

A Fragment-Based Strategy for Investigating and Suppressing the Efflux of Bioactive Small Molecules

Corey L. Compton, Daniel W. Carney, Patrice V. Grooms, Jason K. Sello*

Department of Chemistry, Brown University, 324 Brook Street, Providence, RI 02912

jason_sello@brown.edu

Supporting Information

Table of Contents:

Strains and Growth Conditions	S2
Plasmids and Primers	S3
Construction of Strains	S5
Antibacterial Assays	S5
Toxicity Data for ADEP Potentiator in <i>S. coelicolor</i>	S7
Transcriptional Analyses of Efflux Pump Genes in <i>S. coelicolor</i>	S8
Chemical Synthesis of ADEP Fragments	S9
Spectra	S17

Strains, Media and Culture Conditions. *Streptomyces coelicolor* strains were grown at 30°C on mannitol soy flour medium (SFM), Difco nutrient agar medium (DNA), yeast extract-malt extract medium (YEME), or minimal liquid medium (NMMP). SFM was used for conjugations between *S. coelicolor* and *Escherichia coli* and for generating spore stocks. *Escherichia coli* strains DH5α and ET12567/pUZ8002 were grown on Luria-Bertani medium at 37°C for routine sub-cloning. For the selection of *E. coli*, ampicillin, apramycin, chloramphenicol, hygromycin, and kanamycin were employed at 100, 50, 25, 80, and 50 µg/ml, respectively. Nalidixic acid was used at 20 µg/ml to counterselect *E. coli* in conjugations with *S. coelicolor*. Apramycin and hygromycin were used at 50 µg/ml for the selection of *S. coelicolor* exconjugants. *Streptomyces coelicolor* M145 was grown at 30 °C on mannitol soya flour medium. *Mycobacterium smegmatis* MC2155 was grown in Luria-Bertani medium supplemented with 0.2% (v/v) glycerol at 37 °C. *Mycobacterium tuberculosis* H37Rv was maintained in Middlebrook 7H9 broth supplemented with 0.2% (v/v) glycerol, 0.05% Tween 80, and 10% (v/v) oleic acid-albumin-dextrose-catalase.

Table S1. Strains Used in this Study

Strain	Description	Source/Reference
<i>Streptomyces coelicolor</i> A3(2)		
M145	Prototroph SCP1-, SCP2-	
B836	M145 $\Delta 1718::apr$	This Study
B837	M145 $\Delta 1719-20::apr$	This Study
B838	M145 $\Delta 1718::apr -pJS876$	This Study
B839	M145 $\Delta 1719-20::apr -pJS877$	This Study
B840	M145 WT – PJS 381	This Study
<i>Escherichia coli</i>		
DH5α	F ⁻ $\phi 80lacZ\Delta M15 \Delta(lacZYA-argF)$ U169 recA1 endA1 hsdR17 (r _K ⁻ m _K ⁻) phoA supE44 thi-1 gyrA96 relA1 λ	Invitrogen
ET12567	<i>dam, dcm, hsdS, cat, tet</i>	
BW25113	$\Delta(araD-araB)567$ $\Delta lacZ4787(::rrnB-4) lacIp-4000(lacIQ)$, λ^- , <i>rpoS</i> 369(Am), <i>rph</i> -1, $\Delta(rhaD-rhaB)568$, <i>hsdR</i> 514	
<i>Mycobacterium</i>		
<i>M. smegmatis</i> MC2155		ATCC
<i>M. tuberculosis</i> H37Rv		Broad Institute

Table S2. Plasmids Used in this Study

Plasmid	Description	Source/Reference
<i>pIJ790</i>	<i>[oriR101], [repA101(ts)], araBp-gam-bet-exo, Chl^R</i>	1
<i>plj10257</i>	oriT, Φ BT1 attB-int, Hyg ^r , ermEp*, derivative of pMS81	1
pUZ8002	RP4 Derivative, OriT-, Kan ^R	1
pMS81	<i>OriT, ΦBT1 attB-int, Hyg^R</i>	1
pBlueScript KS+	pUC <i>ori</i> , MCS, Amp ^R	Agilent Technologies (Stratagene)
pJS876	Stl11 Δ SCO1718:: <i>apr</i>	This Study
pJS877	Stl11 Δ SCO1719-20:: <i>apr</i>	This Study
pJS378	<i>SCO1718 cloned by pcr into pBS, Amp^R</i>	This Study
pJS379	SCO 1718 subcloned from pJS378 into <i>plj10257, hyg^R</i>	This Study
PJS380	<i>SCO1719-20 cloned by pcr into pBS, Amp^R</i>	This Study
pJS381	SCO 1719-20 subcloned from PJS380 into <i>plj10257, hyg^R</i>	This Study

Table S3. Primers Used in this Study

Primer Name	Application / Function	Sequence
SCO 1718 KO R	PCR targeting/disruption of <i>SCO 1718</i>	CGCTGCTGTGACCCCCAGCCGGATCGGCTC CCCTGCTCATCTAGATGTAGGCTGGAGCTGC TTC
SCO 1718 KO F	PCR targeting/disruption of <i>SCO 1718</i>	TGTACGTAAGTAGGATGGTGGCCGAGGTGA TGCACGATGTCTAGAATTCCGGGGATCCGT CGACC
SCO 1719-20 KO R	PCR targeting/disruption of <i>SCO 1719-20</i>	TAGTTACGTACACCGTACGTAGTGCGCTATG GTCGGCACTCTAGAATTCCGGGGATCCGTC GACC
SCO 1719-20 KO F	PCR targeting/disruption of <i>SCO 1719-20</i>	CCTCCCCACCGGCGGTGACGCGGTACCCCG GCCCGTTCATCTAGATGTAGGCTGGAGCTG CTTC
SCO 1718 KO Det R	Verification of <i>SCO 1718</i> replacement with <i>apr</i>	GGTCACGGAGATCAC
SCO 1718 KO Det F	Verification of <i>SCO 1718</i> replacement with <i>apr</i>	GTCATGTGCCGACCATAGC
SCO 1719-20 KO Det F	Verification of <i>SCO 1719-20</i> replacement with <i>apr</i>	GACCAGATCACTTCGGGTACG
SCO 1719-20 KO Det R	Verification of <i>SCO 1719-20</i> replacement with <i>apr</i>	GCACTCGTCCGTCATCC
<i>clpP1clpP2</i> RT-PCR F	Detection of <i>clpP1clpP2</i> transcript	CACAGCTGCTGCTCCTTG
<i>clpP1clpP2</i> RT-PCR R	Detection of <i>clpP1clpP2</i> transcript	GTCGATGAGGCCGTACTC
<i>1719-20</i> RT-PCR F	Detection of <i>SCO 1719-20</i> transcript	CACGCAGTACCTGGAGGAG
<i>1719-20</i> RT-PCR R	Detection of <i>SCO 1719-20</i> transcript	GGAGGAAGACGTCGTCGAG
<i>SCO 1719-20 Overexpression F</i>	cloning of <i>SCO1719-20</i> for overexpression	CATATGACTACTACGTAC
<i>SCO 1719-20 Overexpression R</i>	cloning of <i>SCO1719-20</i> for overexpression	CTCGAGTCAGCGGCCCCCGT

Construction of SCO1718 and SCO1719-20 null mutants: Strains of *S. coelicolor* in which either the SCO1718 locus or the SCO1719-20 locus was replaced with an apramycin resistance cassette, *apr*, were constructed via polymerase chain reaction (PCR)-targeted mutagenesis (1). The *apr* resistance cassettes for replacement of each locus were prepared from using the KO F and R primers supplied in Table S3 and the Expand High Fidelity DNA polymerase (Roche). The resistance cassettes were introduced individually into *E. coli* BW25113/pIJ790 containing the appropriate WT (Stl11) cosmid. Arabinose was added to induce expression of the λ RED recombinase. The resulting recombinant cosmids, Stl11 Δ SCO1718::*apr* (pJS876) and Stl11 Δ SCO1719-20::*apr* (pJS877), were transformed into ET12576/pUZ8002, a non-methylating strain of *E. coli*, and introduced into wild-type *S. coelicolor* A3(2) M145 through conjugation as previously described (1), yielding *S. coelicolor* B836 and B837, respectively. Double cross-over exconjugants were selected by apramycin resistance and kanamycin sensitivity. The disruptions of SCO1718 and SCO1719-20 were confirmed via PCR on isolated genomic DNA from *S. coelicolor* using the appropriate KO Det primers (Table S3).

Overexpression of SCO1719-20 genes: The SCO 1719-20 ORF was amplified from wild-type *S. coelicolor* genomic DNA using primers 5'-CATATGACTACTACGTAC-3' and 5'-CTCGAGTCAGCGGCCCCCGT-3'. The resulting 1816 bp PCR products were cloned into pBluescript II KS+ and sequenced to confirm their identities. The genes were transferred into the integrative, constitutive expression vector pIJ10257 using *Spe*I and *Xho*I, yielding pJS390. The resultant plasmids were introduced into *E. coli* strain ET12567/pUZ8002 and then into WT *S. coelicolor* by way of conjugation, as described previously. Exconjugants with pJS390 (strain B840) were identified by selection for hygromycin resistance.

Antibacterial assays. Assays with *S. coelicolor* M145 were performed on Difco nutrient agar medium supplemented with the indicated concentrations of compound. Inocula of 10^6 germinated spores were added to each well and growth was assessed after incubation at 30°C for 48 h. The lowest drug concentration that resulted in visual clearing of the wells was considered to be the MIC. *All experiments were performed six times.*

Assays with *M. smegmatis* were performed on Difco nutrient agar medium supplemented with 0.2% glycerol and the indicated concentrations of compound. After allowing the clumped cells to settle, liquid cultures with an OD of 0.6 were plated onto the DNA plates supplemented with compounds and growth was assessed after incubation at 30°C for 72 h. The lowest drug concentration that resulted in visible clearing of the wells was considered to be the MIC. *All experiments were performed in triplicate.*

To determine the MICs of compounds for *M. tuberculosis*, OD600-based assays were used. Bacteria were grown to midlog phase and plated in 96-well plates at OD600 = 0.025 in the presence of small molecule inhibitors for a 7 day period, and growth was assessed by reading OD600. The MIC value was determined as the lowest concentration that inhibited growth by >90% relative to the DMSO control. *All experiments were performed in triplicate.*

Table S4. ADEP Potentiator Optimization

Pot. : ADEP Molar Ratio	ADEP MIC (µg/mL)				
	0 : 1	2.5 : 1	5 : 1	10 : 1	20 : 1
3	16	13	13	10	9
6	16	16	16	16	16
7	16	16	16	16	16
8	16	16	16	16	16
9	16	16	16	16	16
10	16	16	16	16	16
11	16	16	16	13	13
12	16	16	16	16	16
13	16	16	16	16	16
14	16	13	9	7	5
15	16	16	16	16	16
16	16	16	10	7	5
17	16	16	16	16	16
Reserpine	16	16	16	10	9

[a] ADEP Minimal Inhibitory Concentrations of wild-type *S. coelicolor* when compound is applied in molar excess over ADEP. This is an expanded table from Figure 2.

Table S5. ADEP Susceptibility of *S. coelicolor* mutant strains

Strain	ADEP MIC (µg/mL)
WT M145	16
<i>ΔSCO1718::apr</i>	36
<i>ΔSCO1719-20::apr</i>	16
WT + pIJ10257 SCO 1719-20	36

ADEP Minimal Inhibitory Concentrations of *S. coelicolor* null and overexpression strains

Table S6. Potentiation is Specific to ADEP

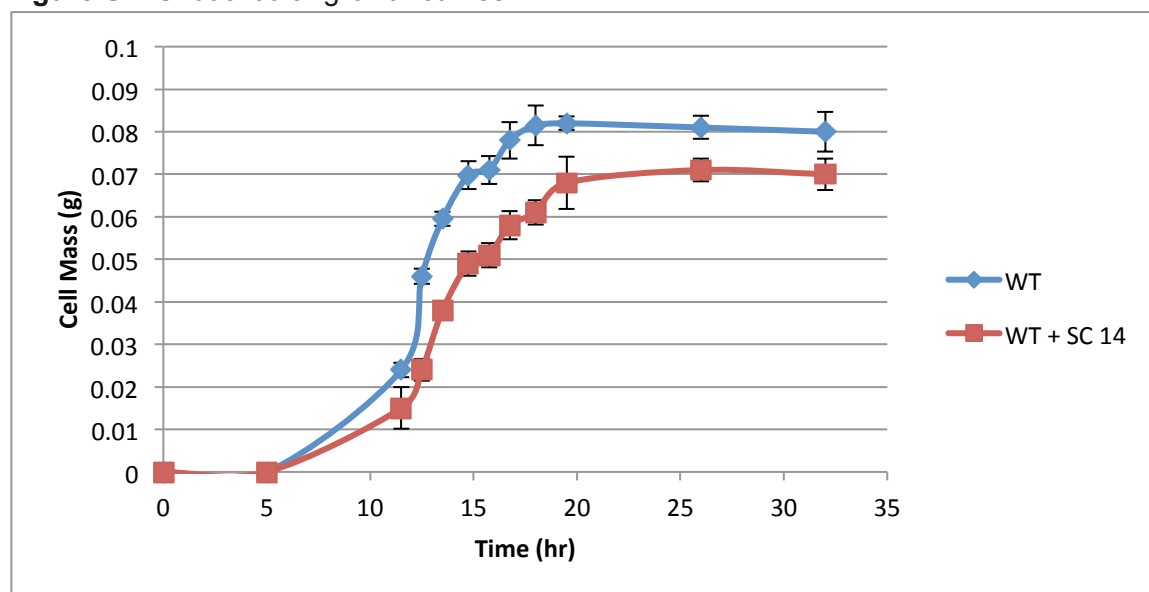
Compound	MIC ($\mu\text{g/mL}$)		
	- Fragment 14	+ Fragment 14	+ Reserpine
ADEP	16	4	8
Chloramphenicol	80	80	20
Spiramycin	150	150	75

[a] Potentiation of Chloramphenicol and Spiramycin against wild-type *S. coelicolor* in solid growth media with fragment 14 or Reserpine at 50 $\mu\text{g/mL}$.

Fragment Toxicity. All ADEP potentiating compounds were found to have no measurable MIC in *S. coelicolor* at concentrations as high as 200 $\mu\text{g/mL}$. The fragments were tested for antibacterial activity in *M. smegmatis* and *M. tuberculosis* at the potentiating concentration of 50 $\mu\text{g/mL}$ and displayed no noticeable growth defects or antibacterial activity at these concentrations.

Growth Curves of *S. coelicolor*. Growth was assessed in Tryptone Soya Broth (TSB). Initially, an equal amount of colony forming units (CFU) were added to 50 mL TSB with or without compound (50 $\mu\text{g/mL}$) in an Erlenmeyer flask appropriately fitted with a spring to prevent clumping. These cultures were incubated at 30° C with constant agitation. 1 mL aliquots were removed from the TSB culture periodically and centrifuged. The supernatant was removed and the pellet washed twice with PBS. The resulting pellet was dried for 45 min and weighed. *These experiments were performed in triplicate.*

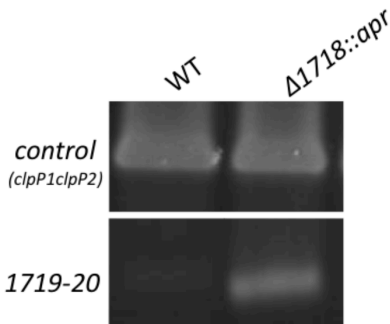
Figure S1. *S. coelicolor* growth curves



Growth of wild-type *S. coelicolor* was assessed in the presence of either DMSO or compound **14** (50 μ g/mL).

Transcriptional Analyses via Reverse Transcription PCR. The transcriptional analyses of the *S. coelicolor* *SCO1719-20* locus were performed as described previously (2). To analyze the transcription of the locus, strains were grown for 24 hrs in NMMP. 1.5mL culture aliquots were obtained and washed once with 10.3% (wt/vol) aqueous sucrose, followed by the addition of 100 μ L of 10 mg/mL lysozyme solution (50 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cells were incubated at 37°C for 15 min. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer. An equal quantity of total RNA (1.0 μ g) was employed in all reverse transcription-PCRs (RT-PCRs). RT-PCR was performed with the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol for transcripts with high GC content. A 340-bp cDNA corresponding to the *SCO1719-20* transcript was detected with *SCO1719-20* RT-PCR primers. A 215-bp cDNA corresponding to the *clpP1clpP2* (*SCO2618-19*) transcript was detected with *clpP1clpP2* RT-PCR primers, as a positive control. All primer sequences are listed in Table S3. The PCR program used for the detection of all transcripts was 50°C for 30 min, 95°C for 15 min, 28 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, and a final elongation at 72°C for 10 min. No signals were detected in control experiments with *Pfu* polymerase, indicating that the RT-PCR signals correspond to the amplification of mRNA transcripts.

Figure S2. Transcriptional Analysis of SCO 1719-20



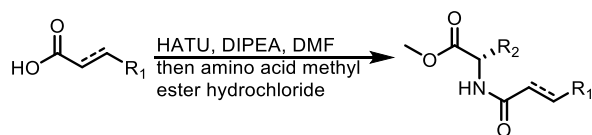
Transcriptional analyses of SCO1719-20 and SCO 2618-19 (*clpP1clpP2*, a positive control) in wild-type *S. coelicolor* M145 and in a mutant strain lacking SCO1718. SCO1719-20 encode components of an ABC transporter that confers ADEP resistance. SCO1718, which is divergently transcribed from the aforementioned genes, encodes a putative transcription factor. The SCO1719-20 genes are constitutively transcribed in a strain lacking a transcription factor encoded by SCO1718.

References

1. Kieser T, Bibb M, Buttner M, Chater K, Hopwood D. The John Innes Foundation; **2000**.
2. Vecchione, J. V., Alexander, B., Jr., Sello, J. K. 2009; *Antimicrob Agents Chemother.* **2009**; 53, 4673-4677.

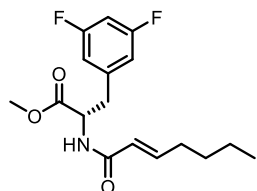
Chemical synthesis of ADEP fragments

All reagents were purchased from Sigma-Aldrich or VWR. Column chromatography was performed using 60 Å (230–400 mesh ASTM) silica gel. NMR analyses were performed on a Bruker Advance Ultrashield Spectrometer (400 or 600 MHz).

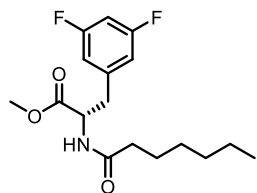


Synthesis of ADEP side chain methyl esters general procedure: The carboxylic acid (1 equivalent) was dissolved in DMF at a concentration of 0.2 M. The acid was then treated with HATU (1.1 equivalents) and DIPEA (2.2 equivalents) and allowed to stir for 15 minutes after which, an amino acid methyl ester hydrochloride was added. The coupling reaction was allowed to proceed for 3 hours, after which the reaction solution was diluted to 5x original volume with ethyl acetate and extracted 3x 1M HCl, 3x sat NaHCO₃, 1x Brine. The organic layer was then

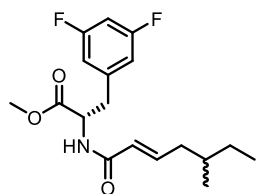
dried over sodium sulfate and purified by silica gel flash chromatography using a hexanes/ethyl acetate solvent gradient.



Methyl (S,E)-3-(3,5-difluorophenyl)-2-(hept-2-enamido)propanoate. Compound isolated as white solid: 80% yield. HRMS (ESI) predicted for $[C_{17}H_{21}F_2NO_3 + H]^+$: 326.1568, found: 326.1559. 1H NMR (400MHz, CHLOROFORM-d) δ = 6.87 (td, J = 7.0, 15.3 Hz, 1 H), 6.73 - 6.66 (m, 1 H), 6.66 - 6.60 (m, 2 H), 6.02 (d, J = 6.8 Hz, 1 H), 5.80 (td, J = 1.5, 15.3 Hz, 1 H), 4.95 (td, J = 5.7, 7.4 Hz, 1 H), 3.79 - 3.73 (m, 3 H), 3.19 (dd, J = 5.8, 13.8 Hz, 1 H), 3.11 (dd, J = 5.3, 13.8 Hz, 1 H), 2.19 (dq, J = 1.4, 7.2 Hz, 2 H), 1.49 - 1.39 (m, 2 H), 1.38 - 1.29 (m, 2 H), 0.91 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (75MHz, CHLOROFORM-d) δ = 171.6, 165.5, 164.6, 164.5, 161.3, 161.2, 146.4, 140.0, 139.9, 139.7, 122.6, 112.4, 112.3, 112.1, 112.0, 103.0, 102.6, 102.3, 52.8, 52.5, 37.6, 31.7, 30.2, 22.2, 13.8.

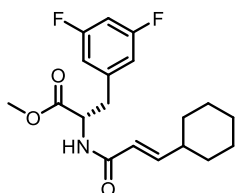


Methyl (S)-3-(3,5-difluorophenyl)-2-heptanamidopropanoate. Compound isolated as white solid: 89% yield. HRMS (ESI) predicted for $[C_{17}H_{23}F_2NO_3 + H]^+$: 328.1724, found: 328.1715. 1H NMR (400 MHz, CHLOROFORM-d) δ = 6.70 (tt, J = 2.1, 8.9 Hz, 1 H), 6.63 (d, J = 6.3 Hz, 2 H), 5.96 (d, J = 7.5 Hz, 1 H), 4.96 - 4.82 (m, 1 H), 3.76 (s, 3 H), 3.17 (dd, J = 5.8, 14.1 Hz, 1 H), 3.07 (dd, J = 5.5, 13.8 Hz, 1 H), 2.21 (t, J = 7.5 Hz, 2 H), 1.67 - 1.55 (m, 2 H), 1.29 (br. s., 6 H), 0.93 - 0.83 (m, 3 H). ^{13}C NMR (101 MHz, CHLOROFORM-d) δ = 172.7, 171.7, 164.2, 161.7, 139.8, 112.3, 112.2, 112.1, 112.0, 102.9, 102.7, 102.4, 52.6, 52.5, 37.6, 36.5, 31.5, 28.9, 25.5, 22.4, 14.0.

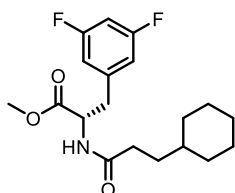


Methyl (2S)-3-(3,5-difluorophenyl)-2-((E)-5-methylhept-2-enamido)propanoate. Compound isolated as white solid: 78% yield. HRMS (ESI) predicted for $[C_{18}H_{23}F_2NO_3 + H]^+$: 340.1724, found: 340.1715. 1H NMR (400MHz, CHLOROFORM-d) δ = 6.87 (td, J = 7.5, 15.1 Hz, 1 H), 6.70 (tt, J = 2.3, 9.0 Hz, 1 H), 6.67 - 6.59 (m, 2 H), 5.99 (d, J = 7.0 Hz, 1 H), 5.80 (d, J = 15.6 Hz, 1 H), 5.00 - 4.89 (m, 1 H), 3.76 (s, 3 H), 3.21 (dd, J = 5.8, 13.8 Hz, 1 H), 3.12 (dd, J = 5.3, 13.8 Hz, 1 H), 2.25 - 2.15 (m, 1 H), 2.08 - 1.98 (m, 1 H), 1.59-1.49 (m, 1 H), 1.44 - 1.32 (m, 1 H), 1.23

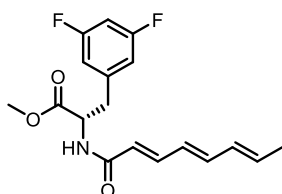
- 1.13 (m, 1 H), 0.92 - 0.86 (m, 6 H). ^{13}C NMR (101MHz, CHLOROFORM-d) δ = 171.6, 165.4, 164.2, 164.1, 161.7, 161.6, 145.4, 139.9, 139.8, 123.7, 112.3, 112.3, 112.2, 112.1, 102.9, 102.7, 102.4, 52.8, 52.6, 39.2, 37.6, 34.2, 29.2, 29.1, 19.1, 11.3



Methyl (S,E)-2-(3-cyclohexylacrylamido)-3-(3,5-difluorophenyl)propanoate. Compound isolated as white solid: 83% yield. HRMS (ESI) predicted for $[\text{C}_{19}\text{H}_{23}\text{F}_2\text{NO}_3 + \text{H}]^+$: 352.1724, found: 352.1716. ^1H NMR (400MHz, CHLOROFORM-d) δ = 6.83 (dd, J = 6.8, 15.3 Hz, 1 H), 6.70 (tt, J = 2.1, 9.0 Hz, 1 H), 6.67 - 6.60 (m, 2 H), 6.00 (d, J = 7.0 Hz, 1 H), 5.74 (dd, J = 1.3, 15.3 Hz, 1 H), 5.00 - 4.91 (m, 1 H), 3.76 (s, 3 H), 3.19 (dd, J = 5.8, 13.8 Hz, 1 H), 3.12 (dd, J = 5.3, 13.8 Hz, 1 H), 2.20 - 2.06 (m, 1 H), 1.80-1.72 (m, 4 H), 1.68 (d, J = 12.5 Hz, 1 H), 1.37 - 1.24 (m, 2 H), 1.24 - 1.07 (m, 3 H). ^{13}C NMR (101MHz, CHLOROFORM-d) δ = 171.6, 165.8, 164.2, 164.1, 161.7, 161.6, 151.4, 139.9, 139.9, 120.2, 112.3, 112.3, 112.2, 112.1, 102.9, 102.7, 102.4, 52.9, 52.5, 40.3, 37.6, 31.8, 25.9, 25.7

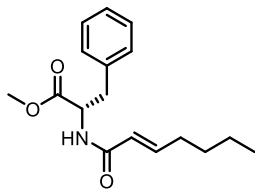


Methyl (S)-2-(3-cyclohexylpropanamido)-3-(3,5-difluorophenyl)propanoate. Compound isolated as white solid: 84% yield. HRMS (ESI) predicted for $[\text{C}_{19}\text{H}_{25}\text{F}_2\text{NO}_3 + \text{H}]^+$: 354.1881, found: 354.1873. ^1H NMR (400MHz, CHLOROFORM-d) δ = 6.74 - 6.68 (m, 1 H), 6.66 - 6.60 (m, 2 H), 5.95 (d, J = 6.8 Hz, 1 H), 4.92 - 4.86 (m, 1 H), 3.77 (s, 3 H), 3.17 (dd, J = 5.8, 13.9 Hz, 1 H), 3.07 (dd, J = 5.4, 13.8 Hz, 1 H), 2.26 - 2.19 (m, 2 H), 1.68 (s, 3 H), 1.71 (s, 2 H), 1.55 - 1.47 (m, 2 H), 1.28 - 1.11 (m, 4 H), 0.89 (q, J = 11.4 Hz, 2 H). ^{13}C NMR (101MHz, CHLOROFORM-d) δ = 173.0, 171.7, 164.2, 161.7, 139.9, 139.8, 112.3, 112.2, 112.1, 112.1, 102.9, 102.7, 102.4, 52.6, 52.6, 37.6, 37.2, 34.0, 33.0, 32.9, 26.5, 26.1.

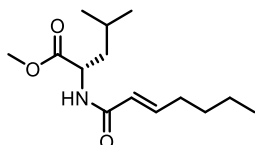


Methyl (S)-3-(3,5-difluorophenyl)-2-((2E,4E,6E)-octa-2,4,6-trienamido)propanoate
Compound isolated as white solid: 77% yield. HRMS (ESI) predicted for $[\text{C}_{18}\text{H}_{19}\text{F}_2\text{NO}_3 + \text{H}]^+$: 336.1411, found: 336.1405. ^1H NMR (400MHz, CHLOROFORM-d) δ = 7.33 - 7.22 (m, 1 H), 6.71 (t, J = 9.0 Hz, 1 H), 6.68 - 6.62 (m, 2 H), 6.53 (dd, J = 10.8, 14.8 Hz, 1 H), 6.24 - 6.11 (m, 2 H), 6.09 (d, J = 7.5 Hz, 1 H), 6.02 - 5.89 (m, 1 H), 5.86 (d, J = 15.1 Hz, 1 H), 5.07 - 4.90 (m, 1 H),

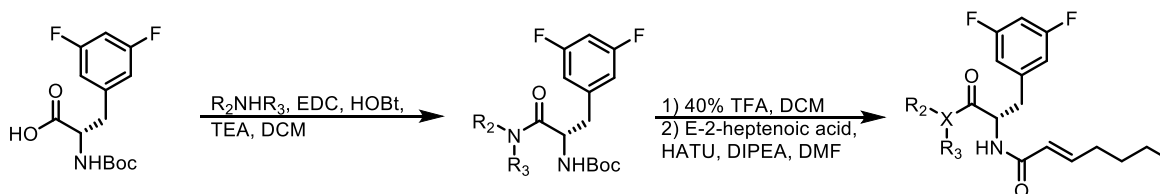
3.77 (s, 3 H), 3.21 (dd, J = 5.8, 13.8 Hz, 1 H), 3.13 (dd, J = 5.3, 13.8 Hz, 1 H), 1.84 (d, J = 6.5 Hz, 3 H). ^{13}C NMR (101MHz, CHLOROFORM- d) δ = 171.6, 165.7, 164.2, 164.1, 161.7, 161.6, 142.4, 140.7, 139.8, 134.7, 131.2, 127.3, 121.4, 112.3, 112.3, 112.1, 112.1, 102.9, 102.7, 102.4, 52.9, 52.5, 37.6, 18.5.



Methyl (E)-hept-2-enoyl-L-phenylalaninate Compound isolated as a white solid: 97% yield. HRMS (ESI) predicted for $[\text{C}_{17}\text{H}_{23}\text{NO}_3 + \text{H}]^+$: 290.1756, found: 290.1750. ^1H NMR (400MHz, CHLOROFORM- d) δ = 7.37 - 7.19 (m, 3 H), 7.14 - 7.05 (m, 2 H), 6.86 (td, J = 7.0, 15.1 Hz, 1 H), 5.96 (d, J = 7.5 Hz, 1 H), 5.77 (td, J = 1.5, 15.1 Hz, 1 H), 4.97 (td, J = 5.6, 7.8 Hz, 1 H), 3.73 (s, 3 H), 3.24 - 3.09 (m, 2 H), 2.25 - 2.10 (m, 2 H), 1.48 - 1.38 (m, 2 H), 1.38 - 1.28 (m, 2 H), 0.90 (t, J = 7.0 Hz, 3 H). ^{13}C NMR (101MHz, CHLOROFORM- d) δ = 172.1, 165.4, 145.9, 135.8, 129.2, 128.5, 127.0, 122.8, 53.0, 52.3, 37.8, 31.7, 30.2, 22.2, 13.8.



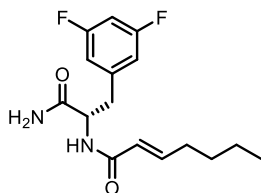
methyl (E)-hept-2-enoyl-L-leucinate Compound isolated as a colorless oil: 95% yield. HRMS (ESI) predicted for $[\text{C}_{14}\text{H}_{25}\text{NO}_3 + \text{H}]^+$: 256.1913, found: 256.1904. ^1H NMR (600MHz, CHLOROFORM- d) δ = 6.87 (td, J = 7.0, 15.4 Hz, 1 H), 5.91 - 5.83 (m, 1 H), 5.81 (td, J = 1.5, 15.3 Hz, 1 H), 4.73 (dt, J = 4.8, 8.6 Hz, 1 H), 3.74 (s, 3 H), 2.19 (dq, J = 1.5, 7.2 Hz, 2 H), 1.70 - 1.65 (m, 2 H), 1.60 - 1.53 (m, 1 H), 1.47 - 1.39 (m, 2 H), 1.39 - 1.30 (m, 2 H), 0.96 (d, J = 5.9 Hz, 3 H), 0.94 (d, J = 6.2 Hz, 3 H), 0.91 (t, J = 7.2 Hz, 3 H). ^{13}C NMR (151MHz, CHLOROFORM- d) δ = 173.8, 165.7, 145.9, 122.9, 52.3, 50.5, 41.9, 31.7, 30.2, 24.8, 22.8, 22.2, 22.0, 13.8.



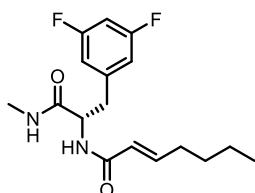
Synthesis of ADEP side chain methyl amides general procedure: Boc-3,5-Difluorophenylalanine (1 equivalent) and either 7 M ammonia in methanol (1 equivalent), 2 M methyl amine in methanol (1 equivalent), or dimethylamine hydrochloride (1 equivalent) were dissolved in dichloromethane at a concentration of 0.1 M with respect to the Boc-amino acid. The solution was then treated with EDC (1.1 equivalents), HOBt (1.1 equivalents), and TEA (1.1 equivalents for free base amines, or 2.1 equivalents for dimethyl amine HCl). The coupling reactions were allowed to run over night after which solvent was removed and the residue was

diluted in ethyl acetate to twice the original reaction volume. The ethyl acetate solution was extracted 3x 1M HCl, 3x sat NaHCO₃, 1x brine, and then dried over sodium sulfate. The concentrated crude Boc-amino amides were advanced without further purification.

Crude Boc-amino amides were deprotected by treatment with 40% TFA in DCM at a concentration of 0.5 M. Upon complete conversion of the starting material, the reaction was concentrated first by blowing with a stream of nitrogen and then by placing the residue under high vacuum. Once dry, the free base amino amides were subjected to HATU mediated coupling with a carboxylic acid and purified as described in the general procedure for the synthesis of side chain methyl esters.

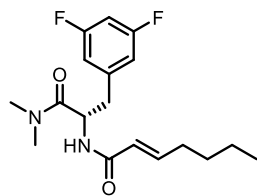


(S,E)-N-(1-amino-3-(3,5-difluorophenyl)-1-oxopropan-2-yl)hept-2-enamide. Compound isolated as white solid: 69% yield over 3 steps. HRMS (ESI) predicted for [C₁₆H₂₀F₂N₂O₂ + H]⁺: 311.1571, found: 311.1562. ¹H NMR (400MHz, DMSO-d₆) δ = 8.12 (d, J = 9.0 Hz, 1 H), 7.50 (s, 1 H), 7.11 (s, 1 H), 7.04 (tt, J = 2.4, 9.4 Hz, 1 H), 7.00 - 6.90 (m, 2 H), 6.54 (td, J = 7.0, 15.1 Hz, 1 H), 6.03 - 5.83 (m, 1 H), 4.61 - 4.42 (m, 1 H), 3.02 (dd, J = 4.5, 13.6 Hz, 1 H), 2.78 (dd, J = 10.0, 14.1 Hz, 1 H), 2.17 - 2.06 (m, 2 H), 1.40 - 1.31 (m, 2 H), 1.31 - 1.22 (m, 2 H), 0.86 (t, J = 7.3 Hz, 3 H). ¹³C NMR (101MHz, DMSO-d₆) δ = 172.7, 164.8, 163.3, 163.2, 160.9, 160.7, 143.0, 142.9, 142.8, 142.7, 124.1, 112.4, 112.3, 112.2, 112.1, 102.0, 101.8, 101.5, 53.3, 37.3, 30.8, 29.9, 21.6, 13.7.



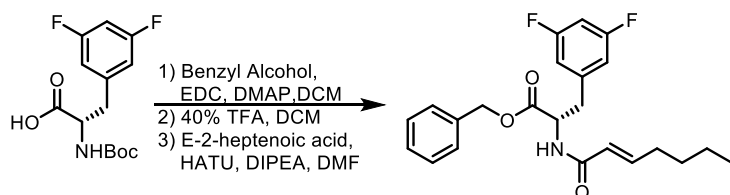
(S,E)-N-(3-(3,5-difluorophenyl)-1-(methylamino)-1-oxopropan-2-yl)hept-2-enamide.

Compound isolated as white solid: 72% yield over 3 steps. HRMS (ESI) predicted for [C₁₇H₂₂F₂N₂O₂ + H]⁺: 325.1727, found: 325.1719. ¹H NMR (400MHz, CHLOROFORM-d) δ = 6.89 - 6.78 (m, 1 H), 6.76 (d, J = 6.3 Hz, 2 H), 6.67 (dt, J = 2.4, 9.0 Hz, 1 H), 6.55 (d, J = 7.5 Hz, 2 H), 5.79 (d, J = 15.3 Hz, 1 H), 4.77 (q, J = 7.5 Hz, 1 H), 3.15 - 2.99 (m, 2 H), 2.74 (d, J = 4.8 Hz, 3 H), 2.18 (q, J = 6.9 Hz, 2 H), 1.48 - 1.38 (m, 2 H), 1.37 - 1.28 (m, 2 H), 0.90 (t, J = 7.3 Hz, 3 H). ¹³C NMR (101MHz, CHLOROFORM-d) δ = 171.3, 166.0, 146.3, 140.7, 140.6, 122.7, 112.3, 112.0, 102.4, 54.1, 38.1, 31.7, 30.2, 26.2, 22.2, 13.8.



(S,E)-N-(3-(3,5-difluorophenyl)-1-(dimethylamino)-1-oxopropan-2-yl)hept-2-enamide.

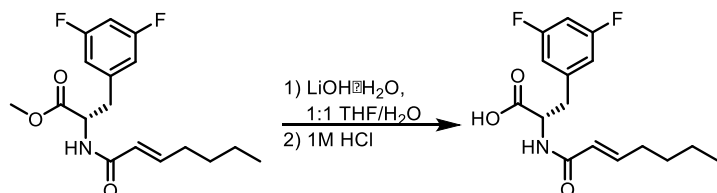
Compound isolated as white solid: 82% yield over 3 steps. HRMS (ESI) predicted for $[C_{18}H_{24}F_2N_2O_2 + H]^+$: 339.1884, found: 339.1874. 1H NMR (400MHz, CHLOROFORM- d) δ = 6.92 - 6.79 (m, 1 H), 6.77 - 6.65 (m, 3 H), 6.47 (d, J = 8.0 Hz, 1 H), 5.80 (d, J = 15.1 Hz, 1 H), 5.22 (dt, J = 6.0, 7.8 Hz, 1 H), 3.01 (t, J = 6.3 Hz, 2 H), 2.92 (s, 3 H), 2.86 - 2.79 (m, 3 H), 3.07-2.95 (m, 2 H), 1.49 - 1.40 (m, 2 H), 1.38 - 1.30 (m, 2 H), 0.91 (t, J = 7.3 Hz, 4 H). ^{13}C NMR (101MHz, CHLOROFORM- d) δ = 170.7, 165.3, 164.2, 164.0, 161.7, 161.6, 145.8, 140.3, 140.2, 123.0, 112.4, 112.3, 112.2, 112.1, 102.8, 102.5, 102.3, 49.6, 39.1, 37.0, 35.6, 31.7, 30.2, 22.2, 13.8.



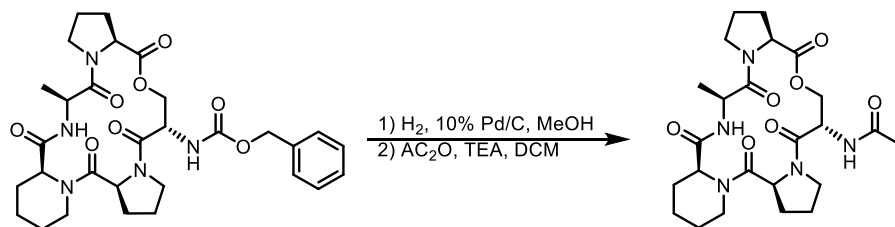
Synthesis of side chain benzyl ester: Boc-3,5-Difluorophenylalanine (110 mg, 0.37 mmol) and benzyl alcohol (38 μ L, 0.37 mmol) were dissolved in DCM (2 mL). The solution was treated with EDC (77 mg, 0.40 mmol) and DMAP (5 mg, 0.04 mmol) and allowed to react for 3 hours, after which solvent was removed and the residue was diluted in ethyl acetate to twice the original reaction volume. The ethyl acetate solution was extracted 3x 1M HCl, 3x sat $NaHCO_3$, 1x brine, and then dried over sodium sulfate. The concentrated crude Boc-amino acid esters were advanced without further purification.

Half of the crude Boc-difluorophenylalanine benzyl ester was deprotected by treatment with 40% TFA in DCM (0.5 mL). Upon complete conversion of the starting material, the reaction was concentrated first by blowing with a stream of nitrogen and then by placing the residue under high vacuum. Once dry, the Boc- deprotected product was treated with a preactivated solution of E-2-heptenoic acid (23 mg, 0.18 mmol), HATU (190 mg, 0.18 mmol) and DIPEA (61 μ L, 0.35 mmol). The coupling reaction was allowed to proceed for 3 hours, after which the reaction solution was diluted to 5x original volume with ethyl acetate and extracted 3x 1M HCl, 3x sat $NaHCO_3$, 1x Brine. The organic layer was then dried over sodium sulfate and purified by silica gel flash chromatography using a hexanes/ethyl acetate solvent gradient. Compound isolated as a white solid: 63 mg, 55% over 3 steps. HRMS (ESI) predicted for $[C_{23}H_{25}F_2NO_3 + H]^+$: 401.1881, found: 401.1870. 1H NMR (400MHz, CHLOROFORM- d) δ = 7.43 - 7.36 (m, 3 H), 7.36 - 7.30 (m, 2 H), 6.88 (td, J = 7.0, 15.6 Hz, 1 H), 6.66 (tt, J = 2.3, 9.0 Hz, 1 H), 6.58 - 6.46 (m, 2 H), 6.01 (d, J = 7.0 Hz, 1 H), 5.80 (td, J = 1.5, 15.1 Hz, 1 H), 5.21 (d, J = 12.0 Hz, 1 H), 5.13 (d, J = 12.0 Hz, 1 H), 4.98 (td, J = 5.4, 7.3 Hz, 1 H), 3.17 (dd, J = 6.0, 14.1 Hz, 1 H), 3.10 (dd, J = 5.0, 14.1 Hz, 1 H), 2.26 - 2.11 (m, 2 H), 1.52 - 1.39 (m, 2 H), 1.39 - 1.29 (m, 2 H), 0.91 (t, J = 7.2 Hz, 3 H). ^{13}C NMR (101MHz, CHLOROFORM- d) δ = 171.0, 165.4, 164.1, 164.0, 161.6, 161.5,

146.4, 139.8, 139.7, 134.7, 128.8, 128.7, 128.7, 122.7, 112.4, 112.3, 112.2, 112.2, 102.8, 102.6, 102.3, 67.6, 52.8, 37.5, 31.7, 30.2, 22.2, 13.8.

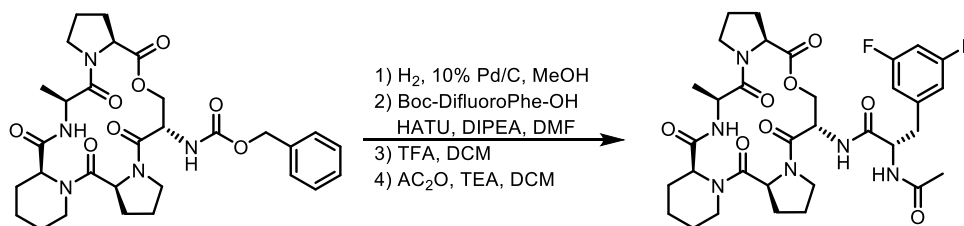


Synthesis of ADEP Side Chain Free Acid: Methyl (S,E)-3-(3,5-difluorophenyl)-2-(hept-2-enamido)propanoate (50 mg, 0.15 mmol) was dissolved in 1:1 THF/H₂O (0.5 mL) and treated with lithium hydroxide monohydrate (17 mg, 0.41 mmol). The reaction was allowed to proceed for 45 minutes, after which 1 M HCl (0.62 mL) was added. The reaction solution was then transferred to a separatory funnel and extracted 3x DCM. The DCM extracts were combined and dried over sodium sulfate. After removal of solvent, the ADEP side chain free acid was revealed as a white solid and used without further purification: 43 mg, 93% yield. HRMS (ESI) predicted for [C₁₆H₁₉F₂NO₃ + H]⁺: 311.1411, found:312.1402. ¹H NMR (600MHz, CHLOROFORM-d) δ = 10.12 (br. s., 1 H), 6.89 (td, *J* = 7.0, 15.4 Hz, 1 H), 6.77 - 6.58 (m, 3 H), 6.27 (d, *J* = 7.0 Hz, 1 H), 5.82 (td, *J* = 1.5, 15.3 Hz, 1 H), 4.93 (td, *J* = 5.7, 7.3 Hz, 1 H), 3.26 (dd, *J* = 5.7, 14.1 Hz, 1 H), 3.15 (dd, *J* = 5.7, 14.1 Hz, 1 H), 2.24 - 2.11 (m, 2 H), 1.48 - 1.37 (m, 2 H), 1.37 - 1.24 (m, 2 H), 0.90 (t, *J* = 7.3 Hz, 3 H). ¹³C NMR (151MHz, CHLOROFORM-d) δ = 173.6, 166.7, 163.8, 163.7, 162.1, 162.0, 147.6, 139.7, 139.6, 122.1, 112.4, 112.4, 112.3, 112.3, 102.9, 102.7, 102.5, 53.1, 37.0, 31.8, 30.1, 22.2, 13.8.



Synthesis of N-acetyl ADEP peptidolactone: Previously reported Cbz ADEP peptidolactone (31) (58 mg, 0.10 mmol) was dissolved in MeOH (1 mL) and treated with 10% Pd/C (13 mg) and 1 M HCl (0.10 mL). The reaction flask was sealed with a septum through which H₂ was delivered from a balloon. Upon complete conversion of the starting material, the reaction was filtered through celite, rinsing with methanol. The crude product was then concentrated and subsequently dissolved in DCM (0.33 mL) and treated with acetic anhydride (20 μL, 0.20 mmol) and triethylamine (28 μL, .20 mmol). The acetylation reaction was allowed to proceed for 3 hours after which the reaction solution was transferred directly to a silica gel column and chromatographed with 80% acetone in ethyl acetate. The product was isolated as a white solid: 39 mg, 78% yield. HRMS (ESI) predicted for [C₂₄H₃₅N₅O₇ + H]⁺: 506.2615, found:506.2607. ¹H NMR (600MHz, CHLOROFORM-d) δ = 8.62 (d, *J* = 9.5 Hz, 1 H), 6.44 (d, *J* = 9.5 Hz, 1 H), 5.22 (dd, *J* = 3.1, 9.0 Hz, 1 H), 4.99 (qd, *J* = 6.6, 9.5 Hz, 1 H), 4.80 (dd, *J* = 1.5, 11.7 Hz, 1 H), 4.73 - 4.64 (m, 1 H), 4.62 - 4.55 (m, 1 H), 4.55 - 4.45 (m, 2 H), 3.78 (ddd, *J* = 5.1, 8.2, 11.6 Hz, 1 H), 3.72 - 3.61 (m, 2 H), 3.58 - 3.45 (m, 2 H), 2.73 (d, *J* = 11.7 Hz, 1 H), 2.63 (dt, *J* = 2.6, 13.3 Hz, 1

H), 2.41 - 2.29 (m, 1 H), 2.26 - 2.10 (m, 2 H), 2.04 - 1.99 (m, 1 H), 1.97 (s, 3 H), 1.96 - 1.92 (m, 3 H), 1.78 - 1.71 (m, 2 H), 1.62 (d, $J = 14.3$ Hz, 1 H), 1.53 - 1.42 (m, 2 H), 1.41 (d, $J = 6.6$ Hz, 3 H), 1.38 - 1.29 (m, 1 H). ^{13}C NMR (151MHz, CHLOROFORM- d) $\delta = 173.5, 171.2, 170.1, 169.8, 168.6, 166.4, 65.5, 58.7, 57.2, 56.5, 51.4, 48.1, 46.9, 46.4, 41.1, 31.0, 30.6, 28.4, 24.8, 23.1, 23.0, 21.3, 21.3, 17.9$.



Synthesis of N-acetyl-3,5-difluorophenylalanyl ADEP peptidolactone: Previously reported Cbz ADEP peptidolactone (31) (58 mg, 0.10 mmol) was dissolved in MeOH (1 mL) and treated with 10% Pd/C (13 mg) and 1 M HCl (0.10 mL). The reaction flask was sealed with a septum through which H_2 was delivered from a balloon. Upon complete conversion of the starting material, the reaction was filtered through celite, rinsing with methanol. The crude product was then concentrated and subsequently dissolved in DMF (0.33 mL) and combined with Boc-3,5-difluorophenylalanine. Once homogeneous, the reaction solution was treated with HATU (38 mg, 0.10 mmol) and DIPEA (35 μL , 0.20 mmol) and allowed to react over night. The reaction was then diluted with ethyl acetate (3 mL) and extracted: 3x 1M HCl, 3x sat NaHCO_3 , 1x brine, and then dried over sodium sulfate. After removal of the solvent, the crude Boc-3,5-difluorophenylalanyl ADEP peptidolactone was used without further purification.

The crude Boc-3,5-difluorophenylalanyl ADEP peptidolactone was deprotected by treatment with 40% TFA in DCM (0.5 mL). Upon complete conversion of the starting material, the reaction was concentrated first by blowing with a stream of nitrogen and then by placing the residue under high vacuum. Once dry, the Boc- deprotected 3,5-difluorophenylalanyl ADEP peptidolactone was dissolved in DCM (0.5 mL) and treated with acetic anhydride (16 μL , 0.17 mmol) and triethylamine (24 μL , 0.17 mmol). The reaction was allowed to proceed for 3 hours, after which the reaction solution was added directly to a silica gel column and chromatographed with 50-100% acetone in ethyl acetate. The final product was isolated as a white solid: 41 mg, 59% over 4 steps. HRMS (ESI) predicted for $[\text{C}_{33}\text{H}_{42}\text{F}_2\text{N}_6\text{O}_8 + \text{H}]^+$: 689.3110, found: 689.3097. ^1H NMR (600MHz, CHLOROFORM- d) $\delta = 8.54$ (d, $J = 9.5$ Hz, 1 H), 7.51 (d, $J = 9.5$ Hz, 1 H), 6.99 (d, $J = 8.1$ Hz, 1 H), 6.76 - 6.68 (m, 2 H), 6.64 (tt, $J = 2.3, 9.0$ Hz, 1 H), 5.16 (dd, $J = 2.9, 8.8$ Hz, 1 H), 5.01 - 4.91 (m, 1 H), 4.79 (dd, $J = 1.7, 11.6$ Hz, 1 H), 4.74 (dt, $J = 5.1, 7.9$ Hz, 1 H), 4.71 - 4.66 (m, 2 H), 4.56 - 4.47 (m, 2 H), 3.77 (ddd, $J = 5.5, 8.1, 11.7$ Hz, 1 H), 3.71 - 3.59 (m, 2 H), 3.56 (dd, $J = 10.1, 11.6$ Hz, 1 H), 3.41 - 3.32 (m, 1 H), 2.95 (dd, $J = 8.4, 13.2$ Hz, 1 H), 2.88 (dd, $J = 4.8, 13.6$ Hz, 1 H), 2.71 (d, $J = 10.3$ Hz, 1 H), 2.65 - 2.57 (m, 1 H), 2.38 (dt, $J = 5.1, 8.8$ Hz, 1 H), 2.26 - 2.16 (m, 2 H), 2.15 (s, 3 H), 2.06 - 1.90 (m, 5 H), 1.79 - 1.74 (m, 1 H), 1.65 (d, $J = 12.5$ Hz, 1 H), 1.53 - 1.44 (m, 2 H), 1.44 - 1.36 (m, 1 H), 1.33 (d, $J = 6.6$ Hz, 3 H). ^{13}C NMR (151MHz, CHLOROFORM- d) $\delta = 172.5, 171.1, 170.8, 170.5, 169.8, 169.4, 165.0, 163.6, 162.0, 140.2, 112.6, 112.6, 112.4, 102.4, 102.3, 65.1, 59.0, 57.1, 56.9, 54.2, 51.2, 47.8, 46.8, 46.5, 41.0, 38.5, 30.8, 30.4, 29.2, 27.9, 25.0, 23.1, 22.8, 21.3, 21.2, 17.9$.

Spectra

