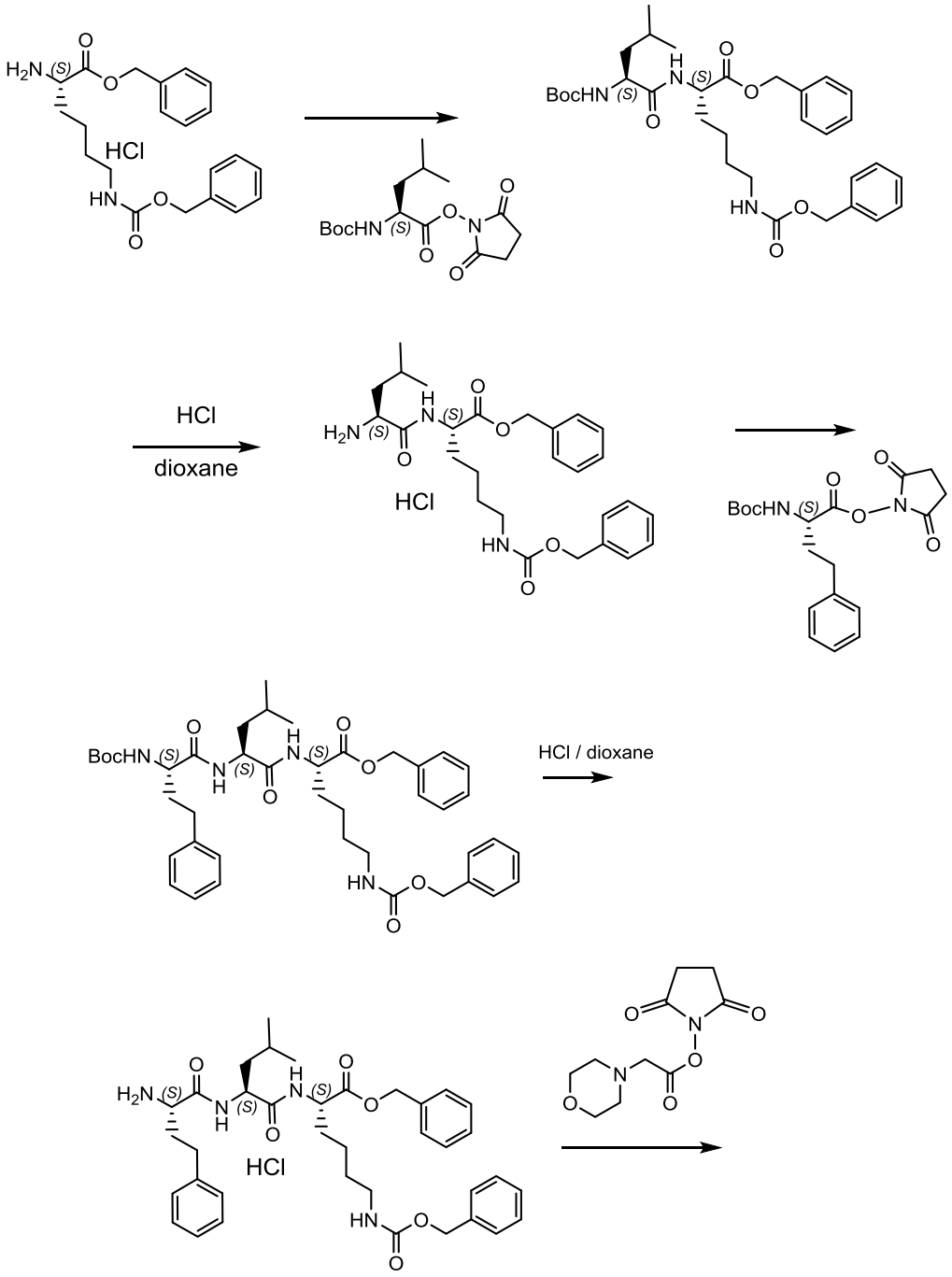
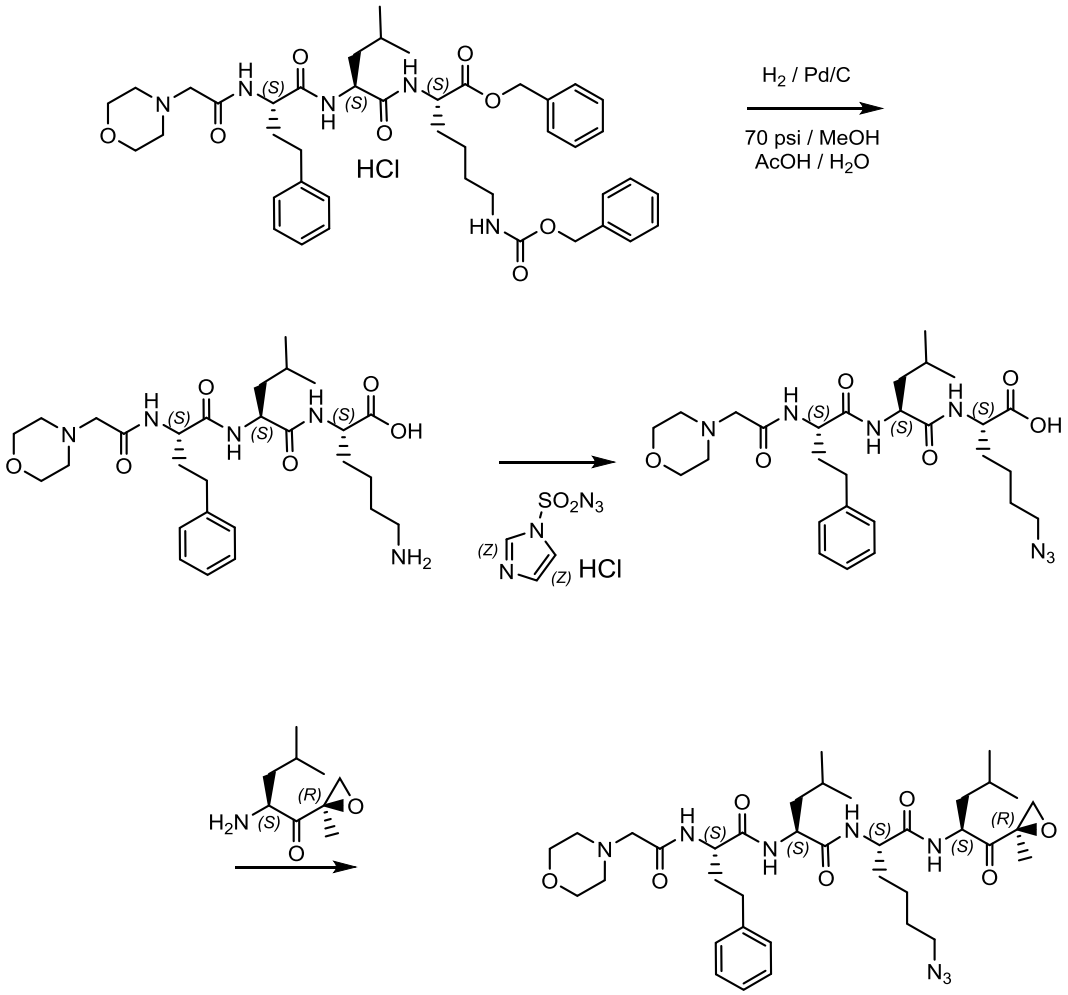
Supplementary Methods to

Specificity of protein covalent modification by the electrophilic proteasome inhibitor carfilzomib in human cells

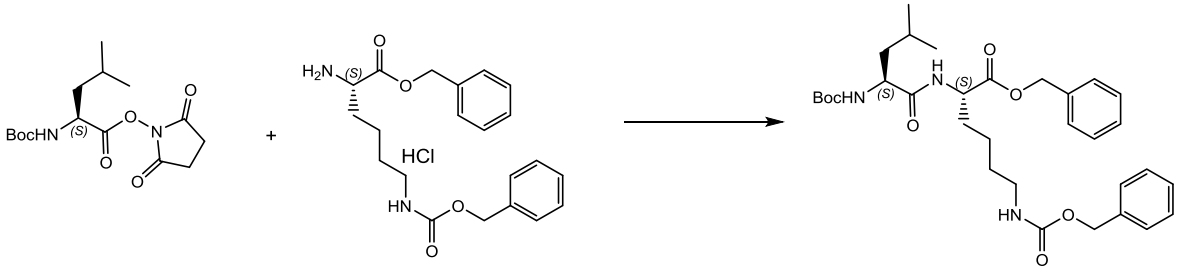
Joel D. Federspiel, Simona G. Codreanu, Sandeep Goyal, Matthew E. Albertolle, Eric Lowe, Juli Teague, Hansen Wong, F. Peter Guengerich, and Daniel C. Liebler

Synthesis of (S)-6-azido-N-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)-2-((S)-4-methyl-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)pentanamido)hexanamide



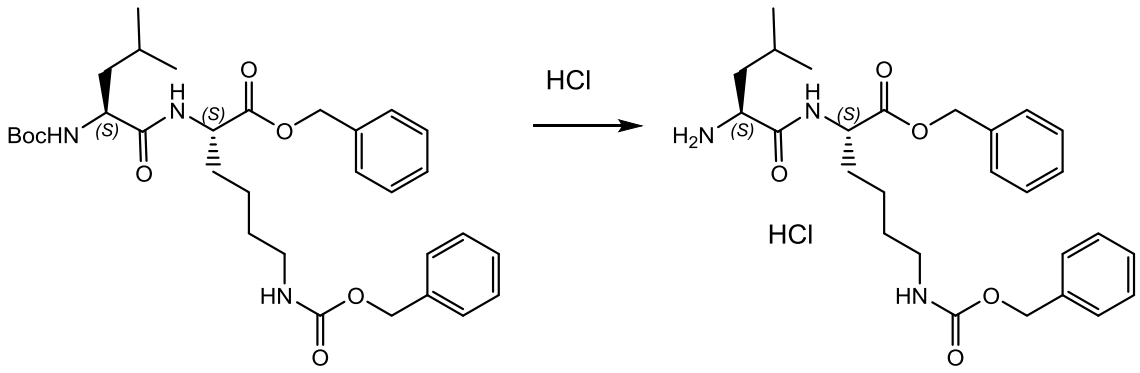

HCl
H2 / Pd/C
70 psi / MeOH
AcOH / H2O
SO2N3
HCl

Step 1: Preparation of Benzyl N^6-((benzyloxy)carbonyl)-N^2-((tert-butoxycarbonyl)-L-leucyl)-L-lysinate



2,5-dioxopyrrolidin-1-yl (tert-butoxycarbonyl)-L-leucinate (8.08 g, 24.57 mmol) was dissolved in anhydrous DMF (50 mL) and treated with benzyl N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (10 g, 24.57 mmol) and diisopropylethylamine (8.98 mL, 51.60 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed at reduced pressure and the residue dissolved in ethyl acetate, washed with water (3 x), dried and evaporated to dryness. The residue was triturated with isopropyl alcohol twice and the product was dried at high vacuum to afford benzyl N^6-((benzyloxy)carbonyl)-N^2-((tert-butoxycarbonyl)-L-leucyl)-L-lysinate (14.01 g, 98% yield). LC/MS = 584.5 $[M+H]^+$.

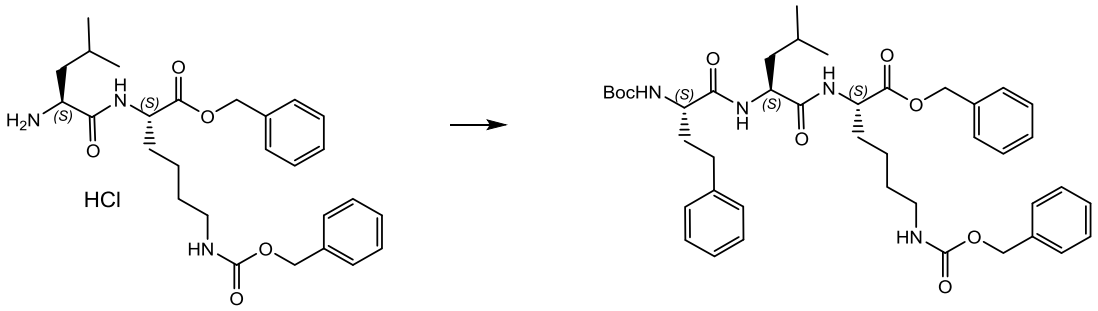
Step 2: Preparation of benzyl N^2-(L-leucyl)-N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride



Benzyl N^6-((benzyloxy)carbonyl)-N^2-((tert-butoxycarbonyl)-L-leucyl)-L-lysinate (14.01 g, 23.99 mmol) was dissolved in anhydrous dioxane (50 mL) and treated with HCl in dioxane (4N, 50 mL). The solution was stirred at room temperature for 90 minutes. The solvent was concentrated at reduced pressure to about 20 mL and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then

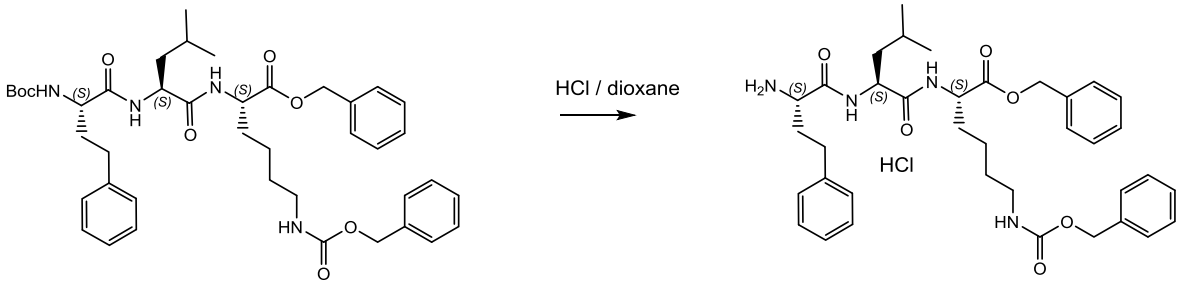
with hexane. The solid was dried overnight at high vaccum to afford benzyl N^2-(L-leucyl)-N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (11.82 g, 95% yield). LC/MS = 484.3 $[M+H]^+$.

Step 3: Preparation of benzyl N^6-((benzyloxy)carbonyl)-N^2-(((S)-2-((tert-butoxycarbonyl)-amino)-4-phenylbutanoyl)-L-leucyl)-L-lysinate



Benzyl N2-(L-leucyl)-N6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (11.82 g, 22.73 mmol) was dissolved in anhydrous DMF (50 mL) and treated with 2,5-dioxopyrrolidin-1-yl (S)-2-((tert-butoxycarbonyl)amino)-4-phenylbutanoate (8.55 g,22.73) and diisopropylethylamine (8.72 mL, 50.01 mmol). The reaction mixture was stirred at room temperature for 4 h. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate, washed with water (3 x), dried and evaporated. The residue was triturated with isopropyl alcohol, filtered and dried at high vacuum overnight to afford benzyl N6-((benzyloxy)carbonyl)-N2-(((S)-2-((tert-butoxycarbonyl)amino)-4-phenylbutanoyl)-L-leucyl)-L-lysinate (16.6 g, 98% yield). LC/MS = 745.6 $[M+H]^+$.

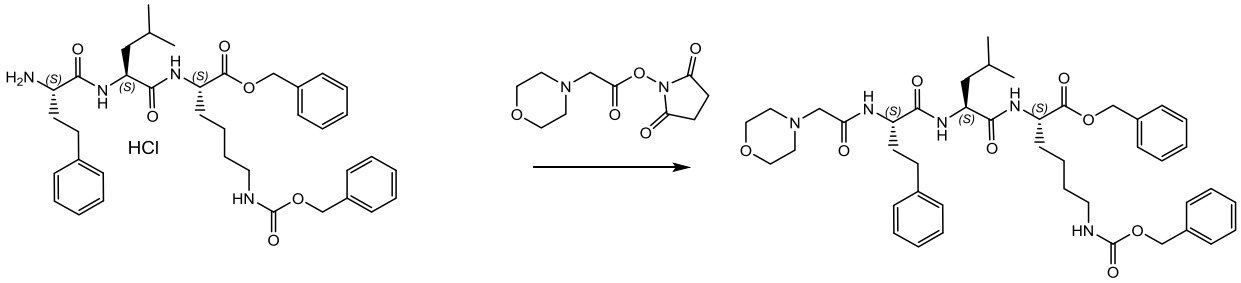
Step 4: Preparation of benzyl N^2-(((S)-2-amino-4-phenylbutanoyl)-L-leucyl)-N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride



Benzyl N^6-((benzyloxy)carbonyl)-N^2-(((S)-2-((tert-butoxycarbonyl)amino)-4-phenylbutanoyl)-L-leucyl)-L-lysinate (16.6 g, 22.28 mmol) was dissolved in anhydrous dioxane (70 mL) and treated

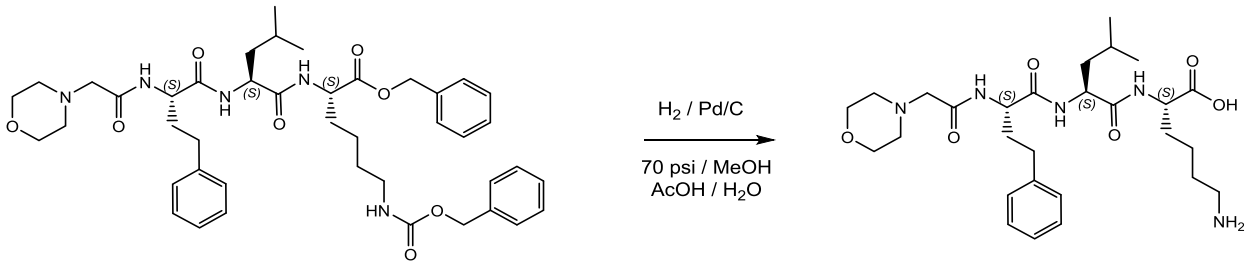
with HCl in dioxane (4N, 70 mL) at room temperature for 2 h. The solvent was concentrated at reduced pressure to about 30 mL and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried overnight at high vaccum to afford benzyl N^2-(L-leucyl)-N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (14.722 g, 97% yield). LC/MS = 645.6 $[M+H]^+$.

Step 5: Preparation of benzyl N^6-((benzyloxy)carbonyl)-N^2-(((S)-2-(2-morpholino-acetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysinate



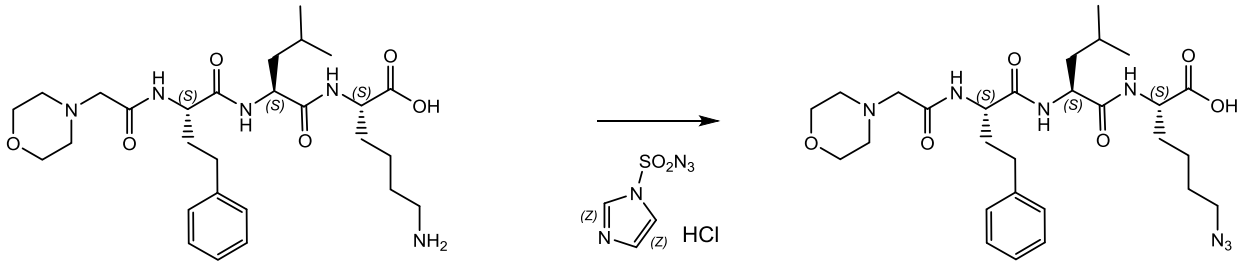
Benzyl N^2-(L-leucyl)-N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (14.72 g, 21.62 mmol) was dissolved in anhydrous DMF (70 mL) and treated with 2,5-dioxopyrrolidin-1-yl 2-morpholinoacetate (18.31 g, 75.67 mmol) and diisopropylethylamine (8.27 mL, 47.56 mmol) at room temperature for 4 h. Another 5 g 2,5-dioxopyrrolidin-1-yl 2-morpholinoacetate was added and the reaction mixture stirred an additional 2 h at which time LC/MS analysis indicated that the reaction was complete. The solvent was removed under reduced pressure and the residue was taken up in ethyl acetate. The precipitate which formed was filtered and discarded. The solution was washed with water (3 x), dried and evaporated. The residue was treated with isopropyl alcohol and evaporated to dryness (3 x). The residual solid was dried overnight at high vacuum to afford benzyl N^6-((benzyloxy)carbonyl)-N^2-(((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysinate (16.98 g, 102% yield). LC/MS = 772.6 $[M+H]^+$. Without further purification, the material thus obtained was used directly in the next step.

Step 6: Preparation of ((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl-L-lysine



Benzyl N^6-((benzyloxy)carbonyl)-N^2-(((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysinate (16.98 g, 21.62 mmol) was dissolved in methanol (200 mL), acetic acid (200 mL) and water (5 mL) and hydrogenated over 10% Pd/C (2.2 g) at room temperature, 70 psi. After 4 h, LC/MS analysis showed the reaction to be complete. The catalyst was filtered off and washed with methanol. The solution was evaporated to dryness and then azeotroped twice with isopropyl alcohol. The residue was triturated with ether and filtered and dried overnight at high vacuum to afford ((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl-L-lysine (12.44 g, 105% yield). LC/MS = 548.5 $[M+H]^+$. Without further purification, the material thus obtained was used directly in the next step.

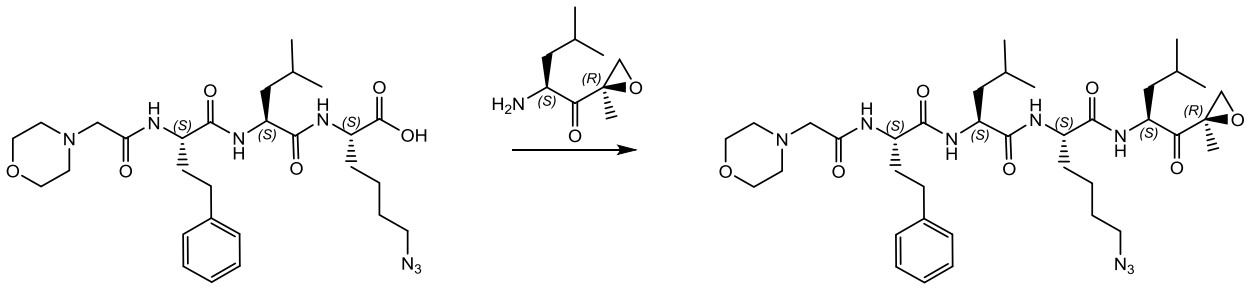
Step 7: Preparation of N^6-diazo-N^2-(((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysine



((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl-L-lysine (12.44 g, 21.6 mmol) was dissolved in methanol (120 mL) and treated with 1H-imidazole-1-sulfonyl azide hydrochloride (Goddard-Borger* and Stick; Organic Letters 2007 Vol. 9, No. 19 3797 – 3800)(5.42 g, 25.92 mmol) and potassium carbonate (7.45 g, 54 mmol). Copper sulfate pentahydrate (5.4 g, 21.6 mmol) was added to the reaction mixture which was stirred at room temperature for 2 h. The solvent was evaporated and the residue was taken up in ethyl acetate and washed with sulfuric

acid (2%) and water. The residue thus obtained after removal of the solvent was dried at high vacuum overnight to afford the title product (6.57 g, 56% yield). A further 3.8 g of the desired product was recovered from the combined aqueous layers by further extraction with ethyl acetate (2 x), which was washed with brine, dried and evaporation. The combined yield of N^6-diazo-N^2-(((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysine was 10.37 g, 88% yield. LC/MS = 574.7 $[M+H]^+$.

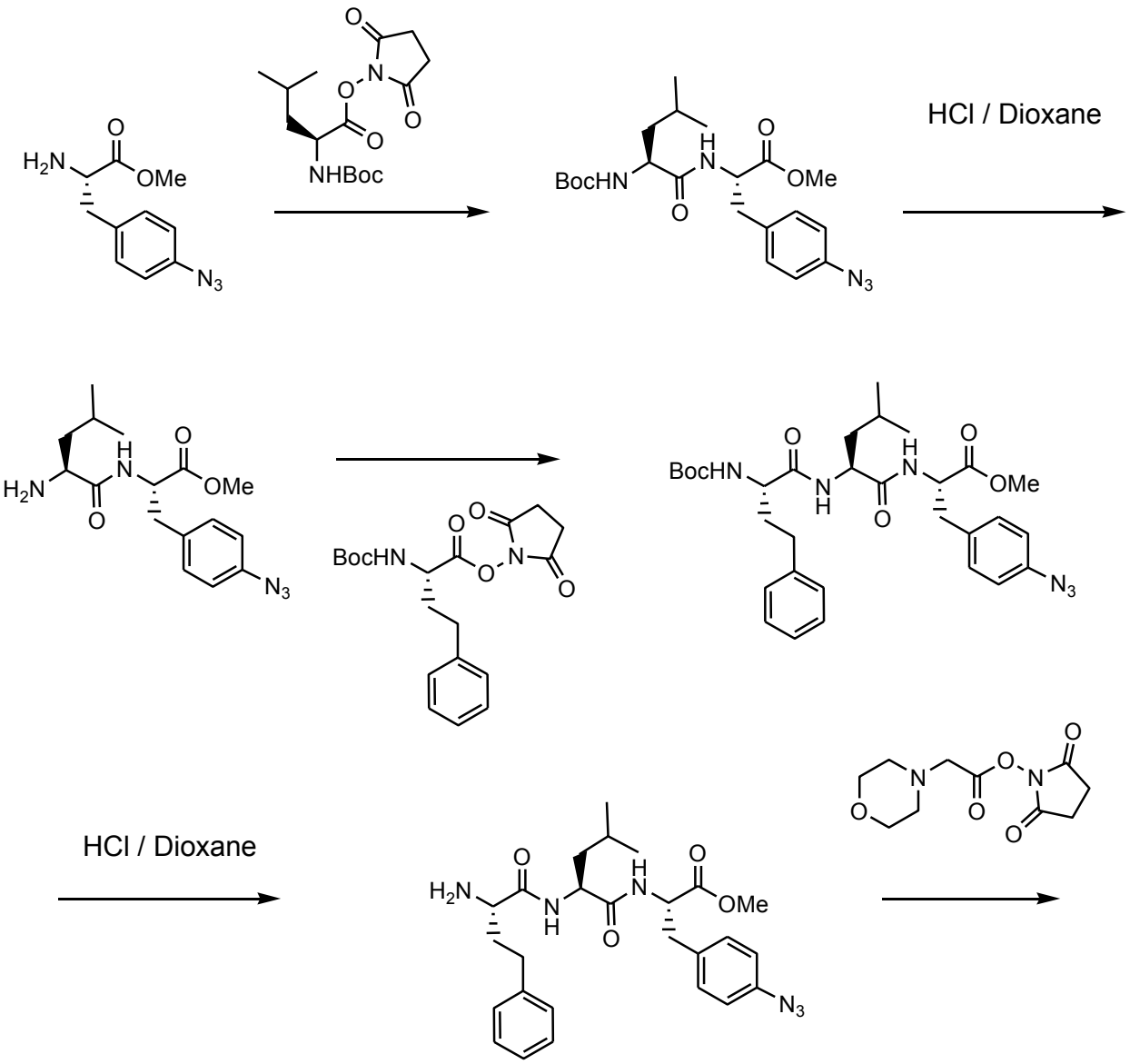
Step 8: Preparation of (S)-6-azido-N-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxo-pentan-2-yl)-2-((S)-4-methyl-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-pentanamido)hexanamide

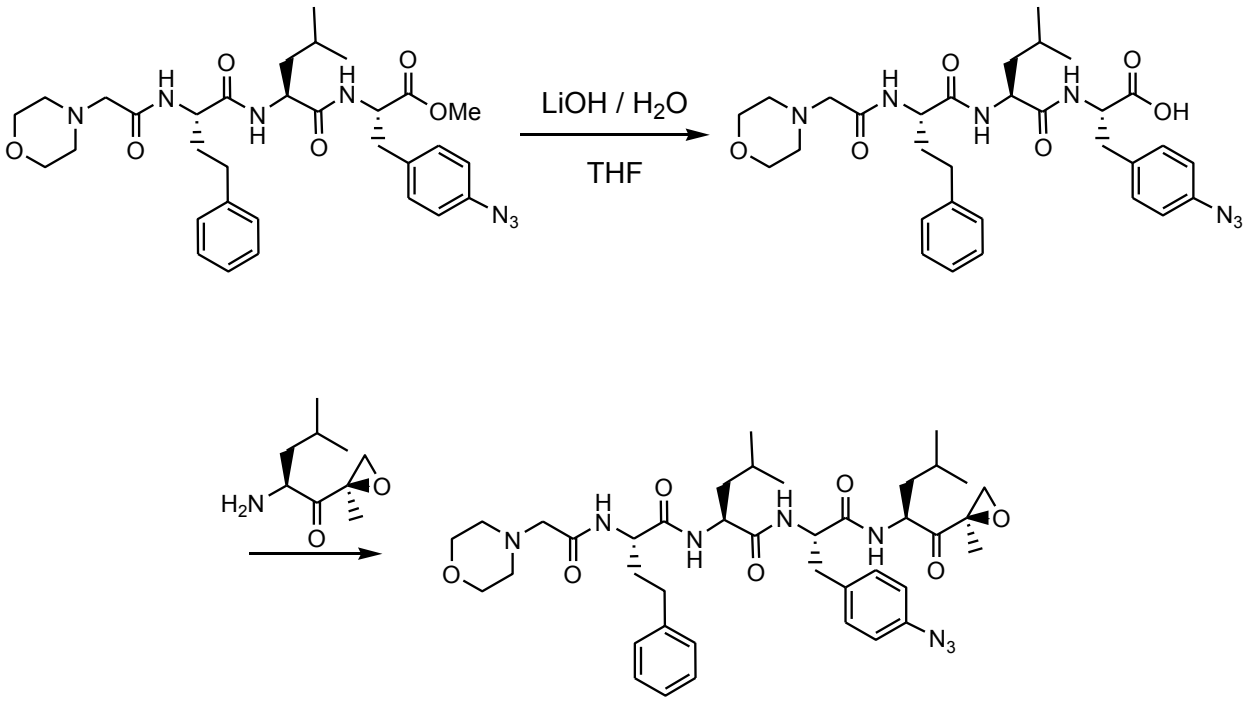


N^6-diazo-N^2-(((S)-2-(2-morpholinoacetamido)-4-phenylbutanoyl)-L-leucyl)-L-lysine (6.53 g, 11.38 mmol) was dissolved in anhydrous DMF (65 mL) and mixed with BOP (5.19 g, 13.66 mmol) and 2-amino-4-methyl-1-(2-methyl-oxiranyl)-pentan-1-one (3.25 g, 11.38 mmol) (Kawamura et al., Bioorganic & Medicinal Chemistry 22,(12), 3091 – 3095 (2014)). At -20 °C diisopropylethylamine (6.33 g, 36.4 mmol) was added dropwise. The solution was stirred and allowed to rise to room temperature over a 1 h period. After stirring overnight at room temperature, the reaction mixture was diluted with ethyl acetate (800 mL) and extracted with water (100 mL x 3). The combined aqueous layers were back extracted with ethyl acetate (100 mL x 2) and the combined organic extracts were dried and evaporated to dryness to afford 9.5 g of the crude product which was purified by preparative HPLC to afford pure (S)-6-azido-N-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)-2-((S)-4-methyl-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-pentanamido)hexanamide. (4.13 g)

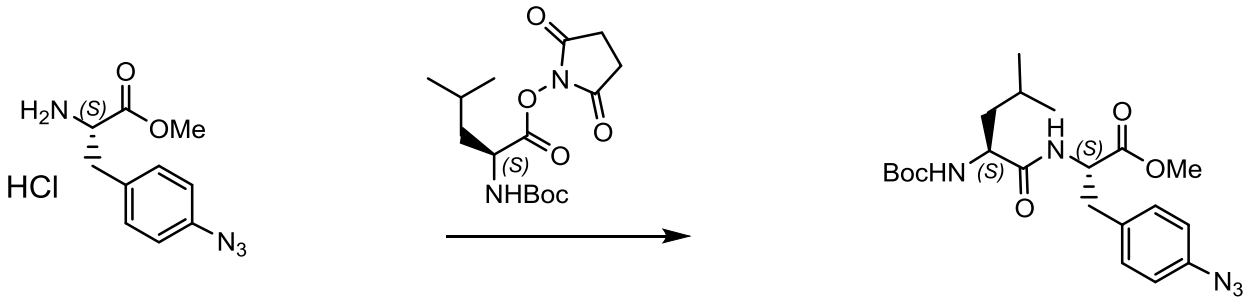
NMR (DMSO-d6): δ 8.16 (d, NH x 2), 7.90 (d, NH x 2), 7.31 – 7.15 (m, ArH x 5), 4.40 – 4.26 (m, N-CHR-CO x 4), 3.63 – 3.59 (m, CH_2-O-CH_2), 3.35 – 2.96 (m, $N(CH_2)_3$), 2.52 – 2.42 (m, CH_2CH_2Ar), 2.44 + 1.88 (m, CH_2-oxirane), 1.64 – 1.23 (m, CH_2 x 6, CH x 2), 1.40 (s, CH_3 x 1), 0.90 – 0.81 (m, CH_3 x 4, isopropyl). LC/MS = 727.7, 98.5% purity.

Synthesis of 4-Methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-azido-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide



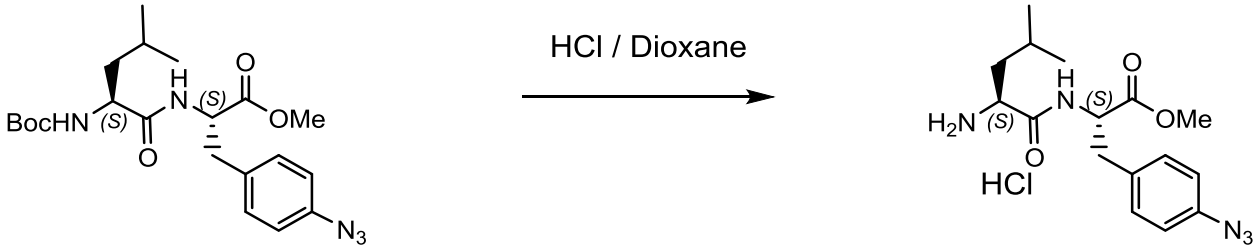

LiOH / H2O
THF
OMe
OH
N3
H2N

Step 1: Preparation of 3-(4-Azido-phenyl)-2-(2-tert-butoxycarbonylamino-4-methyl-pentanoylamino)-propionic acid methyl ester



2-Amino-3-(4-azido-phenyl)-propionic acid methyl ester hydrochloride (WO 2007/131764)(4.1 g, 15.9 mmol) was dissolved in anhydrous DMF (25 mL) and treated with diisopropylethylamine (3.5 mL, ca. 1.2 eqt.). The mixture was stirred for 25 m at room temperature. 2-tert-Butoxycarbonylamino-4-methyl-pentanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (5.0 g, 15.2 mmol) was added and the mixture was stirred at room temperature for 20 h. The solution was poured into water (700 mL) and acidified to pH~2 with 1N H_2SO_4. The product was extracted with dichloromethane (200 mL), washed with water, dried and evaporated to dryness. The residue was purified by flash chromatography to afford 3-(4-azido-phenyl)-2-(2-tert-butoxycarbonyl-amino-4-methyl-pentanoylamino)-propionic acid methyl ester(5.6 g, 85% yield). LC/MS = 434.3 $[M+H]^+$. An additional 0.5 g of less purity was isolated from the column as a mixed fraction.

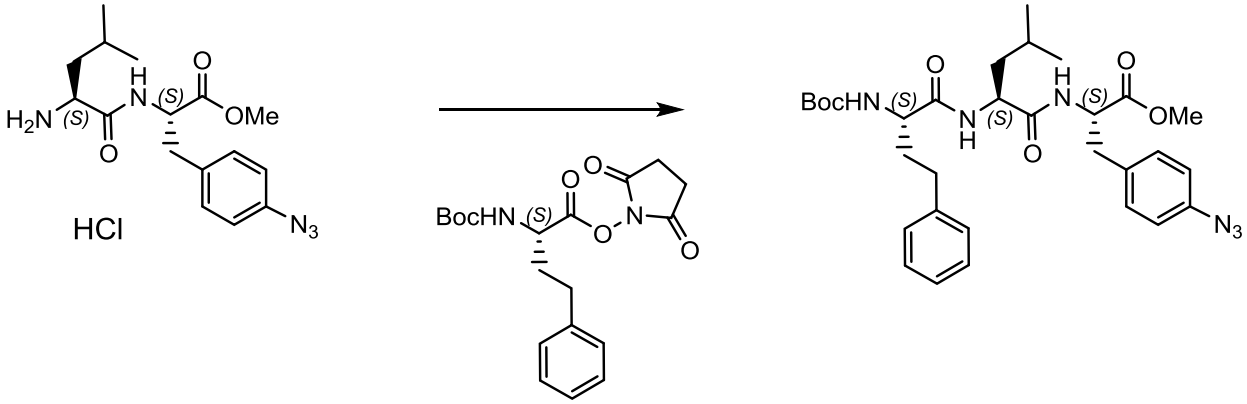
Step 2: Preparation of 2-(2-Amino-4-methyl-pentanoylamino)-3-(4-azido-phenyl)-propionic acid methyl ester



3-(4-Azido-phenyl)-2-(2-tert-butoxycarbonylamino-4-methyl-pentanoylamino)-propionic acid methyl ester (5.7 g, 13.1 mmol) was dissolved in anhydrous dioxane (100 mL) and treated with HCl in dioxane (4N, 25 mL) at room temperature for 2 h, at which time, another 25 mL portion of HCl was added and stirred for another 2 h. The solvent was concentrated at reduced pressure and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried overnight at

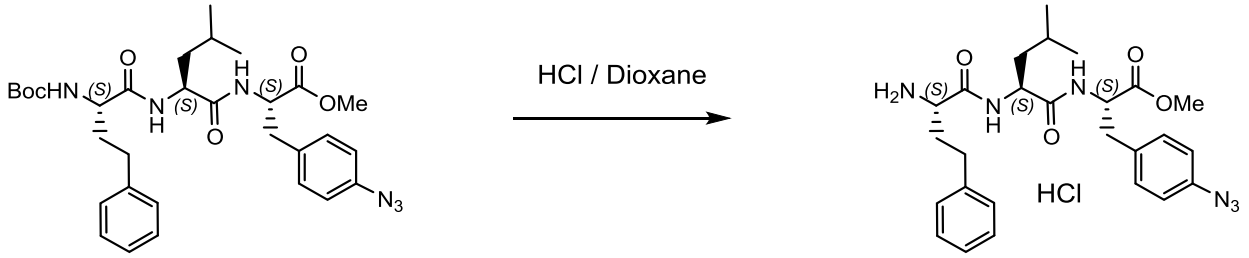
high vacuum to afford 2-(2-Amino-4-methyl-pentanoylamino)-3-(4-azido-phenyl)-propionic acid methyl ester hydrochloride (4.8 g, 98.7% yield). LC/MS = 334.4 $[M+H]^+$.

Step 3: Preparation of 3-(4-Azido-phenyl)-2-[2-(2-tert-butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-propionic acid methyl ester



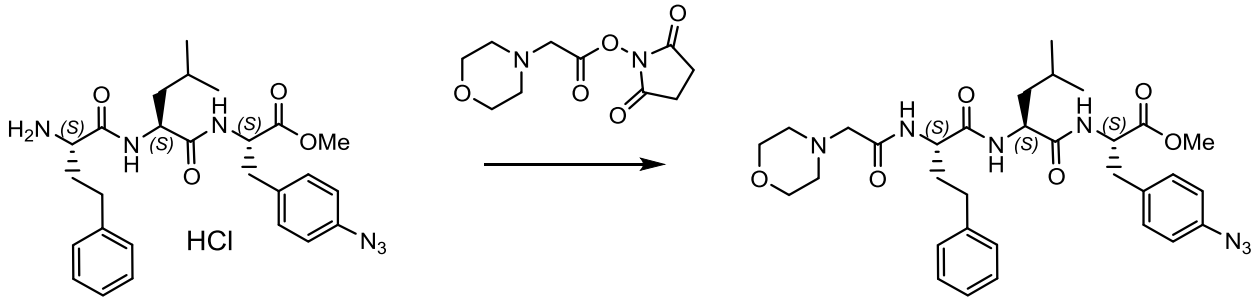
2-(2-Amino-4-methyl-pentanoylamino)-3-(4-azido-phenyl)-propionic acid methyl ester hydrochloride (4.8 g, 12.98 mmol) was dissolved in anhydrous DMF (50 mL) and treated with diisopropylethylamine (5.03 g, 38.9 mmol). The solution was stirred for 30 m at which time, 2-tert-butoxycarbonylamino-4-phenyl-butyric acid 2,5-dioxo-pyrrolidin-1-yl ester (5.37 g, 14.4 mmol). The reaction mixture was stirred at room temperature overnight. The solution was poured into water (800 mL) and acidified to pH~2 with 1N H_2SO_4. The product was extracted with ethyl acetate 3x, washed with water, dried and evaporated to dryness. The residue was purified by flash chromatography to afford 3-(4-Azido-phenyl)-2-[2-(2-tert-butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-propionic acid methyl ester (7.0 g, 91% yield). LC/MS = 595.3 $[M+H]^+$.

Step 4: Preparation of 2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-azido-phenyl)-propionic acid methyl ester



3-(4-Azido-phenyl)-2-[2-(2-tert-butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-propionic acid methyl ester (7.0 g, 11.77 mmol) was dissolved in anhydrous dioxane (80 mL) and treated with HCl in dioxane (4N, 25 mL). After 2 h at room temperature, another 18 mL HCl in dioxane was added and the reaction mixture stirred overnight at room temperature. The solvent was concentrated at reduced pressure and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried overnight at high vacuum to afford 2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-azido-phenyl)-propionic acid methyl ester hydrochloride. (5.82 g, 93% yield). LC/MS = 495.4 $[M+H]^+$.

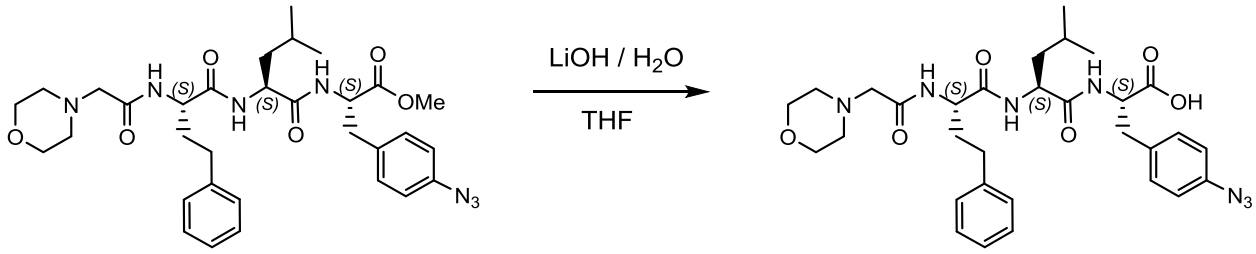
Step 5: Preparation of 3-(4-Azido-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid methyl ester



2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-azido-phenyl)-propionic acid methyl ester hydrochloride (5.82 g, 10.96 mmol) was dissolved in anhydrous DMF (25 mL) and diisopropylethylamine (4.25 g, 33 mmol) was added. After stirring the reaction mixture for 30 m morpholin-4-yl-acetic acid 2,5-dioxo-pyrrolidin-1-yl ester (10 g, 41.3 mmol)was added and the reaction mixture was stirred for 2 days at room temperature at which time LC/MS analysis indicated that the reaction was nearly complete. An additional 0.6 g morpholin-4-yl-acetic acid

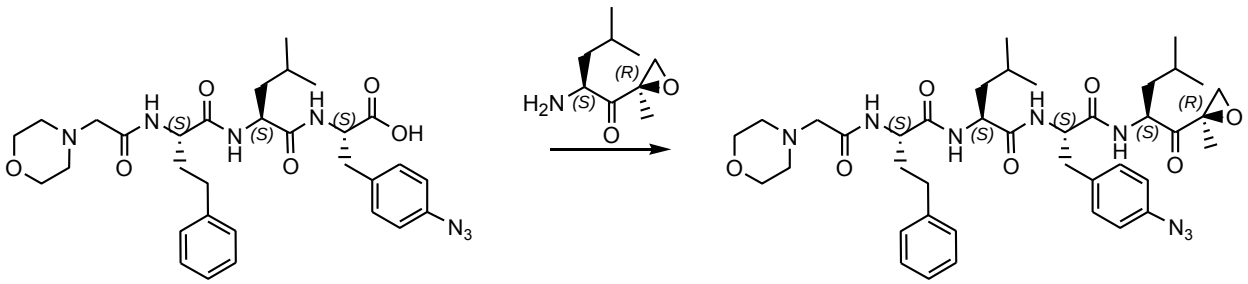
2,5-dioxo-pyrrolidin-1-yl ester was added and after stirring an additional 4 h, the reaction was complete. The solvent was removed under reduced pressure and the residue was taken up in ethyl acetate. The precipitate which formed was filtered and discarded. The solution was washed with water (3 x), dried and evaporated. The residue was treated with isopropyl alcohol and evaporated to dryness (3 x). The residual solid was dried overnight at high vacuum to afford 3-(4-azido-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid methyl ester (6.5 g, 95% yield). LC/MS = 622.4 $[M+H]^+$.

Step 6: Preparation of 3-(4-Azido-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid



3-(4-Azido-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid methyl ester (6.5 g, 10.45 mmol) was dissolved in THF (150 mL) and treated with lithium hydroxide hydrate (1.0 g, 23.8 mmol) in water (20 mL). The reaction mixture was warmed briefly to dissolve all and then stirred at room temperature for 2 h at which time LC/MS analysis indicated that reaction was complete. The solvent was removed and the residue partitioned between ethyl acetate (150 mL) and dilute HCl. The organic layer was washed with water, then brine and dried. Upon evaporation of the solvent, pure (S)-3-(4-azidophenyl)-2-((S)-4-methyl-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-pentanamido)propanoic acid (6.23 g, 98% yield). LC/MS = 608.6 $[M+H]^+$.

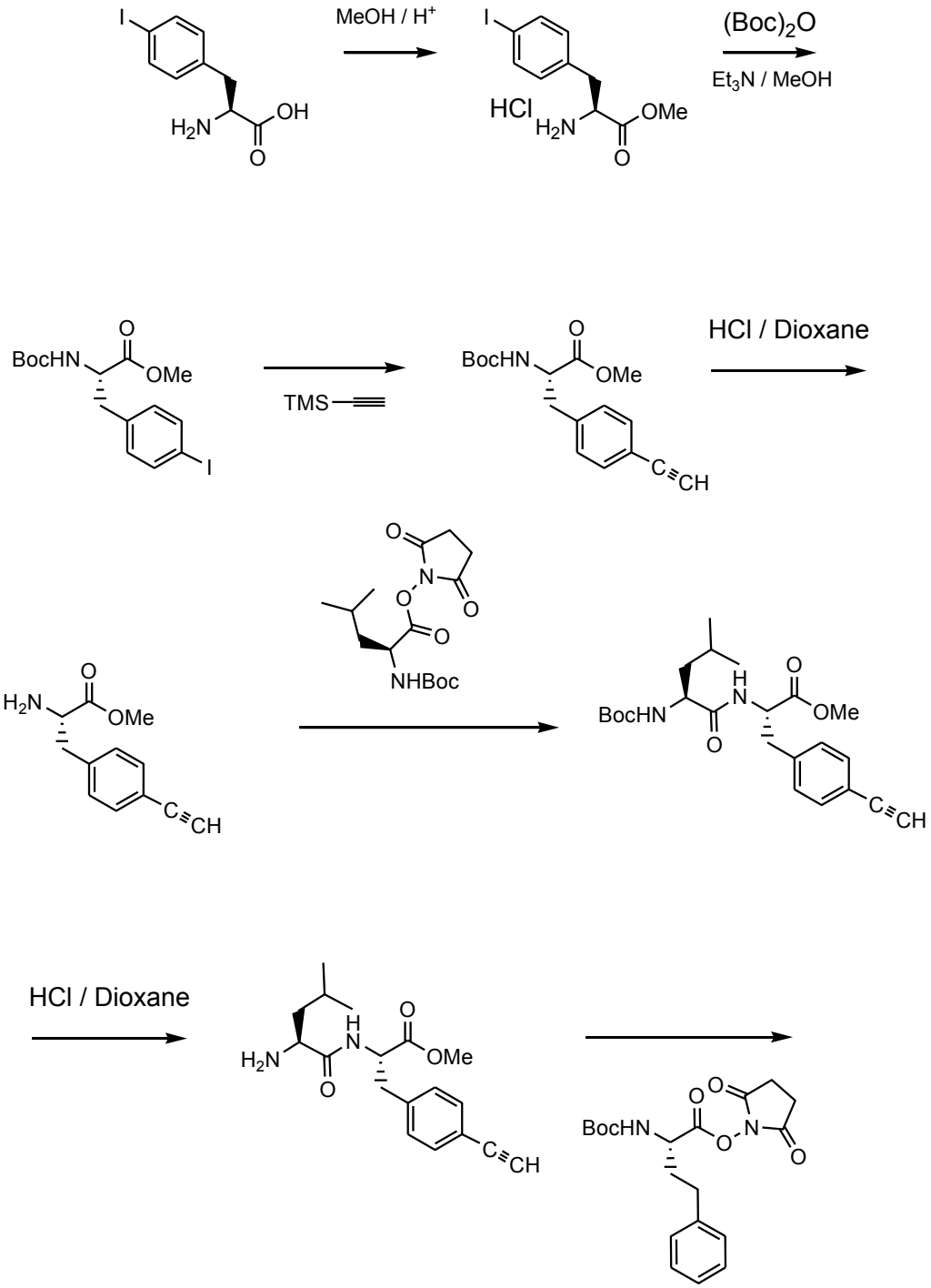
Step 7: Preparation of 4-Methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-azido-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide

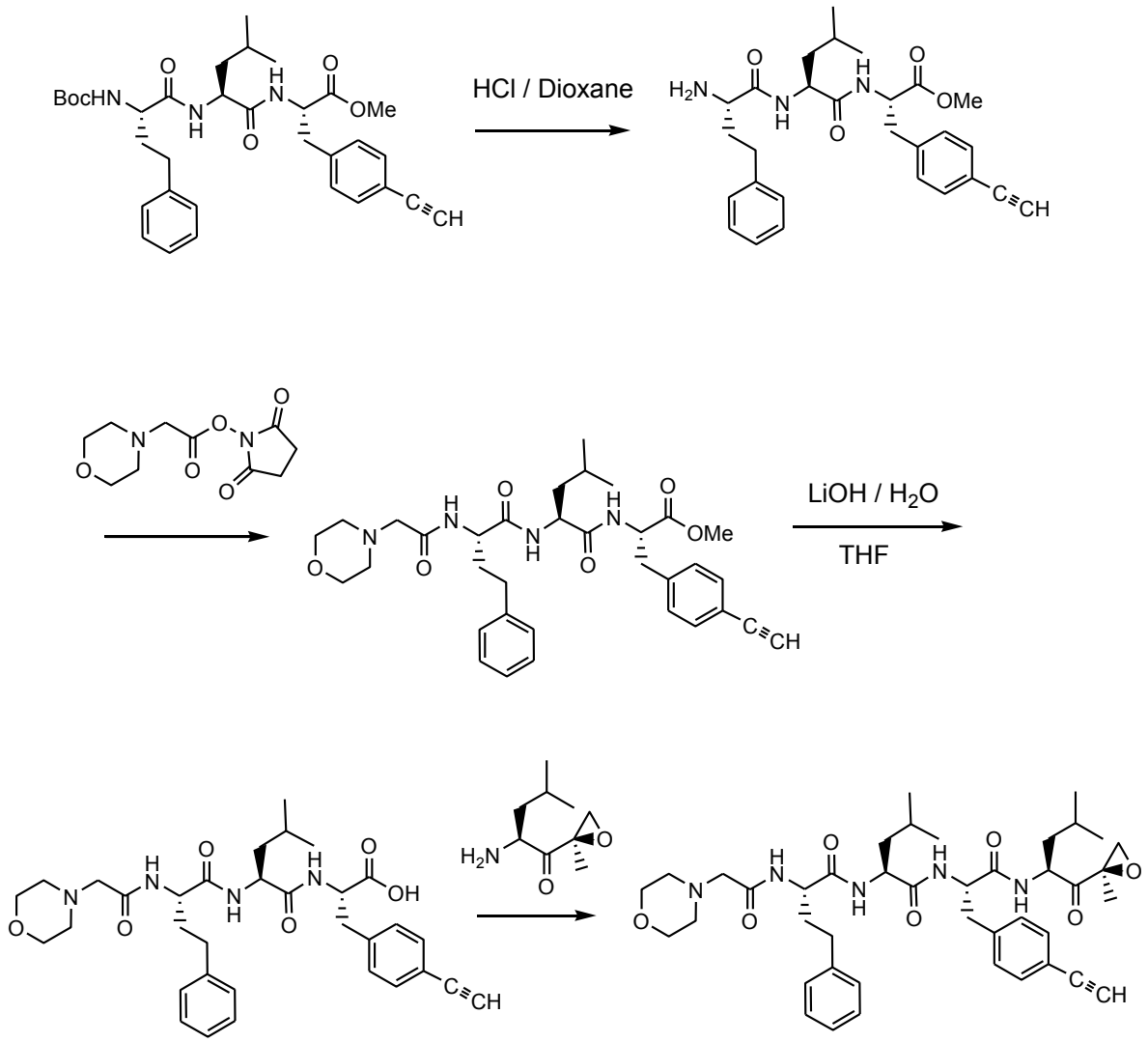


3-(4-Azido-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid (7.21 g, 11.86 mmol) was dissolved in anhydrous DMF (50 mL) and mixed with BOP (5.42 g, 14.25 mmol) and 2-amino-4-methyl-1-(2-methyl-oxiranyl)-pentan-1-one (3.39 g, 11.88 mmol) (Kawamura et al., Bioorganic & Medicinal Chemistry 22,(12), 3091 – 3095 (2014)). At -20 °C diisopropylethylamine (6.613 g, 38.0 mmol) was added dropwise. The solution was stirred and allowed to rise to room temperature over a 1 h period. After stirring overnight at room temperature, the reaction mixture was diluted with ethyl acetate (800 mL) and extracted with water (100 mL x 3). The combined aqueous layers were back extracted with ethyl acetate (100 mL x 2) and the combined organic extracts were dried and evaporated to dryness to afford 12.14 g of the crude product which was purified by preparative HPLC to afford pure 4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-azido-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide. (5.87 g)

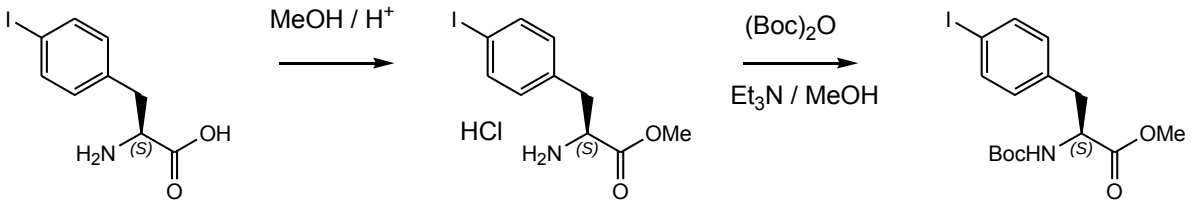
NMR (DMSO-d6): δ 8.26 (d, NH), 8.07 (d, NH), 7.97 (d, NH), 7.89 (d, NH),7.29 – 6.89 (m, ArH x 9), 4.5 – 4.27 (m, N-CHR-CO x 4), 3.62 – 3.59 (m, CH_2-O-CH_2), 3.34 – 2.89 (m, $N(CH_2)_3$), 2.51 – 2.42 (m, $ArCH_2$ x 2), 2.74 + 2.45 (m, CH_2-oxirane),1.87 – 1.26 (m, CH_2 x 3, CH x 2),1.40 (s, CH_3 x 1), 0.90 – 0.79 (m, CH_3 x 4, isopropyl). LC/MS = 761.6, 97.2% purity.

Synthesis of 4-Methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-ethynyl-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide



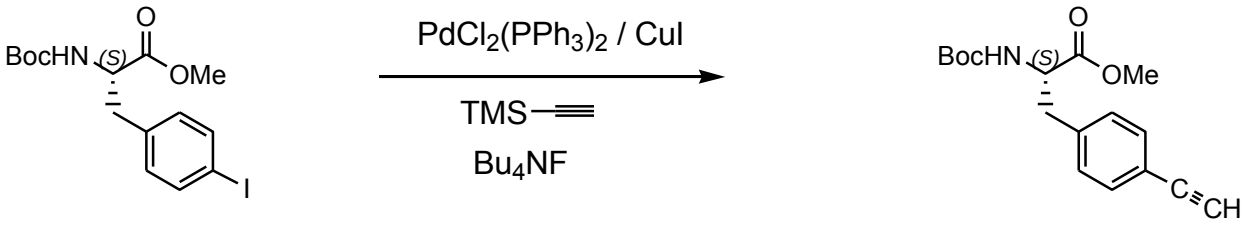

BocHN
HCl / Dioxane
H_2N
OMe
LiOH / H_2O
THF
OH

Step 1: Preparation of 2-tert-Butoxycarbonylamino-3-(4-iodo-phenyl)-propionic acid methyl ester



2-Amino-3-(4-iodo-phenyl)-propionic acid (24.9 g, 85.5 mmol) was slurried in anhydrous methanol (400 mL) and saturated with HCl gas at 0 °C. The mixture was allowed to reach room temperature and the solution thus formed was stirred at room temperature overnight. Evaporation of the solvent gave a residue which was triturated with dry ether and filtered to afford 2-amino-3-(4-iodo-phenyl)-propionic acid methyl ester hydrochloride. Without further purification, the material was taken up in methanol (200 mL) and treated with triethylamine (100 mL). The resulting solution of free base was cooled to 20 °C and treated with Boc anhydride (25 g, 114.5 mmol) and stirred overnight at room temperature. Most of the solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate and water, acidified with dilute HCl. The aqueous layer was back extracted with ethyl acetate and the combined organic extracts washed with water, dilute HCl, water and finally brine. The dried extract was evaporated to afford 2-tert-butoxycarbonylamino-3-(4-iodo-phenyl)-propionic acid methyl ester (34 g, 99% yield). LC/MS = 406.3 $[M+H]^+$.

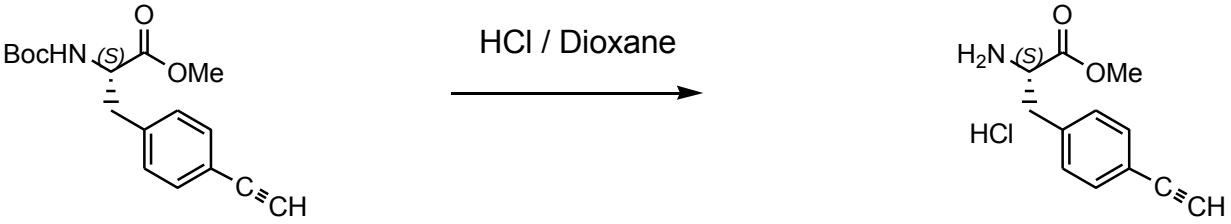
Step 2: Preparation of 2-tert-Butoxycarbonylamino-3-(4-ethynyl-phenyl)-propionic acid methyl ester



2-tert-Butoxycarbonylamino-3-(4-iodo-phenyl)-propionic acid methyl ester (12.7 g, 30.1 mmol) was mixed with triethylamine (200 mL) and then treated with CuI (0.900 g, 4.7 mmol), $PdCl_2(PPh_3)_2$ (0.480 g, 0.68 mmol) and stirred under a nitrogen atmosphere for 15 m at which time ethynyl-trimethyl-silane (9.3 g, 94.7 mmol) was added dropwise. The reaction mixture was stirred overnight at room temperature. The mixture was diluted with water (1 L), the pH was

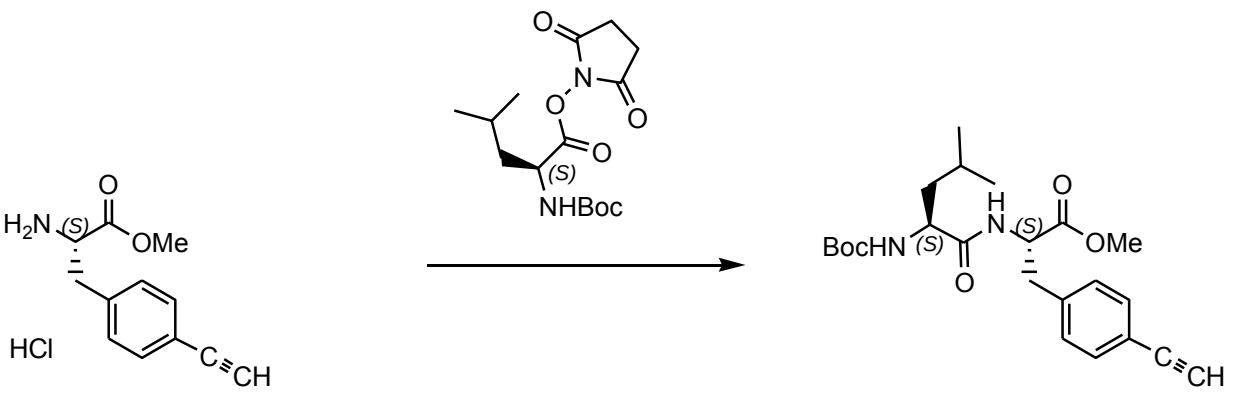
adjusted to ~7 with 1N H_2SO_4, extracted with ether (3 x 100 mL). The combined organic layers were washed with water followed by brine and the solution was dried and the solvent removed under reduced pressure. After drying in vacuo thoroughly, the crude product was dissolved in anhydrous THF (200 mL), cooled to -78 °C and treated with tetrabutylammonium fluoride trihydrate in THF (1M, 120 mL). The reaction was stirred at the same temperature for 2 h and then brought to room temperature, quenched with water (1 L) and extracted with ether (3 x 200 mL). The combined ether layers were washed with brine, dried and evaporated to dryness to afford 2-tert-butoxycarbonylamino-3-(4-ethynyl-phenyl)-propionic acid methyl ester which was purified by flash chromatography (7.5 g, 79% yield). LC/MS = 304.1 $[M+H]^+$.

Step 3: Preparation of 2-Amino-3-(4-ethynyl-phenyl)-propionic acid methyl ester



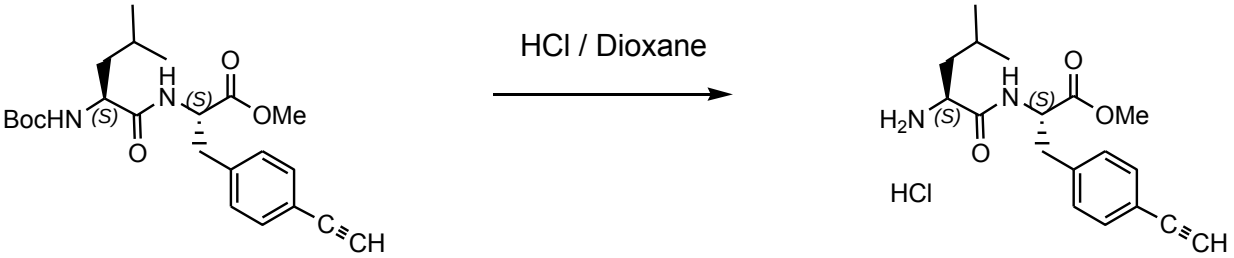
2-tert-Butoxycarbonylamino-3-(4-ethynyl-phenyl)-propionic acid methyl ester (7.5 g, 24.7 mmol) was dissolved in anhydrous dioxane (40 mL) and treated with HCl in dioxane (4N, 40 mL) at room temperature for 2 h. The solvent was concentrated at reduced pressure and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried overnight at high vacuum to afford 2-amino-3-(4-ethynyl-phenyl)-propionic acid methyl ester hydrochloride salt (5.8 g, 98%). LC/MS = 204.3 $[M+H]^+$.

Step 4: Preparation of 2-(2-tert-Butoxycarbonylamino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester



2-Amino-3-(4-ethynyl-phenyl)-propionic acid methyl ester hydrochloride (5.8 g, 24.2 mmol) was dissolved in anhydrous DMF (50 mL) and treated with diisopropylethylamine (9.38 g, 72.6 mmol). After stirring for 30 m, reaction mixture was treated with 2-tert-butoxycarbonylamino-4-methyl-pentanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (8.2 g, 25 mmol). The reaction mixture was stirred at room temperature overnight. The solution was poured into water (800 mL) and acidified to pH~2 with 1N H_2SO_4. The product was extracted with ethyl acetate 3x, washed with water, dried and evaporated to dryness. The residue was purified by flash chromatography to afford 2-(2-tert-Butoxycarbonylamino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester (9.27 g, 92% yield). LC/MS = 417.5 $[M+H]^+$.

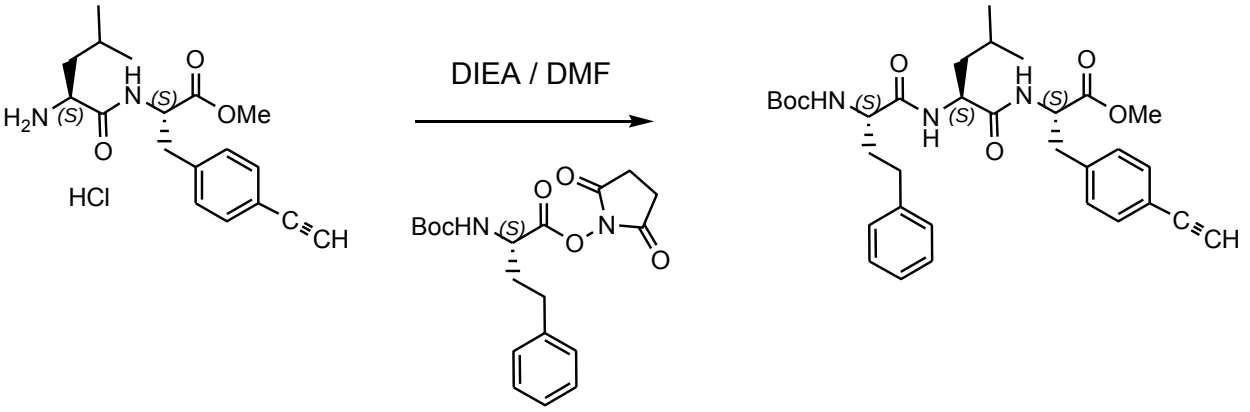
Step 5: Preparation of 2-(2-Amino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester



2-(2-tert-Butoxycarbonylamino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester (9.27 g, 22.26 mmol) was dissolved in anhydrous dioxane (100 mL) and treated with HCl in dioxane (4N, 100 mL) at room temperature for 2 h. The solvent was concentrated at reduced pressure and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried

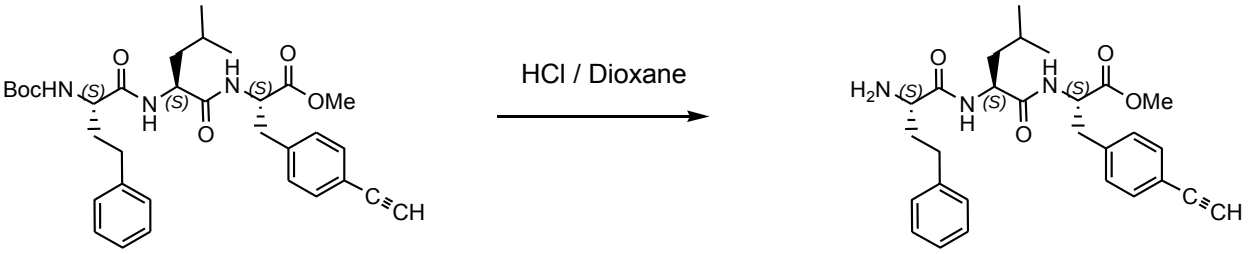
overnight at high vacuum to afford 2-(2-amino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester hydrochloride (7.8 g, 99% yield). LC/MS = 317.3 $[M+H]^+$.

Step 6: Preparation of 2-[2-(2-tert-Butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester



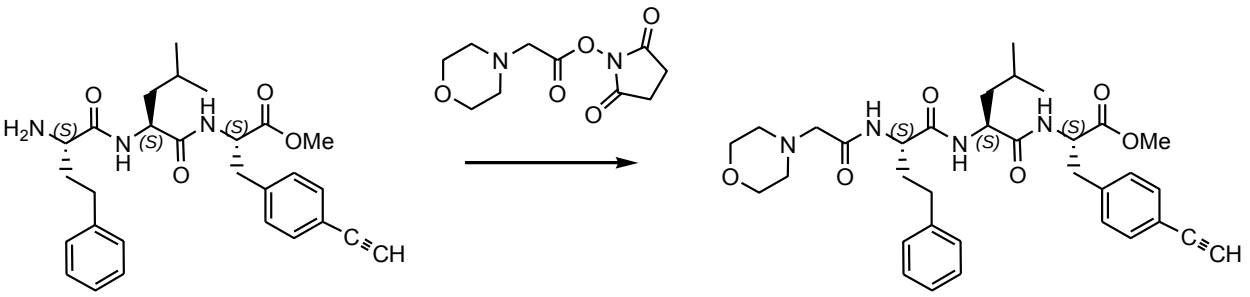
2-(2-Amino-4-methyl-pentanoylamino)-3-(4-ethynyl-phenyl)-propionic acid methyl ester (12 g, 34 mmol) was dissolved in anhydrous DMF (50 mL) and and treated with diisopropylethylamine (13.20 g, 102 mmol). After stirring for 30 m, the reaction mixture was treated with 2-tert-butoxycarbonylamino-4-phenyl-butyric acid 2,5-dioxo-pyrrolidin-1-yl ester (12.8 g, 34 mmol). The reaction mixture was stirred at room temperature overnight. The solution was poured into water (800 mL) and acidified to pH~2 with 1N H_2SO_4. The product was extracted with ethyl acetate 3x, washed with water, dried and evaporated to dryness. The residue was purified by flash chromatography to afford 2-[2-(2-tert-butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester (18.3 g, 93% yield). LC/MS = 578.6 $[M+H]^+$.

Step 7: Preparation of 2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester



2-[2-(2-tert-Butoxycarbonylamino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester (18.3 g, 31.7 mmol) was dissolved in anhydrous dioxane (250 mL) and treated with HCl in dioxane (4N, 100 mL) at room temperature for 4 h. The solvent was concentrated at reduced pressure and the product was precipitated by the addition of ether. The precipitate was filtered and washed first with anhydrous ether, and then with hexane. The solid was dried overnight at high vacuum to afford 2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester hydrochloride (15.6 g, 96% yield). LC/MS = 478.5 $[M+H]^+$.

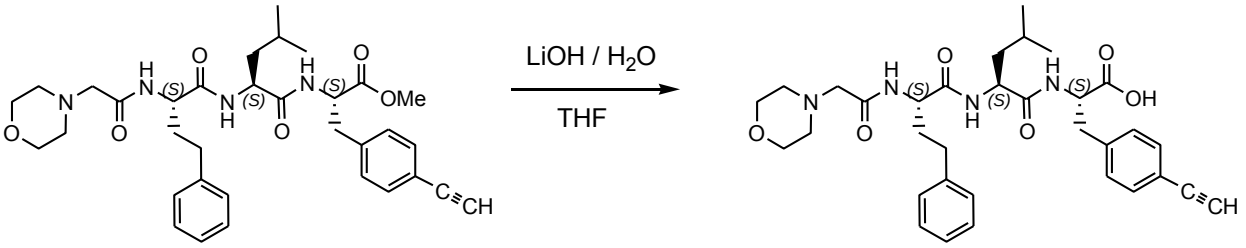
Step 8: Preparation of 3-(4-Ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetyl-amino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid methyl ester



2-[2-(2-Amino-4-phenyl-butyrylamino)-4-methyl-pentanoylamino]-3-(4-ethynyl-phenyl)-propionic acid methyl ester (15.6 g, 30.34 mmol) was dissolved in anhydrous DMF (70 mL) and diisopropylethylamine (7 mL) was added. Morpholin-4-yl-acetic acid 2,5-dioxo-pyrrolidin-1-yl ester (23 g, 95 mmol) was added and the reaction mixture was stirred for 4 h at room temperature at which time LC/MS analysis indicated that the reaction was complete. The

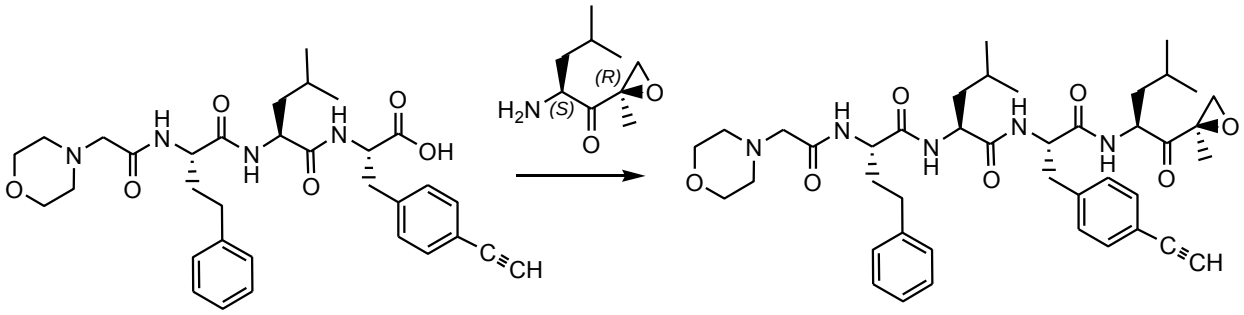
solvent was removed under reduced pressure and the residue was taken up in ethyl acetate. The precipitate which formed was filtered and discarded. The solution was washed with water (3 x), dried and evaporated. The residue was treated with isopropyl alcohol and evaporated to dryness (3 x). The residual solid was dried overnight at high vacuum to afford 3-(4-ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino] pentanoyl-amino}-propionic acid methyl ester (11.5 g, 62% yield) LC/MS = 605.6 $[M+H]^+$. Without further purification, the material thus obtained was used directly in the next step.

Step 9: Preparation of 3-(4-Ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid



3-(4-Ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid methyl ester (11.5 g, 19 mmol) was dissolved in THF (300 mL) and treated with lithium hydroxide hydrate (1.86 g, 41.8 mmol) in water (35 mL). The reaction mixture was stirred at room temperature for 2 h at which time LC/MS analysis indicated that reaction was complete. The solvent was removed and the residue partitioned between ethyl acetate (300 mL) and dilute HCl. The organic layer was washed with water, then brine and dried. Upon evaporation of the solvent, pure 3-(4-ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid was isolated (11 g, 98% yield). LC/MS = 591.3 $[M+H]^+$.

Step 10: Preparation of 4-Methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-ethynyl-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide



3-(4-Ethynyl-phenyl)-2-{4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoylamino}-propionic acid (8 g, 13.55 mmol) was dissolved in anhydrous DMF (80 mL) and mixed with BOP (6.183 g, 16.27 mmol) and 2-amino-4-methyl-1-(2-methyl-oxiranyl)-pentan-1-one (3.87 g, 13.55 mmol) (Kawamura et al., Bioorganic & Medicinal Chemistry 22,(12), 3091 – 3095 (2014)). At -20 °C diisopropylethylamine (7.549 g, 43.4 mmol) was added dropwise. The solution was stirred and allowed to rise to room temperature over a 1 h period. After stirring overnight at room temperature, the reaction mixture was diluted with ethyl acetate (800 mL) and extracted with water (100 mL x 3). The combined aqueous layers were back extracted with ethyl acetate (100 mL x 2) and the combined organic extracts were dried and evaporated to dryness to afford 15.529 of the crude product which was purified by preparative HPLC to afford pure 4-methyl-2-[2-(2-morpholin-4-yl-acetylamino)-4-phenyl-butyrylamino]-pentanoic acid {2-(4-ethynyl-phenyl)-1-[3-methyl-1-(2-methyl-oxiranecarbonyl)-butylcarbamoyl]-ethyl}-amide. (5.41 g)

NMR (DMSO-d6): δ 8.30 (d, NH), 8.08 (d, NH), 7.98 (d, NH), 7.89 (d, NH), 7.31 – 7.13 (m ArH x 9), 4.58 – 4.26 (m, N-CHR-CO x 4),4.10 (s, CH acetylene), 3.63 – 3.60 (m, CH_2-O-CH_2), 3.13 – 2.97 (m, $N(CH_2)_3$), 2.51 – 2.42 (m, $ArCH_2$ x 2), 2.76 + 2.45 (m, CH_2-oxirane),1.92 – 1.28 (m, CH_2 x 3, CH x 2), 1.40 (s, CH_3 x 1), 0.89 – 0.79 (m, CH_3 x 4, isopropyl). LC/MS = 744.5, 96.2% purity.