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Supplemental Information

A Small-Molecule Inhibitor of Bax and Bak Oligomerization Prevents Genotoxic Cell Death and Promotes Neuroprotection

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Figure S1. Effect of different compounds on membrane binding and permeabilization activities of Bax and Bak, Related to Figure 2

(A, B) Nutlin-3a inhibits Bax mediated permeabilization of liposomes and mitochondria.
(A) Inhibition of cBid and Bax mediated ANTS/DPX release from liposomes by the indicated concentrations of Nutlin-3a. Mean ± SD, n=3. **Unless specified otherwise, in all supplementary figures n indicates the number of independent experiments.**
(B) Inhibition of cBid and Bax mediated release of Smac-mCherry from $Bax^{-/-A}/Bak^{-/-}$ mitochondria by the indicated concentrations of Nutlin-3a. Mean ± SD, n=3.
(C) Controls illustrate that 40 μM of the indicated compounds did not permeabilize $Bax^{-/-}/Bak^{-/-}$ or $Bax^{-/-}$mitochondria. Mean ± SD, n=3.
(D-F) DAN004 inhibits but BJ-1 and BJ-BP do not inhibit MOMP.
(D) BJ-1 and BJ-1-BP did not prevent tBid and Bax from releasing Smac-mCherry from mitochondria isolated from $Bax^{-/-}$ $Bak^{-/-}$ BMK cells. Mean ± SD, n=3.
(E) BJ-1 and BJ-1-BP did not prevent exogenous tBid and endogenous Bak from releasing SmacmCherry from $Bax^{-/-}$ mitochondria isolated from BMK cells. Mean ± SD, n=3.
(F) DAN004 and MSN-125 inhibit exogenous tBid and endogenous Bak mediated release of cytochrome c from mitochondria that do not contain Bax, isolated from mouse liver. Permeabilization was measured by % of cytochrome c released from the mitochondria determined by immunoblotting pelleted mitochondria and supernatants. Mean ± SD, n = 3.
(G) Preincubation of Alexa568-labeled BAX-126C (20 nM) and DAN004 (10 μM) at 37°C for 10 minutes before adding reconstituted DiD liposomes does not inhibit Bax binding to liposomes as measured by FRET.
(H) MSN-125 does not inhibit Bax binding to mitochondria isolated from $Bak^{-/-}$ mouse liver. Bax or mitochondria were pre-incubated with 40 μM MSN-125 or DMSO (for - MSN-125 sample) for

15 min at 22 °C as specified and then 0.2 mg/ml mitochondria or 2.5 µl radioactive Bax synthesized by in vitro transcription/translation and 20 nM cBid were added and incubated for 60 min at 37 °C. For coincubation all components were mixed together and incubated accordingly. Both mitochondria pellets and supernatants were collected after centrifugation and subjected to SDS-PAGE and phosphorimaging to detect radioactive Bax. The fractions of Bax bound to mitochondria calculated from band intensities in the pellet and supernatant for two independent experiments are presented below the gel image as circles and squares with dashes for the means. **(I)** DAN004 inhibits membrane permeabilization mediated by heat activated Bax. After incubation of 120 nM of Bax with the indicated concentrations of DAN004 or as a control, Bcl-XL, liposomes were added. The temperature was raised to 55° C for 30 minutes and permeabilization was measured as %ANTS/DPX release from liposomes. Individual data points are means of duplicate samples for two independent experiments.

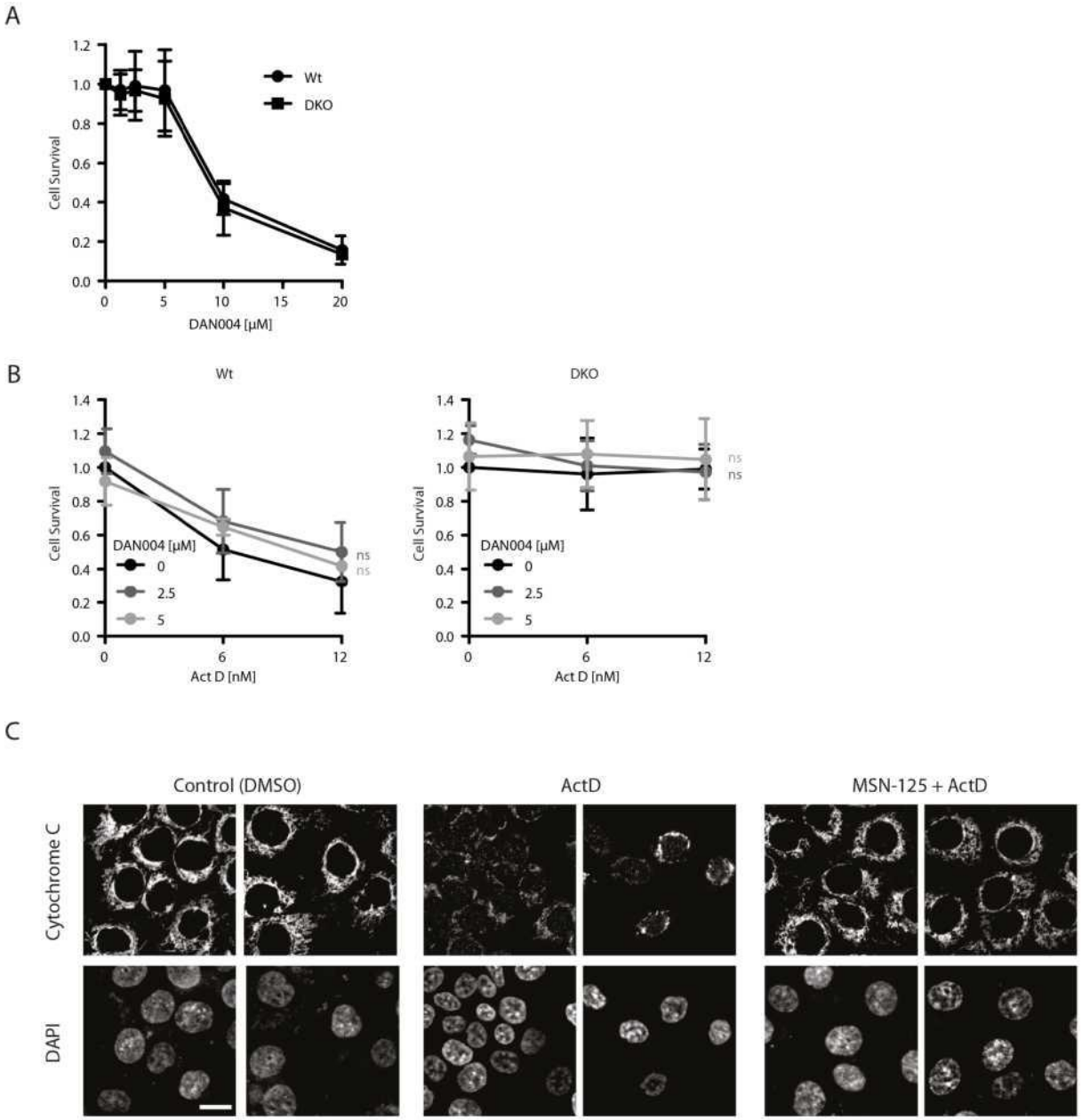


Figure S2. Unlike MSN-125, DAN004 does not protect cells from apoptosis induced by ActD, Related to Figure 3
(A-B) DAN004 kills Wt and DKO cells and does not confer protection against ActD.
(A) Cell viability of HCT-116 Wt and *Bax*$^{-/-}$/*Bak*$^{-/-}$ DKO cells exposed to DAN004 for 8 hours. Cells were replated after 4 days and grown till the "DMSO" treated cells were confluent. Ssurviving cells were stained with crystal violet. Results are normalized to DMSO treated controls. Mean ± SD, n=4 independent experiments.
(B) HCT-116 Wt and DKO cells treated with DAN004 for 4 hours followed by treatment with both DAN004 and the indicated doses of ActD for 4 hours. After 4 days cells were replated and cell

survival was assessed as described above. Statistical analysis was conducted using two-way ANOVA with the Bonferroni post-test comparing DAN004-treated cells versus untreated cells. ns = non-significant for P values >0.05; Mean ± SD, n = 3 independent experiments.
(C) MSN-125 prevents ActD mediated cytochrome c release from ActD treated HCT-116 cells. HCT - 116 cells were treated with 10 μM MSN-125 for 3 hours before ActD treatment for 24 hours. Nuclear shrinkage observed for ActD treated cells is also reduced by MSN-125. Cells were fixed and immunofluorescence was performed with primary antibodies against cytochrome c. Nuclei were visualized using DAPI. The scale bar indicates 10 μm.

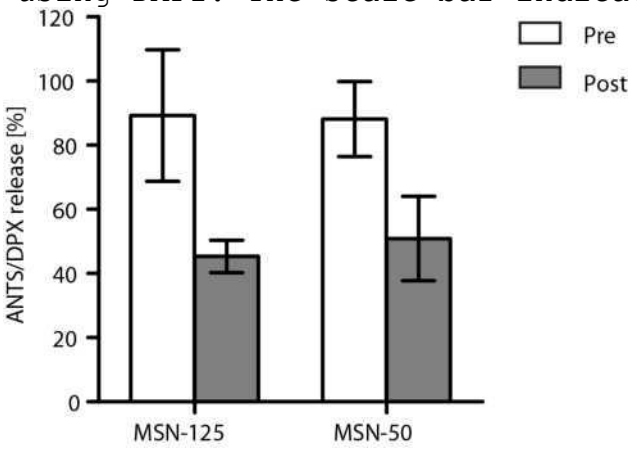


Figure S3. MSN-50 and MSN-125 do not inhibit liposome permeabilization by partitioning into the membrane, Related to Figure 4
Liposomes pre-incubated with 10μMSN-125 or MSN-50 and passed over a gel filtration column to remove unpartitioned compound ("Pre") released ANTS/DPX in response to tBid and Bax. In control reactions in which MSN-125 or MSN-50 was added after liposomes were passed over the column ("Post") ANTS/DPX release was inhibited.

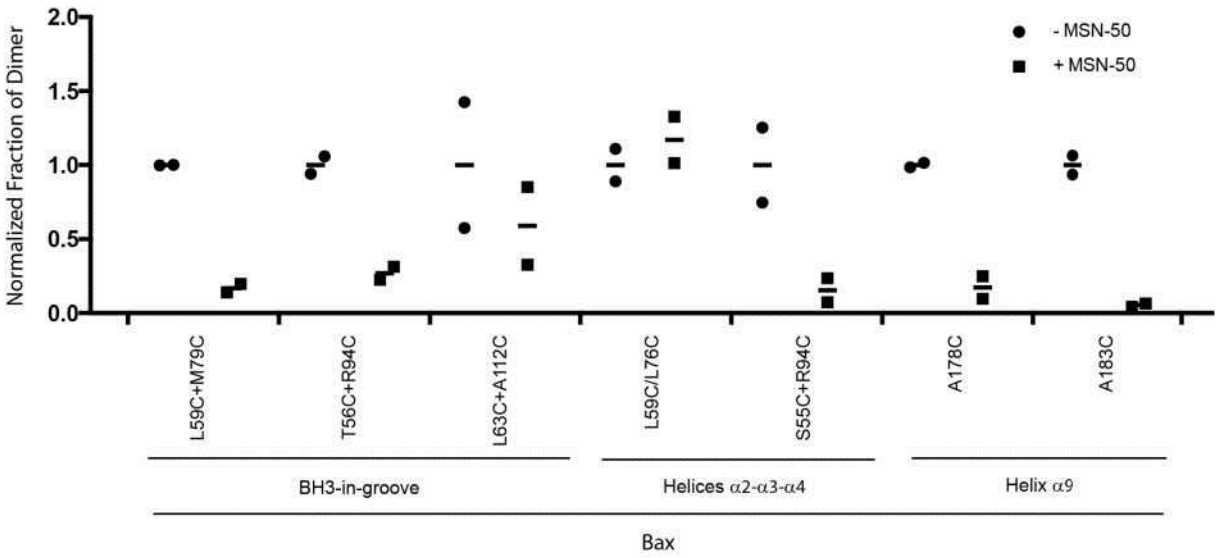


Figure S4. MSN-50 inhibits some, but not all, interactions in Bax

dimers, Related to Figure 5

While disulphide linkage of L59C/L76C was resistant to MSN-50, linkages of the other sites were inhibited by MSN-50, similar to the data for MSN-125 shown in Figure 5B. The only exception is the linkage of L63C with A112C that is partially blocked by MSN-50 but fully blocked by MSN-125. Intensities of both dimer and monomer bands were obtained from phosphorimager data for disulfide crosslinking of single-cysteine Bax mutant pairs (e.g., L59C+M79C) or the double-cysteine Bax mutant (L59C/L76C) incubated with mitochondria lacking endogenous Bax and Bak, isolated from $Bak^{-/-}$ mouse liver, in the absence or presence of MSN-50. This data was used to calculate the mean fraction of dimer in the absence of MSN-50 and the corresponding normalized fraction of dimer after treatment with inhibitor. Circles and squares represent individual data points with the mean indicated by a dash. Data from n = 2 independent experiments is shown.

Supplemental Table S1. Related to Figure 2

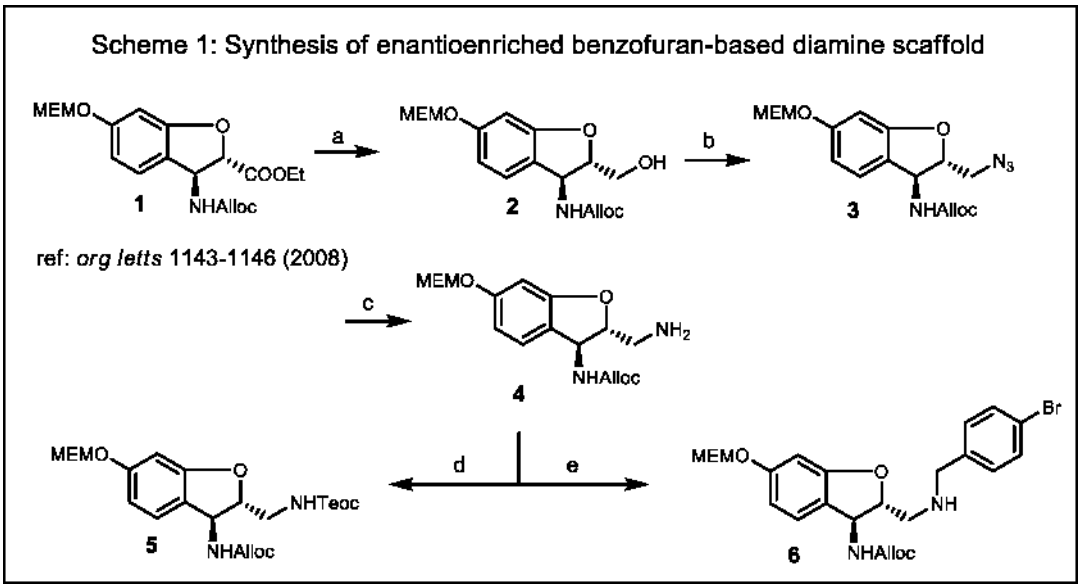
Liposomes permeabilization by 10 pM of the compounds cBid and Bax.

Compound ID:	Structure	ANTS/ DPX Release [%]
ILS-JRK-3-37	MEMO HN^ /O -A. N Ph H	68
ILS-JRK-3-41	MEMO ....z^{OH} HN. .-O O ^N^^Ph H	91
ILS-JRK-3-45	MEMO \^0.....$/^{NH2}$ H N_ .-O aXV,	94
ILS-JRK-3-31	MEMO (\~o o vWV^ NH2 O ^{H}	45
ILS-JRK-3-39	MEMO ,CC^	104
ILS-JRK-3-29	MEMO f\o r'*'-''*' o	94

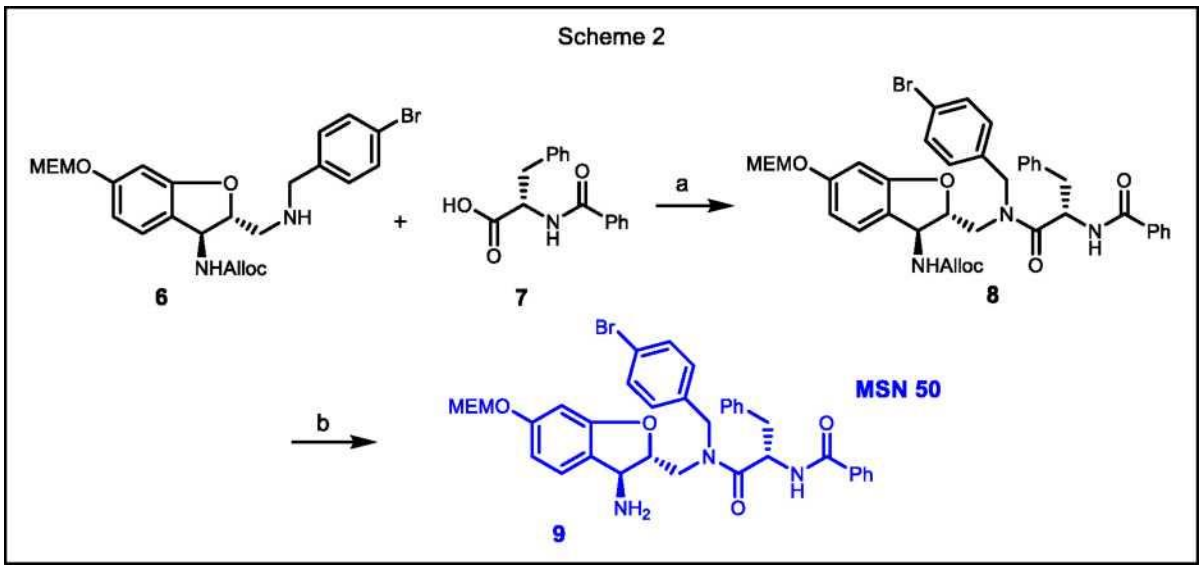
ILS-JRK-3-34	MEMO Ph\ /O	95
ILS-JRK-3-32	MEMO /Ph /Ph **^?-yAx**	145
ILS-JRK-C95-124 (1)	MEMO ^^,Br **th &** NHAlloc J. NHFmoc Ph	93
ILS-JRK-C95-140	**memo** **Br^=b°** Vl **r "cooEt** **^yo** **Ph\A** **NHFmoc**	93
ILS-JRK-C95-182	**MEMO /v.** \^ • ✓**z-O** **' T °** **■"x ■"^i„**	76

METHODS S1. Synthesis of MSN-50, MSN-125 and DAN004

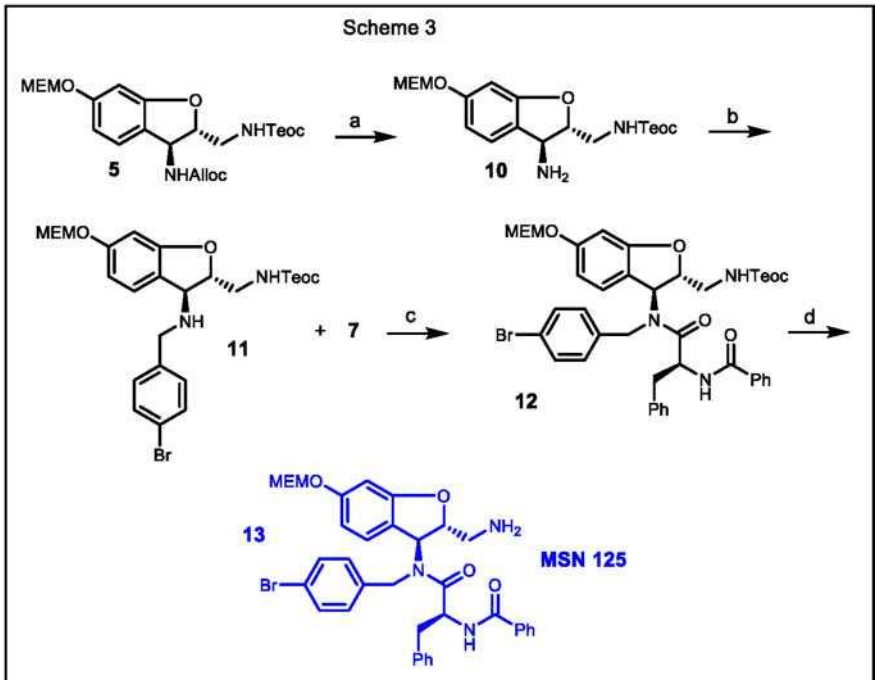
1) Experimental Section for the Synthesis of Small Molecules MSN-50 and MSN-125



Reagents and conditions: a) UBH_4, THF, 0° C to rt, 2 h; b) DPPA, TPP, DEAD, THF, 0 °C, 2h; c) TPP, H_2O, THF, rt, 12 h; d) triphosgene, trimethylsilyl ethanol, py, CH_2Cl_2, 0 °C, 4h; e) *p*-bromobenzaldehyde, $NaCNBH_3$, TMOF, AcOH, MeOH, rt, 4 h.

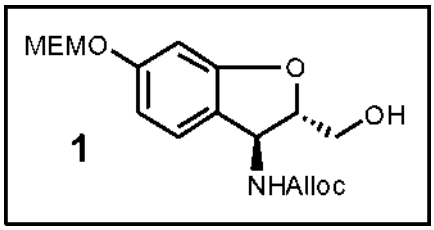


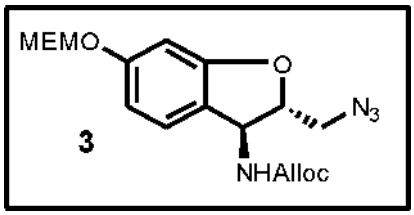
Reagents and conditions: a) PyBop, DIPEA, CH_2Cl_2, rt, 12 h; b) $Pd(PPh_3)_4$, morpholine, CH_2Cl_2, 0 °C to rt, 2 h.



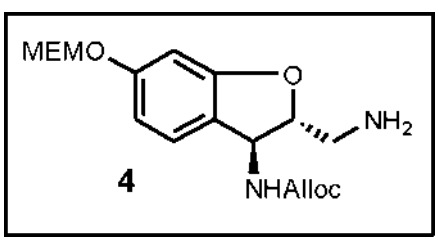
Reagents and conditions: a) $Pd(PPh_3)_4$, morpholine, CH_2Cl_2, 0 °C to rt, 2h; b) *p*-bromobenzaldehyde, $NaCNBH_3$, TMOF, AcOH, MeOH, rt, 4 h; c) PyBop, DIPEA, CH_2Cl_2, rt, 12 h; d) TBAF, THF, 0 °C to rt, 2 h.

Experimental Section

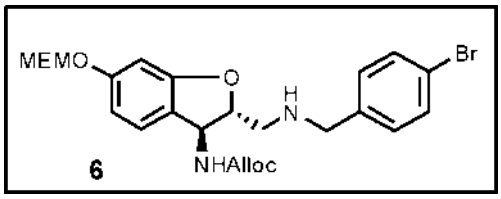
To  °C a stirred solution of compound **1** 0.5 g, 1.26 mmol) in dry THF (10 mL) was added a solution of 2M LiBH4 in THF of (0.62 mL, 1.26 mmol) at 0 under inert atmosphere. The reaction was allowed to warm to room temperature and stirred for 3 hours. The reaction mixture was quenched by addition of a saturated *aq.* NH_4Cl, extracted with ethyl acetate, washed with brine, dried over anhydrous MgSO4 and concentrated *in vacuo.* Purification by flash chromatography (40% ethyl acetate in hexane) afforded compound **2** (0.38 g, 80 %) as a white solid. ^{1}H NMR (CDCla, 400 MHz): 3.21 (s, 3H), 3.39 (m, 2H), 3.81-3.88 (m, 4H), 4.59-4.61 (m, 3H), 5.14-5.35 (m, 6H), 5.90-5.94 (m, 1H), 6.58 (s, 1H), 6.63 (d, *J* = 2.0 Hz, 1H), 7.16 (d, *J* = 2.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): 56.0, 59.4, 63.9, 66.5, 68.1, 71.9, 91.8, 93.9, 98.4, 99.4, 109.9, 118.6, 118.7, 125.8, 132.7, 156.5, 160.0, 161.2; MS: (ES+) m/z = 354 (m+1), HPLC purity (>97%).



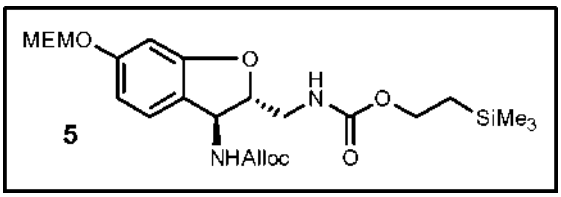
To a solution of alcohol **2** (0.047 g, 0.097 mmol) in 3.88 mL of THF was added triphenylphosphine (0.026 g, 0.099 mmol), DEAD (0.019 mL, 0.099 mmol), and diphenylphosphoryl azide (0.021 mL, 0.099 mmol) at 0 °C and stirred for 3 hours. The reaction was quenched with saturated $NaHCO_3$ and extracted with ethyl acetate (3 x 10 mL). The combined organics were dried over anhydrous Mg_2SO_4, concentrated in vacuo and purified by silica gel column chromatography to afford azide 3 (0.042 g, 85%) as brownish liquid. ^{1}H NMR ($CDCl_3$, 400 MHz):3.39 (s, 3H), 3.50-3.60 (m, 3H), 3.70-3.74 (m, 1H), 3.81-3.84 (m, 2H), 4.61 (d, *J* = 5.52 Hz, 2H), 4.65-4.69 (m, 1H), 5.04-5.07 (m, 1H), 5.12-5.14 (m, 1H), 5.25-5.27 (m, 3H), 5.28-5.35 (d, *J* = 17.5 Hz, 1H), 5.87-5.98 (m, 1H) 6.62 (d, *J* = 2.0 Hz, 1H), 6.65-6.68 (m, 1H), 7.17-7.19 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): 53.8, 56.1, 59.4, 66.3, 68.2, 71.9 90.2, 94.0, 99.5, 110.1, 118.4, 125.7, 132.8, 156.0, 160.2, 161.1; MS: (ES+) m/z = 379 (m+1), HPLC purity (>97%).



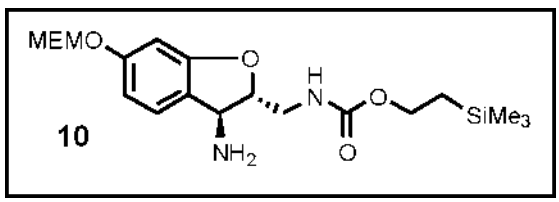
To a stirred solution of azide compound **3** (0.225 g, 0.595 mmol) in THF (3 mL) was added TPP (0.166 g, 0.636 mmol) and water (100 μL). The reaction mixture was left for stirring for 12 h at room temperature. After the completion of the reaction THF was removed under vacuuo and purified by flash column using DCM, MeOH solvent system to afford pure amine **4** as colorless liquid in 80 % yield. ^{1}H NMR ($CDCl_3$, 400 MHz): 2.96-3.03 (m, 1H), 3.09-3.14 (m, 1H), 3.40 (s, 3H), 3.56-3.59 (m, 2H), 3.81-3.84 (m, 2H), 4.48-4.53(m, 1H), 4.62 (d, J = 5.0 Hz, 2H), 5.05-5.12 (m, 1H), 5.25-5.28 (m, 3H), 5.31-5.38 (d, J = 16.5 Hz, 1H), 5.87-5.99 (m, 1H), 6.57 (d, J = 2.0 Hz, 1H), 6.64 (dd, *J* = 2.5 Hz, 1H), 7.18 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): 45.4, 56.1 59.4, 66.2, 68.1, 71.9, 93.2, 94.0, 99.3, 109.6, 118.4, 119.3, 125.9, 132.9, 156.0, 160.0, 161.3. MS: (ES+) m/z = 353 (m+1), HPLC purity (>97%).



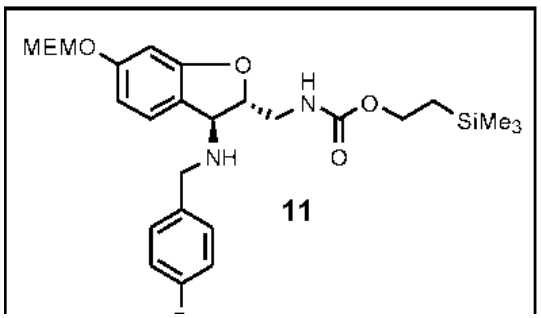
The pure amine **4** (0.318 g, 0.9 mmol) and *p*-bromobenzaldehyde (0.183 g, 0.993 mmol) dissolved in triethyl orthoformate (4.0 mL), and a solution of $NaCNBH_4$ (85 mg, 1.355 mmol) in TMOF/MeOH/AcOH (3 mL/720 μL/80 μL) was added to the above mixture at room temperature. The reaction mixture was stirred for 4 h. The reaction was quenched with saturated NH_4Cl solution and washed with water and brine. The organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (1:1 ethyl acetate/hexanes) to give the product **6** (0.36 g, 77%). ^{1}H NMR ($CDCl_3$, 400 MHz): 2.90-2.95 (m, 1H), 3.0-3.04 (m, 1H), 3.39 (s, 3H), 3.56-3.58 (m, 2H), 3.81-3.83 (m, 4H), 4.60-4.65 (m, 3H) 5.03 (d, *J* = 7.5 Hz, 1H), 5.14-5.17 (m, 1H), 5.23-5.26 (m, 3H), 5.31-5.35 (m, 1H) 5.89-5.99 (m, 1H), 6.56 (d, *J* = 2.0 Hz, 1H), 6.61 (dd, *J* = 2.0 Hz, 1H), 7.16 (d, *J* = 8.5 Hz, 1H), 7.21 (d, *J* = 8.5 Hz, 1H) 7.44 (d, J = 8.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz):52.1, 53.5, 56.6, 59.4, 66.2, 68.1, 71.9 91.0, 94.0, 99.3, 109.7, 118.4, 119.3, 121.1, 125.9, 130.2, 131.8, 132.9, 139.5, 156.0, 160.0, 161.2; MS: (ES+) m/z = 521 (M+1), 523 (M+3) HPLC purity (>97%).

a 

Triphosgene (33 mg, 0.112 mmol) was added to the round bottom flask and cooled to 0 °C. This was then followed by slow addition of 3 mL of anhydrous CH_2Cl_2, and 2-(trimethylsilyl) ethanol (49 μL, 0.34 mmol) was added in one portion to the reaction mixture. Then pyridine (27.6 μL, 0.34 mmol) was added drop wise to the reaction mixture and stirred for 2 h at 0 °C. Then a solution of free amine **4** (60 mg, 0.17 mmol) and pyridine (42 μL, 0.51 mmol) in 3 mL of anhydrous CH_2Cl_2 was added via cannula over a period of 5 min. The mixture was stirred continuously for 2 h at 0 °C. When the TLC showed no starting material, the reaction mixture was quenched via the addition of a saturated solution of $NaHCO_3$, and the aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum, and the crude product was chromatographed on silica gel with (hexane/ ethyl acetate 7/3) to give **5** (77 mg, 91%) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz):1.0 (t, *J* = 8.5Hz 2H), 3.4 (s, 3H), 3.5-3.59 (m, 4H) 3.81-3.84 (m, 2H), 4.18 (t, *J* = 8.5Hz 2H), 4.57-4.63 (m, 1H) 5.03-5.09 (m, 1H), 5.25-5.27 (m, 3H), 5.31-5.35 (d, *J* = 17.0Hz, 1H), 6.57 (d, *J* = 2.0 Hz, 1H), 6.65 (dd, *J* = 2.0 Hz, 1H), 7.18 (d, *J* = 8.0 Hz, 1H); MS: (ES+) m/z = 497 (M+1), 498 (M+2) HPLC purity (>95%).

To 

a solution of **5** (77 mg, 0.155 mmol) in dry CH_2Cl_2 (5 mL) under organ atmosphere at 0 °C, was added morpholine (27.6 μL, 0.317 mmol) and tetrakis(triphenylphosphine) palladium (o) catalyst (17.9 mg, 0.0158 mmol). The round-bottom flask containing the mixture was covered with aluminum foil and stirred for 1 h. TLC showed the completion of the reaction. The solvent was evaporated to dryness and the crude mixture subjected to flash column chromatography to give pure benzylic amine **10** (60 mg, 95% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): 0.05 (s, 9H), 0.99 (t, *J* = 8.53 H_z, 2H), 3.40-3.48 (m, 4H), 3.57-3.66 (m, 2H), 3.82-3.84 (m, 2H), 4.17(t, *J* = 8.51H_z 2H) 4.29(d, *J* = 5.52 H_z, 1H), 4.37-4.41(m, 1H), 5.0 (bs, 1H), 5.25(s, 2H0, 6.54-6.66 (m, 1H), 6.64 (dd,' *J* = 2.0, 2.5H_z, 1H), 7.18 (d, *J* = 8.53 H_z, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): -1.0, 0.4, 18.1 43.5, 56.6, 59.4, 63.7, 68.0, 71.9, 92.0, 94.0, 99.3, 109.6, 124.5, 125.2, 157.3, 159.3, 160.3; MS: (ES+) m/z = 413 (M+1), 395 (M-NH_3) HPLC purity (>96%).

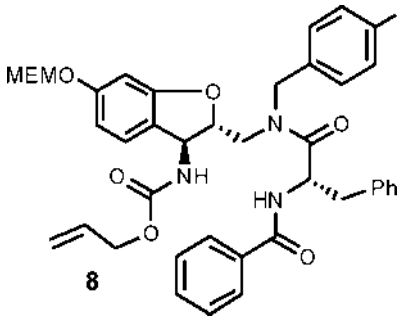


(ES+) m/z = 413 (M+1), 395 (M-NH_3) HPLC purity (>96%).

This compound is prepared following the similar procedure described for the compound **6** using the benzylic amine **10** (63 mg, 0.152 mmol), *p*-

This compound is prepared following the similar procedure described for the compound **6** using the benzylic amine **10** (63 mg, 0.152 mmol), *p*-bromobenzaldehyde (31 mg, 0.168 mmol) and $NaCNBH_3$/AcOH/MeOH (15 mg, 0.228 mmol/12μL/120μL) in TMOF to afford compound **11** (79 mg, 90% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): 0.05 (S, 9H), 1.00(t, *J* = 8.03 H_z) 3.25-3.32 (m, 1H), 3.40 (s, 3H), 3.53-3.59 (m, 3H), 3.82-3.85 (m, 4H), 4.15-4.20 (m, 3H), 4.63-4.66 (m, 1H), 4.97 (m, 1H), 5.25(s, 2H), 4.63-4.66 (m, 1H), 4.97 (m, 1H), 5.25 (s, 2H), 6.57 (d1 *J* = 2.0, 1H), 6.62 (dd, *J* = 2.0, 2.5, 1H), 7.19(d, *J* = 8.03, Hz, 1H), 7.25-7.28 (m, 2H), 7.45-7.48 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): -1.0, 0.4, 18.1, 44.4, 50.2, 59.4, 62.0, 63.7, 68.1, 71.9, 88.7 94.0, 99.4, 109.5, 121.3, 126.0, 130.2, 131.9, 159.5, 160.6; MS: (ES+) m/z = 581 (M+1), 583 (M+3) HPLC purity (>97%).

To a stirred solution of amine **6** (46 mg, 0.088 mmol) and amino acid **7** (28 mg, 0.106 mmol) in dry dichloromethane under organ atmosphere were added PyBrop (49 mg, 0.106 mmol) and DIPEA (31uL, 0.176 mmol) at room temperature. The reaction mixture was allowed to stir for overnight. After completion of the reaction as indicated by the TLC and LC-MS, organic layer evaporated and the crude mixture was dissolved in ethyl acetate (3x20 mL) and combined organic layers were concentrated and the crude mixture was purified by column chromatography using hexane and ethyl acetate (7:3) solvent system to give pure compound **8** (48 mg, 58% yield). ^{1}H NMR ($CDCl_3$, 400 MHz, 58 ^{o}C): 3.11-3.28 (m, 2H), 3.38-3.40 (m, 3H), 3.53-3.60 2H), 3.78-3.85 (m, 3H), 4.26-5.00 (m, 6H), 5.16-5.39 (m, 6H), 5.50 (bs, 1H), 5.83-6.00 (m, 1H), 6.49-6.76 (m, 3H), 6.83-7.00 (m, 2H), 7.11-7.28 (m, 4H), 7.35-7.54 (m, 6H), 7.71-7.82 (m, 2H); MS: (ES+) m/z = 772 (M+1), 774 (M+3) HPLC purity (>95%).



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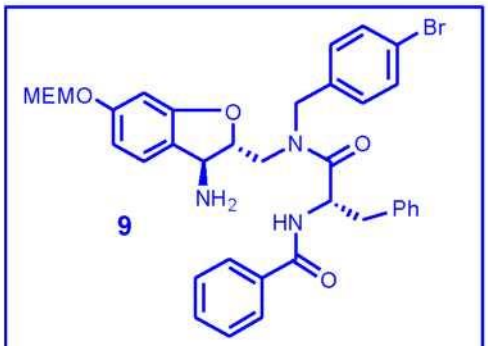
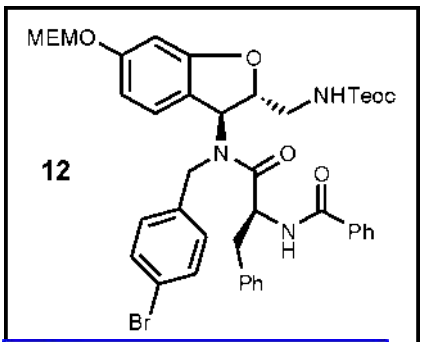
To a solution of **8** (48mg, 0.062 mmol) in dry CH_2Cl_2 (5 mL) under organ atmosphere at 0 °C, was added morpholine (10 μL, 0.124 mmol) and tetrakis(triphenylphosphine) palladium (0) catalyst (7 mg, 0.0062 mmol). The round- bottom flask containing the mixture was covered with aluminum foil and stirred for 1 h. TLC showed the completion of the reaction. The reaction was quenched with saturated NH_4Cl solution and washed with water and brine. The organic layer was dried over anhy. $MgSO_4$, filtered, and concentrated under reduced pressure to give crude compound which upon flash column chromatography using ethyl acetate (100%) to afford amine **9** (40 mg, 95 % yield). ^{1}H NMR ($CDCl_3$, 400 MHz, 58 ^{o}C): 3.02-3.31 (m, 2H), 3.37 (m, 3H), 3.47-3.48 (m, 0.5H), 3.54-3.61 (m, 2.5H), 3.73-3.86 (m, 2.5H), 3.94-3.99 (m, 0.5H), 4.05-4.10 (m, 1H0, 4.14-4.24 (m, 1H), 4.36-4.49 (m, 1H), 4.51-4.63 (m, 1H), 4.70-4.81 (m, 0.5H), 5,02-5.06 (m, 0.5H), 5.19-5.31 (m, 2.5H), 5.36-5.59 (m, 1H), 6.49-6.55 (m, 1H), 6.61-6.65 (m, 1H), 6.79-7.02 (m, 3H), 7.09-7.16 (m, 2H), 7.20-7.34 (m, 3H), 7.39-7.54 (m, 5H), 7.73-7.80 (m, 2H); MS: (ES+) m/z = 671 (M-NH_3+1), 673 (M-NH_3+3) HPLC purity (>97%).

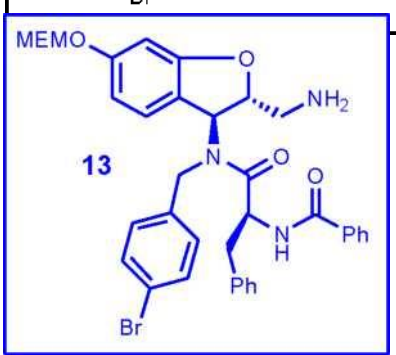


9



12

Compound **12** was prepared following the similar procedure described for the compound **8**, by using amine **11** (60 mg, 0.103 mmol), amino acid **7** (33 mg, 0.123 mmol), PyBrop (58 mg, 0.123 mmol) and DIPEA (36 L, 0.206 mmol) to afford the compound **12** (50 mg) 58 % yield. ^{1}H NMR (DMSO, 400 MHz, 120 ^{o}C): 0.01 (S, 9H), 0.89 (m, 2H), 3.05-3.17 (m, 2H), 3.22-3.34 (m, 5H), 3.48-3.50 (m, 2H), 3.71-3.74 (m, 2H), 4.02-4.06 (m, 2H), 4.20-4.36 (m, 3H), 4.52-4.65 (m, 2H), 5.16 (s, 2H), 5.72 (bs, 1H), 6.37-6.46 (m, 2H), 6.65-6.73 (bs, 1H), 6.89-7.00 (m, 3H), 7.17-7.35 (m, 6H), 7.41-7.53 (m, 4H), 7.78-7.85 (m, 2H), 8.48 (bs, 1H); MS: (ES+) m/z = 832 (M+1), 834 (M+3) HPLC



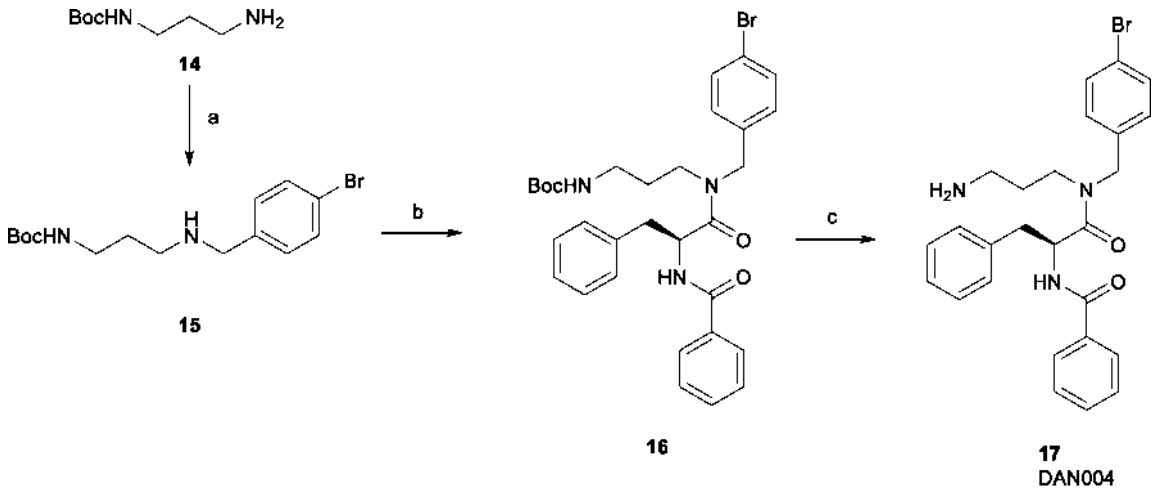
13

To a stirred solution of compound **12** (40 mg, 0.047 mmol) in dry THF under organ atmosphere was added TBAF (0.1 mL, 0.095 mmol) solution in THF at 0° C. The reaction mixture was allowed to warm to rt and stirred for overnight. After completion of the reaction as indicated by TLC and LC-MS, the solvent was evaporated under vacuum to

give crude product which was subjected to flash chromatography using dichloromethane and methanol (9.8:0.2) solvent system on triethylamine neutralized silica gel to give pure amine **13** (30 mg, 93 % yield). ^{1}H NMR ($CDCl_3$, 400 MHz): 2.47-2.67 (m, 0.5 H), 2.83-3.03 (m, 1.5 H), 3.11-3.21 (m, 2H), 3.38-3.41 (m, 3H), 3.56-3.61 (m, 2H), 3.78-3.84 (m, 2H), 3.95-3.98 (m, 0.5), 4.14-4.27 (m, 2H), 4.36-4.47 (m, 0.5H), 5.19-5.28 (m, 2.5H), 5.69-5.83 (m, 1.5H), 6.46-6.54 (m, 2H), 6.69-7.02 (m, 4H), 7.14-7.16 (m, 1H), 7.25-7.35 (m, 4H), 7.45-7.57 (m, 4H), 7.77-7.81 (m, 2H); MS: (ES+) m/z = 688 (M+1), 690 (M+3) HPLC purity (>97%).

2) Experimental Section for the Synthesis of Small Molecule DAN004 (17)

Reductive amination of *fetf*-butyl (3-aminopropyl)carbamate **14** with 4-bromobenzaldehyde gave rise to **15** the secondary amino functionality of which subsequently was coupled to *N*-benzoyl-*L*-phenylalanine yielding **16**. Final deprotection rendered **17** (DAN004) in good overall yield.



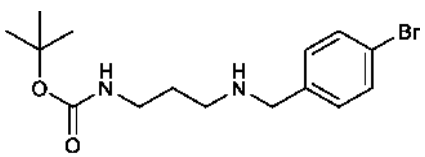
Reagents and conditions', a) 4-bromobenzaldehyde, $NaBH_4$, MeOH, 0°C → RT, 16 h 77%; b) *N*-benzoyl-*L*-phenylalanine, HBTU, N-methylmorpholine, DMF, RT, 14h, 61%; c) TFA, DCM, RT, 72h, 92%.

General synthesis

Commercially available reagents and solvents were used without further purification, except for cyclohexane which was distilled prior to use. Thin layer chromatography (TLC) was performed on precoated TLC-plates (silica gel 60 F254, Merck). Flash column chromatography was performed on prepacked flash chromatography columns (PF 30-SIHPJP/ 12G) purchased from Interchim using a Büchi separation system.

Melting points were determined using the melting point meter MPM-H2 (Schorpp Gerätetechnik, Germany) and are uncorrected. 1H-NMR and 13C-NMR spectra were recorded either on a JEOL ECX-400 or a JEOL ECA-500 spectrometer. Chemical shifts (δ) are given in ppm with the residual solvent signal used as reference [1H, DMSO-d_6 (30°C), quint, δ=2.49ppm; $CDCl_3$ (20°C), s, δ=7.26ppm; 13C, $DMSOd_6$ (30°C), quint, δ=39.50ppm, **$CDCl_3$** (20°C), t, δ=77.16ppm. Coupling constants (J) are reported in hertz (Hz). Peak patterns are abbreviated as follows, s (singlet), d (doublet), dd (double doublet), dt (doublet of triplet), t (triplet), m (multiplet), sm (symmetric multiplet), br (broad), ps (pseudo). Mass spectra were either recorded on a triple quadrupole spectrometer type EP 10+ (MS Vision), a triple quadrupole spectrometer type Q-Trap 2000 (Applied Biosystems), or on a double-focusing sector field spectrometer type AutoSpec (Micromass). Elemental combustion analyses were performed on a vario MICRO cube (Elementar Analysensysteme GmbH, Hanau, Germany).

tert-butyl (3-((4-bromobenzyl)amino)propyl)carbamate (**15**)

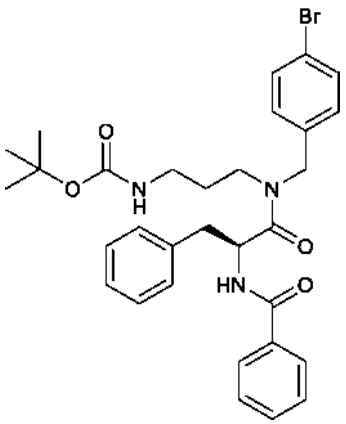


A solution of *ferf*-butyl (3-aminopropyl)carbamate (**14**, 0.435 g, 2.50 mmol) and 4-bromobenzaldehyde (0.509 g, 2.75 mmol, 1.1 equiv.) in MeOH (10 mL) was stirred for 3h at RT under an argon atmosphere. The reaction mixture was subsequently cooled to 0 °C and sodium borohydride (0.378 g, 10.0 mmol, 4 equiv.) was added slowly, followed by further stirring of the mixture for additional 16h at RT. After concentrating in *vacuo,* the remaining residue was taken up in water (20 mL) and the mixture subsequently extracted with EtOAc (3 x 20 mL). The combined organic layers were extracted with aq. 0.5 M HCl (3 x 20 mL) and the separated aqueous layers combined and adjusted to pH 9-10 by addition of an aq. NH_3 solution. After extraction with DCM (3 x 20 mL), the combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and finally concentrated in vacuo to give crude **15** [0.659 g, 77%, 87% purity (qNMR)]

which was used without further purification in the next step.

^{1}H-NMR (500MHz, DMSO-d_6): δ 1.36 (s, 9H), 1.51 (quint, ^{3}J = 6.9 Hz, 2H), 2.44 (t, ^{3}J = 6.7 Hz, 2H), 2.95 (dt, ^{3}J = 6.6 Hz, ^{3}J = 6.3 Hz, 2H), 3.29 (s, 1H), 3.62 (s, 2H), 6.74 (s, 1H), 7.26 (psd, ^{3}J + ^{5}J = 8.6 Hz, 2H), 7.47 (psd, ^{3}J + ^{5}J = 8.3 Hz, 2H). ^{13}C-NMR (125MHz, DMSO-d_6): δ 28.8, 30.3, 38.7, 46.7, 52.7 77.9, 119.9, 130.6, 131.4, 141.1, 156.1. MS (ESI+) *m/z* (%): 342.15 (100) [^{79}Br, *M*+H]+, 687.23 (80) [^{79}Br,^{81}Br, 2*M*+H]+.HRMS (EI): calcd for $C_{15}H_{23}BrN_2O_2$ [^{79}Br, *M*+H]+: 342.094289, found: 342.095397. HRMS-(EI): calcd for $C_{15}H_{23}BrN_2O_2$ [^{81}Br, *M*+H]+: 344.092243, found: 344.093534.

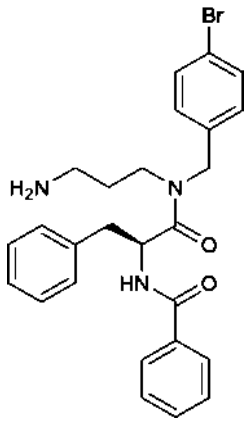
(S)-tert-butyl (3-(2-benzamido-N-(4-bromobenzyl)-3-phenylpropanamido)propyl)carbamate (**16**)



To a solution of *N*-benzoyl-*L*-phenylalanine (0.498 g, 1.85 mmol), HBTU (0.771 g, 2.04 mmol, 1.1 equiv.) and N-methylmorpholine (1.017 ml, 9.25 mmol, 5 equiv.) in 8 ml DMF, **15** (0.699 g, 2.04 mmol,1.1 equiv.) was added and the reaction mixture stirred at RT for 14h under an argon atmosphere. After addition of DCM (100 mL), the separated organic layer was exhaustively washed with an aqueous 5% LiOH solution (5 x 50 ml), dried over $MgSO_4$, filtered, and finally concentrated in *vacuo.* Flash chromatography (cyclohexanes/EtOAc, gradient from 0 → 5% over 15 min) of the remaining crude product gave rise to **16** as colorless solid (0.671 g, 61%).

Mp: 130-133°C. ^{1}H-NMR (500MHz, DMSO-d_6, mixture of rotamers): δ 1.35 (s, 9H), 1.53-1.71 (m, 2H), 2.81-2.96 (m, 2H), 2.98-3.11 (m, 2H), 3.15-3.27 (m, 1H), 4.40-4.73 (m, 2H), 4.97-5.12 (m, 1H), 6.67-6.82 (m, 1H), 7.09-7.31 (m, 6H), 7.32-7.54 (m, 6H), 7.76-7.85 (m, 2H), 8.79-8.82 (m, 1H). ^{13}C-NMR (100MHz, $CDCl_3$, mixture of rotamers): δ 27.5, 28.5, 28.7, 37.3, 37.8, 39.5, 39.9, 43.4, 44.5, 48.4, 50.3, 50.5, 51.2, 79.2, 79.4, 121.5, 121.8, 127.2, 128.5, 128.7, 128.75, 128.77, 129.6, 129.8, 131.8, 131.9 132.1, 133.7, 133.8, 135.2, 135.8, 136.1, 136.3, 156.1, 156.3, 166.8, 167.0, 171.9, 172.5. MS (ESI+) *m/z* (%):594 (100) [^{79}Br, *M*+H]+, 596 (100) [^{81}Br, *M*+H]+. Anal. calcd for: $C_{31}H_{36}BrNaO_4$: C, 62.63; H, 6.10; N, 7.07; found: C: 62.68; H, 6.20; N, 7.27.

(S)-*N*-(1-((3-aminopropyl)(4-bromobenzyl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide (**17**)



To a solution of **16** (0.719 g, 1.21 mmol) in DCM (6 mL), TFA (0.419 mL, 5.45 mmol, 4.5 equiv.) was added and the reaction mixture stirred at RT for 72h. The mixture was then quenched by addition of a sat. $NaHCO_3$ solution (10 mL) and the separated organic layer washed with brine, dried over $MgSO_4$, filtered, and concentrated in *vacuo.* Flash chromatography (DCM/MeOH gradient from 0 → 10% over 15 min) of the crude product

yielded **17** as colorless, hygroscopic solid (0.550 g, 92%).

Mp: 46-50°C (hygroscopic). ^{1}H-NMR (500MHz, DMSO-d_6, mixture of rotamers): δ 1.70-1.83 (sm, 1.5H), 1.90-2.05 (sm, 0.5H), 2.70 (t, ^{3}J = 7.5 Hz, 1H), 2.78 (t, ^{3}J = 7.2 Hz, 1H), 2.96-3.04 (sm, 1H), 3.07-3.15 (m, 1H), 3.23-3.40 (m, 2H), 3.42-3.49 (sm, 1H), 4.40 (d, ^{2}J = 15.5 Hz, 0.5H), 4.60 (d, ^{2}J = 15.5 Hz, 0.5H), 4.61 (d, ^{2}J = 17.5 Hz, 0.5 H), 4.77 (d, ^{2}J = 17.2 Hz, 0.5H), 4.97 (dd, ^{2}J = 14.1 Hz, ^{3}J = 8.2 Hz, 0.5H), 5.12 (dd, ^{2}J = 14.9 Hz, ^{3}J = 8.0 Hz, 0.5H), 7.09-7.94 (m, 5H), 7.27 (pst, ^{3}J = 7.5 Hz, 1H), 7.37-7.55 (m, 6H), 7.78 (d, ^{3}J = 7.2 Hz, 1H), 7.81-7.92 (m, 3H), 8.86 (d, ^{3}J = 7.5 Hz, 1H). ^{13}C-NMR (125MHz, DMSO-d_6): δ 25.8, 27.1, 37.0, 37.2, 37.6, 37.7, 43.7, 45.0, 48.2, 50.2, 51.7, 52.1, 120.5, 120.9 126.95, 127.02, 128.0, 128.1, 128.65, 128.68, 128.7, 128.8, 129.5, 129.8, 130.0, 130.1, 131.7, 1321.9 132.0, 134.2, 134.3, 137.4, 137.8, 138.1, 138.3, 166.8, 167.0, 172.1, 127.6. MS (ESI) *m/z* (%): 494.10 (100) [^{79}Br, *M*+H]+. HRMS (EI): calcd for $C_{26}H_{28}BrN_3O_2$ [^{79}Br, *M*+H]+: 493.136489, found: 493.138102. HRMS (EI): calcd for $C_{26}H_{28}BrN_3O_2$: [^{81}Br, *M*+H]+: 495.134442, found: 495.135035. Anal. calcd for: $C_{26}H_{28}BrN_3O_2$*2.5 H_2O: C, 57.89; H, 6.17; N, 7.79; found: C: 57.55; H, 5.66; N, 7.70.