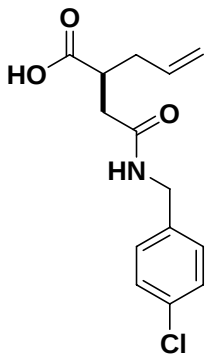


(S)-3-((R)-4-benzyl-2-oxooxazolidine-3-carbonyl)hex-5-enoic acid (14). A round bottom flask was charged with a solution of compound **13** (4.27 g, 11.44 mmol) in dichloromethane (9 mL). TFA (9 mL, 51.42 mmol) was added to this solution at room temperature in one portion. After 1 h, the dichloromethane and excess TFA were removed under reduced pressure. To ensure the removal of all remaining TFA, the residue was azeotroped with benzene 3 times to yield a colorless oil **14**. The material was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.19 (br. s., 1 H) 7.31 - 7.37 (m, 2 H) 7.27 - 7.31 (m, 1 H) 7.22 - 7.26 (m, 2 H) 5.71 - 5.82 (m, 1 H) 5.07 - 5.14 (m, 2 H) 4.25 - 4.33 (m, 1 H) 4.17 (d, *J*=4.9 Hz, 2 H) 3.25 (dd, *J*=13.7, 3.4 Hz, 1 H) 2.96 (dd, *J*=17.6, 10.7 Hz, 1 H) 2.78 (dd, *J*=13.7, 9.3 Hz, 1 H) 2.59 (dd, *J*=17.6, 3.9 Hz, 1 H) 2.39 - 2.47 (m, 1 H) 2.18 - 2.26 (m, 1 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.2, 174.6, 153.0, 135.2, 133.9, 129.4, 128.8, 127.1, 118.12,

65.9, 55.3, 38.6, 37.2, 36.0, 34.86. HRMS calcd for $C_{17}H_{19}NO_5$ (M + H) *m/z* 318.1341, found 318.1339.

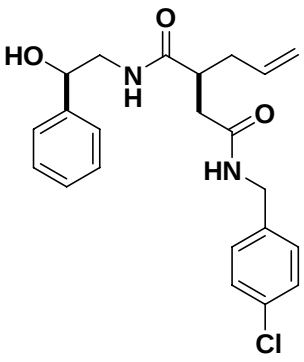


(S)-3-((R)-4-benzyl-2-oxooxazolidine-3-carbonyl)-N-(4-chlorobenzyl)hex-5-enamide (15). A solution of compound **14** (0.402 g, 1.3 mmol), EDC (0.364 g, 1.9 mmol), HOBT (0.256 g, 1.9 mmol), and Hunig's base (0.662 mL, 3.8 mmol) in dichloromethane (17 mL) was cooled to 0°C and stirred for half an hour. To this solution 4-chlorobenzylamine (0.174 mL, 1.43 mmol) and a catalytic amount of DMAP were added. The reaction was stirred overnight and the reaction progress was monitored by TLC. The dichloromethane was removed *in vacuo* and the reaction mixture quenched with aqueous NH_4Cl. The solution was washed with water and brine, dried over Na_2SO_4, and concentrated under reduced pressure to give well formed white crystals of **15** (0.443 g, 79%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 - 7.36 (m, 2 H) 7.21 - 7.29 (m, 5 H) 7.17 - 7.21 (m, 2 H) 6.09 (t, *J*=5.6 Hz, 1 H) 5.73 - 5.82 (m, 1 H) 5.04 - 5.10 (m, 2 H) 4.65 (ddd, *J*=13.3, 6.7, 3.4 Hz 1 H) 4.41 (dd, *J*=15.2, 5.9 Hz, 1 H) 4.34 (dd, *J*=14.9, 5.9 Hz, 1 H) 4.23 - 4.30 (m, 1 H) 4.23 - 4.30 (m, 1 H) 4.14 - 4.18 (m, 2 H) 3.28 (dd, *J*=13.7, 2.9 Hz, 1 H) 2.66 - 2.80 (m, 2 H) 2.41 - 2.51 (m, 2 H) 2.22 - 2.29 (m, 1 H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.1, 171.0, 153.4, 137.1, 135.7, 134.8, 133.4, 129.7, 129.3, 129.1, 129.0, 127.5, 118.1, 66.3, 55.7, 43.1, 40.1, 38.0, 37.8, 36.3. HRMS calcd. for $C_{24}H_{25}ClN_2O_4$ (M + H) *m/z* 441.1581, found 441.1581.

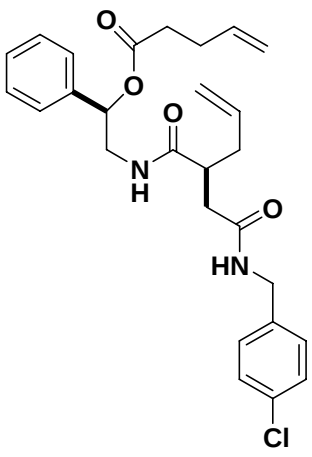


(S)-2-(2-(4-chlorobenzylamino)-2-oxoethyl)pent-4-enoic acid (16). A round bottom flask was charged with a solution of **15** (0.443 g, 1 mmol) in THF/H2O (4:1, 10 mL) and cooled to 0°C. To this solution H_2O_2 (0.45 mL, 4 mmol) was added, followed by aqueous LiOH (0.05 g, 2 mmol/ 2.5 mL H_2O). The reaction

mixture was stirred for 1 hour, followed by an addition of saturated aqueous Na_2SO_3 (4 mL). The reaction mixture was then stirred for an additional 20 minutes. The THF was removed under reduced pressure and the remaining residue was diluted with dichloromethane and water. The organic layer was set aside to recover the hydrolyzed chiral auxiliary within. The aqueous layer was acidified with 3M aqueous HCl followed by extraction with dichloromethane (3 x 60 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated to give **16** (0.26 g, 91%). $[\alpha]_D$ -8.2 (2:1 $CHCl_3$: MeOH) HRMS calcd for $C_{14}H_{16}ClNO_3$ (M + H) *m/z* 282.0897, found 282.0897.

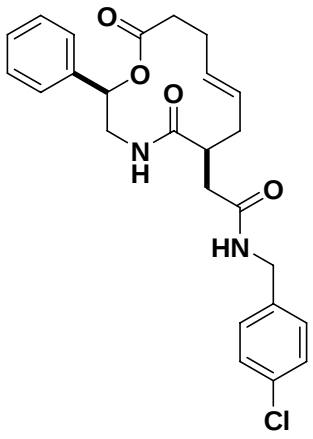


(S)-2-allyl-N4-(4-chlorobenzyl)-N1-((R)-2-hydroxy-2-phenylethyl)succinamide (17). A solution of **16** (0.375 g, 1.33 mmol), EDC (0.382 g, 1.995 mmol), HOBT (0.27 g, 1.995 mmol), and Hunig's base (0.695 mL, 3.99 mmol) in DMF (8.87 mL) was cooled to 0°C and stirred for 30 min. To this solution, (R)-2-amino-1-phenylethanol (0.200 g, 1.46 mmol) and catalytic amount of DMAP were added. Subsequently, the cooling bath was removed, and the reaction mixture was allowed to warm to room temperature overnight. The DMF was removed under reduced pressure. The residue was then quenched with aqueous NH_4Cl, diluted with ethyl acetate, and washed successively with water and brine. This solution was dried over Na_2SO_4, filtered, and concentrated to afford **17** (0.384 g, 72 %). HRMS calcd. for $C_{22}H_{25}ClN_2O_3$ (M + H) *m/z* 401.1632, found 401.1651.



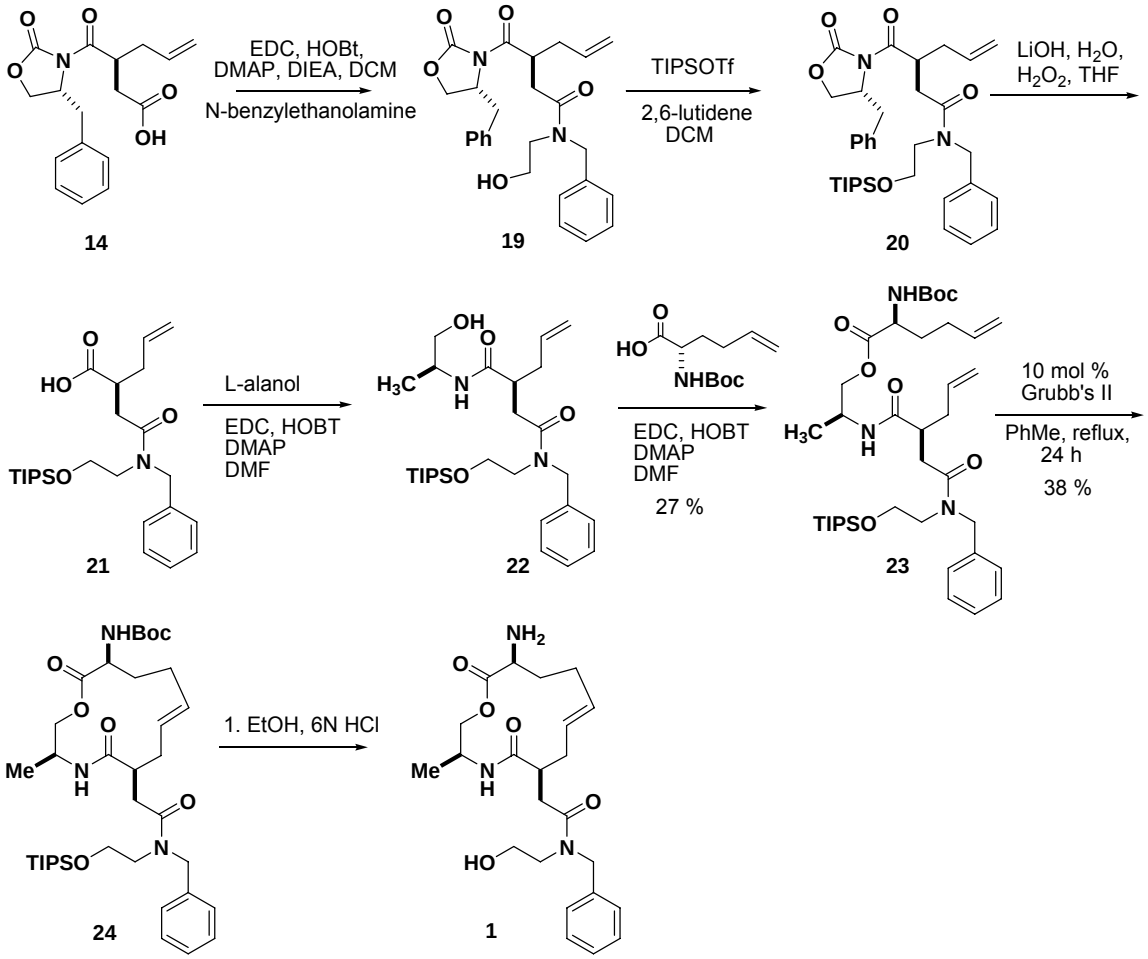
(R)-2-((S)-2-(2-(4-chlorobenzylamino)-2-oxoethyl)pent-4-enamido)-1-phenylethyl pent-4-enoate (18). A flask was charged with a solution of **17** (0.128 g, 0.319 mmol), EDC (0.092 g, 0.479 mmol), Hunig's base (0.124 g, 0.957 mmol) in DMF (5 mL) and stirred for half an hour. To the reaction mixture, 4-pentenoic acid (0.035 g, 0.351 mmol)

and catalytic amount of DMAP were added and allowed to stir overnight. The reaction progress was monitored by LCMS. The reaction mixture was quenched with aqueous NH_4Cl and then washed with water and brine. The solution was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel chromatography and concentrated to afford **18** (0.115 g, 75 %). $[\alpha]_D$ -27.1 ($CHCl_3$). Asterisks denote rotameric peaks. ^1H NMR (400 MHz, $CDCl_3$): δ 7.23 - 7.37 (m, 7 H) 7.14 - 7.18 (m, 2 H) 6.71 (t, *J*=5.9 Hz, 1 H), *6.54 (t, *J*=5.9 Hz, 1 H) 6.41 (t, *J*=5.8 Hz, 1 H), *6.17 (t, *J*=5.8 Hz, 1 H) 5.58 - 5.84 (m, 3 H) 4.96 - 5.09 (m, 4 H) 4.36 (dd, *J*=11.3, 6.2 Hz, 1 H) 4.22 - 4.30 (m, 1 H) 3.55 - 3.64 (m, 1 H) 3.46 - 3.53 (m, 1 H) 2.70 - 2.84 (m, 1 H) 2.44 - 2.53 (m, 3 H) 2.27 - 2.40 (m, 4 H) 2.10 - 2.21 (m, 1 H); ^{13}C NMR (DMSO-d_6 ,126MHz): δ 173.8, *173.6, 171.7, 170.9, *170.7, 138.8, *138.5, 136.9, 135.9, *135.7, 131.2, 129.0, 128.4, 128.1, 126.3, 116.6, *116.5, 115.5, 73.8, *73.7, 43.7, 41.3, 37.2, 37.0, 36.3, 36.1, 32.8, 28.3 ppm. HRMS calcd. for $C_{27}H_{31}ClN_2O_4$ (M + H) *m/z* 483.2050, found 483.2052.

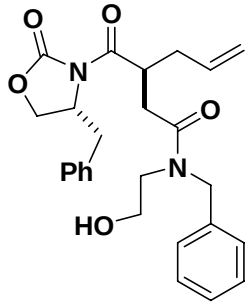


N-(4-chlorobenzyl)-2-((2R,6S,E)-5,12-dioxo-2-phenyl-1-oxa-4-azacyclododec-8-en-6-yl)acetamide (2). To a solution of **18** (0.057 g, 0.119 mmol) in toluene (11 mL) was added Grubbs II catalyst (0.011 g, 0.0129 mmol). The reaction mixture was heated to 65°C and stirred overnight. The reaction progress was monitored by TLC. The reaction mixture was allowed to cool, diluted with dichloromethane to make a 0.1M solution, and stirred with $Pb(OAc)_4$ (0.029 g, 0.065 mmol) overnight. The $Pb(OAc)_4$ was removed by directly subjecting the mixture to silica gel chromatography to afford **2** (0.034 g, 63 %). $[\alpha]_D$ -22.9 (2:1 $CHCl_3$: MeOH). 1.8:1 *E/Z* isomers (*E*: $J_{H,H}$ = 12.6 Hz). Asterisks denote minor isomer. ^1H NMR (DMSO-d_6, 500MHz): δ 8.54 (t, *J*=5.9 Hz, 1 H), *8.41 (t, *J*=5.9 Hz, 1 H), 7.94 (d, *J*=9.8 Hz, 1 H), 7.31 - 7.42 (m, 7 H), 7.24 (dd, *J*=8.3 Hz, 6.5 Hz, 2 H), 5.83 - 5.91 (m, 1 H), *5.40 (d, *J*=2.8 Hz, 2 H), 5.31 (dd, *J*=12.6, 5.7 Hz, 2 H), 4.18 - 4.27 (m, 2 H), 4.00 - 4.12 (m, 1 H), *3.03 (t,

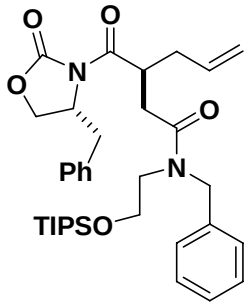
J=10.0 Hz, 1 H), *2.88 (d, *J*=13.7 Hz, 1 H), 2.76 (d, *J*=14.2 Hz, 1 H), 2.58 - 2.66 (m, 1 H), 2.32 - 2.44 (m, 2 H), 2.12 - 2.32 (m, 4 H), 1.97 - 2.11 (m, 2 H); ^{13}C NMR (DMSO-d_6,126 MHz): δ *174.8, 172.8, 172.3, *171.7, 170.43, *170.35, *138.8, 138.7, 138.2, *138.1, 131.2, 130.2, 129.7, 128.94, *128.87, 128.7, *128.6, *128.2, 128.1, 127.9, *126.23, 126.21, 73.3, 43.1, 42.7, 41.3, 38.3, 35.7, 34.4, 29.2 ppm. HRMS calcd. for $C_{25}H_{27}ClN_2O_4$ (M + H) *m/z* 455.1737, found 455.1741.



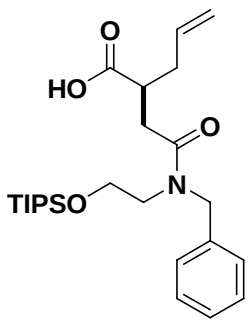
Scheme S2. Synthesis of **1**.



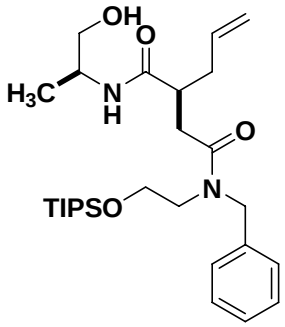
(S)-N-benzyl-3-((R)-4-benzyl-2-oxooxazolidine-3-carbonyl)-N-(2-hydroxyethyl)hex-5-enamide (19). A solution of **14** (1 g, 3.15 mmol) in DMF (21.01 mL) was made in a dry round bottom flask. To this solution, EDC (0.906 g, 4.73 mmol), HOBT (0.724 g, 4.73 mmol) and N,N-diisopropylethylamine (1.651 mL, 9.45 mmol) were added at 0ºC. The reaction was stirred for 30 minutes and then N-benzylethanolamine (0.499 mL, 3.47 mmol) and a catalytic amount of DMAP were added. The reaction was stirred overnight. The reaction was quenched with aqueous NH_4Cl and then diluted with ethyl acetate. The organic layer was then washed successively three times with aqueous NH_4Cl, water, and brine. The organic layer was then dried over sodium sulfate and concentrated under reduced pressure to afford **19**, which was used without further purification. HRMS calcd. for $C_{26}H_{30}N_2O_5$ (M + H) *m/z* 451.2238, found 451.2233.



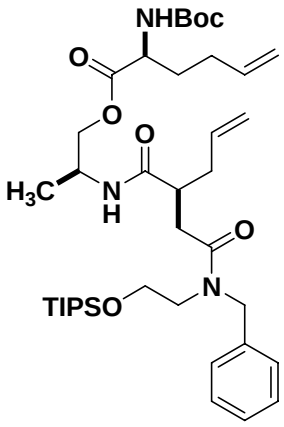
(S)-N-benzyl-3-((R)-4-benzyl-2-oxooxazolidine-3-carbonyl)-N-(2-(triisopropylsilyloxy)ethyl)hex-5-enamide (20). In a round bottom flask, a solution of **19** (0.85g, 1.887 mmol) and 2,6-lutidene (0.549 mL, 4.72 mmol) in DCM (12.58 mL) was cooled to 0ºC. To this solution TIPS-OTf (0.67 mL, 2.453 mmol) was added in portions over 1 minute. The reaction was stirred at room temperature for 2 hours. The reaction was then quenched with aqueous ammonium chloride solution and diluted with DCM. The organic layer was then washed successively with water and brine and dried over sodium sulfate. The material was concentrated under reduced pressure to afford **20** which was used without further purification. HRMS calcd. for $C_{35}H_{50}N_2O_5Si$ (M + H) *m/z* 607.3557, found 607.3567.



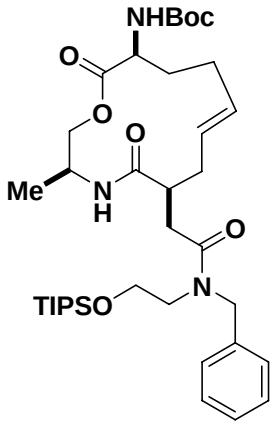
(S)-2-(2-(benzyl(2-(triisopropylsilyloxy)ethyl)amino)-2-oxoethyl)pent-4-enoic acid (21). A solution of **20** (0.9 g, 1.483 mmol) in a 4:1 solution of THF (11.86 mL) and water (2.97 mL) was cooled to 0 ºC. To this solution H_2O_2 (0.606 mL, 5.93 mmol) was added and then followed by a 2% aqueous LiOH (0.057 g, 2.373 mmol) solution (2.85 mL). The reaction was stirred for 1 hour at 0ºC. An aqueous 17% sodium sulfite (0.748 g, 5.93 mmol) solution (4.4 mL) was then added and allowed to stir for an additional 20 minutes. The reaction mixture was concentrated under reduced pressure and then diluted with water. The aqueous solution was acidified with HCl and extracted 3 times with DCM. The combined organic layers were washed once with brine, dried over sodium sulfate and concentrated under reduced pressure to afford **21**, which was used without further purification.



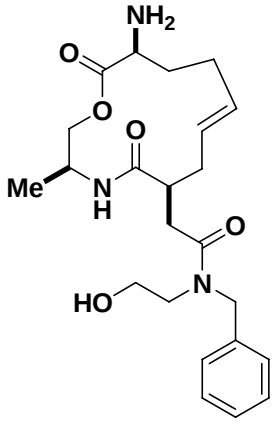
(S)-2-allyl-N4-benzyl-N1-((S)-1-hydroxypropan-2-yl)-N4-(2-(triisopropylsilyloxy)ethyl)succinamide (22). A dry round bottom flask was charged with a solution of **21** (0.3g, 0.670 mmol) in DMF (4.47 mL). To this solution, EDC (0.193 g, 1.01 mmol), HOBT (0.154 g, 1.01 mmol), and N,N-diisopropylehtylamine (0.351 mL, 2.01 mmol) were added at 0ºC. The reaction was stirred for 30 minutes and then (S)-2-aminopropan-1-ol (0.057 mL, 0.737 mmol) and a catalytic amount of DMAP were added. After stirring overnight, the reaction was quenched with aqueous NH_4Cl and then diluted with ethyl acetate. The organic layer was then washed 3 times with aqueous NH_4Cl and then diluted with ethyl acetate. The organic layer was washed 3 more times with aqueous NH_4Cl, water, and brine. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to afford **22**, which was used without further purification.



(S)-((10S,13S)-10-allyl-7-benzyl-3,3-diisopropyl-2,13-dimethyl-8,11-dioxo-4-oxa-7,12-diaza-3 silatetradecan-14-yl) 2-(tert-butoxycarbonylamino)hex-5-enoate (23). A solution of (S)-2-(tert-butoxycarbonylamino)hex-5-enoic acid (0.450 g, 1.85 mmol) in DMF (25 mL) was made in dry round bottom flask. To this solution, EDC (0.978 g, 5.1 mmol) and Et_3N (4 mL, 29 mmol) were added at 0ºC. The reaction was stirred for 30 minutes and then **22** (0.863 g, 1.7 mmol) and a catalytic amount of DMAP were added. The reaction was quenched with aqueous ammonium chloride solution and then diluted with ethyl acetate. The organic layer was then washed successively 3 times with aqueous NH_4Cl and then diluted with ethyl acetate. The organic layer was then washed 3 more times with aqueous NH_4Cl, water, and brine. The organic layer was then dried over sodium sulfate and concentrated under reduced pressure and purified by silica gel flash chromatography to afford **23** (0.228g, 27 %). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.34 - 7.39 (m, 1 H), 7.26 - 7.32 (m, 1 H), 7.22 - 7.26 (m, 1 H), 7.13 - 7.20 (m, 1 H), 6.31 (d, *J*=6.8 Hz, 1 H), 5.65 - 5.84 (m, 2 H), 5.58 (d, *J*=6.8 Hz, 1 H), 4.93 - 5.13 (m, 4 H), 4.66 - 4.77 (m, 2 H), 4.50 - 4.58 (m, 1 H), 4.30 - 4.40 (m, 1 H), 4.25 (dt, *J*=11.0, 4.0 Hz, 1 H), 3.98 - 4.20 (m, 2 H), 3.83 - 3.90 (m, 1 H), 3.72 - 3.83 (m, 1 H), 3.50 - 3.62 (m, 1 H), 3.38 (ddd, *J*=13.7, 6.8, 4.4 Hz, 1 H), 3.21 - 3.31 (m, 1 H), 2.71 - 2.90 (m, 1 H), 2.58 (d, *J*=13.7 Hz, 1 H), 2.41 - 2.50 (m, 1 H), 2.38 (dt, *J*=13.9, 6.7 Hz, 1 H), 2.21 (dt, *J*=13.9, 6.7 Hz, 1 H), 2.10 - 2.17 (m, 2 H), 2.03 - 2.10 (m, 1 H), 1.83 - 2.01 (m, 1 H), 1.60 - 1.77 (m, 2 H), 1.43 - 1.47 (m, 9 H), 1.16 - 1.20 (m, 3 H), 1.02 - 1.06 (m, 18 H); ^{13}C NMR ($CDCl_3$,126MHz): δ 175.4, 175.2, 172.7, 172.5, 137.1, 136.4, 135.3, 135.2, 128.9, 128.6, 127.7, 127.6, 127.3, 126.3, 117.3, 66.04, 65.96, 62.0, 61.1, 52.8, 49.2, 49.1, 48.8, 47.5, 43.3, 43.0, 36.6, 36.3, 36.0, 35.9, 17.9, 16.8, 11.8 ppm. HRMS calcd. for $C_{39}H_{65}N_3O_7Si$ (M + H) *m/z* 716.46645, found 716.46455.



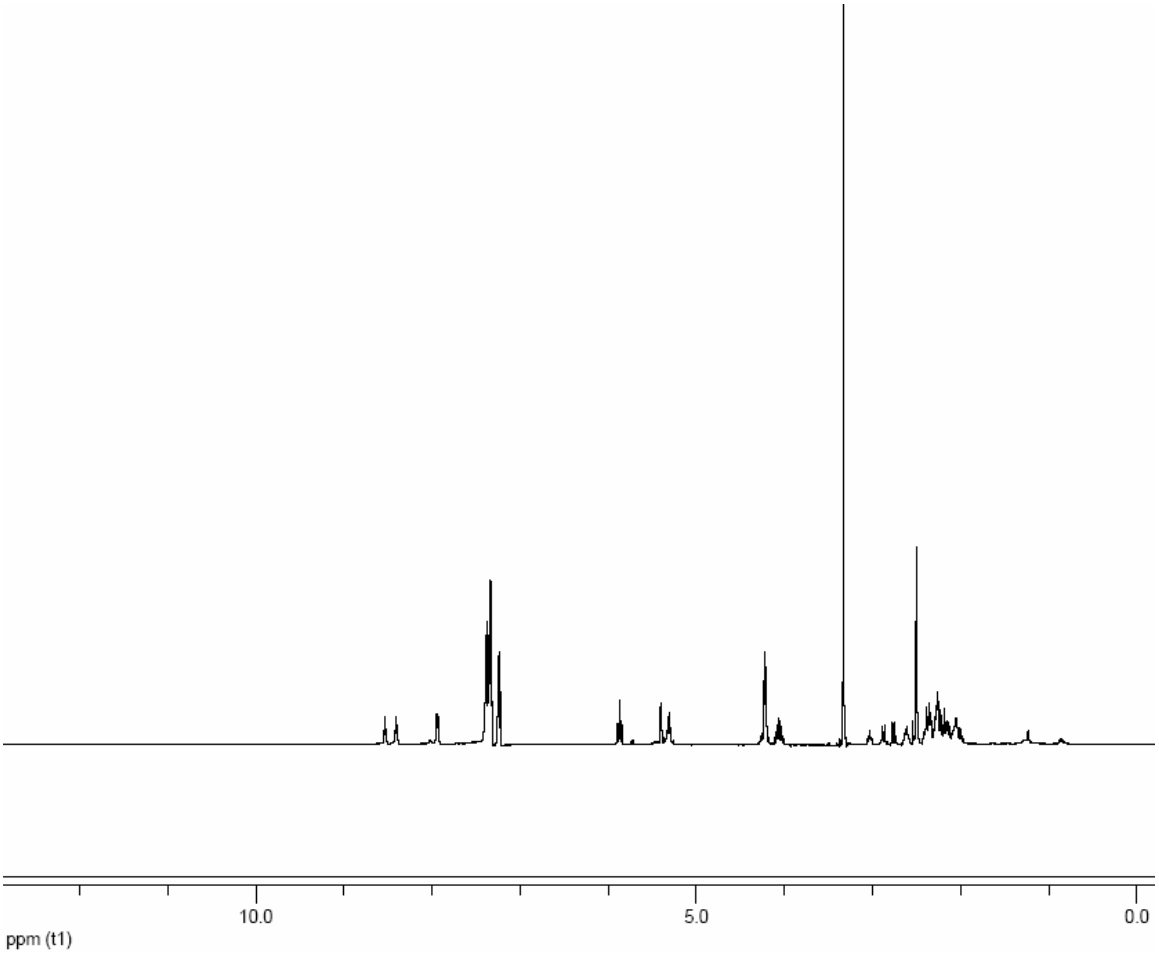
tert-butyl (3S,6S,12S,E)-6-(2-(benzyl(2-(triisopropylsilyloxy)ethyl)amino)-2-oxoethyl)-3-methyl-5,13-dioxo-1-oxa-4-azacyclotridec-8-en-12-ylcarbamate (24). To a solution of **23** (0.091 g, 0.126 mmol) in toluene (9 mL) was added Grubbs II catalyst (0.011 g, 0.0129 mmol). The reaction mixture was heated to 65°C and stirred overnight. The reaction mixture was allowed to cool, diluted with dichloromethane to make a 0.1M solution, and stirred with $Pb(OAc)_4$ (0.029g, 0.065 mmol) overnight. The $Pb(OAc)_4$ was removed by directly subjecting the mixture to silica gel chromatography to afford **24** (0.033 g, 38 %). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.12 - 7.39 (m, 5 H), 6.33 (br. s., 1 H), 5.26 - 5.50 (m, 2 H), 4.68 - 4.94 (m, 2 H), 4.52 (d, *J*=16.1 Hz, 1 H), 4.31 (br. s., 1 H), 4.12 (d, *J*=6.8 Hz, 1 H), 3.68 - 3.90 (m, 3 H), 3.54 (br. s., 1 H), 3.43 (d, *J*=16.6 Hz, 1 H), 3.29 (d, *J*=17.1 Hz, 1 H), 2.93 (dd, *J*=15.4, 8.1 Hz, 1 H), 2.70 - 2.83 (m, 2 H), 2.55 (d, *J*=17.1 Hz, 1 H), 2.39 (d, *J*=14.2 Hz, 1 H), 2.26 (d, *J*=7.3 Hz, 1 H), 2.12 - 2.23 (m, 4 H), 2.02 - 2.09 (m, 2 H), 1.44 (br. s., 9 H), 1.26 (br. s., 3 H), 1.04 (br. s., 18 H); ^{13}C NMR ($CDCl_3$,126 MHz): δ 171.8, 171.4, 137.5, 136.9, 130.8, 128.9, 128.5, 127.7, 127.5, 127.2, 126.2, 62.1, 61.4, 60.3, 52.8, 52.4, 49.3, 49.0, 44.0, 43.6, 35.7, 34.9, 34.4, 28.3, 27.2, 17.9, 14.3, 11.8. HRMS calcd. for $C_{37}H_{61}N_3O_7Si$ (M + H) *m/z* 688.43515, found 688.43478.



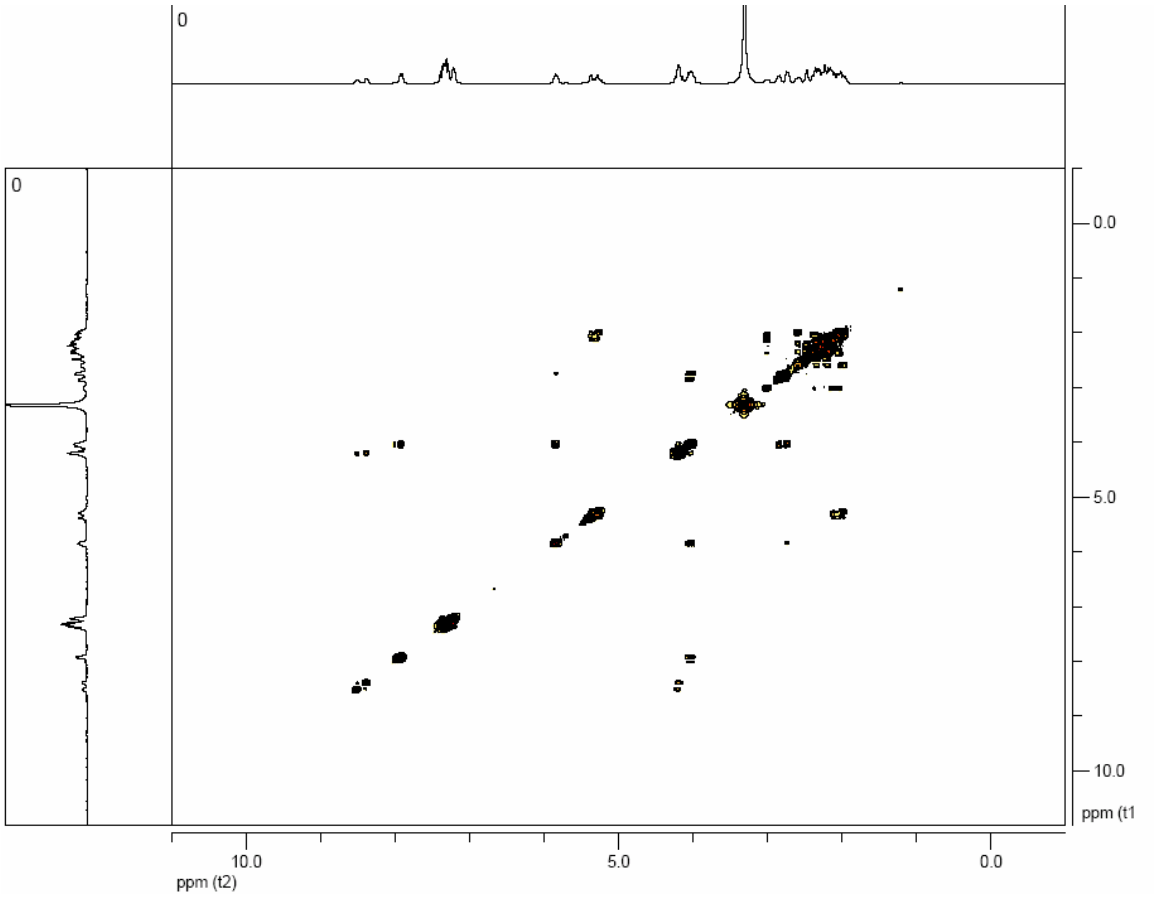
2-((3S,6S,12S,E)-12-amino-3-methyl-5,13-dioxo-1-oxa-4-azacyclotridec-8-en-6-yl)-N-benzyl-N-(2-hydroxyethyl)acetamide (1). A round bottom flask was charged with a solution of **24** (0.016 g, 0.023 mmol) in EtOH (1 mL) and 6N HCl (10 µL) and was allowed to stir at room temperature for 24 hours. The material was diluted with water, and extracted with ethyl acetate three times to remove organic impurities. The aqueous layer was diluted with aqueous $NaHCO_3$ and was extracted with ethyl acetate six times. The resulting material was dried with sodium sulfate, and concentrated under reduced

pressure (0.001 g, 10 %). ^{1}H NMR (CD_3OD, 500MHz): δ 7.19 - 7.39 (m, 5 H), 5.31 - 5.55 (m, 2 H), 4.68 - 4.81 (m, 2 H), 4.06 - 4.21 (m, 1 H), 3.86 - 4.01 (m, 1 H), 3.79 (d, *J*=11.2 Hz, 1 H), 3.65 (d, *J*=15.1 Hz, 2 H), 3.37 - 3.51 (m, 3 H), 2.70 - 2.85 (m, 1 H), 2.35 - 2.50 (m, 1 H), 2.17 - 2.32 (m, 2 H), 1.99 - 2.15 (m, 2 H), 1.70 - 1.83 (m, 1 H), 1.57 - 1.68 (m, 1 H), 1.37 - 1.50 (m, 1 H), 1.20 - 1.31 (m, 3 H), 1.12 (dd, *J*=6.1, 2.2 Hz, 1 H). ^{13}C NMR (CD_3OD, 126 MHz): δ 169.6, 131.2, 130.2, 129.7, 129.0, 128.8, 128.7, 68.4, 60.9, 60.6, 53.7, 53.2, 50.4, 46.8, 46.7, 31.5, 30.9, 29.1, 29.0. HRMS calcd. for $C_{23}H_{33}N_3O_5$ (M + H) *m/z* 432.24930, found 432.24947.

IV. NMR spectra

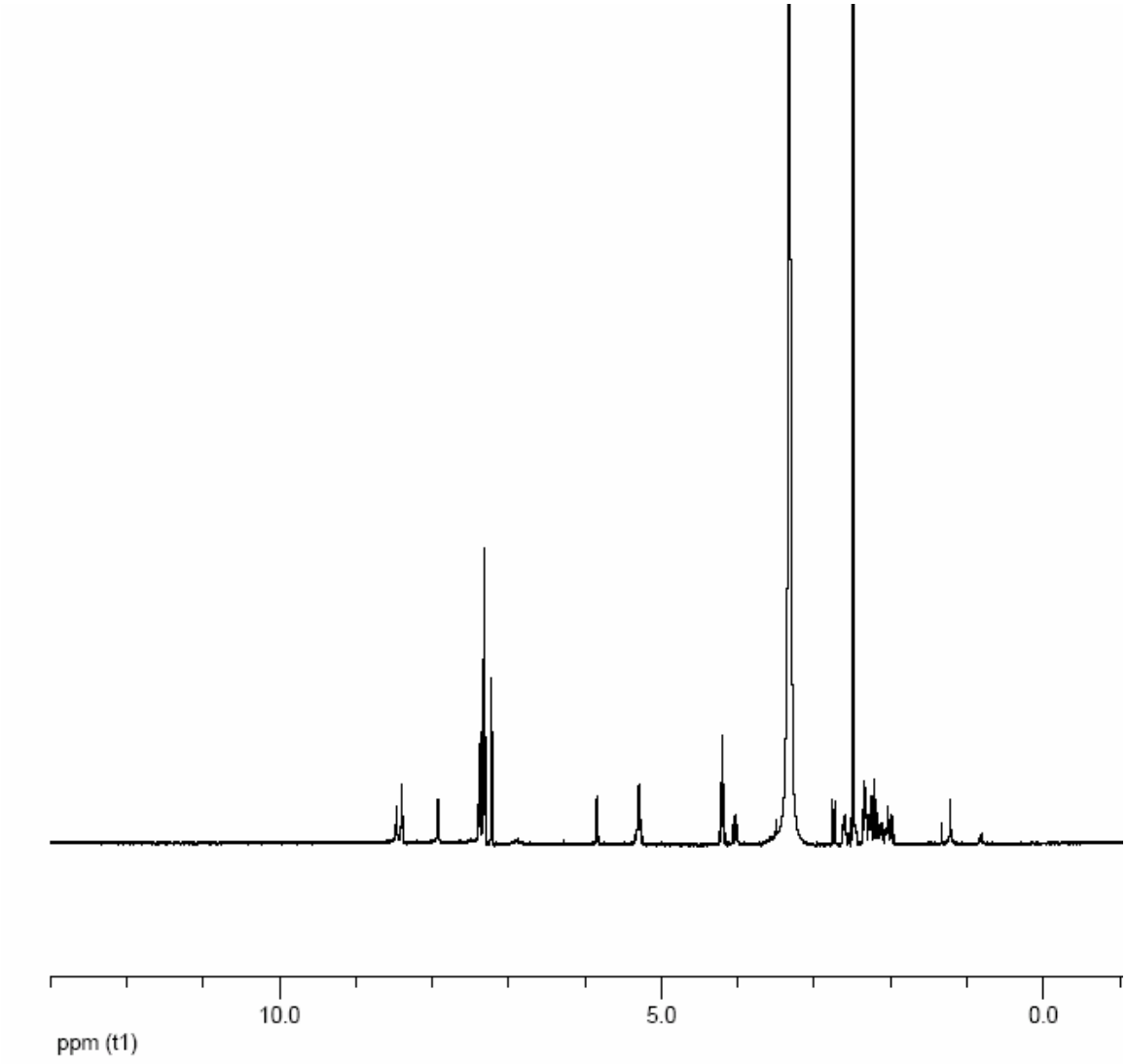


Robotnikinin ^{1}H NMR (DMSO-d_6, 500MHz)



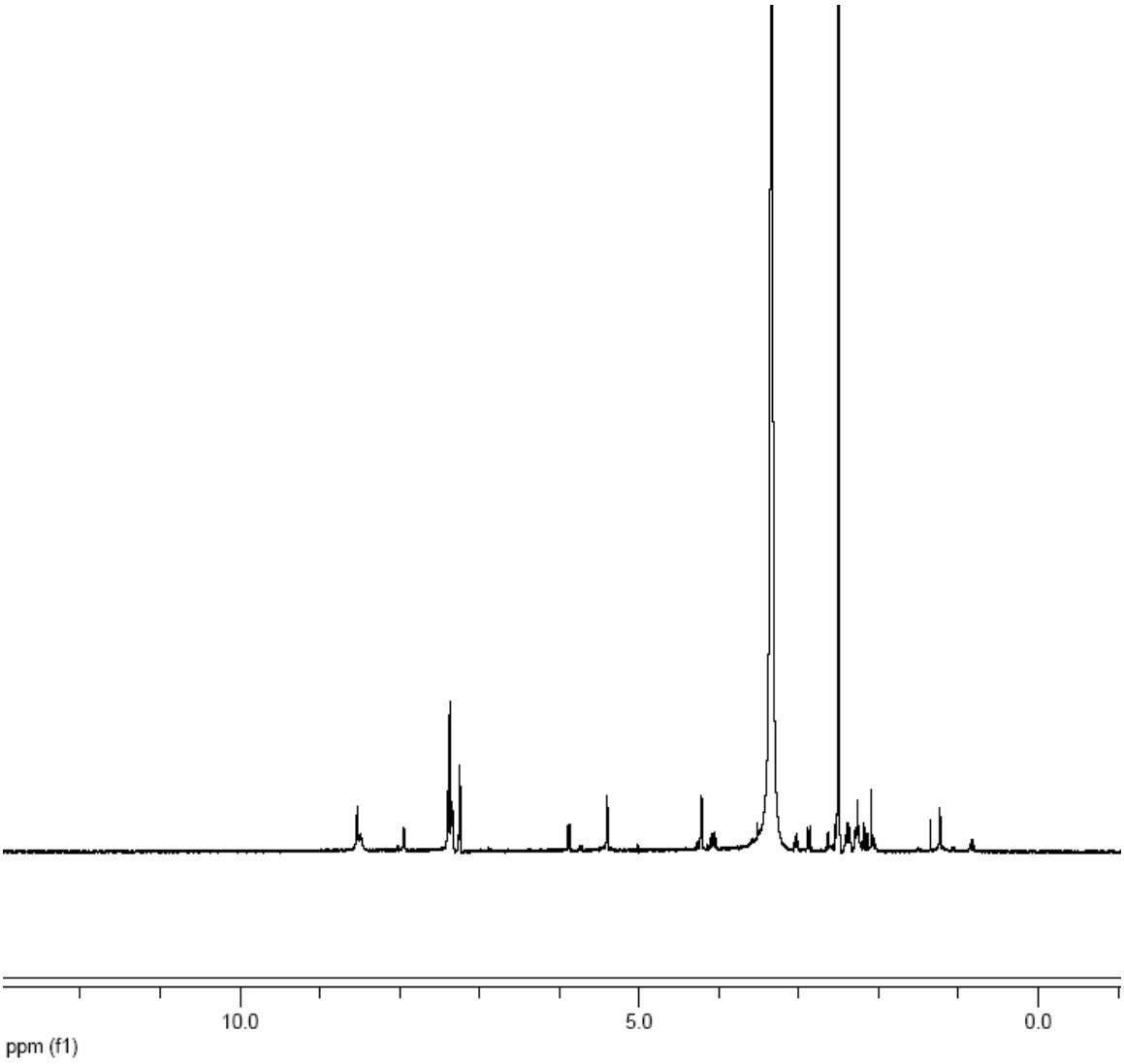
Robotnikinin ^{1}H COSY NMR (DMSO-d_6, 500MHz)

Robotnikinin Major (*E*-isomer) product HPLC purified. ^{1}H NMR (DMSO-d_6, 500MHz): δ 7- 8.39 (m, 1 H) 7.92 (d, *J*=9.7 Hz, 1 H) 7.36 (m, 7 H) 7.22 (d, *J*=8.4 Hz, 2 H) 5.84 (dd, *J*=11.5, 2.0 Hz, 1 H) 5.29 (dd, *J*=12.4, 5.5 Hz, 2 H) 4.20 (t, *J*=6.2 Hz, 2 H) 4.03 (ddd, *J*=14.0, 11.5, 10.0 Hz, 1 H) 2.74 (dd, *J*=12.2, 1.9 Hz, 1 H) 2.61 (m, 1 H) 2.34 (m, 2 H) 2.20 (m, 4 H) 2.01 (m, 2 H). HRMS calcd. for $C_{25}H_{27}ClN_2O_4$ (M + Na) *m/z* 477.15516 , found 477.15583.

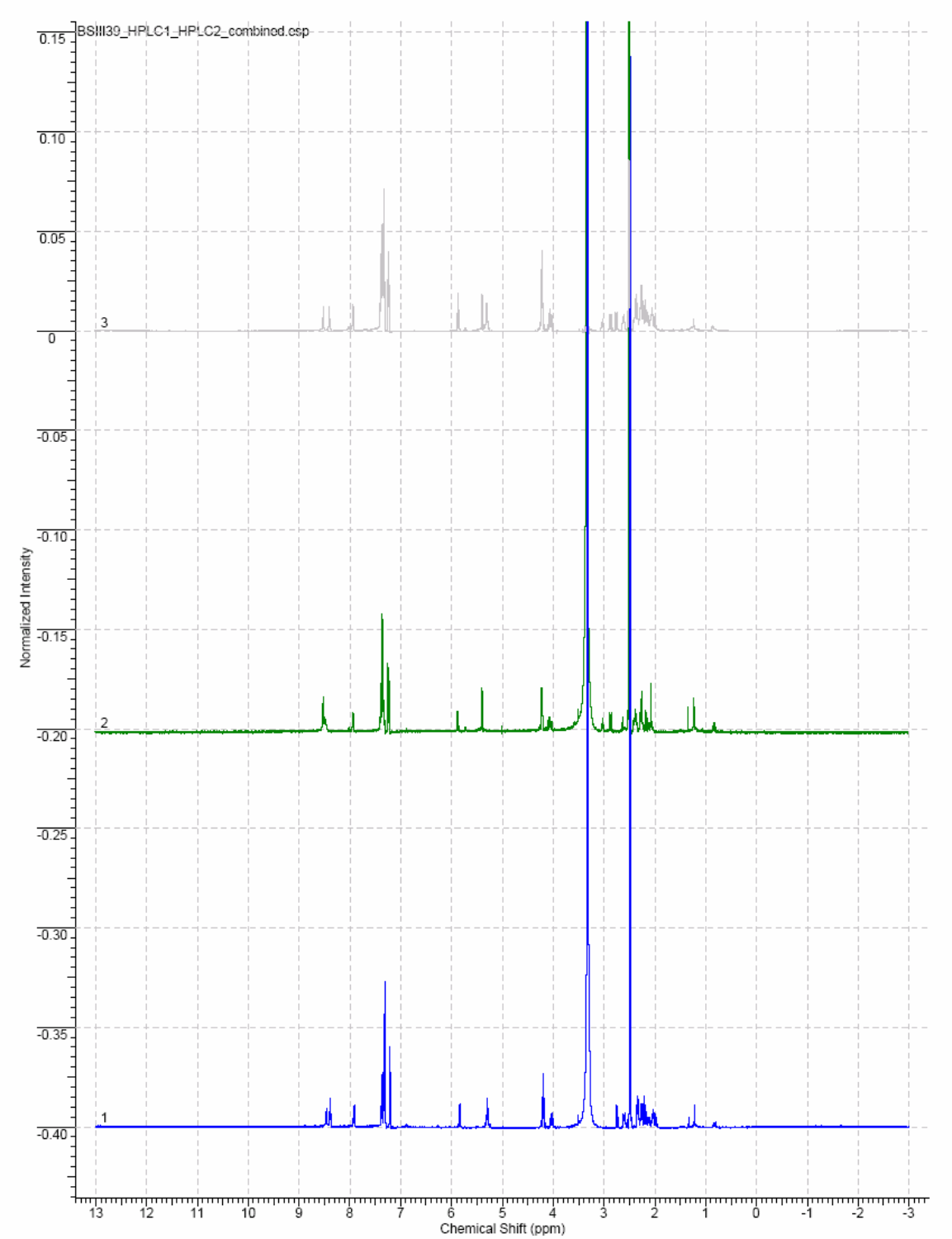


Robotnikinin ^{1}H NMR (DMSO-d_6, 500MHz)**, Major (*E*-isomer) product HPLC purified.**

Robotnikinin Minor product (*Z*-isomer) HPLC purified. ^{1}H NMR (DMSO-d_6, 500MHz): δ 8.52 (m, 1 H) 7.94 (d, *J*=8.7 Hz, 1 H) 7.37 (m, 7 H) 7.24 (d, *J*=8.5 Hz, 2 H) 5.88 (dd, *J*=11.5, 2.1 Hz, 1 H) 5.40 (d, *J*=2.6Hz, 2 H) 4.22 (d, *J*=6.2Hz, 2 H) 4.07 (ddd, , *J*=13.8, 11.4, 9.9 Hz, 1 H) 3.03 (t, *J*=9.6 Hz, 1 H) 2.87 (d, *J*=13.3 Hz, 1 H) 2.39 (m, 2 H) 2.28 (m, 4 H) 2.16 (m, 2 H). HRMS calcd. for $C_{25}H_{27}ClN_2O_4$ (M + H) *m/z* 455.17321, found 455.17481.



Robotnikinin ^{1}H NMR (DMSO-d_6, 500MHz)**, Minor product (*Z*-isomer) HPLC purified**


BSIII39_HPLC1_HPLC2_combinod.csp
Normalized Intensity
Chemical Shift (ppm)
1
2
3

Robotnikinin spectra: ^{1}H NMR (DMSO-d_6, 500MHz) Top: E/Z mixture, middle: minor product, bottom: major product from HPLC purification.

V. Assay protocols

Cell-based Shh pathway assays

Shh Light 2 cells are NIH3T3 cells that have been transfected with a Gli-luciferase construct, as previously reported.[1,2] Shh-LIGHT2 cells (ATCC, Manassas VA) were cultured with DMEM (GIBCO, Carlsbad CA), 10% Calf Bovine Serum (ATCC Manassas VA), 0.4 mg/ml G-418 (ATCC, Manassas VA), 0.15 mg/ml Zeocin (Invitrogen, Carlsbad CA, Cat. No. R25001) at 37 ^{o}C. When confluent, the cells were plated 1:5 into white 96-well optical-bottom plates and allowed to reach confluence over approximately 5-7 days. When confluent, the medium was removed and the Shh-LIGHT2 cells were treated with robotnikinin or cyclopamine (EMD biosciences 10394 Pacific Center Court, San Diego, CA 92121, USA) in DMEM with 0.5% Calf Bovine Serum. After incubation for 30 hours, each well was treated with 100 µL of Bright-Glo luciferase assay reagent (Promega, Madison WI 53711) and the plate was read with an Envision Multi-Label Reader 2102 luminometer (Perkin Elmer, Waltham MA) Wells treated in identical fashion were then treated with Cell Titer-Glo reagent (Promega, Madison WI) and read with an Envision Multi-Label Reader 2102 luminometer after being allowed to stand 10 minutes at room temperature.

The *Ptc1*$^{-/-}$ cell line was derived from an embryonic mouse fibroblast cell line transfected with a β-galactosidase construct.[2] The cell line was cultured in DMEM with 10% FBS until confluent, and then cells were plated 1:5 into white 96-well optical-bottom plates and allowed to reach a confluent state over 3-4 days while incubating at 37 ^{o}C. After reaching confluency, the medium was removed from each well, and the cells were treated with robotnikinin, or cyclopamine (EMD biosciences, San Diego CA) in DMEM with 0.5% FBS. After a 30 hour incubation, the wells were treated with Beta-Glo assay reagent (Promega, Madison WI) and allowed to be gently agitated for 30 minutes at room temperature. After the agitation was complete, plates were read with an Envision Multi-Label Reader 2102 luminometer.

Recombinant Shh Proteins

Recombinant human sonic hedgehog 1845-CF, N-terminal peptide (rhShh) was obtained from *R & D Systems* (Minneapolis, MN) in lyophilized form. Purity was > 95% based on SDS Page electrophoresis visualized with silver stain. Recombinant mouse amino-terminal peptide 461-CF (rmShh) was also obtained from *R & D Systems* in lyophilized form, and purity was > 97 % based on SDS Page electrophoresis visualized with silver stain.

Surface Plasmon Resonance (SPR) protein binding assays

Biacore™ T100 (GE Healthcare, Uppsala, Sweden) was used to perform the experiments reported herein. Sensor surface preparation and interaction analyses experiments were performed at 25^0C. Prior to surface preparation, lyophilized ShhN protein (R&D Systems) was dissolved in either water or PBS buffer, at pH 7.4 and protein purity determined by Nu-Page 4-12% Bis-Tris gel in MOPS buffer with a silver stain sensor preparation.

ShhN was immobilized onto series S sensor chip CM4 via a standard N-ethyl-N'-(dimethylaminopropyl)carbodiimide/ N-hydroxysuccinimide (EDC/NHS) amine coupling procedures.[3] Shh was diluted to 10 μg/mL in 10mM sodium acetate pH5.5 for these procedures and resultant immobilization levels were 1000 – 1200 R.U.s. Control surfaces were prepared similarly without protein derivatization and utilized as a reference surfaces for compound binding experiments.

For compound interaction analyses, 0.01 M Hepes, pH7.4, 0.15 M NaCl, 0.05% Surfactant P20 and 5% DMSO was used for both running and sample buffers. Compound samples were prepared by serial dilution in the range 0.78uM-25uM and flowed over control and derivatized surfaces for two minutes at a flow-rate of 80μL/min. Zero concentration blank buffer cycles were included as negative control samples. Solvent correction procedures were included to compensate for any DMSO related bulk refractive index variations and performed

as described previously.[4] Non-specific binding effects to sensor surface CM4 were absent for all analyses reported.

Data analysis was carried out using Biacore T100 evaluation software v1.1.1. Data were prepared by subtraction of reference surface data and blank buffer sample data, a procedure commonly referred to as 'double referencing'.[5] Solvent correction was then applied as described previously.[4]

SMM screening

The three experimental SMM slides were incubated on parafilm (Alcon, Menasha WI) with 300 µL of a 25 µg/mL rmShh solution in a 0.1 % BSA in PBS buffer. The negative control slides were incubated with 300 µL of 0.1 % BSA in PBS buffer alone. All slides were allowed to incubate for one hour at room temperature followed by washing each slide with PBS buffer three times. Each slide was then incubated with 300 µL of a 5 µg/mL biotin-labeled Shh antibody (R & D Systems, Minneapolis MN) solution in TBS with 0.1% BSA. After one hour of incubation, all five slides were washed with TBS buffer three times. Each slide was then incubated at room temperature for one hour with 300 µL of a 20 pg/mL solution of streptavidin Alexa 647 (Invitrogen, Carlsbad CA) in TBST with 0.1 % BSA followed by three washes with PBST. Slides were dried by centrifugation and scanned with a GenePix 4000B microarray plate reader (Axon Instruments, Sunnyvale CA). Data analysis was performed with GenePix Pro software (Axon Instruments, Sunnyvale CA).

BODIPY-cyclopamine/Smo binding assay.

Smo-binding assays were conducted with BODIPY-cyclopamine and Smo-overexpressing cells as previously described[6,7], using an CMV-promoter-based SV40 origin-containing expression construct for Smo-Myc_3 (murine Smo containing three consecutive Myc epitopes at the C-terminus). HEK-293T cells were dispensed in DMEM containing 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin into 24-well tissue culture plates (seeded 30,000 cells/well) containing poly-D-lysine-treated glass

coverslips. These cells were cultured for 14-18 h until they reached 55 to 65% confluency, after which they were transfected with the Smo-Myc_3 expression construct with FuGENE 6 (Roche) according to the manufacturer's protocols. 24 h after transfection, the cells were washed with 1X PBS buffer and cultured in DMEM containing 0.5% FBS, 5 nM BODIPY-cyclopamine, and the Hh pathway inhibitors at a concentration of 20 μM. After 30 min, 10 μM Hoescht 33342 was added to each well, and the compounds were incubated with the cells for an additional 60 min. The cells were then washed two times with 1X PBS, mounted in Prolong Gold (Invitrogen) and immediately imaged using a DM4500B compound microscope (Leica).

Primary keratinocyte cell culture and artificial skin equivalents

Culturing of primary human keratinocytes was previously described.[8] The full thickness skin model, EpiDermFT System (MatTek, Ashland), consists of normal, human-derived epidermal keratinocytes and normal, human-derived dermal fibroblasts which have been cultured to form a multilayered, highly differentiated model of the human dermis and epidermis.

Analysis of Gene Expression by real time RT-PCR

Gene expression was compared by quantifying mRNA levels by real time RT-PCR. For this, total RNA preparations (1μg) were used in a reverse transcriptase reaction with a mix of oligonucleotide dT and random hexamer primers, followed by real time PCR with gene-specific primers (Gli1: Qiagen Quantitect PrimerAssay QT00060501; Gli2: Qiagen Quantitect PrimerAssay QT00018648), using an Icycler IQTM real time detection system (Bio-Rad) according to the manufacturer's recommendation, with SYBR Green (Bio-Rad) for detection. Each sample was tested in triplicate, and the results were normalized by real time PCR of the same cDNA with 36B4 primers (36B4 forward primer: 5'- GCA ATG TTG CCA GTG TCT GT-3; reverse primer, 5'- GCC TTG ACC TTT TCA GCA AG -3').

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