Photoaffinity labeling is a powerful tool to identify protein targets of biologically active small molecules and probe the structure of ligand binding sites, especially in the case of integral membrane proteins. A difficulty with photoaffinity labeling is that the small molecule must retain biological activity after derivatization with a photoreactive group such as benzophenone or 3-trifluoromethyl-3-phenyl diazirine. While these substituents are effective photocross-linkers and have been incorporated into small molecules, peptides, and proteins, their large size can potentially interfere with small molecule/protein interactions. The amino acid isosteres, photo-leucine and photo-methionine, have smaller alkyl diazirine side chains and were recently used to probe protein-protein interactions after random biosynthetic incorporation in cells or via site-specific native protein ligation. By contrast, the potential for incorporating these novel photoreactive amino acids into natural product scaffolds has not been explored. In this communication we report (1) an improved synthesis of photo-leucine, (2) incorporation of photo-leucine and an alkyne click chemistry tag into a cyclodepsipeptide inhibitor of protein secretion, and (3) identification of the inhibitor’s target by photoaffinity labeling.

HUN-7293 (1) is a fungal cyclodepsipeptide that was first identified as an inhibitor of vascular cell adhesion molecule (VCAM) expression. Recently, we and others discovered that related cyclodepsipeptides potently block the cotranslational translocation of VCAM and a subset of other proteins into the endoplasmic reticulum (ER), an early step in the biogenesis of secretory and membrane proteins. Biochemical experiments revealed that these compounds act at the ER membrane to perturb interactions between nascent ribosome-associated VCAM chains and the translocation channel. A heterotrimeric membrane protein, the Sec61 complex, forms the structural core of this channel and thus emerged as a potential direct target of 1.

To identify the target, we designed photoaffinity probe 2, in which photo-leucine replaces leucine at position 4 of 1. We substituted N-methylphenylalanine at position 5, previously shown to have a negligible effect on potency. A propargyl substituent was installed at position 1 to enable Cu(I)-catalyzed conjugation of a rhodamine−azide reporter (click chemistry) after photo-cross-linking under native conditions (Figure 1). These conservative modifications were aimed at preserving the biological potency of 1 while providing the necessary functionality for target identification.

Synthesis of 2 required Boc-(S)-photo-leucine (6), which we prepared via ozonolysis of commercially available 4, followed by formation of the diazirine by the method of Church and Weiss (Scheme 1). Boc-(S)-photo-leucine was efficiently coupled to peptides (EDCI-HOAt) or deprotected (4 N HCl) to give the free amino acid in quantitative yield. This route is a significant improvement over the original six-step synthesis of (S)-photo-leucine, which proceeded in low yield and required enzymatic resolution of a racemic intermediate.

We incubated a crude ER microsome fraction with 500 nM 2 and irradiated the mixture with 350 nm light for 1 min. Proteins were denatured in 1% sodium dodecylsulfate (SDS) and subjected to standard click chemistry conditions using a rhodamine−azide reporter (see Supporting Information). Following electrophoresis, in-gel fluorescent scanning revealed a major rhodamine-labeled protein with an apparent molecular weight of ~50 kDa (Figure 2A, lane 1). Labeling of this protein required both UV light (lane 2) and irradiation with ~350 nm light for 1 min. Proteins were denatured in 1% sodium dodecylsulfate (SDS) and subjected to standard click chemistry conditions using a rhodamine−azide reporter (see Supporting Information). Following electrophoresis, in-gel fluorescent scanning revealed a major rhodamine-labeled protein with an apparent molecular weight of ~50 kDa (Figure 2A, lane 1).
 Antibody raised against Sec61 of detergent extract of ER microsomes, a Sec61-depleted extract, or protein, we prepared proteoliposomes reconstituted from either a same relative mobility as Sec61 observed (lanes 5 and 6). To independently confirm that Sec61 is the photo-cross-linked protein, we prepared proteoliposomes reconstituted from either a detergent extract of ER microsomes, a Sec61-depleted extract, or a depleted extract replenished with purified Sec61 (lane 2) were photolyzed in the presence of 2 and analyzed by click chemistry/in-gel fluorescent scanning. The major cross-linked protein at ~50 kDa is shown (Figure 2B). To independently confirm that Sec61 is the photo-cross-linked protein, we prepared proteoliposomes reconstituted from either a detergent extract of ER microsomes, a Sec61-depleted extract, or a depleted extract replenished with purified Sec61 complex. Depletion of the intact Sec61 complex from the detergent extract by the rhodamine labeling of two additional proteins (lanes 2–4). Weak labeling of two additional proteins (~60 and ~40 kDa) was not competed by excess 3 and is likely nonspecific. Background labeling by the rhodamine–azide, independent of UV light or 2, was also observed (lanes 5 and 6).

The major cross-linked protein migrated on SDS gels with the same relative mobility as Sec61, the largest subunit of the Sec61 complex (Figure S2). Consistent with Sec61α as the primary target of 2, the ~50 kDa rhodamine-labeled protein was immuno-precipitated directly from the click reaction mixture with an antibody raised against Sec61α, but not with a control antibody (Figure 2B).

Photo-cross-linking of 2 to Sec61α is remarkably selective given the proteome complexity of the ER, which contains hundreds of membrane and luminal proteins; Sec61α is a relatively minor constituent (~0.7% of total ER protein). We attribute this high selectivity to two factors. First, due to its similar size, shape, and hydrophobicity, photo-leucine likely forms intimate contacts with Sec61α in a manner similar to the leucine side chain of 1. Second, the short lifetime (nanoseconds) of the carbene derived from 2 (Figure 1) ensures that molecules not bound to Sec61α are rapidly quenched by intramolecular rearrangement, solvent, or membrane lipids. Despite the short lifetime, the photo-cross-linking yield of 2 to Sec61α was estimated to be ~23% (Figure S4). Thus, depending on the specific application, alkyl diazirines may offer advantages over the widely used benzenophene cross-linker. The modular cyclodepsipeptide scaffold of 1 should facilitate installation of a diazirine at multiple positions. High-resolution mapping of the cross-linking site(s) by mass spectrometry will likely shed light on the mechanism by which these compounds selectively inhibit cotranslational translocation.

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Supporting Information Available: Detailed experimental section, synthesis, and spectral characterization of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References


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Supporting Information

Photo-leucine incorporation reveals the target of a cyclodepsipeptide inhibitor of cotranslational translocation

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I. Cell biological and photo-crosslinking experiments

Photo-crosslinking reactions in ER microsomes and reconstituted proteoliposomes

Canine ER microsomes that were stripped of ribosomes (prepared as previously described)\(^1\) were incubated with or without \(2\) (500 nM) and \(3\) (0–20 µM, final concentration of 4% DMSO in all samples) in physiological salt buffer (PSB, 50 mM Hepes pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate) containing 250 mM sucrose (37.5 µL reaction volume, 1 mg total protein/mL) for 1 h at RT in the dark. Samples were transferred to a single well of separate 96-well plates and photolyzed at RT. Photolysis was conducted using a Hg(Xe) lamp (Oriel Instruments, model 66923) operated at 1000 W using a filter with a maximum transmittance at 350 nm (#59810, Oriel Instruments) and a filter to absorb heat (#59044, Oriel Instruments). Samples were irradiated 6 cm from the source for 1 min (longer irradiation times did not increase the yield of crosslinking). A control sample was left in the dark. Following photolysis, the concentration of \(3\) was equalized in all samples (20 µM for Figure 2 and Figure S2) to control for the total concentration of alkyne present in the subsequent click reaction. Samples were then diluted to 50 µL with PSB and sedimented through a sucrose cushion (0.5 M sucrose in PSB, 200 µL) by centrifugation at 100,000 rpm for 45 min at 4 °C in a TLA 100 rotor. The cushion was removed, the membrane pellet was resuspended in
31.5 µL of phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, pH 7.4) and the membranes were transferred to a fresh tube for the click reaction (described below). Photo-crosslinking in reconstituted proteoliposomes was conducted as follows: proteoliposomes were incubated with 2 (250 nM) in PBS (20 µL reaction volume, 0.5 mg total protein/mL) for 1 h at RT in the dark. Samples were photolyzed as described above and 19.5 µL of each sample was transferred directly to a fresh tube for the click reaction (described below).

**Click Chemistry**

Click reactions were performed essentially as previously described.² Tris(benzyltriazolylmethyl) amine (TBTA) was synthesized as previously described.³ The rhodamine-azide reporter (Rox-N₃) was synthesized from 5-(and-6)-carboxy-X-rhodamine succinimidyl ester (Invitrogen) and 3-azido propylamine as previously described.² To the photo-crosslinked microsomes (prepared as described above) were added the following reagents in order: 1% SDS (10% stock in H₂O), 50 µM Rox-N₃ (2.5 mM stock in DMSO), 1 mM tris(carboxyethyl) phosphine (TCEP, 50 mM stock in H₂O, pH 7), 100 µM TBTA (1.7 mM stock in 4:1 tert-BuOH/H₂O), and lastly 1 mM CuSO₄ (50 mM stock in H₂O). Reactions were mixed gently and incubated at RT for 1 h. Laemmli sample buffer (5×, 7.5 µL) was added and the reactions were resolved by SDS-electrophoresis on 12% acrylamide mini-gels (BioRad). Gels were scanned for fluorescence (610 nm) using a Typhoon 9400 phosphorimager (Amersham).
Immunoprecipitation

ER microsomes were incubated with a saturating concentration of 2 (5 µM) in PSB containing 0.25 M sucrose (25 µL total reaction volume, 2 mg total protein/mL) for 1 h and subjected to photo-crosslinking and click reactions as described above. Following the click reaction, samples (25 µL) were diluted ten-fold with IP buffer (1% Triton X-100, 50 mM Hepes pH 7.4, 100 mM sodium chloride) and incubated with rabbit antiserum raised against Sec61α⁴ or non-immune rabbit serum (Animal Pharm Services) for 2 h at 4 °C. Protein A beads (50 µL of a 50% slurry, Amersham), pre-equilibrated in IP buffer, were added and samples were rotated overnight (18 h) at 4 °C. The beads were sedimented by centrifugation (2000 rpm, 1 min), the supernatants removed, and the beads washed (5×) with ice cold IP buffer (0.5 mL). Proteins were eluted with Laemmli sample buffer (1×, 50 µL) at RT for 1 h and analyzed by SDS-PAGE and in-gel fluorescent scanning.
Figure S1. Validation of 2 and 3 as inhibitors of VCAM expression. VCAM expression analysis in COS-7 cells was performed as previously described. Briefly, cells were transfected with a VCAM expression plasmid using Lipofectamine 2000 (Invitrogen), or not transfected (NT). 5 h after transfection, the media was changed to include HUN-7293 (1, a generous gift from Dale Boger, The Scripps Research Institute), 2, or 3 at the indicated concentrations. After 24 h, cells were harvested in lysis buffer (1% SDS, 0.1 M Tris-HCl pH 8.0) and equal amounts of total protein were resolved by SDS-PAGE and analyzed by immunoblotting with VCAM antibodies (α-VCAM, Santa Cruz Biotechnology). Under these conditions, compounds 1–3 had no obvious effect on cell viability.
Figure S2. Photo-crosslinking in ER microsomes. Photo-crosslinking and click reactions were conducted as described above. A portion (30 µL) of each reaction was resolved by SDS-PAGE and analyzed by in-gel fluorescent scanning (left panel) followed by Coomassie staining (right panel). A smaller portion (5 µL) of each reaction was separated by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blot (center panel) with Sec61α antibodies. Note that Sec61α runs at the same relative mobility as the major fluorescent band at ~50 kDa, but does not represent a major Coomassie-stained band.
Figure S3. Characterization of reconstituted proteoliposomes. A detergent extract of ER microsomes was reconstituted directly into proteoliposomes (control, lane 3) or first immunodepleted of the Sec61 complex using Sec61β antibodies as previously described. The Sec61-depleted extract was either reconstituted directly into proteoliposomes (lane 1) or first replenished with Sec61 complex (purified from canine pancreatic ER microsomes as previously described) (lane 2). Reconstituted proteoliposomes were analyzed by Coomassie staining (top panel) and immunoblotting against the indicated antigens (bottom panels). The Sec61 complex is depleted by ~90% (as judged by α and β subunits), while none of the other translocon components or total proteins are appreciably
affected. SRα (SRP receptor α, antibody from ref. 5), RbI (Ribophorin I, antibody was a gift from Reid Gilmore, University of Massachusetts Medical School, Worcester), CNX (Calnexin, antibody from Stressgen Biotechnologies), TRAPα (Translocon-associated protein complex subunit α, antibody from ref. 5), TRAM (Translocating-chain associating membrane protein, antibody was a gift from Peter Walter, University of California, San Francisco), Sec61α (antibody from ref. 4), Sec61β (antibody from ref. 5).
(A) Photo-crosslinking reaction

<table>
<thead>
<tr>
<th>Propargylated-RSK-1 CTD (pmol)</th>
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<td>0.91</td>
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(B) Standard Curve

- **Propargylated-RSK-1 CTD**
- **Sec61α**

\[ y = 2612.5x + 154.75 \]

\[ R^2 = 0.9983 \]
Figure S4. Estimated yield of photo-crosslinked Sec61α in ER microsomes. (A) Photo-crosslinking and click reactions were performed as described above. The total amount of Sec61 complex in the photo-crosslinking reaction (5.88 pmol) was calculated based on a previous estimate of 1.7 pmol Sec61 per "equivalent" of microsomes⁶ (defined in ref. 7). Propargylated RSK-1 CTD (C-terminal kinase domain, amino acids 410-735) was generated by incubating RSK-1 CTD (230 nM) with 1.5 equiv fmk-propargylamine (ref. 8) in PBS for 1.5 h at RT. These labeling conditions effect quantitative, stoichiometric modification of RSK-1 CTD with the propargylated fluoromethylketone inhibitor as determined by ESI-MS (ref. 8 and unpublished data). The propargylated RSK-1 CTD reference protein and the photo-crosslinking reaction were then subjected to the click reaction under standard conditions and the reactions quenched with Laemmli sample buffer. A dilution series of the Rox-labeled RSK-1 CTD was then analyzed by SDS-PAGE and in-gel fluorescence scanning (Lanes 2-8) and the background-corrected fluorescence intensities (ImageJ) were used to construct a standard curve (B). Using the fluorescence intensity of labeled Sec61α (Lane 1, asterisk) and the linear fitted equation to the standard curve, the amount of photo-crosslinked Sec61α was estimated at 1.32 pmol, corresponding to a photo-crosslinking yield of 23%.
II. Chemical synthesis

General

Materials obtained from commercial sources were reagent grade and used without further purification. 1-Ethyl-3-(3’-dimethylaminopropyl) carbodiimide (EDCI) and 1-hydroxy-7-azabenzotriazole (HOAt) were from AKSci. All other commercially available reagents were obtained from Sigma-Aldrich unless otherwise noted. (S)-2-hydroxypent-4-ynoic acid was prepared by diazotization of (S)-propargyl-glycine (Bachem) and was consistent with published data. Anhydrous solvents were obtained from a commercial drying/purification system (Glass Contour, Inc., Laguna Beach, CA).

Air- and water-sensitive reactions were conducted under an inert argon atmosphere in flame-dried glassware. Reactions were monitored by analytical thin layer chromatography (TLC) on silica gel 60 F₂₅₄ glass plates (EM Science, 0.25 mm) and by LC-ESI-MS using a Waters 2695 Separations Module (Xterra MS C18 column (Waters), flow rate 0.2 mL/min) connected inline to a Waters ZQ mass detector. Preparative reverse phase high performance liquid chromatography (HPLC) was carried out on a Peeke Scientific Combi-A 5 µM preparative C18 column (50 × 22 mm, flow rate 10 mL/min) using a Varian Prostar 210 solvent delivery system equipped with a UV/vis detector (monitoring at 220 and 245 nm). Silica gel chromatography was conducted with 230-400 mesh silica gel (EM Scientific). All ¹H and ¹³C NMR spectra were recorded on a Varian 400 MHz spectrometer. ¹H spectra are referenced to TMS at 0.00 ppm, and ¹³C spectra are referenced to the center of the CDCl₃ triplet at 77.16 ppm. High resolution MS data were collected at the UCSF Mass Spectrometry Facility (HR-EI-MS spectra were acquired on a VG70-SE instrument from MicroMass, Manchester, UK; HR-FT-MS
spectra were acquired on a Finnigan LTQ-FTICR instrument from Thermoscientific, Waltham, MA).

Solution phase synthesis of 2 and 3 was conducted as previously described for the synthesis HUN-7293 (1) and its analogs,\textsuperscript{11} with the exception of the linear heptadepsipeptide as described below.

\[(S)-\text{Boc-photo-leucine, 6}\]

\((S)\)-Boc-4-oxo-norvaline (5) was synthesized by the method of Cornish \textit{et al.}\textsuperscript{12} (S)-Boc-4,5-dehydro-leucine (4, ChemImpex, 1.616 g, 7.05 mmol) was dissolved in DCM (45 mL, 0.16 M) and cooled to \(-78\) °C. \(\text{O}_3/\text{O}_2\) gas was slowly bubbled through the solution (0.5 L/min, \(~0.5\) g \(\text{O}_3/h\)) until a purple color formed (\(~1.5\) h). Nitrogen was then passed through the solution until it returned to a colorless mixture. Dimethylsulfide (1.095 g, 17.62 mmol) was added and the reaction was warmed to RT and stirred overnight (18 h). The solvent and excess dimethylsulfide were removed \textit{in vacuo} and the amber colored residue was chromatographed on silica gel (5\% MeOH in DCM, 0.1\% AcOH) to yield 1.30 g (80\%) of (S)-Boc-4-oxo-norvaline (5) as a transparent oil. \(^1\text{H}\) and \(^{13}\text{C}\) NMR of 5 were consistent with published data.\textsuperscript{13}

Diazirine formation followed the general procedure described by Church and Weiss.\textsuperscript{14} (S)-Boc-4-oxo-norvaline (5, 0.480 g, 2.08 mmol) was charged into a three-necked flask and azeotroped (3×) with dry toluene. The flask was then fitted with a dry-ice condenser and ammonia (15 mL, 0.14 M) was slowly condensed into the flask. The
solution was refluxed for 5 h with stirring, cooled to –78 °C, and a solution of hydroxylamine-\(O\)-sulfonic acid (HOSA) in MeOH (0.270 g, 2.39 mmol, 1.84 M) was added drop-wise over 20 min. The ice bath was then removed and the mixture was held at reflux temperature for an additional 1 h. Anhydrous MeOH (3 mL) was then added, the reaction was warmed to RT and stirred overnight. The following day, the slurry was filtered, the filter cake was washed with MeOH (25 mL), and the combined washes were treated with triethylamine (0.210 g, 2.08 mmol) and concentrated to 8 mL. An additional equivalent of triethylamine (0.210 g, 2.08 mmol) was then added, the solution was cooled to 0 °C and titrated with a solution of \(I_2\) in MeOH (0.1 M). When a slight orange color persisted, the solvent was removed, the residue brought up in dH\(2\)O (20 mL) and acidified to pH 2 with 1 M HCl. The aqueous phase was extracted with three portions of EtOAc (20 mL) and the combined organic fractions were washed with saturated NaCl solution (1×), dried over Na\(_2\)SO\(_4\), filtered, and the solvent removed. The resulting orange solid was purified by silica chromatography (gradient elution 0%–2.5% MeOH in DCM, 0.1% AcOH) to yield 0.280 g (55%) of 6 as a slightly yellow gum. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 5.07 (d, \(J = 7.4\) Hz, 1H), 4.28–4.38 (m, 1H), 2.06 (dd, \(J = 15.1, 4.1\) Hz, 1H), 1.61 (dd, \(J = 15.1, 8.8\) Hz, 1H), 1.48 (s, 9H), 1.10 (s, 3H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 176.2, 155.5, 80.8, 50.4, 37.7, 28.4, 23.9, 19.8. HR-FT-MS \(m/z\) 244.1292 ([M+H]\(^+\), \(C_{10}H_{18}N_3O_4\) requires 244.1297).
To a stirred solution of Boc-(S)-photo-leucine (6, 119.5 mg, 0.491 mmol) and N-methyl-L-phenylalanine methyl ester hydrochloride (Bachem) (124.1 mg, 0.540 mmol) in DCM/DMF (5:1, 5 mL, 0.1 M) at 0 °C was added HOAt (73.5 mg, 0.540 mmol), EDCI (188.2 mg, 0.982 mmol) and NaHCO₃ (45.4 mg, 0.540 mmol). The ice bath was removed and the reaction was warmed to RT and stirred overnight (18 h). Workup and removal of the solvent as previously described,¹¹ yielded a residue that was chromatographed on silica gel (20% EtOAc in hexanes) to yield 124.3 mg (60%) of 7 as a transparent gum that solidified at −20 °C. ¹H NMR (400 MHz, CDCl₃, major rotamer): δ 7.15–7.38 (m, 5H), 5.31 (dd, J = 10.5, 5.5 Hz, 1H), 4.83 (br d, J = 9.5 Hz, 1H), 4.51–4.57 (m, 1H), 3.73 (s, 3H), 3.39 (dd, J = 14.6, 5.5 Hz, 1H), 3.02 (dd, J = 14.6, 10.5 Hz, 1H), 2.93 (s, 3H), 1.78 (dd, J = 15.0, 5.3 Hz, 1H), 1.44 (s, 9H), 1.32 (dd, J = 15.0, 8.6 Hz, 1H), 1.05 (s, 3H). ¹³C NMR (100 MHz, CDCl₃, major rotamer): δ 171.9, 170.9, 155.0, 136.7, 128.9, 128.6, 127.0, 80.0, 58.6, 52.5, 46.8, 38.5 34.6, 32.7, 28.4, 23.7, 20.0. HR-EI-MS m/z 419.2281 ([M+H]+, C₂₁H₃₁N₄O₅ requires 419.2294).
To a stirred solution of $N$-[(2S,4R)-2-$N$-(tert-Butoxycarbonyl)amino]-4-methyloctanoyl]-$N$-methyl-L-leucine tert-butyl ester hydrochloride$^{11}$ (138.0 mg 0.351 mmol) and (S)-2-hydroxypent-4-ynoic acid (40.0 mg, 0.351 mmol) in DCM/DMF (5:1, 3.5 mL, 0.1 M) at 0 °C was added HOAt (52.5 mg, 0.386 mmol), EDCI (134.6 mg, 0.704 mmol), and 2,6-lutidine (41.0 µL, 0.354 mmol). The solution was warmed to RT and stirred overnight (18 h). After workup and removal of the solvent as previously described,$^{11}$ the residue was chromatographed on silica gel (30% EtOAc in hexane) to yield 109.5 mg (72%) of 8 as a transparent oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.35 (br d, $J = 8.8$ Hz, 1H), 5.18 (dd, $J = 10.5$, 5.2 Hz, 1H), 4.98–5.04 (m, 1H), 4.25 (q, $J = 5.5$ Hz, 1H), 3.85 (d, $J = 5.5$ Hz, 1H), 2.98 (s, 3H), 2.63–2.78 (m, 2H), 2.06 (t, $J = 2.6$ Hz, 1H), 1.43–1.76 (m, 6H), 1.44 (s, 9H), 1.14–1.35 (m, 6H), 1.0 (d, $J = 6.4$ Hz, 3H), 0.93 (d, $J = 6.7$ Hz, 3H), 0.82–0.91 (m, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 173.5, 171.8, 170.7, 81.8, 79.8, 71.4, 70.0, 55.4, 47.5, 40.1, 37.5, 37.2, 31.0, 29.5, 29.3, 28.2, 25.3, 25.0, 23.4, 23.0, 21.6, 19.2, 14.2. HR-EI-MS $m/z$ 452.3250 ([M]$^+$, C$_{25}$H$_{44}$N$_2$O$_5$ requires 452.3250).
To a stirred solution of 7 (120.0 mg, 0.287 mmol) in tert-BuOH/H₂O (2:1, 2.8 mL, 0.1 M) at 0 °C was added LiOH·H₂O powder (24.1 mg, 0.573 mmol) and the reaction was stirred until no starting material was detected by TLC (2 h). Workup of the reaction as previously described¹¹ yielded 110 mg (95%) of the carboxylic acid of 7 as a white solid that was used without further purification. To a stirred solution of the carboxylic acid derived from 7 (72.6 mg, 0.179 mmol) and N-[(2S,4R)-2-amino-4-methyloctanoyl]-N-methyl-L-alanine methyl ester hydrochloride¹¹ (63.0 mg, 0.204 mmol) in DCM/DMF (5:1, 2 mL, 0.1 M) at −30 °C was added HOAt (29.2 mg, 0.215 mmol), EDCI (68.8 mg, 0.359 mmol), and 2,6-lutidine (23.0 µL, 0.204 mmol). The mixture was stirred at −30 °C for 5 h and was then warmed to RT and stirred overnight (18 h). After workup and removal of the solvent as previously described,¹¹ the residue was purified by silica gel chromatography (33% EtOAc in hexane) to yield 82.1 mg (70%) of 9 as a transparent gum. ¹H NMR (400 MHz, CDCl₃, two rotamers): δ 8.13 (d, J = 8.5 Hz, 1H), 8.09 (d, J = 8.5 Hz, 1H), 7.15–7.42 (m, 10H), 6.70 (d, J = 8.8 Hz, 1H), 6.68 (d, J = 9.1 Hz, 1H), 5.34–5.46 (m, 2H), 5.18–5.30 (m, 1H), 5.06 (dt, J = 8.8, 4.1, 1H), 4.97–5.02 (m, 1H), 4.72–4.90 (m, 2H), 4.42 (q, J = 7.4 Hz, 1H), 3.71 (s, 3H), 3.70 (s, 3H), 3.63–3.70 (m, 1H, partially overlapped), 3.30 (dd, J = 14.5, 6.5 Hz, 1H), 3.07 (s, 3H), 2.96–3.02 (m, 1H, partially overlapped), 2.98 (s, 3H), 2.96 (s, 3H), 2.89 (s, 3H), 1.91 (br s, 1H), 1.83 (dd, J = 14.6, 6.5 Hz, 1H), 1.55–1.67 (m, 2H), 1.46–1.53 (m, 2H), 1.45 (s, 9H), 1.44 (s, 9H),
1.38–1.43 (m, 10H), 1.12–1.33 (m, 12H), 1.06 (s, 3H), 1.00 (d, J = 5.8 Hz, 3H), 0.94 (d, J = 6.3 Hz, 3H), 0.85–0.91 (m, 6H), 0.76 (s, 3H), –0.07–0.06 (m, 1H). 13C NMR (100 MHz, CDCl3, two rotamers): δ 172.9, 172.8, 172.6, 172.4, 172.2, 172.0, 169.4, 168.9, 156.0, 154.9, 137.7, 136.9, 129.7, 129.5, 129.1, 128.6, 127.1, 126.9, 81.2, 80.2, 62.3, 58.0, 52.4, 52.3, 52.1, 51.9, 47.7, 47.6, 46.8, 45.7, 40.3, 39.6, 38.2, 37.5, 37.3, 36.0, 34.5, 34.1, 31.4, 31.1, 31.0, 29.8, 29.6, 29.5, 29.4, 29.3, 28.5, 28.5, 24.0, 23.4, 23.1, 20.2, 19.7, 19.2, 19.1, 14.5, 14.4, 14.2, 14.2. HR-EI-MS m/z 658.4047 ([M]+, C34H54N6O7 requires 658.4053).

To a stirred solution of 9 (47.0 mg, 73.1 µmol) in tert-BuOH/H2O (2:1, 0.75 mL, 0.1 M) at 0 °C was added LiOH·H2O powder (6.0 mg, 143.0 µmol) and the reaction was stirred until no starting material was detected by TLC (1 h). Workup of the reaction as previously described11 yielded the carboxylic acid of 9 as a transparent gum that was used directly in the next step without further purification. Polystyrene-immobilized triphenyl phosphine (Fluka, cat. #93093) was washed alternately (3×) with THF (2 mL) and MeOH (2 mL) followed by two additional washes with THF (2 mL) and was dried under vacuum. The washed beads (104.0 mg, 312 µmol) were then added to a stirred solution of the carboxylic acid derived from 9 (46.0 mg, 71.3 µmol) and tripeptide 8 (32.3 mg, 71.3 µmol) in dry THF (1 mL, 0.07 M) and the mixture was stirred at RT for 45 min.
Diisopropyl azodicarboxylate (DIAD) (63.0 mg, 312 \( \mu \)mols) was added and the mixture was stirred overnight at RT (18 h). The following day the mixture was filtered, the polystyrene beads were washed with THF (4 mL), and the solvent was removed. The residue was purified by preparative HPLC (MeOH/H\(_2\)O, linear gradient elution: 30/70 to 65/35 at 5 min, then 65/35 to 90/10 at 40 min, then 95/5 to 100/0 at 45 min, \( R_t = 44.4 \) min) to yield 53.0 mg (70%) of 10 as a white solid. \(^1\)H NMR (CDCl\(_3\), 400 MHz, two rotamers): \( \delta \) 8.11 (d, \( J = 8.1 \) Hz, 1H), 8.07 (d, \( J = 8.5 \) Hz, 1H), 7.70 (d, \( J = 9.2 \) Hz, 1H), 7.50 (d, \( J = 9.0 \) Hz, 1H), 7.10–7.45 (m, 10H), 7.03 (d, \( J = 7.6 \) Hz, 1H), 7.00 (d, \( J = 5.0 \) Hz, 1H), 5.42–5.51 (m, 1H), 5.17–5.31 (m, 3H), 4.80–5.12 (m, 6H), 4.43–4.56 (m, 2H), 4.20–4.32 (m, 1H), 3.70–3.77 (m, 1H), 3.17–3.32 (m, 2H), 3.14 (s, 3H), 3.12 (s, 3H), 2.99–3.11 (m, 2H, overlapped), 2.98 (s, 3H), 2.96 (s, 3H), 2.94 (s, 3H), 2.89 (s, 3H), 2.73–2.85 (m, 3H), 2.59 (ddd, \( J = 15.0, 8.2, 2.4 \) Hz, 1H), 1.95 (t, \( J = 2.5 \) Hz, 1H), 1.94 (t, \( J = 2.7 \) Hz, 1H), 1.53–1.71 (m, 21H), 1.49 (d, \( J = 7.3 \) Hz, 6H), 1.45 (s, 18H), 1.44 (s, 18H), 1.12–1.32 (m, 24H), 1.05 (s, 3H), 0.87–1.03 (m, 36H), 0.76 (s, 3H), 0.11 (d, \( J = 15.5 \) Hz, 1H). HR-FT-MS \( m/z \) 1079.7141 ([M+H]\(^+\), \( C_{58}H_{95}N_8O_{11} \) requires 1079.7120).
To a stirred solution of 10 (15.0 mg, 13.9 µmol) in DCM (2.3 mL, 6 mM) at 0 °C were added TFA (0.460 mL) and anisole (three drops) and the reaction was stirred at 0 °C until the N-Boc and tert-butyl ester protecting groups were removed (as determined by LC-ESI-MS) (2 h). The solvent was removed with a stream of argon and the residue was dried under vacuum. The resulting solid was then treated at 0 °C with 4.0 M HCl/EtOAc (3 mL) for 30 sec. The solvent was again removed with a stream of argon and the residue was azeotroped (2×) with toluene. The residue was dissolved in DMF (14 mL, 1 mM), cooled to 0 °C, and treated with DIPEA (4.9 µL, 27.8 µmol) and diphenylphosphorylazide (DPPA) (6.0 µL, 27.8 µmol) and the reaction was stirred at 4 °C for 100 h. After removal of the solvent by rotary evaporation, the residue was purified by preparative HPLC (MeOH/H₂O linear gradient elution: 30/70 to 70/30 at 5 min, then 70/30 to 90/10 at 40 min, then 90/10 to 100/0 at 50 min. Rₜ = 34.5 min) to yield 4.7 mg (37%) of 2 as a white solid. ¹H NMR (CDCl₃, 400 MHz, major rotamer): δ 8.44 (d, J = 10.0 Hz, 1H), 7.81 (d, J = 9.7 Hz 1H), 7.09–7.27 (m, 5H), 6.03 (d, J = 5.9 Hz, 1H), 5.21–5.27 (m, 1H), 5.13 (dd, J = 11.8, 3.2 Hz, 1H), 4.95–5.07 (m, 2H), 4.79–4.93 (m, 2H), 4.55 (dd, J = 7.1, 4.0 Hz, 1H), 3.58–3.80 (m, 2H), 3.20 (s, 3H), 2.91–3.09 (m, 2H), 2.88 (s, 3H), 2.51 (s, 3H), 2.10 (t, J = 2.4 Hz, 1H), 1.74–1.91 (m, 3H), 1.58 (d, J
= 7.0 Hz, 3H), 1.45–1.55 (m, 7H, overlapped), 1.14–1.37 (m, 12H), 1.11 (d, J = 6.3 Hz, 3H), 1.02 (d, J = 6.6 Hz, 3H), 0.92–0.99 (m, 6H), 0.86–0.91 (m, 6H), 0.78 (s, 3H), 0.19 (dd, J = 15.7, 2.9 Hz, 1H). HR-FT-MS m/z 905.5884 ([M+H]⁺, C₄₉H₇₇N₈O₈ requires 905.5864).

To a stirred solution of N-[(2S,4R)-2-[N-[N-(tert-Butoxycarbonyl)-L-leucinyl]-N-methyl-L-phenylalanyl]amino-4-methyloctanoyl]-L-alanine methyl ester¹¹ (106.0 mg, 164.0 µmol) in tert-BuOH/H₂O (2:1, 1.7 mL, 0.1 M) at 0 °C was added LiOH·H₂O powder (14.0 mg, 328 µmol) and the reaction was stirred until no starting material was detected by TLC (2 h). Workup of the reaction as previously described¹¹ yielded the carboxylic acid that was used directly without further purification. To a stirred solution of the crude carboxylic acid (95.0 mg, 150 µmol) and tripeptide 8 (68.0 mg, 150 µmol) in toluene (3 mL, 0.05 M) was added pre-washed polystyrene-immobilized triphenyl phosphine (350.0 mg, 1.05 mmol, washing done as described above) and the reaction was stirred at RT for 45 min. Diisopropyl azodicarboxylate (DIAD) (212.3 mg, 1.05 mmol) was then added and the mixture was stirred overnight at RT (18 h). The following day the mixture was filtered, the polystyrene beads were washed with toluene and the solvent was removed. The residue was purified by preparative HPLC (MeOH/H₂O linear gradient
elution: 60/40 to 100/0 over 23 min, $R_t = 18.5$ min) to yield 69.0 mg (43%) of 11 as an off-white solid. $^1$H NMR (400 MHz, CDCl$_3$ two rotamers): $\delta$ 8.58 (d, $J = 9.5$ Hz, 1H), 8.21 (d, $J = 8.3$ Hz, 1H), 7.85 (d, $J = 8.6$ Hz, 1H), 7.43 (d, $J = 8.2$ Hz, 1H), 7.03–7.38 (m, 10H), 6.67 (d, $J = 6.6$ Hz, 1H), 6.65 (d, $J = 7.5$ Hz, 1H), 5.37–5.48 (m, 1H), 5.09–5.32 (m, 4H), 5.32–4.74 (m, 6H), 4.37–4.59 (m, 2H), 4.03–4.19 (m, 1H), 3.24 (s, 3H), 3.13–3.30 (m, 4H, partially overlapped), 3.11 (s, 3H), 2.99 (s, 3H), 2.98 (s, 3H), 2.95 (s, 3H), 2.94 (s, 3H), 2.74–2.93 (m, 3H), 2.56 (ddd, $J = 17.4$, 8.2, 2.5 Hz, 1H), 1.94 (t, $J = 2.5$ Hz, 1H), 1.90 (t, $J = 2.1$ Hz, 1H), 1.23–1.77 (m, 23H, partially overlapped), 1.50 (d, $J = 7.1$ Hz, 3H), 1.47 (d, $J = 7.3$, 3H), 1.45 (s, 18H), 1.41 (s, 18H), 1.18–1.26 (m, 24H), 0.83–1.03 (m, 36H), 0.78 (d, $J = 6.3$ Hz, 6H), 0.66 (d, $J = 6.6$ Hz, 3H), 0.62 (d, $J = 6.5$ Hz, 3H), –0.31– –0.17 (m, 1H). HR-FT-MS $m/z$ 1067.7371 ([M+1]$^+$, C$_{59}$H$_{99}$N$_{6}$O$_{11}$ requires 1067.7371).

![Chemical Structure](image)

To a stirred solution of 11 (28.8 mg, 27.0 µmol) in DCM (4.2 mL, 6 mM) at 0 °C were added TFA (0.833 mL) and anisole (three drops) and the reaction was stirred until the N-Boc and tert-butyl ester protecting groups were removed (as determined by LC-ESI-MS) (2 h). The solvent was removed with a stream of argon, the residue was dried under vacuum and then brought up in 4.0 M HCl/EtOAc (1 mL) for 30 sec. The solvent was again removed with a stream of argon and the residue azeotroped (2x) with toluene. The residue was then dissolved in DMF (25 mL, 1 mM), cooled to 0 °C, and treated with
DIPEA (28.2 µL, 162 µmol) and diphenylphosphorylazide (DPPA) (17.4 µL, 81.0 µmol) and the reaction was stirred at 4 °C for 190 h. After removal of the solvent by rotary evaporation, the residue was purified by preparative HPLC (MeOH/H₂O linear gradient elution: 50/50 to 100/0 over 30 minutes, %R: 21.8 min) to yield 8.9 mg (37%) of 3 as a white solid. ¹H NMR (400 MHz, CDCl₃, major rotamer): δ 8.53 (d, J = 10.3 Hz, 1H), 7.81 (d, 9.5 Hz, 1H), 7.07–7.38 (m, 5H), 6.07 (d, J = 6.3 Hz, 1H), 5.12 (dd, J = 11.6, 2.9 Hz, 1H), 4.93–5.03 (m, 2H), 4.83–4.92 (m, 2H), 4.54 (dd, J = 11.0, 3.7 Hz, 1H), 4.22–4.32 (m, 1H), 3.71–3.78 (m, 1H), 3.55–3.63 (m, 1H), 3.07–3.17 (m, 2H), 3.06 (s, 3H), 2.91 (s, 3H), 2.54 (s, 3H), 2.01 (t, J = 2.5 Hz, 1H), 1.79–1.90 (m, 4H), 1.55 (d, J = 6.9 Hz, 3H), 1.37–1.65 (m, 7H), 1.16–1.37 (m, 12H), 1.07 (d, J = 6.5 Hz, 3H), 1.00 (d, J = 6.7 Hz, 3H), 0.97 (d, J = 6.3 Hz, 3H), 0.85–0.95 (m, 9H), 0.77 (d, J = 6.3 Hz, 3H), 0.64 (d, J = 6.4 Hz, 3H), 0.03–0.09 (m, 1H). HR-FT-MS m/z 893.6132 ([M+H]⁺, C₅₀H₈₁N₆O₈ requires 893.6116).

III. References Cited


