Supplementary Material

Trapping biosynthetic acyl-enzyme intermediates with encoded 2,3-diaminopropionic acid

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Supplementary Discussion 1: The direction of the oligomerisation pathway catalysed by Vlm TE_{wt}

There are two possible pathways for oligomerisation of NRPS intermediates by TE domains such as Vlm TE, as illustrated in **Fig. 3b** and **Extended Data Fig. 5a-d**. Vlm TE_{wt} could oligomerise tetradepsipetidyl *D*-hiv–*D*-val-*L*-lac–*L*-val moieties by ester bond formation between the distal hydroxyl of *D*-hiv in tetradepsipeptidyl-O-TE and the carbonyl of *L*-val from tetradepsipeptidyl-S-PCP ("forward transfer"; **Extended Data Fig. 5a, c**), or else by ester bond formation between the distal hydroxyl of *D*-hiv in tetradepsipeptidyl-S-PCP and the carbonyl of *L*-val from tetradepsipeptidyl-O-TE ("reverse transfer" (**Extended Data Fig. 5b, d)**. The reverse transfer pathway is so called because the intermediate is passed from a more C-terminal domain of the NRPS (TE domain) to a more N-terminal domain (PCP domain), whereas typically in NRPS synthesis intermediates are progressively elongated and transferred from more N- to more C- terminal domains (**Fig. 1c, Extended Data Fig. 1)**. In addition, the octadepsipeptide would later be transferred again to the TE domain, as seen in the far right of panels and **Extended Data Fig. 5a-d**.

The synthetic intermediates detected in the Vlm TE_{wt}-mediated synthesis of valinomycin differentiate between two possible oligomerisation pathways[1,2]. LC-MS analysis of Vlm TE_{wt} reactions with tetradepsipeptidyl-SNAC **7** showed formation of valinomycin, as well as the octadepsipeptidyl-SNAC **11** and dodecadepsipeptidyl-SNAC **15**, (**Fig. 3a, Extended Data Fig. 5g**). Intermediates **11** and **15** are only produced in the reverse transfer oligomerisation pathway (**Extended Data Fig. 5b, d**) and not the forward transfer pathway, indicating Vlm TE catalyses the reverse transfer pathway. Consistent with the reverse pathway, experiments using a mixture of tetradepsipeptidyl-SNAC **7** and tetradepsipeptidyl-SNAC missing the terminal hydroxyl (deoxy-tetradepsipeptidyl-SNAC **8**), showed peaks for deoxy-octadepsipeptidyl-SNAC **12** and deoxy-dodecadepsipeptidyl-SNAC **16** (**Extended Data Fig. 5j**). In addition, the valinomycin synthesis assay also shows small peaks corresponding to the 16-mer depsipeptidyl-SNAC **19**, the 20-mer depsipeptidyl-SNAC **23** and the cyclic 16-mer depsipeptide **29,** (**Fig. 3a, Extended Data Fig. 5i**) indicating that a small percentage of the time, the oligomerisation (again, by the reverse pathway) by Vlm TE domain proceeds past the dodecadepsipeptidyl intermediate, and that Vlm TE has somewhat more flexibility in final product than previously thought.

Our biochemical experiments with TE_{DAP} also supports the conclusion that synthesis occurs via the reverse transfer pathway. As discussed in the main text, incubation of tetradepsipeptidyl-SNAC with TE_{DAP} produces tetradepsipeptidyl-*N*-TE_{DAP}, where the tetradepsipeptidyl is attached to TE by a stable amine bond (**Fig. 4**), but all other atoms are the same as in the native transient tetradepsipeptidyl-*O*-TE_{wt} intermediate. If the synthesis

occurs by the forward transfer pathway, the distal hydroxyl of *D*-hiv in tetradepsipeptidyl-*N*-TE_{DAP} should attack the carbonyl of *L*-val from the next tetradepsipeptidyl-SNAC molecule, making octadepsipeptidyl-*N*-TE_{DAP} (like **Extended Data Fig. 5a, c**). This should then undergo the next oligomerisation step analogously, producing dodecadepsipeptidyl-*N*-TE_{DAP}. This dodecadepsipeptidyl-*N*-TE_{DAP} intermediate should accumulate because the amide link will stall cyclisation. Thus, in the forward transfer case, incubation of tetradepsipeptidyl-SNAC with TE_{DAP} should result in dodecadepsipeptidyl-*N*-TE_{DAP} (with smaller amounts of tetradepsipeptidyl-*N*-TE_{DAP} and octadepsipeptidyl-*N*-TE_{DAP}). In contrast, if the synthesis occurs by the reverse transfer pathway, the distal hydroxyl of *D*-hiv in tetradepsipeptidyl-SNAC will attempt to attack the carbonyl of *L*-val in the amide group of tetradepsipeptidyl-*N*-TE, but will be stalled by the enhanced stability of the amide bond. Thus, in the reverse transfer case, incubation of tetradepsipeptidyl-SNAC with TE_{DAP} should result in tetradepsipeptidyl-*N*-TE_{DAP}, and negligible amounts of other acyl-TE species. Our data shows formation of tetradepsipeptidyl-*N*-TE_{DAP}, with no octadepsipeptidyl-*N*-TE_{DAP} or dodecadepsipeptidyl-*N*-TE_{DAP} detected, fully consistent with the reverse transfer pathway.

That this oligomerising-cyclising depsipeptide synthetases uses an analogous reverse pathway to the more canonical gramicidin S synthetase[1,2] and the dimerising-cyclising elaiophylin synthase[3] suggests that all oligomerising-cyclising NRPSs and PKSs will use this synthetic scheme.

Supplementary Discussion 2: Putative model of oligomerisation and cyclisation catalysed by Vlm TE

Several NRPS pathways feature TE domains that oligomerise and cyclise linear peptidyl or depsipeptidyl substrates. They are involved in biosynthesis of the antibiotic gramicidin S[2], the emetic toxin cereulide[4,5], the siderophores enterobactin and bacillibactin[6,7], the anticancer conglobatin[8], the DNA bis-intercalator thiocoraline[9] and valinomycin, a potassium ionophore depsipeptide with antimicrobial, antitumoural and cytotoxic properties[5,10]. These TE domains perform a challenging synthetic task: They oligomerise their (depsi)peptidyl intermediates up to, but not beyond, the number of copies found in the biologically active compound, and then change their reactivity mode to catalyse cyclisation and release of the completed product.

The structures of TE_{wt}, tetradepsipeptidyl-TE_{DAP} and dodecadepsipeptidyl-TE_{DAP} can provide insight into the oligomerisation steps, change in reactivity, and cyclisation step. While the rest of the TE domain does not alter its conformation substantially between any of our structures, there is a major difference in lid conformations of the early (apo TE_{wt}, tetradepsipeptidyl-TE_{DAP}) and late (dodecadepsipeptidyl-TE_{DAP}) stage structures (**Fig. 4h-i**). We do not believe the crystal packing environment unduly influences the conformation of the lid because all the structures are derived from crystallisation in very similar conditions, and because the dodecadepsipeptidyl-TE_{DAP} conformation is seen in several different crystal packing environments. However, it is likely that the presence of a particular length of depsipeptidyl- moiety does not "lock" the lid into one particular conformation. Rather, the lid will remain dynamic throughout the catalytic cycle, with the presence of the various substrates influencing which conformations predominate. Indeed, the dodecadepsipeptidyl-TE_{DAP} structures are actually a clustered of similar conformations, rather than one single conformation (**Fig. 4h**). When for simplicity and clarity only one dodecadepsipeptidyl-TE_{DAP} (or tetradepsipeptidyl-TE_{DAP}) structure is shown (**Fig. 4c-g, j, i, Extended Data Fig. 9, Supplementary Video 1, 2**) it is meant to be representative of the family of similar conformations, rather than "the dodecadepsipeptidyl-TE_{DAP} conformation".

As described in the main text, to transition from the early type of lid conformation (apo TE_{wt}, tetradepsipeptidyl-TE_{DAP}) to the late (dodecadepsipeptidyl-TE_{DAP}), the lid undergoes a dramatic, non-rigid body movement where some helices rotate >90° and others translocate by ~25 Å (**Fig. 4j, Supplementary Video 1, 2**). The mobility of the lid has been observed in other studies (often because of disorder and lack of electron density), but the conformational changes observed with TE_{DAP} are substantially more dramatic and more informative. Lid conformations vary between apo TE structures from open conformations, suggesting ease of access of the incoming substrate to the active site pocket, to closed conformation,

presumably restricting access to the active sites, and lastly to a channel, restricting but not eliminating access to the active site. Examination of the few TEs with apo and holo structures show little change in lid conformation upon formation of the acyl-enzyme intermediate. For example the structure of pikromycin TE with a non-hydrolysable phosphonate based analog of the polyketide acyl chain shows the lid in a channel conformation very similar to the channel seen in the apo TE structure. Similarly, the deoxyerythronolide B TE domain shows subtle movement of the lid upon formation of a phosphonate ester. This underscores the significant changes seen in the lid conformation of the Vlm TE upon formation of the dodecodepsipeptidyl-intermediate.

In dodecadepsipeptidyl-TE_{DAP}, the lid helices form a concave pocket, which seems likely to be important during the cyclisation step in the thioesterase cycle. This pocket is mainly made up of hydrophobic residues, and it provides a steric barrier that prevents a dodecadepsipeptide attached to Ser/DAP2463 from extending out in a linear fashion (**Fig. 4j)**. Rather, this lid conformation favours curling back of the substrate's free end towards the acyl linkage between TE and the substrate**.** Thus, cyclisation of the dodecadepsipeptide to valinomycin may be thought of as entropically controlled by the pocket, with the dodecapeptide conformations dictated by partial confinement in the pocket and TE domain active site.

The PCP domain, a key player in the TE domain catalytic cycle, is absent from the structures we have determined. Its binding site can be inferred from the informative dead-end inhibitor trapped PCP-TE structure of EntF[11] (**Extended Data Fig. 9a**). The PCP domain docks at αE of the TE, and the PPE extends the ~15 Å to position the thiol near Ser/DAP2463 (**Extended Data Fig. 9a, b-iii**). The position of the PPE in the EntF structure is compatible with the dodecapeptide-bound conformation of the lid, but not with the apo/tetrapeptide-bound conformation in our Vlm TE structures. (The lid in the EntF structure is partially disordered.) This EntF structure showed how the PCP and TE domains can position the thioester of the depsipeptidyl-PPE near Ser/DAP2463, but in Vlm, these domains must also be able to position the terminal hydroxyl of tetradepsipeptidyl-PPE near Ser/DAP2463 for the oligomerisation step. To do so, another ~15 Å of length of tetradepsipeptide (between terminal hydroxyl and PPE sulphur) must be accommodated in the TE domain (compare **Extended Data Fig. 9b-ii** and **b-iii**). The lid likely facilities this, perhaps using a pocket similar to the one we observe in the dodecapeptidyl-TE_{DAP} structures.

One can thus assemble the known structures into a hypothetical pathway for oligomerisation and cyclisation (**Extended Data Fig. 9b**). When in the observed apo/tetradepsipeptide-bound conformation, Lα1 of Vlm TE may inhibit any attached depsipeptide from curling around for cyclisation (**Extended Data Fig. 9b-i**). PCP binding

could induce a TE conformation similar to those we observe for the dodecadepsipeptide-bound TE, which could accommodate the ~30 Å tetradepsipeptidyl-PPE bound to the PCP domain and guide it towards the active site (**Extended Data Fig. 9b-ii**). A transition to an open / largely disordered lid (as seen in EntF PCP-TE) could allow the PCP to present the thioester for transfer back to the Ser2463 (**Extended Data Fig. 9b-iii**). Finally, the lid conformation observed in the dodecadepsipeptide-TE_{DAP} structures, with its semi-sphere-like pocket, could help curl the dodecadepsipeptide back towards Ser2463 for cyclisation. (**Extended Data Fig. 9b-iv**).

The observation that there are multiple similar conformations of Vlm TE even when it is covalently bound with bona fide substrates, and the paucity of specific interactions between the lid and the rest of the TE domain make it unlikely that there is a single, fully defined conformation at any of these steps of the synthetic cycle. Formation of a pre-defined / templated conformation of the cyclisation substrate has been proposed to facilitate cyclisation in tyrocidine synthetase[1,12], while specific interaction between the lid and the polyketide substrate was proposed to do this in pikromycin synthase, but there is no evidence for these mechanisms in Vlm TE. Indeed, specific and strong binding interactions could slow the synthetic cycle, as the tetradepsipeptide must transition back and forth between being ligated to the PCP domain and to the TE domain, and the same tetradepsipeptide must assume multiple different positions in the course of a cycle. Rather, the lid conformation likely fluctuates rapidly through the cycle, "breathing" and transiently visiting reaction-competent conformations. Interestingly, a novel inhibitor to a *Mycobacterium tuberculosis* polyketide synthase TE domain binds between the cluster of lid helices[13]. It is proposed to compete with substrate binding, but such an inhibitor could also act by preventing structural rearrangements in the lid similar to those we observe here.

Supplementary Methods

Supplementary Table 1: List of primers used in this study. Mutated residues are depicted in uppercase.

Primer name	Primers sequence
MbY271f	ggaaaggtctcgaccctgNNKaactatctgcgtaaactggatcgtattc
MbY271r	ggaaaggtctcagggtcggggccagcatcggacgcag
MbN311f	ggaaaggtctccatggttNNKttttgccaaatgggcagcggctgcacc
MbN311r	ggaaaggtctcaccatggtgaattcttccaggtgttctttgc
MbY349f	ggaaaggtctccatggtgNNKggcgataccctggatattatgcatgg
MbY349r	ggaaaggtctcaccatgcagctatcgcccacaatttcgaagtc
MbV366f	ggaaaggtctccagcgcgNNKgtgggtccggttagcctggatcgtg
MbV366r	ggaaaggtctcgcgctgctcagttccagatcgccatgc
MbW382f	ggaaaggtctctaaaccgNNKattggcgcgggttttggcctggaacg
MbW382r	ggaaaggtctcggtttatcaatgccccattcacgatcc
TEV_Amb_fw	ggaaaggtctcgTAGggcagtccattagtatcaactagagatgg
TEV_Amb_rev	ggaaaggtctcccCTActgcccatccttggtttgaatccaatgc
TEV_Ala_fw	ggaaaggtctcgGCTggcagtccattagtatcaactagagatgg
TEV_Ala_rev	ggaaaggtctcccAGCctgcccatccttggtttgaatccaatgc
TE_for_pNHD_fw	ttattacatatgcatcatcatcaccaccatc
TE_for_pNHD_rev	ataataactcgagttagccacgcg
Vlm2_TE_Amb_Fw	gtgtatatcggtggtcacTAGctgggtggccatat
Vlm2_TE_Amb_Rev	atatggccacccagCTAgtgaccaccgatatacac

Creation of DAPRSlib library by inverse PCR

Using the plasmid pBK-*pylS* as a template[14], the library (DAPRSlib) for amino acid **6** was generated by five consecutive rounds of inverse PCR reactions using the PrimeSTAR HS DNA Polymerase (Takara Bio) following manufacturer's guidelines. Primers randomised the codons for positions Y271, N311, Y349, V366 and W382 of the *pylS* gene to the codons for all 20 natural amino acids (all primer are listed in **Supplementary Table 1**). The resulting PCR products were digested with BsaI-HF and DpnI, and circularised with T4 DNA ligase. DNA was transformed into Eletrocompetent MegaX DH10B™ T1R Electrocomp™ *E.coli* cells (Invitrogen) following the manufacturer's instructions and inoculated into overnight culture with appropriate antibiotic to prepare plasmid DNA. Diversity was estimated by plating serial dilutions of the transformation rescue culture on LB-agar plates with appropriate antibiotic. A library of 10^8 transformants that was isolated covered the theoretical diversity of the library with 97% confidence.

Selections of active aaRS with DAP derivatives

Selections of synthetase mutants specific for amino acids **2**-**6** were carried out as previously reported[14] using the following libraries: DAPRSlib (Y271, N311, Y349, V366, W382), D3 (L270, Y271, L274, N311, C313), PylS fwd (A267, Y271, L274, C313, M315), Susan 1(A267, Y271, Y349, V366, W382), Susan 2 (N311, C313, V366, W382, G386), Susan 4 (A267, Y349, S364, V366, G386). Briefly, *Mb*PylRS libraries in pBK vectors were subjected to five rounds of alternating positive and negative selection. The positive selections were performed in the presence of the desired ncAA (1 mM) using a chloramphenicol acetyl transferase reporter with an amber codon at a permissive position (codon 112) and expressing the cognate tRNA. Cells that survived the positive selection on chloramphenicol (typically 50 g/mL) LB agar are predicted to use either a natural amino acid that is constitutively present in the cell or the ncAA added to the cell. The negative selection used a barnase reporter containing amber codons and providing the cognate tRNA, in the absence of ncAA, to remove synthetase variants that use natural amino acids.

***GFP(150TAG)His6* expression and purification**

Superfolder green fluorescent protein (sfGFP) with **6** incorporated at position 150 was expressed from p15A-sfGFP150TAG in MegaX DH10B T1R cells containing pBK_DAPRS or pBK_PylRS vector. LB broth supplemented with 12.5 µg/mL tetracycline, 25 µg/mL kanamycin and 1 mM of **6** or N^{ε}-*tert*-butyloxycarbonyl-lysine (BocK) was inoculated with the transformed cells. Expression was induced with 0.2% (w/v) L-(+)-arabinose (Sigma) for 16 h at 37˚C whilst shaking at 220 rpm. Bacteria were then harvested and the protein purified by polyhistidine affinity chromatography.

His6-lipoyl-TEV-Strep expression and purification

BL21 (DE3) cells were transformed with pNHD-His6-lipoyl-TEV$_{wt}$-Strep, pNHD-His6-lipoyl-TEV$_{Ala}$-Strep (gene is a gift from Mark Allen)[15] or co-transformed with pSF-DAPRS-PylT[16] pNHD-His6-lipoyl-TEV$_{Amber}$-Strep and grown on TB-agar plates containing 25 µg/mL tetracycline and (and 50 µg/mL kanamycin for co-transformed cells) overnight at 37˚C (TB media containing 25 µg/mL tetracycline (and 50 µg/mL kanamycin for co-transformed cells) was inoculated with some transformed colonies. The cultures were diluted 1:100 into TB media containing 12.5 µg/mL tetracycline (and 25 µg/mL kanamycin and 100 μM of **6** for co-transformed cells) and incubated at 37˚C; once the OD_{600} reached 0.5-0.7, the cultures were moved to 20˚C. After 30 min of further incubation, the cultures were induced using 250 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and protein expression was carried out at 20˚C for 16 h. Cells were harvested by centrifugation and resuspended in 50 mM tris-HCl pH 7.5, 150 mM NaCl, 2 mM β-Mercaptoethanol, 1 Roche Inhibitor Cocktail tablet / 50 mL, 0.5 mg/mL lysozyme (Sigma), 50 µg/mL DNase (Sigma) and lysed by sonication. The lysate was clarified by centrifugation at 39'000 × *g* for 30 min and filtered through a 0.4 µm polyethersulfone (PES) membrane. His6-lipoyl-TEV-Strep was purified using nickel affinity chromatography (HisTrap HP column, GE Healthcare) with a linear gradient of imidazole (0 mM to 500 mM). Fractions containing the protein were further purified by Strep-tag affinity purification using a 5 mL StrepTrap HP column (GE Healthcare). After sample loading, the column was washed with strep binding buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 5 mM dithiothreitol [DTT]). The protein was eluted using a linear gradient of desthiobiotin (0 mM to 1.25 mM). For His6-lipoyl-TEV$_{Amber}$-Strep, the protein was irradiated with UV light (365 nm, 35 mWcm^{-2}, 1 min) at the end of the purification.

Ub$_{tev}$ expression and purification

BL21 (DE3) cells were transformed with pNHD-Ub-tev-His6 and grown on LB-agar plates containing 25 µg/mL tetracycline overnight at 37˚C. LB media containing 25 µg/mL tetracycline was inoculated with the some colonies resulting from the transformation. The culture was diluted 1:100 into fresh LB media containing 12.5 µg/mL tetracycline; once the OD_{600} reached 0.5, the cultures were induced using 1 mM IPTG and protein expression was carried out at 37˚C for 6 h. Cells were harvested by centrifugation and resuspended 50 mM tris-HCl pH 7.5, 150 mM NaCl, 2 mM β-Mercaptoethanol, 1 Roche Inhibitor Cocktail tablet / 50 mL, 0.5 mg/mL lysozyme (Sigma), 50 µg/mL DNase (Sigma) and lysed by sonication. The lysate was clarified by centrifugation at 39'000 × *g* for 30 min and filtration through a 0.4 µm PES membrane. Ub was purified using nickel affinity chromatography (HisTrap HP column, GE Healthcare) with a linear gradient of imidazole (30 mM to 500 mM). The protein was dialysed overnight against 10 mM tris-HCl at 4˚C and Ub was further purified by ion exchange

chromatography (HiTrapS 5mL column, GE Healthcare) using a NaCl gradient (0-1 M mM) in 50 mM ammonium acetate, pH 4.5. Pure fractions were pooled before overnight dialysis against 20 mM tris-HCl pH 7.4. The sample was then concentrated to ~15 mg/mL using an Amicon Ultra-15 (3 kDa MWCO) centrifugal filter device (Millipore).

Reactions of TEV with Ub_{tev}

15 μg of His6-lipoyl-TEV-Strep were incubated at 30˚C with 60 μg of Ub_{tev} and allowed to react overnight in 150 μL of 50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM DTT. 20 μL of the reaction were loaded on a 4-12% NuPAGE Bis-Tris gel (Invitrogen) and allowed to run for 45 min in 2-(n-morpholino)-ethanesulfonic acid (MES) buffer. Protein was transferred on a polyvinylidene fluoride (PVSF) membrane (Roche) using 25 mM Tris pH 8.2, 192 mM glycine, 10% (v/v) methanol. Membranes were subsequently blocked for 1h in TBST buffer (25 mM Tris pH pH 7.4, 150 mM NaCl, 0.05% [v/v] Tween 20) containing 5% (w/v) milk powder at room temperature. Antibodies (Strep-Tactin-HRP conjugate (α Strep) [IBA Lifesciences] or P4D1 antibody (α Ub) [Enzo Life Sciences]) were added in 5% TBST-milk and incubated at 4˚C overnight. Secondary antibody (for α Ub antibody) was added in 5% TBST-milk and incubated at room temperature for 1 h. Blots were developed using Amersham enhanced chemiluminescence (ECL) (GE Healthcare) and a ChemiDoc XRS+ gel imaging system (Bio-Rad).

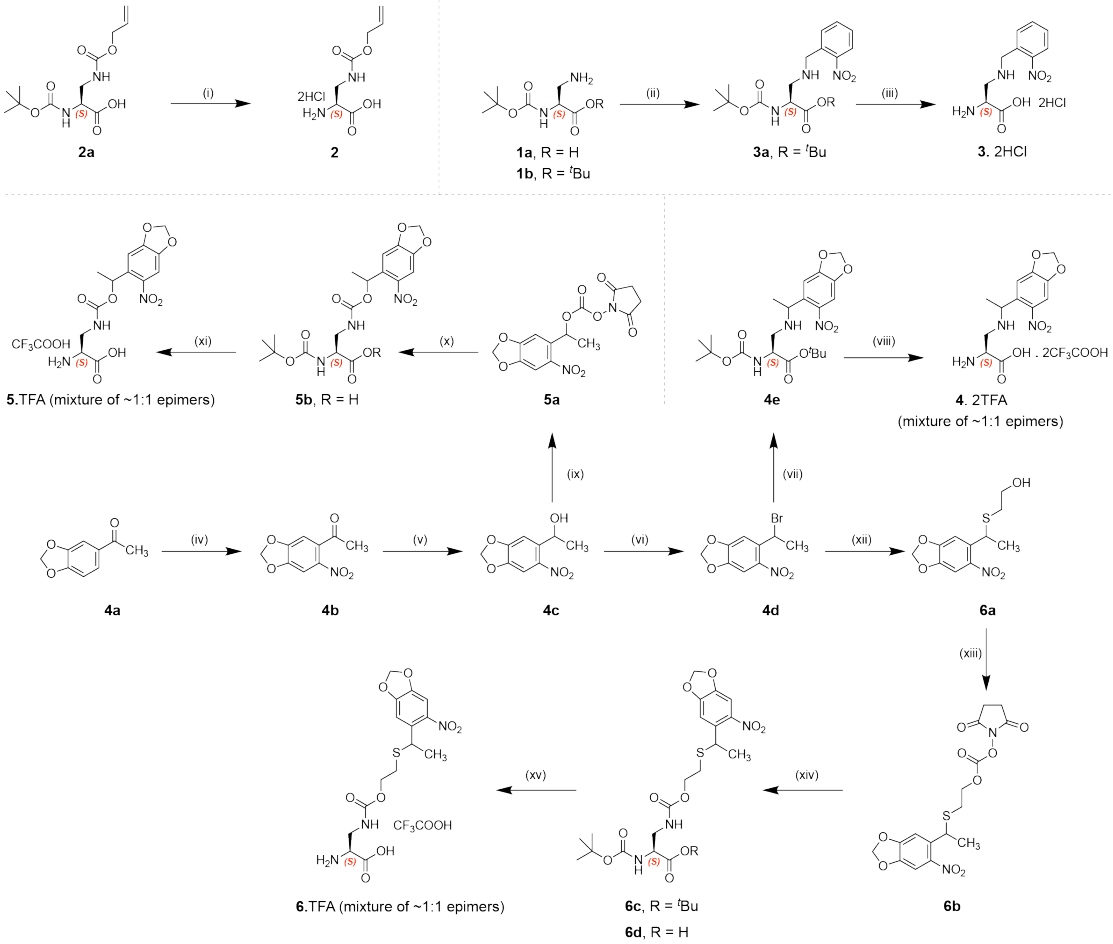
Analysis of intracellular concentration of DAP derivatives

The analysis of intracellular concentration of DAP derivatives was performed as previously described[16]. In short, DAP derivatives were added to a 5 mL solution of LB media to a final concentration of 1 mM. A control sample was also prepared with 5 mL of unsupplemented LB media. Each solution was inoculated with DH10B cells. The cultures were agitated at 220 rpm in the dark at 37˚C for 12 h. The OD_{600} of each sample was determined, and the cells from each culture were harvested. The cell pellets were washed three times with 1 mL of fresh ice-cold LB media by cycles of resuspension and centrifugation. The washed cell pellets were resuspended in a methanol:water solution (60:40). Zirconium beads (0.1 mm) were added to each suspension. The suspensions were vortexed for 12 min to lyse the cells. The lysate was centrifuged at 21000 x *g* for 30 min at 4˚C. The supernatant was carefully removed, and placed into a fresh 1.5 mL Eppendorf tube. The solutions were centrifuged again at 21000 x *g* for 2 h at 4˚C. A 100 μl aliquot of the supernatant from the resulting sample was analysed by LC-ESI-MS. A gradient of 0.5% to 95% acetonitrile in water was applied to elute the clarified lysates from a Zorbax C18 (4.6 x 150 mm) column. The concentrations were estimated using an estimate of 8×10^8 cells per 1 OD_{600} unit and a cell volume of 0.6×10^{-15} L.

Chemical synthesis

1H and 13C spectra of all new compounds used in this study are provided in **Appendix 1**.

Synthesis of amino acids



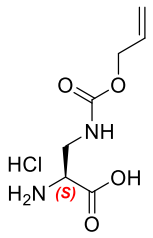
Supplementary Scheme 1: Synthesis scheme of amino acids presented in this study.

Reagents and Conditions: (i) **2a** (10.0 mmol), HCl (4M in 1,4-dioxane) (8.0 eq.), Et_3SiH (2 eq.), r.t., 1 h, 90% (**2**); (ii) **1b** (19.2 mmol), 2-nitrobenzyl bromide (1.2 eq.), DIPEA (2.0 eq.), dry THF, r.t., 10 h, 67% (**3a**); (iii) **3a** (11.6 mmol), HCl (4M in 1,4-dioxane) (8.62 eq.), Et_3SiH (2.7 eq.), r.t., 24 h, 99% (**3** • 2HCl); (iv) **4a** (20.0 mmol) in glacial CH_3COOH (1.563 M), dropwise addition to conc. HNO_3 (70%), 0 °C, 1 h of dropwise addition, then 40 °C, 2.5 h, 58% (**4b**); (v) **4b** (209.0 mmol), $NaBH_4$ (0.9 eq.), portion-wise addition (8 × 15 min), r.t., CH_3OH-C_2H_5OH-CH_2Cl_2 (44:29:27), then 4 h (total time = 6 h), r.t., 99% (**4c**); (vi) **4c** (75.0 mmol), PBr_3 (0.4 eq., dropwise addition), dry CH_2Cl_2, 0 °C, then dry pyridine (cat.), 0 °C, 15 min, then r.t., 1.5 h, 89% (**4d**); (vii) Boc-L-Dap-O^{t}Bu **1b** (15.0 mmol), 13 (1.2 eq.), DIPEA (3.0 eq.), dry THF, r.t., 64 h, 82% (**4e**); (viii) **4e** (9.49 mmol), TFA (20.64 eq.), Et_3SiH (6.60 eq.), dry CH_2Cl_2, 73% (**4** • 2CF_3COOH); (ix) **4c** (100.0 mmol), Su_2O (1.5 eq.), DIPEA (3.0 eq.), dry CH_3CN, r.t., 16 h, 92% (**5a**); (x) *Method 1*: Boc-L-Dap-O^{t}Bu **1b** (14.05 mmol), **5a** (1.2 eq.), DIPEA (3.0 eq.), dry CH_2Cl_2 / dry THF (2:1), r.t. 20 h, 94% (**5c**); *Method 2*: **5a** (12.5 mmol), Boc-L-Dap-OH **1a** (1.25 eq.), DIPEA (3.0 eq.), dry THF / dry CH_3CN (9:1), r.t., 24 h, 99% (**5b**); (xi) **5b** (9.2 mmol), TFA (21.3 eq.), dry CH_2Cl_2, r.t., 99% (**5**•CF_3COOH); (xii) **4d** (21.0 mmol), 2-mercaptoethanol (1.05 eq.), 1,4-dioxane (degassed), aq. NaOH (0.5 M in degassed H_2O, 1.0 eq.), r.t., 12 h, dark, argon atm., 95% (**6a**); (xiii) **6a** (61.0 mmol), Su_2O (1.4 eq.), dry DIPEA (4.0 eq.), dry CH_3CN, r.t., dark, argon atm., 14 h, quant. conv. (**6b**); (xiv) *Method 1*: **6b** (18.0 mmol), Boc-L-Dap-O^{t}Bu.HCl **1b** (1.6 eq.), dry DIPEA (3.0 eq.), dry CH_3CN, r.t., 10 h, dark, argon atm., 94% (**6c**); *Method 2*: **6b** (60.0 mmol), Boc-L-Dap-OH.HCl **1a** (1.1 eq.), dry DIPEA

(4.0 eq.), dry CH_3CN, r.t., 14 h, dark, argon atm., 96% (**6d**); (xv) *Method 1*: **6c** (12.066 mmol), TFA (21.647 eq.), dry Et_3SiH (10.378 eq.), dry CH_2Cl_2, r.t., dark, 24 h, monitored by LC-MS instrument (reverse phase, H_2O-CH_3CN as mobile phase), product purified by trituration (CH_3OH/Et_2O), 64% (**6**•TFA); *Method 2*: **6d** (57.626 mmol), TFA (9.065 eq.), dry Et_3SiH (2.173 eq.), dry CH_2Cl_2, r.t., dark, 5 h, monitored by LC-MS instrument (reverse phase, H_2O-CH_3CN as mobile phase), if reaction was incomplete it was left stirring longer with additional TFA (up to 2 eq.), product purified by trituration (dry CH_3OH/Et_2O), 63% (**6**•TFA);

Abbreviations: M = Molarity; conc. = concentrated; mol = moles; mmol = millimoles; ˚C = degree Celcius; eq. = equivalents; h = hour; min = minutes; r.t. = room temperature; cat. = catalytic, aq. = aqueous; Su = succinimidyl; DIPEA = diisopropylethylamine; atm = atmosphere, Boc = *tert*-butoxycarbonyl; ^{t}Bu = *tert*-butyl; Et = ethyl; TFA = trifluoroacetic acid; THF = tetrahydrofuran; LC-MS = liquid chromatography-mass spectrometry; ELS = evaporative light scattering.

(*S*)-3-{[(Allyloxy)carbonyl]amino}-2-aminopropanoic acid (2)



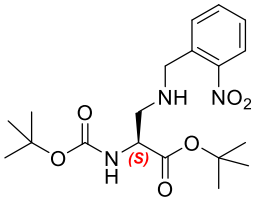
Chemical Formula: $C_7H_{12}N_2O_4$. HCl
Exact Mass: 188.07971 (without HCl)
Molecular Weight: 224.64100 (with HCl)

2

Boc-Dap(Alloc)-OH **2a** (4.325 g, 15.0 mmol, 1.0 eq., procured from Bachem Ltd.) was loaded onto a dry 250 mL single-necked round-bottomed flask and dissolved in HCl (4M in 1,4-dioxane, 60.0 mL, 240.0 mmol, 16.0 eq.). Dry Et_3SiH (10.0 mL, 62.6075 mmol, 4.174 eq.) was added to the solution at r.t. and a faint white precipitate appeared immediately, which intensified with time upon stirring at r.t. under a nitrogen atmosphere. After 24 h, an intense white precipitate was observed and the reaction was judged to be complete by LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase, gradient). The mixture was then evaporated under reduced pressure and the product was dissolved in dry CH_3OH (100 mL) followed by evaporation to dryness under reduced pressure. This was repeated thrice to remove bulk of 1,4-dioxane by azeotropic evaporation. The residue was re-dissolved in dry CH_3OH (10 mL) and triturated with dry Et_2O (500 mL) to precipitate the product. This was filtered and washed with further Et_2O (2 ×125 mL) and dried in high vacuum (<0.1 mbar) overnight to obtain (*S*)-3-{[(allyloxy)carbonyl]amino}-2-aminopropanoic acid HCl salt **2** as a bright white powder (3.03 g, 90%); ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 3.42-3.56 (m, 2H), 4.48 (d, J = 5.3 Hz, 2H), 5.15-5.36 (m, 2H), 5.80-5.98 (m, 1H), 7.44-7.60 (m, 1H), 8.24-8.70 (broad s, 3H); ^{13}C NMR (100.61 MHz, DMSO-d_6) δ 40.4 (CH_2), 52.4 (CH), 64.7 (CH_2), 117.2 (CH_2), 133.4 (CH), 156.2 (C), 169.1 (C); MS (ESI+) *m/z* (rel intensity) 189 [$(M+H)^+$, 100], 134

(4), 81 (9); HRMS (ESI +) *m/z* calc'd for $C_7H_{13}O_4N_2$ $[M+H]^+$: 189.0870, found 189.0866 (Δ = -2.19 ppm)

tert-Butyl (*S*)-2-[(_tert_-butoxycarbonyl)amino]-3-[(2-nitrobenzyl)amino]propanoate (3a)

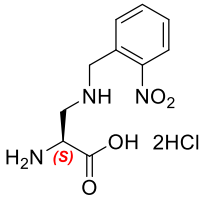


Chemical Formula: $C_{19}H_{29}N_3O_6$
Exact Mass: 395.20564
Molecular Weight: 395.45600

3a

Boc-L-Dap-O^{t}Bu·HCl **1b** (5.0 g, 19.206 mmol, 1.0 eq.) was loaded into a dry 500 mL 2-neck round-bottomed flask and dry THF (75 mL) was added to it, followed by dry DIPEA (6.69 mL, 38.412 mmol, 2.0 eq.). The contents were left stirring under an argon atmosphere at 0˚C. 2-nitrobenzyl bromide (4.979 g, 23.048 mmol, 1.2 eq.) was loaded on to a separate 250 mL dry single-neck round bottomed flask, dissolved in dry THF (125 mL), and the resulting solution then transferred to the main flask containing Boc-L-Dap-O^{t}Bu by cannula under a positive pressure of argon over 5 min at 0˚C. The mixture was then warmed to r.t. and left stirring at r.t. for 10 h. The reaction mixture was then concentrated under reduced pressure and the crude mixture extracted with EtOAc (200 mL) and washed with brine solution (3 × 250 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to obtain a brown viscous oil. Product was purified by flash chromatography on SiO_2 (gradient; eluent: EtOAc/*n*-hexane = 1:9 → 1:4) to obtain the desired product, *tert-butyl (S)-2-[(tert-butoxycarbonyl)amino]-3-[(2-nitrobenzyl)amino]propanoate* **3a** as a faint yellow viscous oil (5.05 g, 67%): R_f = 0.27 (SiO_2 plate, EtOAc/*n*-hexane = 1:4); ^{1}H NMR (400.13 MHz, $CDCl_3$ with TMS as internal standard) δ 1.44 (s, 9H), 1.46 (s, 9H), 2.85-3.40 (m, 2H), 4.02 (d, *J* = 14.5 Hz, 1H), 4.07 (d, *J* = 14.5 Hz, 1H), 4.22-4.35 (m, 1H), 5.20-5.55 (m, 1H), 7.41 (ddd, *J* = 8.4, 8.4, 2.0 Hz, 1H), 7.50-7.67 (m, 2H), 7.94 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$ with TMS as internal standard) δ 28.1 (CH_3), 28.5 (CH_3), 50.7 (CH_2), 50.9 (CH_2), 54.4 (CH), 79.9 (C), 82.3 (C), 124.9 (CH), 128.2 (CH), 131.3 (CH), 133.3 (CH), 135.5 (C), 149.2 (C), 155.7 (C), 170.9 (C).

(*S*)-2-Amino-3-[(2-nitrobenzyl)amino]propanoic acid 3



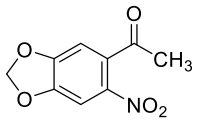
Chemical Formula: $C_{10}H_{13}N_3O_4$. 2HCl
Exact Mass: 239.09061
Molecular Weight: 312.14700 (as 2HCl salt)

3. 2HCl

A 100 mL dry single necked round-bottomed flask was charged with tert-butyl (*S*)-2-[(tert-butoxycarbonyl)amino]-3-[(2-nitrobenzyl)amino]propanoate **3a** (4.59 g, 11.607 mmol, 1.0 eq.).

HCl (25 mL, 4 M in 1,4-dioxane, 100.0 mmol, 8.615 eq.) was added, followed by dry Et_3SiH (5.0 mL, 31.304 mmol, 2.697 eq.). The contents were stirred in the dark under an argon atmosphere. The progress of the reaction was periodically monitored by TLC analysis (SiO_2 plate, EtOAc/*n*-hexane = 3:7). After 48 h, an intense white precipitate was formed and the reaction was judged to be complete by TLC and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase, gradient). The contents were evaporated to dryness under reduced pressure. Remaining 1,4-dioxane was removed by azeotropic evaporation 3x dry CH_3OH (25 mL). The contents were then re-dissolved in dry CH_3OH (25 mL), cooled to 0˚C, triturated with dry Et_2O (400 mL) and vigorously stirred in the dark at room temperature to obtain an intense precipitate. The precipitate was filtered, washed with further dry Et_2O (150 mL), followed by dry *n*-hexane (50 mL), and then evaporated to dryness under high vacuum (<0.1 mbar) for 14 h in dark to obtain the desired product, *(S)-2-amino-3-[(2-nitrobenzyl)amino]propanoic acid HCl salt* **3**, as an off-white powder (3.575 g, 99%): ^{1}H NMR (400.13 MHz, CD_3OD) δ 3.68 (dd, *J* = 13.2, 5.6 Hz, 1H), 3.81 (dd, *J* = 13.2, 7.7 Hz, 1H); 4.48 (dd, *J* = 7.7, 5.6 Hz, 1H), 4.65 (d, *J* = 13.2 Hz, 1H), 4.69 (d, *J* = 13.2 Hz, 1H), 7.74-7.82 (m, 1H), 7.84-7.95 (m, 2H), 8.30 (app. d, *J* = 8.1 Hz, 1H); ^{13}C NMR (100.61 MHz, CD_3OD) δ 47.8 (CH_2), 50.2 (CH), 50.7 (CH_2), 127.1 (CH), 127.3 (C), 132.8 (CH), 135.3 (CH), 136.0 (CH), 150.3 (C), 169.0 (C); MS (ESI+, LC-MS) *m/z* (rel intensity) 240 [$(M+H)^+$, 100%].

4',5'-Methylenedioxy-2'-nitroacetophenone (4b)



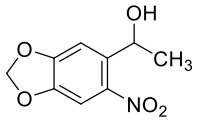
Chemical Formula: $C_9H_7NO_5$
Exact Mass: 209.03242
Molecular Weight: 209.15700

4b

Following a slightly modified procedure described by McGall *et al.*[17], a solution of 3',4'-(methylenedioxy)acetophenone **4a** (16.416 g, 0.1 mol) in glacial CH_3COOH (64 mL) was added drop-wise to a 2 litre three-necked round-bottomed flask containing conc. HNO_3 (136 mL, 70% strength) at 0˚C over 1 h. The reaction mixture was maintained at 0˚C during addition and for a further 1 h with stirring under an argon atmosphere. The mixture was then warmed to 40˚C and stirred for additional 2.5 h. Finally, the mixture was cooled to r.t. and poured slowly into crushed ice (1 litre) in a beaker. A yellow precipitate appeared which was stirred for 15 min and then filtered. The yellow solid was washed with water (3 × 200 mL) and dried in vacuum. The crude yellow solid was then purified by recrystallisation (THF/*n*-hexane) and then by flash chromatography on SiO_2 [eluent: CH_2Cl_2/*n*-hexane (1:1) to 100%CH_2Cl_2] to obtain 4',5'-methylenedioxy-2'-nitroacetophenone[17-19] **4b** as yellow crystals (12.141 g, 58%): R_f = 0.52 (CH_2Cl_2); m.p. 122.8-124.0˚C([19] m.p. 112 °C); ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 2.45 (s, 3H), 6.16 (s, 2H), 6.71 (s, 1H), 7.48 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 30.2 (CH_3), 103.8 (CH_2), 104.8 (CH), 106.2 (CH), 135.1 (C), 140.1 (C), 148.9 (C), 152.8 (C), 199.3 (C); IR

(CH_2Cl_2) ν_{max} 2980, 1708, 1525, 1506, 1484, 1424, 1362, 1338, 1271, 1152, 1038, 932, 875, 819 cm^{-1}; MS (ESI+) *m/z* (rel intensity) 232 [$(M+Na)^+$, 10%], 210 (2), 209 (7), 194 (100), 171 (45), 130 (32), 111 (9).

(*R*,*S*)-1-[4',5'-(Methylenedioxy)-2'-nitrophenyl]ethanol (4c)

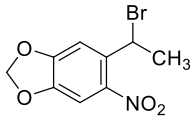


Chemical Formula: $C_9H_9NO_5$
Exact Mass: 211.04807
Molecular Weight: 211.17300

4c

4',5'-Methylenedioxy-2'-nitroacetophenone **4b** (43.714 g, 0.209 mol, 1.0 eq.) was suspended in CH_2Cl_2 (400 mL), CH_3OH (650 mL) and absolute CH_3CH_2OH (425 mL) in a 2 litre, single-necked, round-bottomed flask. The mixture was sonicated for 10 min at r.t. to dissolve the majority of the yellow solid. $NaBH_4$ granules (7.116 g, 0.188 mol, 0.9 eq.) were added in 8 portions (each 0.890 g) to the yellow suspension every 15 min (total time = 2 h addition) at 15 °C, NOTE: effervescence appeared as $NaBH_4$ dissolved and the reaction mixture became a homogeneous yellow solution. After the addition was complete, the reaction mixture was stirred at r.t. for a further 4 h. After this time the reaction was judged complete by TLC analysis (SiO_2, TLC eluent: 100% CH_2Cl_2), and the reaction quenched by addition of dry acetone (100 mL) stirring at r.t. for a further 2 h. The mixture was then evaporated to dryness under reduced pressure to obtain a yellow solid. The solid was subsequently re-dissolved in CH_2Cl_2 (800 mL) and washed sequentially with saturated aq. NH_4Cl solution (3 × 500 mL) and finally with saturated aq. NaCl solution (6 × 800 mL) The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness in high vacuum to obtain (*R*,*S*)-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethanol[17,18] **4c** as a yellow solid (43.563 g, 99%): R_f = 0.19 (CH_2Cl_2); m.p. 76.5-77.5 °C; ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 1.50 (d, *J* = 6.3 Hz, 3H), 2.54 (d, *J* = 3.0 Hz, 1H), 5.42 (qd, *J* = 6.3, 3.0 Hz, 2H), 6.096 (app. d, $^2J_{HH}$ = 3.5 Hz, 1 H, diastereotopic OCH_2O), 6.104 (app. d, $^2J_{HH}$ = 3.5 Hz, 1 H, diastereotopic OCH_2O), 7.24 (s, 1H), 7.42 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 24.3 (CH_3), 65.8 (CH), 103.1 (CH_2), 105.2 (CH), 106.4 (CH), 139.2 (C), 141.5 (C), 147.0 (C), 152.5 (C); IR (CH_2Cl_2) ν_{max} 3649, 2980, 2889, 2360, 2343, 1521, 1506, 1482, 1393, 1340, 1253, 1135, 1090, 1038, 934, 819 cm^{-1}; MS (ESI+) *m/z* (rel intensity) 234 [$(M+Na)^+$, 1%], 194 [$(M-OH)^+$, 100], 130 (20); HRMS (ESI +) *m/z* calc'd for $C_9H_9NO_5$ $[M+Na]^+$: 234.0373, found 234.0364 (Δ = -3.95 ppm).

(*R*,*S*)-1-Bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane (4d)

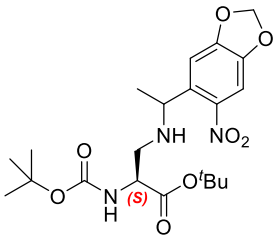


Chemical Formula: $C_9H_8BrNO_4$
Exact Mass: 272.96367
Molecular Weight: 274.07000

4d

A 1 litre, 3-necked round-bottomed flask was dried *in vacuo* for 15 min at >100˚C using a heat gun and purged with dry argon gas and allowed to cool to room temperature. To this was added (*R*,*S*)-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethanol, **4c** (15.838 g, 75.0 mmol, 1.0 eq.). **4c** was dissolved in dry CH_2Cl_2 (375 mL, sonication required for complete solubility), cooled to 0˚C under an argon atmosphere, and the round-bottomed flask was wrapped with aluminium foil to shield it from light. After 20 min, PBr_3 (2.82 mL, 30.0 mmol, 0.4 eq.) was added dropwise for 10 min using a syringe pump at 0 °C, followed by addition of dry pyridine (0.5 mL). The yellow reaction mixture was stirred at 0˚C for 15 min, then brought to r.t. and stirred continuously for 1.5 h. The reaction was judged to be complete by TLC analysis (SiO_2, TLC eluent: 100% CH_2Cl_2), cooled to 0 °C, quenched by the addition of dry CH_3OH (15 mL), warmed to r.t. and stirred for 30 min under an argon atmosphere. After the quenching was complete, the reaction mixture was evaporated to dryness under reduced pressure using a rotary evaporator. The resulting yellow gum was dissolved in CH_2Cl_2 (300 mL) and saturated aq. $NaHCO_3$ solution (300 mL). The contents were loaded into a separating funnel, the aqueous phase was discarded and the organic phase was washed sequentially with further saturated aq. $NaHCO_3$ solution (1 × 300 mL) and saturated aq. NaCl solution (3 × 300 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to obtain a yellow solid. The crude product was purified by flash chromatography on SiO_2 [eluent: CH_2Cl_2/*n*-hexane (1:1), then 100%CH_2Cl_2] to obtain a pure sample of (*R*,*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane[18] **4d** (18.330 g, 89%) as shining yellow crystals. The sample was stored in a freezer at -20˚C in a dry atmosphere and in the dark for several months without any significant decomposition: R_f = 0.17 (CH_2Cl_2/*n*-hexane, 1:4); m.p. 76.1-77.8 °C; ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 2.04 (d, J = 6.8 Hz, 3H), 5.89 (q, J = 6.8 Hz, 1H), 6.13 (s, 2H), 7.27 (s, 1H), 7.35 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 27.6 (CH_3), 42.9 (CH), 103.3 (CH_2), 105.1 (CH), 108.8 (CH), 134.8 (C), 141.6 (C), 147.7 (C), 152.1 (C); IR (CH_2Cl_2) ν_{max} 2981, 2970, 2930, 1615, 1504, 1481, 1420, 1395, 1385, 1328, 1305, 1257, 1156, 1141, 1057, 1028, 1014, 957, 925, 872, 815, 752, 730, 719, 698 cm^{-1}; HRMS (ESI+) *m/z* calc'd for $C_9H_8{}^{79}BrNO_4$ $[M+Na]^+$: 295.9529, found 295.9519 (Δ = -3.45ppm).

***tert*-Butyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl] amino}propanoate (4e)**

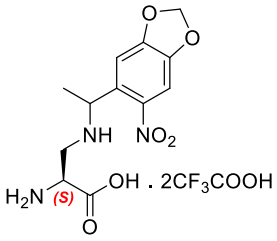


Chemical Formula: $C_{21}H_{31}N_3O_8$
Exact Mass: 453.21111
Molecular Weight: 453.49200

4e

Boc-L-Dap-O^{t}Bu·HCl **1b** (6.233 g, 21.0 mmol, 1.1 eq.) was suspended in dry THF (275 mL) in a dry 1 litre 3-neck round-bottomed flask and dry DIPEA (9.98 mL, 57.273 mmol, 3.0 eq.) was added. The contents were stirred for 10 min at r.t. under a nitrogen atmosphere. The flask was wrapped with aluminium foil and the contents were kept in the dark. (*R*,*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane **4d** (5.232 g, 19.091 mmol, 1.0 eq.) was then added to the reaction mixture. The homogeneous yellow solution and was left stirring in the dark at r.t. under a nitrogen atmosphere for a period of 68 h. The reaction was judged to be complete by TLC analysis (SiO_2 plate; CH_2Cl_2/*n*-hexane = 3:7) and evaporated to dryness under reduced pressure to obtain a dark brown oil. The crude reaction oil was dissolved in CH_2Cl_2 (250 mL) and washed with saturated brine solution (3 × 500 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to obtain a dark brown viscous oil. This was then purified by flash chromatography on SiO_2 (gradient; eluent: 100% CH_2Cl_2, then CH_2Cl_2/CH_3OH/NEt_3 = 94:5:1) to afford the desired product, *tert-butyl (2S)-2-[(tert-butoxycarbonyl)amino]-3-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl) ethyl]amino}propanoate* **4e,** as a yellow-brown sticky gum (7.49 g, 87%): R_f = 0.13 (SiO_2 plate, CH_2Cl_2); ^{1}H NMR (400.13 MHz, $CDCl_3$ with TMS as internal standard) δ 1.34 and 1.36 (2 × d, *J* = 3.6 and 3.6 Hz, 3H), 1.42 and 1.450 (2 × s, 9H), 1.454 and 1.47 (2 × s, 9H), 2.54-2.74 (m, 1H), 2.75-2.89 (m, 1H), 4.03-4.27 (m, 1H), 4.28-4.53 (m, 1H), 5.15-5.43 (m, 1H), 6.05-6.10 (m, 2H), 7.21 (app. broad s, 1H), 7.345 and 7.352 (2 × s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$ with TMS as internal standard) δ (mixture of diastereoisomers) 23.9 (CH_3), 24.0 (CH_3), 28.12 (CH_3), 28.15 (CH_3), 28.41 (CH_3), 28.46 (CH_3), 49.3 (CH_2), 49.4 (CH_2), 53.1 (CH), 53.2 (CH), 54.3 (CH), 54.5 (CH), 79.9 (C), 80.1 (C), 82.3 (C), 82.4 (C), 102.8 (2 × CH_2), 105.2 (2 × CH), 106.77 (CH), 106.83 (CH), 138.1 (C), 143.30 (C), 143.37 (C), 146.7 (C), 152.1 (C), 152.2 (C), 155.5 (C), 155.6 (C), 170.7 (C), 170.8 (C); MS (ESI+) *m/z* (rel intensity) 454 [$(M+H)^+$, 86%], 301 (70), 261 (100), 205 (7), 203 (10), 186 (7), 147 (10); HRMS (ESI+) *m/z* calc'd for $C_{21}H_{32}O_8N_3$ $[M+H]^+$: 454.2184, found 454.2201 (Δ = 3.65 ppm).

(2*S*)-2-Amino-3-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]amino}propanoic acid (4)



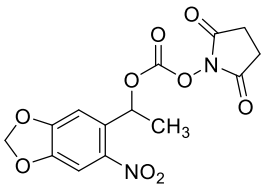
4. 2TFA

(mixture of ~1:1 epimers)

Chemical Formula: $C_{12}H_{15}N_3O_6$. $2CF_3COOH$
Exact Mass: 297.09609
Molecular Weight: 525.31342 (as disalt of CF_3COOH)

tert-Butyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl) ethyl]amino} propanoate **4e** (4.303 g, 9.489 mmol, 1.0 eq.) was dissolved in dry CH_2Cl_2 (30 mL) in a dry 250 mL round-bottomed flask wrapped with aluminium foil to exclude light. Freshly distilled CF_3COOH (15 mL, 195.887 mmol, 20.644 eq.) was added and the yellow solution turned brown. Dry Et_3SiH (10.0 mL, 62.608 mmol, 6.598 eq.) was added and the reaction mixture stirred at r.t. in the dark. The reaction was periodically monitored by LC-MS analysis. After 48 h, the reaction was judged to be complete by LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase, gradient), and the mixture then evaporated to dryness to obtain a dark brown gum. The gum was dissolved in anhydrous CH_3OH (10 mL) and cooled to 0˚C under an argon atmosphere in a dry 2 L round-bottomed flask. This was triturated by addition of dry Et_2O (900 mL) at 0˚C and then stirred vigorously at r.t. for 1 h to obtain a pale-yellow precipitate. The precipitate was filtered, washed with additional dry Et_2O (2 × 200 mL), followed by *n*-hexane (150 mL). The pale yellow powder was transferred to a 100 mL round-bottomed flask and dried in high vacuum (<0.1 mbar) for 40 h in the dark. *(2S)-2-Amino-3-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]amino} propanoic acid* **4** was obtained as a free-flowing pale yellow powder (3.646 g, 73%). The product is a salt of CF_3COOH and a ~1:1 mixture of epimers. The product was stored under argon in the dark at -20 °C: ^{1}H NMR (400.13 MHz, DMSO-d_6 with TMS as internal standard) δ 1.36 and 1.38 (2 × d, J = 3.8 and 3.8 Hz, 3H), 2.65-2.95 and 2.96-3.20 (2 × m, 1H), 3.07-3.25 (m, 1H), 3.60-3.70 (m, 1H), 3.71-3.85 and 4.18-4.44 (2 × m, 1H), 6.21 and 6.23 (2 × d, J = 3.5 and 2.9 Hz, 2H), 7.41 and 7.42 (2 × s, 1H), 7.52 and 7.53 (2 × s, 1H); ^{13}C NMR (100.61 MHz, DMSO-d_6 with TMS as internal standard) δ (mixture of diastereoisomers) 22.6 (CH_3), 22.7 (CH_3), 38.5 (CH_2), 45.8 (CH_2), 51.6 (CH), 51.8 (CH), 52.3 (CH), 52.6 (CH), 103.2 (CH_2), 103.3 (CH_2), 104.5 (CH), 104.6 (CH), 106.4 (CH), 106.6 (CH), 117.1 (C, q, $^1J_{C\text{-}F}$ = 299.0 Hz), 135.4 (C), 135.6 (C), 142.96 (C), 143.01 (C), 146.61 (C), 146.66 (C), 151.93 (C), 151.96 (C), 158.56 (C, q, $^2J_{C\text{-}F}$ = 31.5 Hz), 168.7 (2 × C), 169.6 (2 × C); MS (ESI+) *m/z* (rel intensity) 298 [$(M+H)^+$, 100%], 261 (10), 225 (10), 211 (4), 147 (12), 144 (9), 134 (6), 105 (9), 82 (31); HRMS (ESI+) *m/z* calc'd for $C_{12}H_{16}O_6N_3$ $[M+H]^+$: 298.1034, found 298.1039 (Δ = 1.74 ppm).

2,5-Dioxopyrrolidin-1-yl (1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl) carbonate (5a)

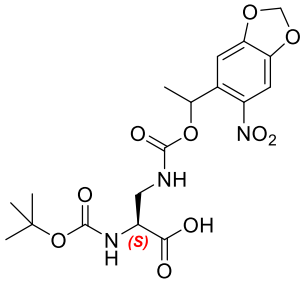


Chemical Formula: $C_{14}H_{12}N_2O_9$
Exact Mass: 352.05428
Molecular Weight: 352.25500

5a

(*R*,*S*)-1-[4',5'-(Methylenedioxy)-2'-nitrophenyl]ethanol **4c** (42.234 g, 200.0 mmol, 1.0 eq.) was charged onto a dry 2 litre 3-neck round-bottomed flask and dissolved in dry CH_3CN (1 L). Dry DIPEA (104.5 mL, 600.0 mmol, 3.0 eq.) was added to the solution, followed by *N,N*-disuccinimidyl carbonate (80.896 g of ≥ 95% purity, 300 mmol, 1.5 eq.). The flask was wrapped with aluminium foil to keep the contents in the dark. The yellow heterogeneous reaction mixture was stirred at r.t. under an argon atmosphere in the dark. After 16h, the reaction mixture was homogeneous and reaction was judged to be complete by TLC analysis (SiO_2 plate, CH_3CN/CH_2Cl_2 = 1:19). The yellow reaction mixture was then adsorbed on to Biotage® Isolute HM-N sorbent and dried under reduced pressure. This was then quickly subjected to flash chromatography on SiO_2 [eluent: CH_2Cl_2, then CH_3CN/CH_2Cl_2 = 1:19] in the dark to obtain the desired product, *2,5-dioxopyrrolidin-1-yl-(1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl) carbonate* **5a** as yellow crystalline needles (65.051 g, 92%) [NOTE: The flash column must be performed quickly to avoid decomposition of the product on prolonged exposure to SiO_2]. The product **5a** was used for the subsequent step immediately. It can be stored in the dark at -20˚Cin a freezer: R_f = 0.6 (SiO_2 plate, CH_3CN/CH_2Cl_2 = 1:19); ^{1}H NMR (400.13 MHz, $CDCl_3$ with TMS as internal standard) δ 1.75 (d, J = 6.4 Hz, 3H), 2.81 (s, 4H), 6.15 (d, J = 3.0 Hz, 2H), 6.42 (q, J = 6.4 Hz, 1H), 7.11 (s, 1H), 7.51 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$ with TMS as internal standard) δ 22.2 (CH_3), 25.6 (CH_2), 76.4 (CH), 103.5 (CH_2), 105.5 (CH), 105.8 (CH), 133.1 (C), 141.6 (C), 148.0 (C), 150.7 (C), 153.0 (C), 168.6 (C).

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-({[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy]carbonyl}- amino)propanoic acid (5b)



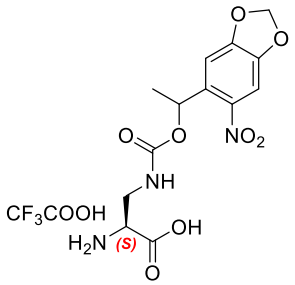
Chemical Formula: $C_{18}H_{23}N_3O_{10}$
Exact Mass: 441.13834
Molecular Weight: 441.39300

5b

Boc-L-Dap-OH **1a** (2.553 g, 12.5 mmol, 1.25 eq.) was suspended in dry THF (180 mL) and dry CH_3CN (20 mL) in a dry 1 litre single-necked round-bottomed flask wrapped with aluminium foil to exclude light. Dry DIPEA (5.23 mL, 30.0 mmol, 3.0 eq.) was added to the

mixture and the contents were stirred for 20 min at r.t. under an argon atmosphere, after which 2,5-dioxopyrrolidin-1-yl-(1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl) carbonate **5a** (3.523 g, 10.0 mmol, 1.0 eq.) was added. The heterogeneous mixture was stirred at r.t. under an argon atmosphere in the dark and the progress of the reaction was periodically monitored by LC-MS analysis. After a few hours the heterogeneous mixture started to be a homogeneous yellow solution. After 24 h, the reaction was judged to be complete and the contents were adsorbed onto Biotage® Isolute HM-N sorbent and dried under reduced pressure. This was then quickly subjected to flash chromatography on SiO_2 [eluent: CH_2Cl_2, then CH_2Cl_2/CH_3OH/CH_3COOH = 94:5:1] in the dark to obtain the desired product, *(2S)-2-[(tert-butoxycarbonyl)amino]-3-({[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethoxy]carbonyl}amino) propanoic acid* **5b** as a brown-yellow gum. This was subjected to azeotropic evaporation using CH_2Cl_2 / cyclohexane (1:1) under reduced pressure to remove residual CH_3COOH from the product **5b**. The product was dried in high vacuum to obtain a pure sample of **5b** as a yellow solid (4.360 g, 99%) and a ~1:1 mixture of epimers; R_f = 0.41 (SiO_2 plate, CH_2Cl_2/CH_3OH/CH_3COOH = 94:5:1); MS (ESI-, LC-MS) *m/z* (rel intensity) 440 [$(M-H)^-$, 100%].

(2*S*)-2-Amino-3-({[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy]carbonyl}amino)propanoic acid (5)



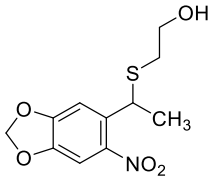
Chemical Formula: $C_{13}H_{15}N_3O_8$. CF_3COOH
Exact Mass: 341.08591
Molecular Weight: 455.29921 (with TFA)

5.TFA (mixture of ~1:1 epimers)

Freshly distilled CF_3COOH (15 mL, 195.894 mmol, 21.293 eq.) was added to a solution of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-({[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethoxy] carbonyl}amino)propanoic acid **5b** (4.061 g, 9.2 mmol, 1.0 eq.) in dry CH_2Cl_2 (50 mL) in a dry 1 L single-necked round-bottomed flask wrapped with aluminium foil. Upon addition of CF_3COOH the yellow solution turned to dark brown, the reaction was stirred at r.t. in the dark and monitored by TLC analysis. After 2h, the reaction was judged to be complete by both TLC (SiO_2 plate; CH_2Cl_2/CH_3OH/CH_3COOH = 94:5:1) and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase, gradient). The reaction mixture was evaporated to dryness under reduced pressure to obtain a dark brown gum. The gum was dissolved in dry CH_3OH (5 mL), cooled to 0˚C and triturated with dry Et_2O (0.9 L) to obtain a pale yellow precipitate. The mixture was left stirring vigorously in the dark under an atmosphere of argon at r.t. The pale yellow precipitate was then filtered and washed with Et_2O (2 × 100 mL) and dry hexane (50 mL). This was dried in vacuo (<0.1 mbar) for 2 days in the dark to obtain the

desired *(2S)-2-amino-3-({[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethoxy]carbonyl}amino)propanoic acid* TFA salt **5** as a fine, pale yellow powder (4.132 g, 99%) and a ~1:1 mixture of epimers as observed by ^{1}H and ^{13}C NMR spectroscopic analysis: ^{1}H NMR (400.13 MHz, CD_3OD) δ 1.57 (d, J = 6.2 Hz, 3H), 2.68 (s, 2H), 3.41-3.58 (m, 1H), 3.59-3.74 (m, 1H), 3.78-4.15 (m, 1H), 6.14 (s, 2H), 6.22 (q, J = 6.2 Hz, 1H), 7.12 (app. d, J = 5.2 Hz, 1H), 7.47 (s, 1H); ^{13}C NMR (100.61 MHz, CD_3OD) δ 22.4 (CH_3), 22.5 (CH_3), 42.1 (CH_2), 42.3 (CH_2), 55.6 (CH), 55.9 (CH), 70.4 (2 × CH), 104.8 (2 × CH_2), 105.7 (2 × CH), 106.7 (CH), 106.9 (CH), 118.2 (C, q, $^{1}J_{C\text{-}F}$ = 292.6 Hz), 136.8 (C), 137.1 (C), 142.8 (C), 142.9 (C), 148.8 (C), 154.0 (C), 158.5 (C), 158.6 (C), 163.1 (C, q, $^{2}J_{C\text{-}F}$ = 34.4 Hz), 170.9 (2 × C), 174.9 (2 × C); MS (ESI+) *m/z* (rel intensity) 342 [$(M+H)^+$, 100%], 311 (10), 233 (5), 189 (9), 130 (19); HRMS (ESI+) *m/z* calc'd for $C_{13}H_{16}O_8N_3$ $[M+H]^+$: 342.0932, found 342.0923 (Δ = -2.63 ppm).

2-{[1-(6-Nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]thio}ethan-1-ol (6a)



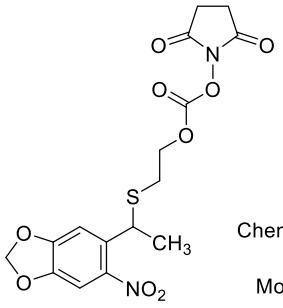
Chemical Formula: $C_{11}H_{13}NO_5S$
Exact Mass: 271.05144
Molecular Weight: 271.28700

6a

A freshly prepared solution of NaOH (0.5 M, 8 g in 40 mL of deionised H_2O, 20.0 mmol, 1 eq.) was loaded onto a 500 mL round 3-necked round-bottomed flask and the solution was degassed by bubbling through a stream of argon gas at r.t. After 30 min, mercaptoethanol (1.47 mL, 21.0 mmol, 1.05 eq.) was added to the flask and degassing was continued for a further 15 min. Separately, freshly (*R*,*S*)-1-bromo-1-[4',5'-(methylenedioxy)-2'-nitrophenyl]ethane **13** (5.481 g, 20.0 mmol, 1.0 eq) was dissolved in 1,4-dioxane (20 mL) in a 100 mL round-bottomed flask wrapped with aluminium foil and degassed by bubbling a stream of argon gas for 15 min in the dark. The degassed solution of **13** in 1,4-dioxane was transferred onto the flask containing aq. NaOH and mercaptoethanol solution, dropwise, for a period of 90 min at r.t. using a cannula under a positive pressure of argon gas. A yellow precipitate formed, which was then dissolved by addition of degassed 1,4-dioxane (60 mL), followed by sonication for 30 min until a homogenous clear yellow solution was obtained. The contents were then left stirring for 12 h at r.t. in the dark under an argon atmosphere, after which time the reaction was judged to be complete by TLC and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase, gradient). The mixture was then evaporated under reduced pressure to remove the volatile organic components. The yellow aqueous content was then extracted with EtOAc (2 × 175 mL) and the combined organic phase was washed with saturated NH_4Cl solution (1 × 500 mL), followed by brine solution (3 × 500 mL). The organic layer was then separated, dried over anhydrous Na_2SO_4, filtered and evaporated

to dryness to obtain a yellow oil. The product was purified by flash chromatography on SiO_2 (eluent: EtOAc/*n*-hexane = 3:7) in the dark to obtain *2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethan-1-ol* **6a** as a sticky yellow oil (5.179 g, 95%): R_f = 0.33 (SiO_2 plate, EtOAc/*n*-hexane = 3:7); ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 1.55 (d, *J* = 7.0 Hz, 3H), 1.96 (t, J = 5.9 Hz, 1H), 2.44-2.65 (m, 2H), 3.52-3.74 (m, 2H), 4.78 (q, *J* = 7.0 Hz, 1H), 6.10 (dd, *J* = 3.8, 1.0 Hz, 2H), 7.27 (s, 1H), 7.28 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 23.2 (CH_3), 34.9 (CH_2), 38.4 (CH), 60.9 (CH_2), 103.1 (CH_2), 104.8 (CH), 108.0 (CH), 136.1 (C), 143.3 (C), 146.9 (C), 152.0 (C); IR (neat) ν_{max} 3393, 2980, 1617, 1518, 1503, 1480, 1418, 1375, 1332, 1252, 1156, 1031, 928, 872, 817, 759; *m/z* (ESI-, LC-MS) 270.1 [$(M-H)^-$, 100%].

2,5-Dioxopyrrolidin-1-yl-(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethyl) carbonate (6b)

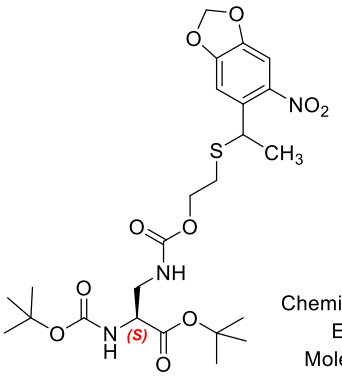


Chemical Formula: $C_{16}H_{16}N_2O_9S$
Exact Mass: 412.05765
Molecular Weight: 412.36900

6b

Intermediate **6b** used for the subsequent reaction for the synthesis of DAP derivatives **6c** and **6d** was synthesised *in situ* starting from the alcohol **6a**. A 3-necked 500 mL round-bottomed flask was dried *in vacuo* using a heat gun and purged with argon gas; this procedure was repeated three times prior to use. The dry flask was charged with 2-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]thio}ethan-1-ol **6a** (4.883 g, 18.0 mmol, 1.0 eq.) dissolved in dry CH_3CN (90 mL). Dry DIPEA (9.41 mL, 54.0 mmol, 3.0 eq.), followed by *N,N'*-disuccinimidyl carbonate (6.796 g of ≥95% purity, 25.2 mmol, 1.4 eq.), was added to the reaction mixture at r.t. in the dark under an argon atmosphere. The reaction mixture turned to turbid yellow and a white precipitate began to form, and after 1 h, the reaction mixture became a homogeneous yellow-brown solution. The reaction was left stirring at r.t. for 12 h and was judged to be complete by TLC analysis (SiO_2 plate, EtOAc/*n*-hexane = 3:7) after this time. The *2,5-dioxopyrrolidin-1-yl-(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethyl) carbonate* **6b** was immediately carried to the next step without further purification: R_f = 0.12 (SiO_2 plate, EtOAc/*n*-hexane = 3:7).

***tert*-Butyl (2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]thio}ethoxy)carbonyl]amino}propanoate (6c)**

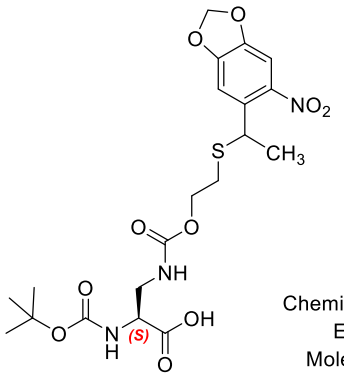


Chemical Formula: $C_{24}H_{35}N_3O_{10}S$
Exact Mass: 557.20432
Molecular Weight: 557.61500

6c

Boc-L-Dap-O^{t}Bu·HCl (8.548 g, 28.8 mmol, 1.6 eq.) was added in one portion to a solution of **6b**, prepared as described above. The yellow reaction mixture turned homogeneous in few minutes and the contents were stirred in dark under an argon atmosphere at After 10 h the reaction was judged to be complete by both TLC (SiO_2 plate, R_f = 0.39, EtOAc/n-hexane = 3:7) and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase), confirming consumption of **6b**. The reaction mixture was then adsorbed onto Biotage® Isolute HM-N sorbent and dried under reduced pressure. This was then subjected to flash chromatography on SiO_2 [eluent: EtOAc/*n*-hexane = 3:7] in the dark to obtain the desired *tert-butyl (2S)-2-[(tert-butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethoxy)carbonyl] amino}propanoate* **6c** as a thick yellow gum (9.405 g, 94%) and a mixture of ~1:1 epimers: R_f= 0.39 (EtOAc/*n*-hexane = 3:7); ^{1}H NMR (400.13 MHz, $CDCl_3$) δ (Mixture of epimers) 1.44 (s, 9H), 1.46 (s, 9H), 1.54 (d, *J* = 6.8 Hz, 3H), 2.36-2.61 (m, 2H), 3.41-3.68 (m, 2H), 4.00-4.18 (m, 2H), 4.24 (broad s, 1H), 4.85 (q, *J* = 6.8 Hz, 1H), 5.15 (broad s, 1H), 5.41 (broad s, 1 H), 6.10 (d, *J* = 6.8, 2H), 7.27 (s, 1H), 7.29 (s, 1H); ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 23.1 (CH_3), 28.1 (CH_3), 28.4 (CH_3), 30.5 (CH_2), 39.0 (CH), 43.2 (CH_2), 54.6 (CH), 65.2 (CH_2), 80.1 (C), 82.9 (C), 103.0 (CH_2), 104.7 (CH), 108.2 (CH), 136.3 (C), 143.5 (C), 146.9 (C), 152.1 (C), 155.6 (C), 156.4 (C), 169.7 (C); *m/z* (ESI+, LC-MS) 558.2 [$(M+H)^+$, 100%]

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl] thio}ethoxy)carbonyl]amino}propanoic acid (6d)

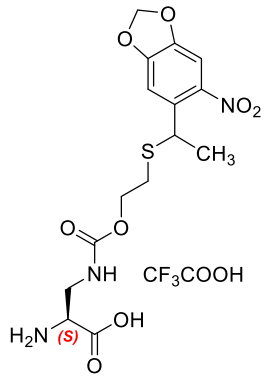


Chemical Formula: $C_{20}H_{27}N_3O_{10}S$
Exact Mass: 501.14171
Molecular Weight: 501.50700

6d

Boc-L-Dap-OH (13.479 g, 66.0 mmol, 1.082 eq.) was added in one portion to a solution of **6b** (26.70 g, prepared as described above) in dry CH_3CN (305 mL) under argon and stirred for 12 h at r.t. After this time the reaction was judged to be complete by LC-MS (C18 reverse phase column, H_2O-CH_3CN as mobile phase) and the contents were adsorbed on to Biotage® Isolute HM-N sorbent and dried under reduced pressure. This was then subjected to flash chromatography on spherical SiO_2 [Supelco®, procured from Sigma Aldrich Ltd., 40-75 µm particle size; gradient; eluent: EtOAc/*n*-hexane = 1:1 → 7:3 → 1:0] in the dark to obtain the desired *(2S)-2-[(tert-butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethoxy)- carbonyl]amino}propanoic acid* **6d** as a thick yellow gum (28.995 g, 95%) and a mixture of ~1:1 epimers: ^{1}H NMR [400.13 MHz, $CDCl_3$ with 0.1% v/v TMS as internal standard] δ (Mixture of epimers) 1.43 (s, 9H), 1.52 (d, J = 6.8 Hz, 3H), 2.30-2.95 (m, 2H), 3.33-3.82 (broad m, 2H), 3.86-4.18 (m, 2H), 4.20-4.48 (m, 1H), 4.64-4.97 (m, 1H), 5.34-5.58 (broad s, 1H), 5.60-5.84 (broad s, 1H), 6.20 (d, J = 8.3 Hz, 2H), 7.10-7.39 (m, 2H), 8.47 (broad s, 1H); ^{13}C NMR [100.61 MHz, $CDCl_3$ with 0.1% v/v TMS as internal standard] δ 23.1 (CH_3), 28.4 (3 × CH_3), 30.5 (CH_2), 39.0 (CH), 42.7 (CH_2), 54.4 (CH), 65.3 (CH_2), 80.8 (C), 103.1 (CH_2), 104.7 (CH), 108.1 (CH), 136.2 (C), 143.4 (C), 146.9 (C), 152.1 (C), 156.3 (C), 157.2 (C), 173.5 (C); *m/z* (ESI-, LC-MS) 500.1 [$(M-H)^-$, 100%]

(2*S*)-2-Amino-3-{[(2-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]thio}ethoxy)carbonyl] amino}propanoic acid (6)



Chemical Formula: $C_{15}H_{19}N_3O_8S$. CF_3COOH
Exact Mass: 401.08929 (without TFA)
Molecular Weight: 515.41321 (with TFA)

6.TFA (mixture of ~1:1 epimers)

Method I (prepared from 6c):

A dry sample of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)ethyl]thio}-ethoxy)carbonyl]amino}propanoate **6c** (6.728 g, 12.066 mmol, 1.0 eq.) was loaded to a dry 250 mL single-necked round-bottomed flask and dissolved in dry CH_2Cl_2 (50 mL), the flask was wrapped in foil to exclude light. Dry Et_3SiH (20 mL, 125.215 mmol, 10.378 eq.) was added to the solution followed by the drop-wise addition of freshly distilled CF_3COOH (20 mL, 261.182 mmol, 21.647 eq.) using a syringe over 15 min at r.t. The reaction mixture turned from yellow to a brown-green colour and was left stirring at r.t. in the dark. After 24 h, the reaction was judged to be complete by TLC (SiO_2 plate, EtOAc/*n*-hexane = 3:7) and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase). The reaction mixture was concentrated under reduced pressure in the dark to obtain a yellow-brown gum. This was dissolved in anhydrous CH_3OH (20 mL) and evaporated to dryness under reduced pressure; this was repeated three times and dried in high vacuum ($<$0.1 mbar) to remove any residual CF_3COOH, Et_3SiH and H_2O. The yellow-brown gum was then dissolved in dry CH_3OH (40 mL) and transferred to a dry 2 L round-bottomed flask under an argon atmosphere and cooled to 0˚C. Dry Et_2O (2 L) was added to the solution *via* cannula under a positive pressure of argon gas in the dark and the contents were vigorously stirred. A yellow precipitate formed and the contents stirred vigorously at 0˚C for 15 min, then at r.t. for a further 2 h. The pale yellow precipitate was filtered and washed with dry Et_2O (3 × 250 mL) and finally with dry *n*-hexane (50 mL). The product was dried in vacuum ($<$0.1 mbar) overnight for 14 h in the dark to obtain *(2S)-2-amino-3-{[(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethoxy)carbonyl]amino}propanoic acid TFA salt* **6** as a pale yellow powder (3.940 g, 63%) and a 1:1 mixture of epimers: ^{1}H NMR [400.13 MHz, CD_3OD/CF_3COOD (5:1) with 1% v/v TMS as internal standard] δ (Mixture of epimers) 1.55 (d, J = 7.0 Hz, 3H), 2.49-2.73 (m, 2H), 3.63 (dd, J = 15.0, 6.4 Hz, 1H), 3.78 (ddd, J = 15.0, 3.6, 2.3 Hz, 1H), 4.0-4.24 (m, 3H),

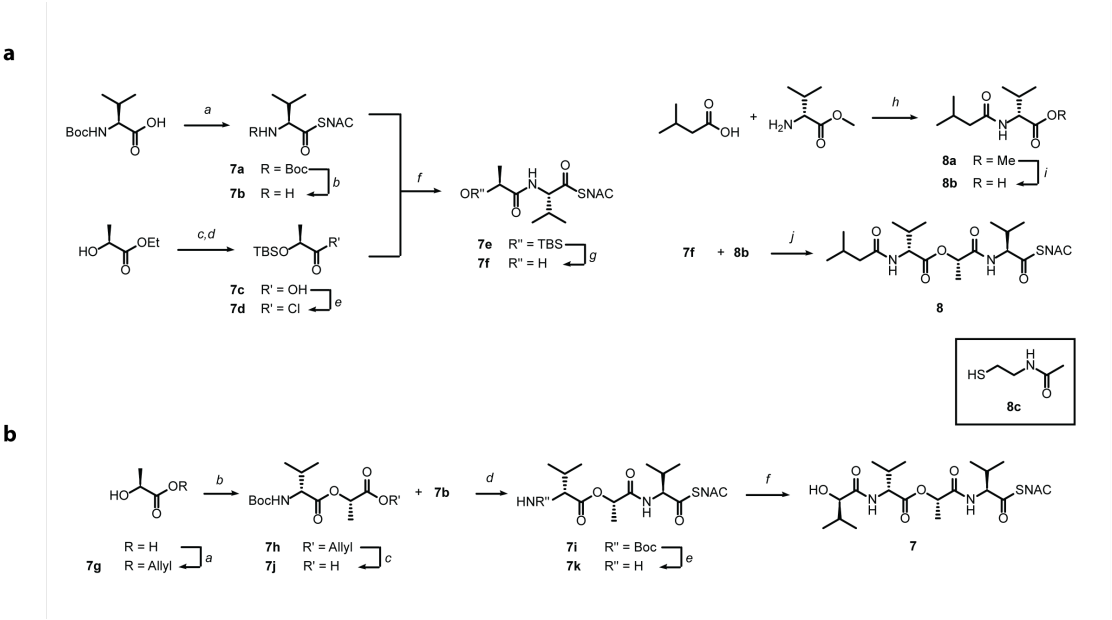
4.81 (q, J = 7.0 Hz, 1H), 6.11 and 6.13 (2 × s, 1 H), 6.40 (s, 1H), 7.29 and 7.33 (2 × s, 1H); ^{13}C NMR [100.61 MHz, CD_3OD/CF_3COOD (5:1) with 1% v/v TMS as internal standard] δ 23.2 (CH_3), 31.4 (CH_2), 40.0 (CH), 42.1 (CH_2), 55.1 (CH), 65.8 (CH_2), 104.8 (CH_2), 105.6 (CH), 109.0 (CH), 117.0 (C, $^1J_{C\text{-}F}$ = 286.5 Hz), 137.1 (C), 144.9 (C), 148.7 (C), 153.7 (C), 160.7 (C), 160.8 (C, $^1J_{C\text{-}F}$ = 38.1 Hz), 170.2 (C); MS (ESI+) *m/z* (rel intensity) 402 [$(M+H)^+$, 100%], 386 (20), 224 (9), 208 (11), 151 (11); HRMS (ESI+) *m/z* calc'd for $C_{15}H_{20}N_3O_8S$ $[M+H]^+$: 402.0971, found 402.0974 (Δ = 0.7 ppm).

Storage: The dry sample of DAP amino acid **6**·TFA was stored in air-tight dark glass vials in a cold, dry and dark environment and was stable for over 3 years without decomposition.
Handling: DAP amino acid **6**·TFA is light sensitive and slightly hygroscopic upon exposure to moist air, hence the sample of it in a vial was always handled in a dark and dry atmosphere. It is noteworthy that the vial containing **6**·TFA taken out from the fridge or freezer was always allowed to warm to r.t. prior to opening and handling.

Method II (prepared from 6d):

A dry sample of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-{[(2-{[1-(6-nitrobenzo[*d*][1,3] dioxol-5-yl)ethyl]thio}ethoxy)carbonyl]amino}propanoic acid **6d** (26.70 g, 53.2395 mmol, 1.0 eq.) was loaded on to a dry 1 litre single-necked round-bottomed flask and dissolved in dry CH_2Cl_2 (300 mL). The flask was wrapped with aluminium foil to exclude light, and dry Et_3SiH (84.69 mL, 530.24 mmol, 10.0 eq.) was added to the solution. After 5 min, freshly distilled CF_3COOH (81.54 mL, 1.0648 mol, 20.0 eq.) was added to the solution drop-wise for 15 min. The solution turned yellow-brown and was left stirring at r.t. in the dark. After 5 h, the reaction was judged to be complete by TLC (SiO2 plate, EtOAc/CH_3COOH = 98:2) and LC-MS analysis (C18 reverse phase column, H_2O-CH_3CN as mobile phase) and the solution was concentrated to dryness under reduced pressure to obtain a yellow-brown gum. This was dissolved in dry CH_3OH (40 mL) and evaporated to dryness under reduced pressure; this was repeated three times and product dried in high vacuum (<0.1 mbar) to remove any residual CF_3COOH, Et_3SiH and H_2O. The yellow-brown gum was dissolved in dry CH_3OH (40 mL), transferred to a dry 3 L round-bottomed flask under an argon atmosphere and cooled to 0 ^oC. Dry Et_2O (2.5 L) was added to the flask *via* cannula under a positive pressure of argon gas while the contents were vigorously stirred. A pale yellow precipitate was formed and the contents stirred vigorously at 0°C for 15 min and then at r.t. for 2 h. The precipitate was then filtered and washed with dry Et_2O (3 × 500 mL), followed by dry *n*-hexane (150 mL). The product was dried in high vacuum (<0.1 mbar) overnight for 14 h in the dark to obtain *(2S)-2-amino-3-{[(2-{[1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethyl]thio}ethoxy)carbonyl]amino}propanoic acid TFA salt* **6** as a pale-yellow powder (20.465 g, 75%) and a mixture of ~1:1 epimers.

VlmTE substrate syntheses

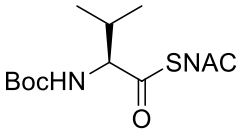


Supplementary Scheme 2: Synthesis scheme for depsipeptidyl-SNAC compounds 7 and 8.

a, Synthesis of deoxytetradepsipeptidyl-SNAC **8**. *a)* **8c**, EDC, DMAP, 72%; *b)* TFA, DCM, 99%; *c)* TBSCl, Imid., DCM; *d)* LiOH, THF, 78%, 2 steps; *e)* $(COCl)_2$, DMF, DCM; *f)* TEA, DCM, 53%; *g)* HF, Pyr., MeCN, 84%; *h)* EDC, DMAP, TEA, DCM, 60%; *i)* LiOH, MeOH, THF, 60%; *j)* EDC, DMAP, DMF, 5:4 *dr*, 92%. **b,** Synthesis of tetradepsipeptidyl-SNAC **7.** *a)* AllylBr, Cs_2CO_3, DMF, 95%; *b)* Boc-d-Val, EDC, DMAP, DCM, 84%; *c)* $Pd(PPh_3)_4$, Morpholine, DCM; *d)* EDC, HOBt, DIPEA, DCM, 94%; *e)* HCl, Dioxane; *f)* d-HIV, EDC, HOBt, DIPEA, DCM, 95%.

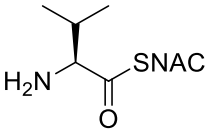
General Synthetic Procedures for SNAC synthesis.

All reagents were purchased from Sigma-Aldrich with the following exceptions: L-lactic acid was purchased from Fisher Scientific, EDC was purchased from Oakwood Chemicals (Estill, SC) at the highest available purity and used without further purification. Valinomycin was purchased from Sigma-Aldrich and BioShop Canada. All solvents were purchased from Fisher Scientific. All reactions were conducted using dry solvents under an argon atmosphere unless otherwise noted. NMR spectroscopy was performed with a Bruker AVANCE II, operating at 400 MHz for ^{1}H spectra, and 100 MHz for ^{13}C spectra and a Bruker AVANCE 300, operating at 300 MHz for ^{1}H spectra, and 75 MHz for ^{13}C spectra. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I for ESI measurements (John L. Holmes Mass Spectroscopy Facility).



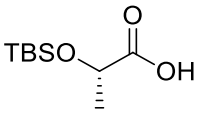
7a

(*S*)-*S*-(2-acetamidoethyl) 2-((*tert*-butoxycarbonyl)amino)-3-methylbutanethioate (7a). Boc-L-Valine (7.29 g, 33.56 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2. *N*-Acetylcysteamine (4.00 g, 33.56 mmol, 1.0 equiv.). *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC, 7.72 g, 40.27 mmol, 1.2 equiv.) and 4-(dimethylamino)pyridine (DMAP, 410 mg, 3.36 mmol, 0.1 equiv.) were added to the mixture. The reaction was stirred for 16 h at ambient temperature. The reaction was quenched with NH_4Cl(aq) and extracted 3 × with EtOAc. The organic fractions were combined, washed with brine, dried over Na_2SO_4 and concentrated. The desired product (7.69 g, 24.16 mmol, 72 % yield) was purified with silica column chromatography (5 % MeOH in CH_2Cl_2). R_f = 0.37 (2:3 acetone:hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.95 (s, 1H), 4.97 (d, *J* = 8.8 Hz, 1H), 4.21 (dd, *J* = 8.9, 4.8 Hz, 1H), 3.48 – 3.30 (m, 2H), 3.08 – 2.94 (m, 2H), 2.22 (td, *J* = 13.4, 6.7 Hz, 1H), 1.43 (s, 9H), 0.96 (d, *J* = 6.9 Hz, 3H), 0.85 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.74, 170.35, 155.66, 80.42, 65.68, 39.38, 30.77, 28.38, 28.33, 23.16, 19.40, 17.01. HRMS (ESI+) Calculated Mass ($C_{14}H_{26}N_2O_4SNa$) 341.1511, found 341.1512.



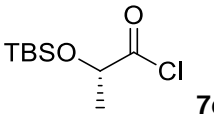
7b

(*S*)-*S*-(2-acetamidoethyl) 2-amino-3-methylbutanethioate (7b). In a round-bottom flask, **7a** (0.5 g, 1.57 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (3 mL). The solution was cooled to 0˚C using an ice bath and trifluoroacetic acid (3 mL) was added. The reaction was allowed to proceed at ambient temperature for 45 min. The reaction mixture was concentrated and the desired product (341 mg, 1.56 mmol, >99 % yield) was purified by silica column chromatography (5 % to 10 % MeOH in CH_2Cl_2). ^{1}H NMR (300 MHz, DMSO) δ 8.45 (s, 157 2H), 8.10 (t, J = 5.5 Hz, 1H), 4.15 (d, J = 4.8 Hz, 1H), 3.27 – 3.17 (m, 2H), 3.13 – 2.98 (m, 2H), 2.28 – 2.09 (m, 1H), 0.99 (d, J = 6.9 Hz, 3H), 0.95 (d, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 196.18, 169.35, 63.48, 37.78, 30.10, 28.40, 22.50, 18.03, 17.26.



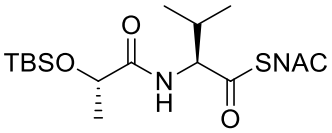
7c

(*S*)-2-((tert-butyldimethylsilyl)oxy)propanoic acid (7c). In a round-bottom flask, ethyl L-lactate (5.08 g, 43.0 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (55 mL) and the solution was cooled to 0˚C using an ice bath. *tert*-Butyldimethylsilyl chloride (6.48 g, 45.15 mmol, 1.05 equiv.) and imidazole (3.51 g, 51.6 mmol, 1.2 equiv.) was added to this mixture, after which the reaction was allowed to proceed at ambient temperature for 2 h. The reaction mixture was then diluted with H_2O and extracted 3 × with CH_2Cl_2. The organic fractions were combined, washed with ice cold 5 % HCl(aq), washed with brine, dried over Na_2SO_4 and concentrated. The crude intermediate (*S*)-ethyl 2-(*tert*-butyldimethylsilyloxy)propanoate was dissolved in THF (215 mL). The mixture was cooled to 0˚C using an ice bath, and a cooled solution of LiOH (0.4 M, 215 mL) was added dropwise over 20 min. The reaction mixture was stirred for 4 h at ambient temperature. The resulting reaction mixture was concentrated to half its original volume, and the resulting aqueous solution was extracted 3 × with Et_2O. The organic fractions were combined and extracted 3 × with a saturated solution of $NaHCO_3$(aq). The aqueous fractions were combined, acidified to pH 4 with 1 M $KHSO_4$(aq) and extracted 3 × with Et_2O. The organic fractions were combined, dried over Na_2SO_4 and concentrated. The desired product (6.88 g, 33.7 mmol, 78 % yield over two steps) was obtained and used without further purification. The NMR data were consistent with literature values[20]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.36 (q, J = 6.8 Hz, 1H), 1.45 (d, J = 6.8 Hz, 3H), 0.92 (s, 9H), 0.13 (s, 6H).



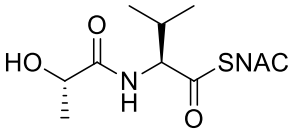
7d

(*S*)-2-((*tert*-butyldimethylsilyl)oxy)propanoyl chloride (7d). In a round-bottom flask, **7c** (3.7 g, 18 mmol, 1.0 equiv.) was dissolved in DMF (45 mL) and the solution was cooled to 0˚C using an ice bath. Oxalyl chloride (13.6 mL of a 2.0 M solution in DCM, 10.0 equiv.) and a catalytic amount of DMF were added. The reaction proceeded for 2 h from 0˚C to ambient temperature. The reaction mixture was concentrated and the crude oil was used in subsequent reactions without purification.



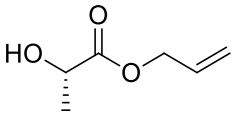
7e

TBSO-L-Lac-L-Val-SNAC (7e). In a round-bottom flask, **7b** (1.95 g, 9 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (40 mL). The crude oil **7d** (18 mmol, 2.0 equiv.) was dissolved in CH_2Cl_2 (5 mL) and added to the mixture. Et_3N (2.5 mL, 18 mmol, 2.0 equiv.) was added and the reaction was allowed to proceed for 4 h. The reaction mixture was quenched with NH_4Cl(aq), extracted 3 × with EtOAc, washed with brine and concentrated. The desired product (1.93 g, 4.77 mmol, 53 % yield) was purified from the crude mixture by silica column chromatography (50 % to 90 % EtOAc in hexanes). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22 (d, J = 9.3 Hz, 1H), 6.03 (s, 1H), 4.53 (dd, J = 9.3, 4.5 Hz, 1H), 4.25 (q, J = 6.7 Hz, 1H), 3.38 (q, J = 6.2 Hz, 2H), 3.07 – 2.98 (m, 2H), 2.40 – 2.21 (m, 1H), 1.93 (s, 3H), 1.38 (d, J = 159 6.7 Hz, 3H), 1.01 – 0.82 (m, 15H), 0.13 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.34, 174.90, 170.47, 70.03, 63.48, 39.47, 31.04, 28.51, 25.82, 23.23, 22.04, 19.47, 18.00, 16.83, -4.54, -5.03.



7f

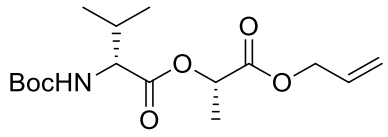
HO-L-Lac-L-Val-SNAC (7f). Compound **7e** (250 mg, 0.617 mmol, 1.0 equiv.) was dissolved in acetonitrile (20 mL) in a 50 mL polypropylene Falcon tube. Pyridine (249 µL, 3.09 mmol, 5 equiv.) and HF (48 wt. % aq. 533 µL, 30.9 mmol, 50 equiv.) were added. The reaction was stirred at ambient temperature for 16 h. The reaction mixture was quenched with NH_4Cl(aq), extracted 3 × with EtOAc, washed with brine, dried over Na_2SO_4 and concentrated. The desired product (150.1 mg, 0.517 mmol, 84 % yield) was purified with silica column chromatography (2 % to 8 % MeOH in CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 9.2 Hz, 1H), 6.20 (s, 1H), 4.54 (dd, J = 9.2, 5.4 Hz, 1H), 4.30 (q, J = 6.8 Hz, 1H), 4.15 (s, 1H), 3.52 – 3.32 (m, 2H), 3.12 – 2.94 (m, 2H), 2.36 – 2.21 (m, 1H), 1.95 (s, 3H), 1.44 (t, J = 6.3 Hz, 3H), 0.97 (d, J = 6.8 Hz, 3H), 0.91 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.20, 175.47, 170.94, 68.66, 63.77, 39.24, 30.90, 28.71, 23.25, 21.28, 19.45, 17.27.



7g

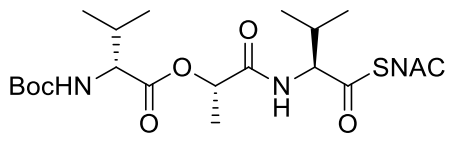
(*S*)-allyl 2-hydroxypropanoate (7g). In a round bottom flask, 1 g L-lactic acid (11.11 mmol, 1 equiv.), and 3.8 g caesium carbonate (11.67 mmol, 1.05 equiv.) were dissolved in 13 mL

DMF. Allyl bromide (3.75 mL, 5.37 g, 44.44 mmol, 4 equiv.) was added dropwise at ambient temperature. Upon complete addition the reaction was stirred at ambient temperature for 48 h. At completion the excess allyl bromide was removed by rotary evaporation and the remaining solution diluted with water then extracted 3 x with Et_2O. The combined organic fractions were washed twice with water, once with brine, dried over Na_2SO_4 and concentrated to the title compound (1.47 g, 95 %) as a pale yellow oil. Characterization data is consistent with reported values[21]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99 – 5.82 (m, 1H), 5.40 – 5.18 (m, 2H), 4.71 – 4.59 (m, 2H), 4.29 (q, *J* = 6.9 Hz, 1H), 2.75 (s, 1H), 1.42 (d, *J* = 6.9 Hz, 3H).



7h

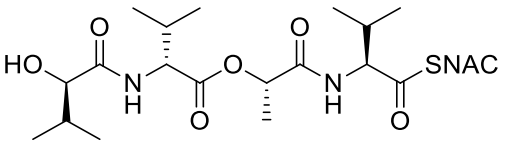
(*R*)-(*S*)-1-(allyloxy)-1-oxopropan-2-yl 2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoate (7h). In a round bottom flask, 1 g of **7g** (7.69 mmol, 1 equiv.) and 1.67 g of Boc-D-Val (8.46 mmol, 1.1 equiv.) were dissolved in 39 mL of CH_2Cl_2. To this solution 2.21 g EDC (11.54 mmol, 1.5 equiv.) and 1.03 g DMAP (8.46 mmol, 1 equiv.) were added at ambient temperature. The resulting solution was stirred for 20 h at ambient temperature. The reaction was quenched with NH_4Cl(aq), extracted 3 × with CH_2Cl_2, washed with $NaHCO_3$(aq), washed with brine, dried over Na_2SO_4, and concentrated. The title compound (2.12 g, 84 %) was purified by silica column chromatography (20 % EtOAc in hexanes). R_f = 0.41 (1:3 EtOAc:Hexanes) ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.95 – 5.81 (m, 1H), 5.29 (dddd, *J* = 21.3, 11.7, 6.6, 1.3 Hz, 2H), 5.13 (q, *J* = 7.0 Hz, 1H), 4.97 (d, *J* = 8.9 Hz, 1H), 4.67 – 4.59 (m, 2H), 4.28 (dd, *J* = 8.9, 4.8 Hz, 1H), 2.25 – 2.11 (m, 1H), 1.50 (d, *J* = 7.1 Hz, 3H), 1.43 (s, 9H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.91 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.50, 169.95, 155.56, 131.43, 118.83, 79.77, 69.17, 65.93, 58.60, 31.28, 28.32, 18.99, 17.49, 17.00. HRMS (ESI+): Exact mass calculated for $C_{16}H_{27}NNaO_6$: 352.1736. Found: 352.1721.



7i

Boc-D-Val-L-Lac-L-Val-SNAC (7i). In a round bottom flask, 250 mg of **7h** (0.76 mmol, 1 equiv.) was dissolved in 4 mL of CH_2Cl_2 under a nitrogen atmosphere. To this solution 86 µL of morpholine (87 mg, 0.99 mmol, 1.3 equiv.) and 62 mg of $Pd(PPh_3)_4$ was added in a single portion. The reaction was stirred at ambient temperature and monitored by TLC. At completion the reaction was quenched by the addition of 10 % aq. HCl, the organic layer was removed and the remaining aqueous fraction was extracted 3 x with CH_2Cl_2. The combined

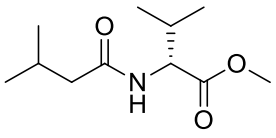
organic fractions were washed with brine, dried over Na_2SO_4 and concentrated, this intermediate, **7j**, was used immediately in the subsequent reaction. To a flame dried round bottom flask was added 194 mg of **7b** (as HCl salt, 0.76 mmol, 1 equiv.) and the crude **7j** (0.76 mmol, 1 equiv.) in 4 mL of CH_2Cl_2. To the resulting solution was added 400 µL of Hünig's base (295 mg, 2.28 mmol, 3 equiv.), 154 mg HOBt (1.14 mmol, 1.5 equiv.) and 220 mg EDC (1.14 mmol, 1.5 equiv.). The reaction was stirred under argon at ambient temperature for 20 h. The reaction was quenched with NH_4Cl(aq), extracted 3 × with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (350 mg, 94 % over 2 steps) was purified by silica column chromatography (40 % acetone in hexanes). R_f = 0.35 (2:3 acetone:hexanes) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.2 Hz, 1H), 6.07 (s, 1H), 5.38 (q, *J* = 6.8 Hz, 1H), 5.02 (d, *J* = 7.0 Hz, 1H), 4.46 – 4.39 (m, 1H), 3.99 (t, *J* = 6.9 Hz, 1H), 3.45 – 3.30 (m, 2H), 3.11 – 2.89 (m, 2H), 2.30 (dq, *J* = 13.4, 6.7 Hz, 1H), 2.11 – 2.01 (m, 1H), 1.92 (s, 3H), 1.49 (d, *J* = 6.9 Hz, 3H), 1.39 (s, 9H), 1.01 – 0.91 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.14, 171.72, 170.89, 170.48, 155.92, 80.45, 70.58, 64.74, 59.74, 39.30, 30.47, 30.27, 28.46, 28.26, 23.10, 19.33, 18.90, 18.49, 17.85, 17.53. HRMS (ESI+): Exact mass calculated for $C_{22}H_{39}N_3NaO_7S$: 512.2406. Found: 512.2391.



7

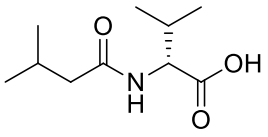
HO-D-Hiv-D-Val-L-Lac-L-Val-SNAC (7). To a round bottom flask was added 118 mg **7i** (0.24 mmol, 1 equiv.) in a minimal amount of THF and this was cooled to 0˚C. To this was added 1 mL of 4M HCl in dioxane (Sigma), and the reaction allowed to warm to ambient temperature. The reaction was monitored by TLC and at completion all solvent was removed by rotary evaporation. The unpurified intermediate **7k** was used immediately in the subsequent reaction. Intermediate **7k** was dissolved in 2 mL of CH_2Cl_2 and to this was added sequentially 125 µL of Hünig's base (93 mg, 0.72 mmol, 3 equiv.), 32 mg of D-α-hydroxyisovaleric acid (0.27 mmol, 1.1 equiv.), 49 mg HOBt (0.36 mmol, 1.5 equiv.), and 70 mg EDC (0.36 mmol, 1.5 equiv.). The reaction was stirred at ambient temperature for 24 h and at completion was quenched with NH_4Cl(aq), extracted 5 × with CH_2Cl_2, washed with $NaHCO_3$(aq), then with brine, dried over Na_2SO_4, and concentrated. The title compound (111 mg, 95%) was purified by silica column chromatography (50 % Acetone in hexanes). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (s, 1H), 6.20 (t, J = 5.7 Hz, 1H), 5.26 (q, J = 7.0 Hz, 1H), 4.55 (br, 1H), 4.47 (dd, J = 9.0, 6.5 Hz, 1H), 4.26 (t, J = 7.7 Hz, 1H), 3.99 (d, J = 2.9 Hz, 1H), 3.52 – 3.25 (m, 2H), 2.99 (ddt, J = 20.4, 13.3, 6.5 Hz, 2H), 2.39 – 2.25 (m, 1H), 2.20 – 2.06 (m, 2H), 1.97 (s, 3H), 1.54 (d, J = 7.0 Hz, 3H), 1.06 – 0.93 (m, 15H), 0.88 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.05, 175.25, 171.69, 171.43 (2C), 76.33, 71.14, 64.55, 58.39, 38.95, 31.95, 30.25, 30.12,

28.64, 23.22, 19.49, 19.17, 19.13, 18.83, 18.18, 18.04, 16.15. HRMS (ESI+): Exact mass calculated for $C_{22}H_{39}N_3NaO_7S$: 512.2401. Found: 512.2406.



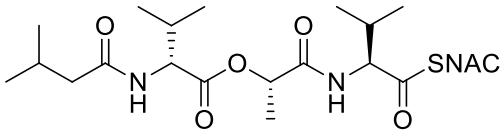
8a

(*R*)-methyl 3-methyl-2-(3-methylbutanamido)butanoate (8a). In a round-bottom flask, D-valine methyl ester hydrochloride (250 mg, 1.5 mmol, 1.0 equiv.) was dissolved in CH_2Cl_2 (15 mL). Isovaleric acid (230 mg, 2.25 mmol, 1.5 equiv.), EDC (430 mg, 2.25 mmol, 1.5 equiv.), DMAP (276 mg, 2.25 mmol, 1.5 equiv.), and Et_3N (420 µL, 3.00 mmol, 2.0 equiv.) were added and the reaction was allowed to mix at ambient temperature for 16 h. The reaction was quenched with NH_4Cl(aq), extracted 3 × with CH_2Cl_2, washed with $NaHCO_3$(aq), washed with brine, dried over Na_2SO_4, and concentrated. The desired compound (193.7 mg, 0.90 mmol, 60 % yield) was purified by silica column chromatography (20 to 50 % EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.96 (d, J = 8.0 Hz, 1H), 4.57 (dd, J = 8.8, 4.9 Hz, 1H), 3.71 (s, 3H), 2.19 – 2.04 (m, 4H), 0.97 – 0.86 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.85, 172.49, 56.91, 52.19, 46.14, 31.35, 26.29, 22.56, 22.53, 19.07, 17.93.

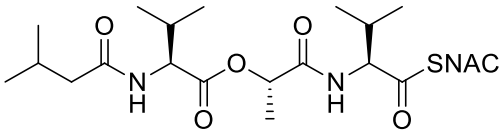


8b

(*R*)-3-methyl-2-(3-methylbutanamido)butanoic acid (8b). In a round-bottom flask, **8a** (180 mg, 1.2 mmol, 1.0 equiv.) was dissolved in MeOH (24 mL) and THF (24 mL) and the solution was cooled to 0˚C using an ice bath. LiOH (1 M, 24 mL) was added dropwise and the solution was allowed to proceed from 0˚C to ambient temperature over 4 h. The solution was concentrated to one-third volume and the resulting aqueous solution was acidified to pH 3 with 10 % HCl. The solution was extracted 3 × with CH_2Cl_2, dried over Na_2SO_4 and concentrated. The desired product (145 mg, 0.72 mmol, 60 % yield) was purified by silica column chromatography (5 % MeOH in CH_2Cl_2 + 0.5 % acetic acid). ^{1}H NMR (300 MHz, MeOD) δ 4.32 (d, J = 5.8 Hz, 1H), 2.23 – 2.01 (m, 4H), 1.01 – 0.92 (m, 12H). ^{13}C NMR (75 MHz, MeOD) δ 175.84, 174.93, 59.00, 45.90, 31.53, 27.50, 22.76, 22.72, 19.65, 18.41.



A



B

8 (A) and 8c (B)

(*R*)-(*S*)-1-(((*S*)-1-((2-acetamidoethyl)thio)-3-methyl-1-oxobutan-2-yl)amino)-1-oxopropan-2-yl 3-methyl-2-(3-methylbutanamido)butanoate (8). In a round bottom flask, the alcohol **7f** (25.2 mg, 0.087 mmol, 1.0 equiv.) and carboxylic acid **8b** (35 mg, 0.174 mmol, 2.0 equiv.) were dissolved in DMF (1 mL). The solution was cooled to –20˚C using a dry ice / acetone bath and EDC (67 mg, 0.35 mmol, 4.0 equiv.) and DMAP (21 mg, 0.174 mmol, 2.0 equiv.) were added. The mixture was allowed to warm to ambient temperature and the reaction proceeded for 16 h. The reaction was quenched with NH_4Cl(aq) and extracted 3 × with EtOAc. The organic fractions were combined, washed with brine, dried over Na_2SO_4 and concentrated. A mixture of C-2.2 diastereomers (37.9 mg, 0.08 mmol, 92 % yield) in a 5:4 ratio (A : B) was purified from the crude residue by silica column chromatography (1 % to 5 % MeOH in CH2Cl2). The diastereomers were separated with preparatory-TLC. **8 (A)** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 8.2 Hz, 1H), 6.15 (s, 1H), 5.93 (d, J = 6.8 Hz, 1H), 5.35 (q, J = 7.0 Hz, 1H), 4.44 167 (dd, J = 8.3, 6.4 Hz, 1H), 4.29 (t, J = 7.0 Hz, 1H), 3.50 – 3.32 (m, 2H), 3.08 – 2.95 (m, 2H), 2.41 – 2.29 (m, 1H), 2.19 – 2.00 (m, 4H), 1.96 (s, 3H), 1.53 (d, J = 6.9 Hz, 3H), 1.05 – 0.92 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.14, 173.48, 171.62, 171.04, 170.65, 71.03, 64.93, 58.71, 45.66, 39.33, 30.37, 30.35, 28.75, 26.31, 23.27, 22.63, 22.57, 19.48, 19.07, 18.79, 18.11, 17.91. **8c (B)** ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (d, J = 8.7 Hz, 1H), 6.04 (s, 1H), 5.81 (d, J = 7.2 Hz, 1H), 5.25 (q, J = 6.8 Hz, 1H), 4.54 (dd, J = 8.8, 5.9 Hz, 1H), 4.49 (dd, J = 7.3, 4.7 Hz, 1H), 3.47 – 3.36 (m, 2H), 3.11 – 2.98 (m, 2H), 2.38 – 2.26 (m, 2H), 2.20 – 2.09 (m, 3H), 1.95 (s, 3H), 1.51 (d, J = 6.9 Hz, 3H), 1.04 (d, J = 6.9 Hz, 3H), 0.99 (dd, J = 6.7, 2.5 Hz, 12H), 0.94 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.74, 173.66, 170.84, 170.68, 170.49, 71.63, 64.29, 57.90, 46.06, 39.32, 30.72, 30.55, 28.91, 26.35, 23.30, 22.64, 22.57, 19.42 (2C), 18.16, 17.94, 17.70. HRMS (ESI+): Exact mass calculated for $C_{22}H_{39}N_3NaO_6S$: 496.2452. Found: 496.2457.

Cloning, expression and purification of Vlm TE constructs

A codon-optimised construct containing *vlm2*$_{PCP4\text{-}TE}$(encoding residues 2290-2655 of Vlm2 from *Streptomyces tsusimaensis*, GenBank: ABA59548.1) was synthesised by ATUM (formerly DNA 2.0) in a *pJExpress411* vector, with an N-terminal hexahistidine tag followed by a tobacco etch virus protease (TEV) cleavage recognition sequence (*pJExpress411-vlm2-PCP4-TE*$_{wt}$). Two BamHI recognition sequences were included in *pJExpress411-vlm2-PCP4-TE*$_{wt}$, at nucleotide positions 2024-2025 and 2267-2268. Digestion with BamHI followed by ligation with T4 DNA ligase (New England Biolabs) excised the PCP4 domain sequence,

yielding the plasmid *pJExpress411-vlm2-TE$_{wt}$* which encodes residues 2368-2655 of Vlm2. To generate an expression vector for $TE_{DAP,}$ the TE_{wt} coding sequence was PCR-amplified from *pJExpress411-vlm2-TE$_{wt}$* with primers TE_for_pNHD_fw and TE_for_pNHD_rev. The PCR product was digested with *NdeI* and *XhoI* and ligated into similarly digested pNHD plasmid using T4 DNA ligase, generating plasmid *pNHD-vlm2-TE$_{wt}$*. Next, an amber stop codon was introduced in place of the codon for serine 2463 by site-directed mutagenesis using primers Vlm2_TE_Amb_Fw and Vlm2_TE_Amb_Rev, generating *pNHD-vlm2-TE$_{amber2463}$*.

TE domains were heterologously expressed in *E. coli* BL21(DE3) cells transformed with *pJExpress411-vlm2-TE$_{wt}$* (TE_{wt}) or co-transformed with *pNHD-Vlm2-TE$_{amber2463}$* and pSF-DAPRS-PylT (TE_{DAP}). Cultures expressing TE_{wt} were grown in LB media supplemented with 17 mg L^{-1} of kanamycin. Those expressing TE_{DAP} were grown in TB media supplemented with 25 mg L^{-1} of kanamycin, 12.5 mg L^{-1} of tetracycline, 0.1 mM of **6** (a 100 mM stock solution of **6** was prepared in 0.4 M NaOH, added to the culture and neutralised using 5 M HCl). Cultures were incubated at 37˚C, with agitation at 220 r.p.m, until they reached an OD_{600nm} = 0.6, after which they were incubated at 16˚C for 30 min, and then expression was induced with 100 µM IPTG. Cultures were incubated for an additional 16 hours at 16˚C before harvesting by centrifugation at 5000 *g* for 20 min. Cell pellets were stored at -80˚C.

For protein purification, cell pellets of TE_{wt} were resuspended in 5 mL of buffer wt-A (50 mM TRIS pH 7.4, 150 mM NaCl, 50 mM imidazole, 2 mM β-mercaptoethanol [βME]) plus DNAseI (Bioshop) per g of wet cells, and lysed by sonication. Lysate was clarified by centrifugation at 40 000 *g* for 20 min. Clarified lysate was applied to two 5 mL HiTrap IMAC FF (GE Healthcare Life Sciences) columns connected in series on an AKTA Prime system (GE Healthcare Life Sciences). Bound protein was eluted with buffer wt-B (buffer wt-A plus 150 mM imidazole). Fractions containing TE_{wt} (as determined by SDS-PAGE analysis) were pooled and incubated with TEV protease in a 1:100 (TE:TEV) mass/mass ratio and dialysed against buffer wt-C (50 mM TRIS pH 7.4, 10 mM NaCl, 2 mM βME) for 16 hours at 4˚C. The dialysed sample was applied to two 5 mL HiTrap IMAC FF columns connected in series, pre-equilibrated in buffer wt-A. Cleaved protein was recovered from the flow through and applied to two 5 mL HiTrap Q HP columns connected in series, pre-equilibrated in buffer Q-A (50 mM TRIS pH 7.4, 10 mM NaCl, 2 mM βME). Protein was eluted by a gradient of 0 to 100% buffer Q-B (50 mM TRIS pH 7.4, 500 mM NaCl, 2 mM βME) over 240 mL. TE_{wt}-containing fractions were concentrated in a 10 kDa molecular weight cut off Amicon® Ultra centrifugal filter (Millipore) and injected onto a Superdex S-200 16/60 PG column (GE-Healthcare) pre-equilibrated in SEC buffer (25 mM HEPES pH 7.4 or pH 8.0, 100 mM NaCl, 0.2 mM tris(2-carboxyethyl)phosphine [TCEP]). Fractions containing purified TE_{wt} were pooled, concentrated and flash frozen.

Cell resuspension, lysis, clarification and Ni-IMAC purification for TE_{DAP} were performed as described for Te_{wt}, except that prolonged exposure to light was avoided. After elution from the Ni-IMAC column, the sample was irradiated with UV light (365 nm, 35 $mWcm^{-2}$, 1 min). TEV cleavage and the subsequent IMAC column were performed as described for TE_{wt}, except that a 1:1 TE:TEV ratio was used. Anion exchange was performed as described for $Te_{wt,}$ except that 25 mM HEPES replaced TRIS as the buffer and 0.2 mM TCEP replaced βME as the reducing agent in the mobile phases. Relevant fractions were concentrated and injected onto a Superdex S-75 10/300 column pre-equilibrated in buffer T (25 mM HEPES pH 8.0, 100 mM NaCl, 0.2 mM TCEP). Fractions containing purified TE_{DAP} were pooled, concentrated, and immediately used for further experiments. The yield of purified TE_{wt} was 30-60 mg per L, the yield of purified TE_{DAP} was 0.1-0.5 mg per L.

Crystallography

Crystallisation conditions for TE_{wt} structure 1 were found in vapour diffusion crystallisation trials using commercially available screens (Qiagen) and a protein concentration of 10 mg mL^{-1}. Optimisation of an initial crystallisation hit in 24-well plates led to a final crystallisation condition where 3.2 µL of 10 mg mL^{-1} TE_{wt}, 4.0 µL 1.65 M DL-malic acid pH 9.5, and 0.8 µL of 17 % m/v IPTG were incubated against a reservoir solution of 500 µL of 1.65 M DL-malic acid pH 9.5. TE_{wt} structure 2 crystals were grown in similar conditions, where 0.5 µL of purified TE_{wt} at 22.4 mg mL^{-1} and 0.5 µL of 1.65 M DL-malic acid pH 8.1 were incubated against a reservoir solution of 500 µL of DL-malic acid pH 8.1. Crystals appeared between 24 and 48 hours and reached their maximum size in approximately one week.

Crystals of unliganded TE_{DAP} were grown in similar conditions to TE_{wt}, with a reservoir solution of 1.65 M DL-malic acid pH 8.0. In order to obtain the tetradepsipeptidyl-TE_{DAP} complex structure, TE_{DAP} crystals were incubated with deoxytetradepsipeptidyl-SNAC **8** once they achieved their maximum size. The reservoir solution was exchanged to 2.66 M DL-malic acid, pH 9.5, and 32 µL of a solution of 1 mM deoxytetradepsipeptidyl-SNAC**,** 2.66 M DL-malic acid pH 9.5, 100 mM NaCl, 25 mM HEPES pH 9.2, 10% DMSO was added to the drop. Crystals were incubated in this condition for 9 days at room temperature.

For dodecadepsipeptidyl-TE_{DAP} complex crystals, TE_{DAP} (0.1 mg mL^{-1}) was incubated in a 1.1 mg mL^{-1} suspension of valinomycin in buffer T for 16 hours at room temperature. The sample was centrifuged at 20 000 *g* and applied to a Superdex S-75 10/300 column preequilibrated in buffer T to remove excess valinomycin. Relevant fractions were pooled and complex formation was evaluated by LC-ESI-MS (see below). The sample was concentrated to 13.4 mg mL^{-1} and diffraction-quality crystals, with a different morphology from the TE_{wt} crystals, were obtained in sitting drops consisting of 1 µL of dodecadepsipeptidyl-TE_{DAP} complex plus 1 µL reservoir solution (1.30 to 1.45 M DL-malic acid pH 8.1) equilibrated against 500 µL

reservoir solution. In an attempt to improve the occupancy of the ligand, a subset of these crystals were further incubated with valinomycin, by addition of 20 µL of a solution containing 555 µM valinomycin, 2 M DL-malic acid pH 8.1, 11 mM HEPES pH 8.0, 44 mM NaCl, 0.088 mM TCEP for 24 hours.

TE_{wt} and dodecadepsipeptidyl-TE_{DAP} crystals were cryo-protected by addition of 10 µL (TE_{wt} structure 1 and dodecadepsipeptidyl-TE_{DAP}) or 20 µL (TE_{wt} structure 2) 3.6 M DL-malic acid pH 8.1 to the crystallisation drop. For dodecadepsipeptidyl-Te_{DAP} crystals that had been incubated with valinomycin, the drop solution was removed and replaced by 10 µL of 3.6 M DL-malic acid. Crystals were equilibrated for at least two minutes and then flash cooled in liquid nitrogen. Tetradepsipeptidyl-TE_{DAP} complex crystals were looped and flash cooled directly from the incubating solution. TE_{wt} data were first collected at the Centre for Structural Biology at McGill University, Montreal, Canada, on a Rigaku RUH3R generator and a R-AXIS IV++ detector. Higher resolution data for TE_{wt} and depsipeptidyl-TE_{DAP} complexes were collected at the Canadian Light Source (CLS) 08ID-1 beamline or at the Advanced Photon Source (APS) NE-CAT 24-ID-C beamline using a Pilatus detector (**Extended Data Table 1**).

TE_{wt} structure determination

Diffraction data from TE_{wt} structure 1 crystals were indexed and integrated in the space group P432 using iMosflm[22] or DIALS[23]. Further space group determination and scaling were performed using the programs POINTLESS and SCALA[24]. The structure was solved by molecular replacement using PHASER[25], with a SCULPTOR[26] modified version of the TE domain of srfA-C (PDB ID 2VSQ)[27] as a search model. The structure was iteratively refined and built with the programs Phenix[28] and AUTOBUILD[29]. Coot[30] was used for iterative model building. Topology diagrams were generated using TopDraw[31] based on results from PDBsum generate (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) using the TE_{wt} structure as input.

Diffraction data sets collected from dodecadepsipeptidyl-TE_{DAP} complex crystals were indexed into either P1 or H3 space groups using iMosflm[22] or DIALS[23]. Most crystals belonging to the H3 group showed evidence of twinning, and only non-twinned diffraction data were used for structure determination. Structures in both the P1 and H3 space groups were solved by molecular replacement using PHASER[25], with the TE_{wt} structure lacking residues 2500-2647 used as a search model. The P1 structure had six molecules in the asymmetric unit whereas the H3 structure contained 2 molecules in the asymmetric unit. All depsipeptidyl-TE_{DAP} models were refined, and mF_o-F_c maps were generated before depsipeptide residues were built in the model (**Fig. 4c, d and Extended Data Fig. 8**). Depsipeptides were built from individual monomers of the PDB Chemical Component Dictionary (DPP, VAL, 2OP, DVA, VAD). Monomer libraries and restraints for the links between the monomers (namely DPP ->

VAL, VAL -> 2OP, 2OP -> DVA, DVA -> VAD and VAD -> VAL) were calculated using AceDRG[32] and merged using LIBCHECK[33]. The resulting merged dictionary was then used for substrate building in Coot[30] and refinement in REFMAC5[34] and phenix.refine[28]. Final statistics are shown in **Extended Data Table 1**.

Depsipeptidyl-TE complex formation

TE_{DAP} or TE_{wt} at a final concentration of 0.2 mg mL^{-1} was incubated with tetradepsipeptidyl-SNAC **7** (1.7 mM), or valinomycin (50 µM) in buffer T containing 1.7% or 1% v/v DMSO for 16 hours. Reactions were concentrated in a 10 kDa molecular weight cut off Amicon® Ultra centrifugal filter (Millipore), clarified by centrifugation at 20 000 *g* and applied to a Superdex S-75 10/300 column pre-equilibrated in buffer T to remove excess depsipeptidyl-SNACs or valinomycin before final LC-ESI-MS analysis. To form the deoxytetradepsipeptidyl-TE_{DAP} complex, TE_{DAP} at a final concentration of 8.7 mg mL^{-1} was incubated with deoxytetradepsipeptidyl-SNAC **8** (2.6 mM) in 25 mM HEPES pH 8.6, 100 mM NaCl, 3.8% v/v DMSO for 40 hours. The sample was diluted in 100 mM ammonium bicarbonate pH 8.0 before final LC-ESI-MS analysis. All incubations were performed at room temperature.

LC-ESI-MS analysis of intact proteins

For experiments shown in **Fig. 2c**, **Extended Data Fig. 7b**, protein samples were subjected to a liquid chromatography (LC) system (Agilent 1200 series) followed by in-line electrospray ionisation mass spectrometry (ESI-MS) on a 6130 Quadrupole spectrometer. Using a Jupiter 5 μ C4 300A column, 150 mm x 2.00 mm (Phenomenex), proteins were run through the LC system using water with 0.1% (v/v) formic acid (solvent A) and a gradient (10% to 75% in 6 min and 75% to 95% in 1,5 min) of acetonitrile with 0.1% (v/v) formic acid (solvent B). Proteins were detected by monitoring UV absorbance at 200 and 280 nm. Protein masses were calculated by deconvolution from the MS acquisition in positive ion mode, using the OpenLAB CDS software (Agilent Technologies).

For experiments shown in **Fig. 4a, b** and **Extended Data Fig. 6c**, **7c, d**, protein concentration was adjusted to 0.1 mg mL^{-1} in buffer T, and 16 uL was injected onto an Agilent PLRP-S (1000 A 5 µM, 50 x 2.1 mm ID) column pre-equilibrated in 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in 100% acetonitrile) on an Agilent Technologies 1260 Infinity HPLC system coupled to a Bruker Amazon Speed ETD ion trap mass spectrometer. MS data was collected with ExtremeScan mass range mode in positive ion polarity, scan range from 50 to 3000 m/z, accumulation time of 1586 μs, RF level of 96%, trap drive 69.8, PSP target Mass 922 m/z, and averaging over 5 spectra. External instrument calibration was performed using the Agilent ESI tune mix. The column compartment temperature was set up at 80˚C throughout the run. After injection, the column was washed for 5 minutes in initial HPLC conditions with the sample compartment diversion valve in the

waste position. Next, a 5-minute gradient from 5% to 100% mobile phase B was performed, followed by an isocratic step of 100% mobile phase B for 8 minutes. Proteins were detected by monitoring UV absorbance at 280 nm. Data was analyzed using the Bruker DataAnalysis software (Bruker). Mass spectra were integrated from 10.5 to 13 minutes, and deconvoluted using a window between 10 000 to 40 000 m/z.

Tandem MS/MS analysis

Proteins were run on 4-12% NuPAGE Bis-Tris gel (Invitrogen) with MES buffer and briefly stained using InstantBlue (Expedeon). The bands were excised and stored in 20 mM Tris pH 7.4. Tryptic digestion and tandem MS/MS analyses were performed by Kate Heesom (Proteomics Facility, University of Bristol).

LC-ESI-MS analysis of VIm TE reaction products

Purified TE_{wt} or TE_{DAP} at 0.2 mg mL^{-1} (6.5 µM) was incubated with tetradepsipeptidyl-SNAC **7** (1.7 mM), or a mix of tetradepsipeptidyl-SNAC **7** and deoxytetradepsipeptidyl-SNAC **8** (1.7 mM each) in buffer T. Samples were incubated at room temperature for 24 hours, and then quenched with one volume of 0.1% formic acid in acetonitrile. Next, samples were centrifuged at 20,000 *g*, flash frozen in liquid nitrogen and stored at -80˚C before HPLC analysis. For HPLC-MS analysis, frozen samples were thawed at room temperature, vortexed and clarified by centrifugation at 20,000 *g* before injection. HR-LC-ESI-MS was performed at the Mass Spectroscopy Facility (Department of Chemistry, McGill University) with an Agilent XDB-C8 (5 µm, 4.6 x 150 mm) column in a Dionex Ultimate 3000 UHPLC system coupled to a Bruker maXis impact QTOF mass spectrometer in positive ESI mode. Ion-trap LC-ESI-MS analysis was performed in an Agilent Technologies 1260 Infinity HPLC system coupled to a Bruker Amazon Speed ETD ion trap mass spectrometer in positive ESI mode. The column compartment was set up at 40˚C throughout the runs. Starting HPLC conditions were 50% mobile phase A (0.1% formic acid in H_2O), 50% mobile phase B (0.1% formic acid in acetonitrile). After injection (1µL for HR-LC-ESI-MS and 5 µL for ion-trap LC-ESI-MS), a gradient from 50% to 98% mobile phase B in 5 minutes was performed, followed by an isocratic step of 98% mobile phase B, run for 20 minutes. For HR-LC-ESI-MS, internal calibration was performed with an intra-run infusion at the beginning of the first analysis using Na^+ formate, and the resulting calibration was used as an external calibration for subsequent analysis. Ion trap external calibration was performed using the Agilent ESI tune mix. Data was analysed using the Bruker DataAnalysis software and the SmartFormula tool (Bruker).

Statistics and Reproducibility

Supplementary Table 2: SmartFormula analysis of the reactions shown Fig. 3a (a) and Extended Data Fig. 5j (b). Experiment on **Fig. 3a** was replicated twice with similar results. HR-MS analysis of experiment on **Extended Data Fig. 5j** was done once.

a. TE_{wt}+tetradepsipeptidyl-SNAC **7.**

Molecule	Adduct	Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	eØ Conf	mSigma	Std I
tetradepsipeptidyl-SNAC **7**	H^+	490.2593	1	C22H40N3O7S	490.2581	-2.4	-3.4	4.5	ok	even	32.2	66.4
	$[NH_4]^+$	507.2878	1	C22H43N4O7S	507.2847	-6.1	1681	3.5	ok	even	50.2	108.7
	Na^+	512.2414	1	C22H39N3NaO7S	512.2401	-2.6	-4.4	4.5	ok	even	33.7	67.6
tetradepsipeptide **9**	H^+	389.2296	1	C18H33N2O7	389.2282	-3.5	-4.4	3.5	ok	even	32.5	59.9
	$[NH_4]^+$	406.2571	1	C18H36N3O7	406.2548	-5.7	1018	2.5	ok	even	24	48.7
	Na^+	411.2120	1	C18H32N2NaO7	411.2102	-4.6	-5.2	3.5	ok	even	21.6	38.1
octadepsipeptidyl-SNAC **11**	H^+	860.4695	1	C40H70N5O13S	860.4685	-1.1	-2.8	8.5	ok	even	35.4	55.8
	$[NH_4]^+$	877.4972	1	C40H73N6O13S	877.4951	-2.4	-0.7	7.5	ok	even	29.5	44
	Na^+	882.4508	1	C40H69N5NaO13S	882.4505	-0.4	-2.1	8.5	ok	even	32.9	52.4
octadepsipeptide **13**	H^+	759.4409	1	C36H63N4O13	759.4386	-3	-3.8	7.5	ok	even	30.4	44.7
	$[NH_4]^+$	776.4686	1	C36H66N5O13	776.4652	-4.5	-2.3	6.5	ok	even	19.4	26.7
	Na^+	781.4227	1	C36H62N4NaO13	781.4206	-2.7	-3.8	7.5	ok	even	28.1	42.1
dodecadepsipeptidyl-SNAC **15**	H^+	1230.6842	1	C58H100N7O19S	1230.6789	-4.3	-5.8	12.5	ok	even	38.3	44
	$[NH_4]^+$	1247.7096	1	C58H103N8O19S	1247.7055	-3.3	-4.4	11.5	ok	even	34.5	40.2
	Na^+	1252.6680	1	C58H99N7NaO19S	1252.6609	-5.7	-6.7	12.5	ok	even	44.5	49.8
16-mer depsipeptidyl-SNAC **19**	H^+	1600.9055	1	C76H130N9O25S	1600.8893	-10.1	799.8	16.5	ok	even	89.4	107.7
	$[NH_4]^+$	1617.9319	1	C76H133N10O25S	1617.9159	-9.9	790.7	15.5	ok	even	91.4	110.2
	Na^+	1622.8891	1	C76H129N9NaO25S	1622.8713	-11	788.5	16.5	ok	even	92	110.5
montanastatin **27**	H^+	741.4309	1	C36H61N4O12	741.4281	-3.8	644.9	8.5	ok	even	22.2	36.8
	$[NH_4]^+$	758.4574	1	C36H64N5O12	758.4546	-3.7	628.9	7.5	ok	even	25.7	41.9
	Na^+	763.4120	1	C36H60N4NaO12	763.4100	-2.6	-5.4	8.5	ok	even	29.3	43.7
valinomycin **28**	$[NH_4]^+$	1128.6673	1	C54H94N7O18	1128.6650	-2.1	0.7	11.5	ok	even	78.2	72.3

b. TE_{wt}+tetradepsipeptidyl-SNAC **7**+ deoxytetradepsipeptidyl-SNAC **8**

Molecule	Adduct	Meas. m/z	#	Ion Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	eØ Conf	mSigma	Std I
tetradepsipeptidyl-SNAC **7**	H^+	490.2607	1	C22H40N3O7S	490.2581	-5.2	-10.1	4.5	ok	even	17	35.1
	$[NH_4]^+$	507.2884	1	C22H43N4O7S	507.2847	-7.3	15.7	3.5	ok	even	25.7	38.4
	Na^+	512.2408	1	C22H39N3NaO7S	512.2401	-1.3	-3.5	4.5	ok	even	33.1	67
deoxytetradepsipeptidyl-SNAC **8**	H^+	474.2649	1	C22H40N3O6S	474.2632	-3.5	-7.9	4.5	ok	even	34.5	49.2
	$[NH_4]^+$	496.2462	1	C22H39N3NaO6S	496.2452	-2	-7	4.5	ok	even	29.9	61.8
tetradepsipeptide **9**	H^+	389.2307	1	C18H33N2O7	389.2282	-6.2	5.6	3.5	ok	even	7.6	12.1
	$[NH_4]^+$	406.2565	1	C18H36N3O7	406.2548	-4.1	19	2.5	ok	even	97	158.9
	Na^+	411.2126	1	C18H32N2NaO7	411.2102	-5.9	3.2	3.5	ok	even	7.9	12.8
deoxyoctadepsipeptide **10**	H^+	743.4482	1	C36H63N4O12	743.4437	-6	-7.5	7.5	ok	even	34.4	55.6
	$[NH_4]^+$	760.4719	1	C36H66N5O12	760.4702	-2.2	627.4	6.5	ok	even	39.4	52.3
	Na^+	765.4321	1	C36H62N4NaO12	765.4256	-6.6	622.1	7.5	ok	even	39.7	51.7
octadepsipeptidyl-SNAC **11**	H^+	860.4731	1	C40H70N5O13S	860.4685	-5.4	-21.3	8.5	ok	even	128.9	140.4
	$[NH_4]^+$	877.5009	1	C40H73N6O13S	877.4951	-6.6	-7	7.5	ok	even	26.4	37.9
	Na^+	882.4517	1	C40H69N5NaO13S	882.4505	-1.4	-4.1	8.5	ok	even	26.8	42
deoxyoctadepsipeptidyl-SNAC **12**	H^+	844.4781	1	C40H70N5O12S	844.4736	-5.2	-9.3	8.5	ok	even	28.4	46.5
	$[NH_4]^+$	861.5006	1	C40H73N6O12S	861.5002	-0.5	-7.1	7.5	ok	even	27.1	41.4
	Na^+	866.4591	1	C40H69N5NaO12S	866.4556	-4	-9.3	8.5	ok	even	30.1	48.4
octadepsipeptide **13**	H^+	759.4466	1	C36H63N4O13	759.4386	-10.5	-25.8	7.5	ok	even	354.4	364.2
	$[NH_4]^+$	776.4740	1	C36H66N5O13	776.4652	-11.4	0.1	6.5	ok	even	108.7	156.6
	Na^+	781.4250	1	C36H62N4NaO13	781.4206	-5.6	-0.7	7.5	ok	even	123.1	169.9
dodecadepsipeptidyl-SNAC **15**	H^+	1230.6992	1	C58H100N7O19S	1230.6789	-16.5	-28.4	12.5	ok	even	481.7	518
	$[NH_4]^+$	1247.7206	1	C58H103N8O19S	1247.7055	-12.1	719.1	11.5	ok	even	58.3	78.3
	Na^+	1252.6585	1	C58H99N7NaO19S	1252.6609	1.9	727.4	12.5	ok	even	51.6	64.5
deoxydodecadepsipeptidyl-SNAC **16**	H^+	1214.6971	1	C58H100N7O18S	1214.6840	-10.7	-19.9	12.5	ok	even	11.1	11.5
	$[NH_4]^+$	1231.7221	1	C58H103N8O18S	1231.7106	-9.4	-17.9	11.5	ok	even	29.4	34.6
	Na^+	1236.6816	1	C58H99N7NaO18S	1236.6660	-12.6	-13.1	12.5	ok	even	14.3	14.8
16-mer depsipeptidyl-SNAC **19**	$[NH_4]^+$	1617.9346	1	C76H133N10O25S	1617.9159	-11.6	683.6	15.5	ok	even	465.4	437.1
	Na^+	1622.8725	1	C76H129N9NaO25S	1622.8713	-0.8	994.2	16.5	ok	even	216	286.2
deoxy 16-mer depsipeptidyl-SNAC **20**	H^+	1584.9076	1	C76H130N9O24S	1584.8944	-8.3	794.8	16.5	ok	even	86	100
	$[NH_4]^+$	1601.9337	1	C76H133N10O24S	1601.9209	-8	288.2	15.5	ok	even	14.2	13.1
	Na^+	1606.8919	1	C76H129N9NaO24S	1606.8763	-9.7	554.5	16.5	ok	even	29	31
valinomycin **28**	$[NH_4]^+$	1128.6752	1	C54H94N7O18	1128.6650	-9	445.7	11.5	ok	even	24	29.7

Full Acknowledgments to Synchrotron Sources

CLS: Research described in this paper was performed using beamline 08ID-1 at the Canadian Light Source, which is supported by the Canada Foundation for Innovation, Natural Sciences and Engineering Research Council of Canada, the University of Saskatchewan, the Government of Saskatchewan, Western Economic Diversification Canada, the National Research Council Canada, and the Canadian Institutes of Health Research.

APS: This work is based upon research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by the National Institute of General Medical Sciences from the National Institutes of Health (P41 GM103403). The Pilatus 6M detector on 24-ID-C beam line is funded by a NIH-ORIP HEI grant (S10 RR029205). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

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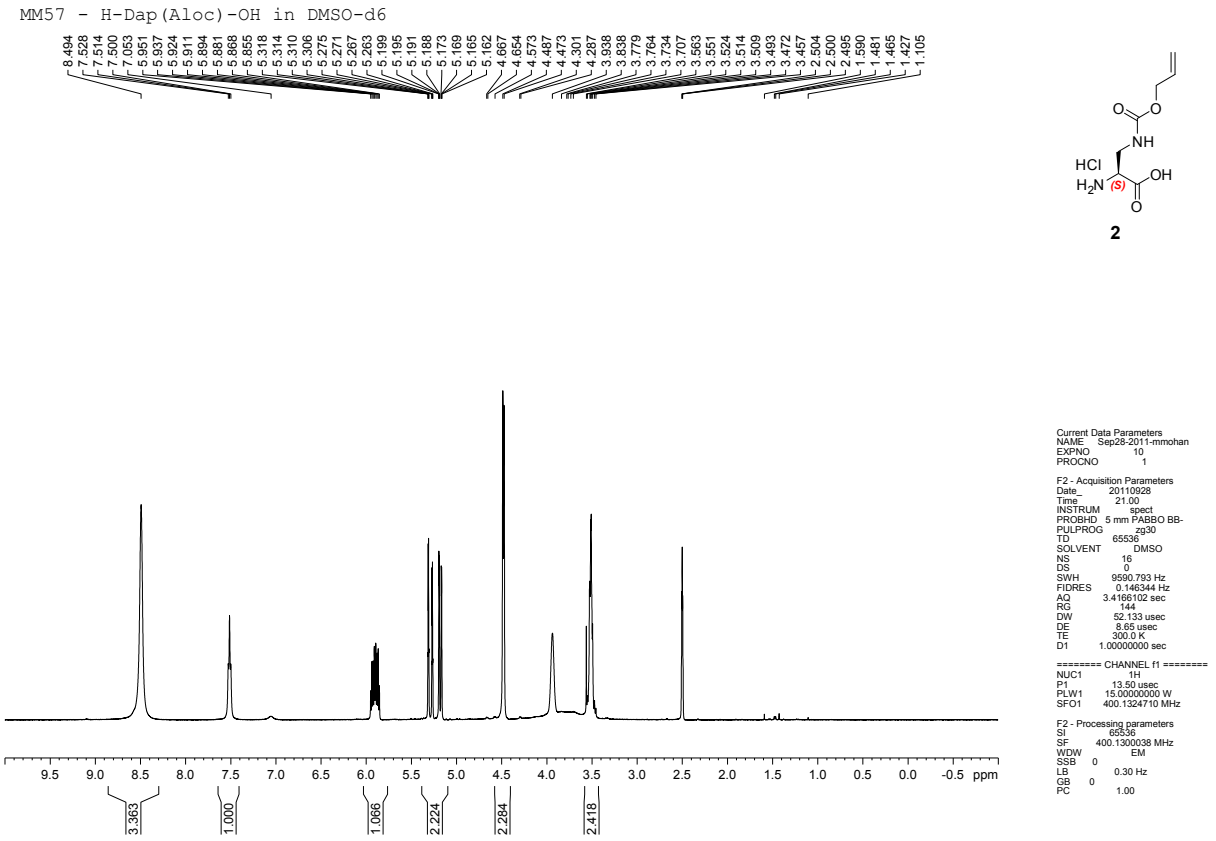
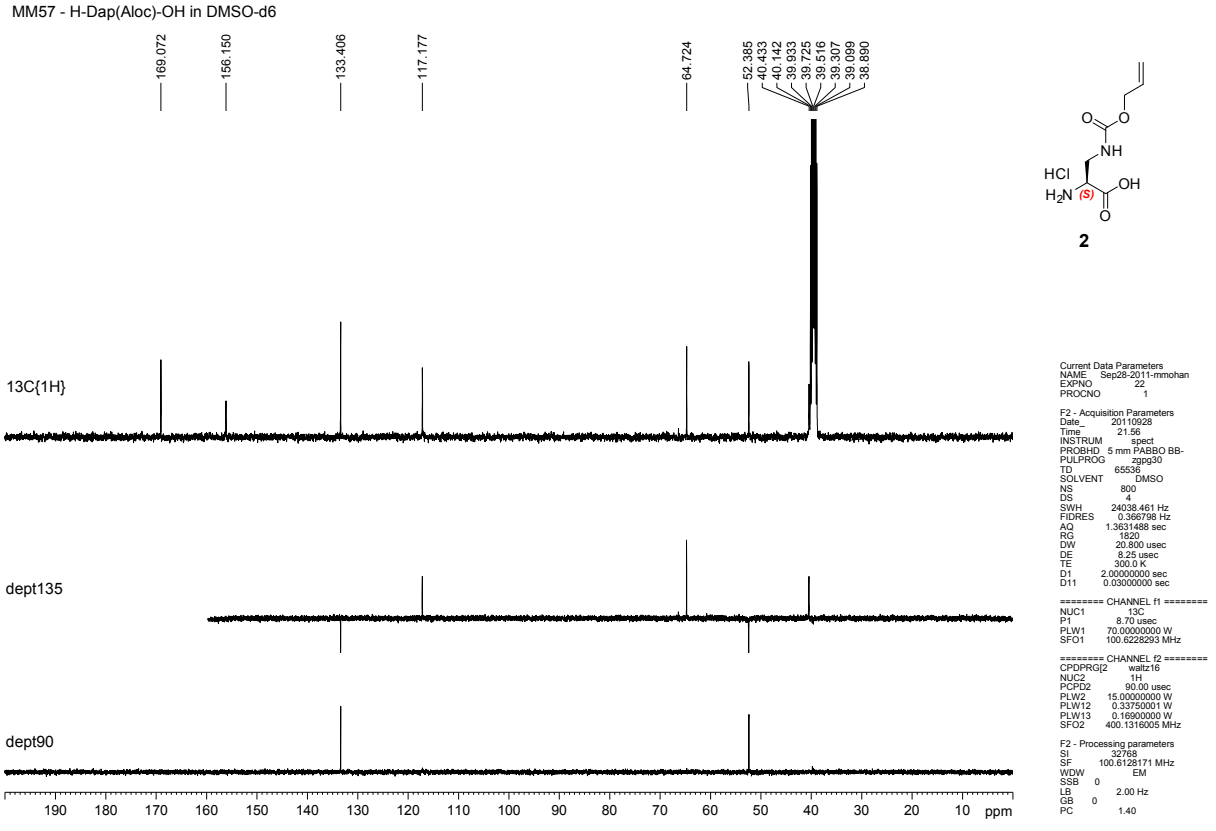
Appendix 1

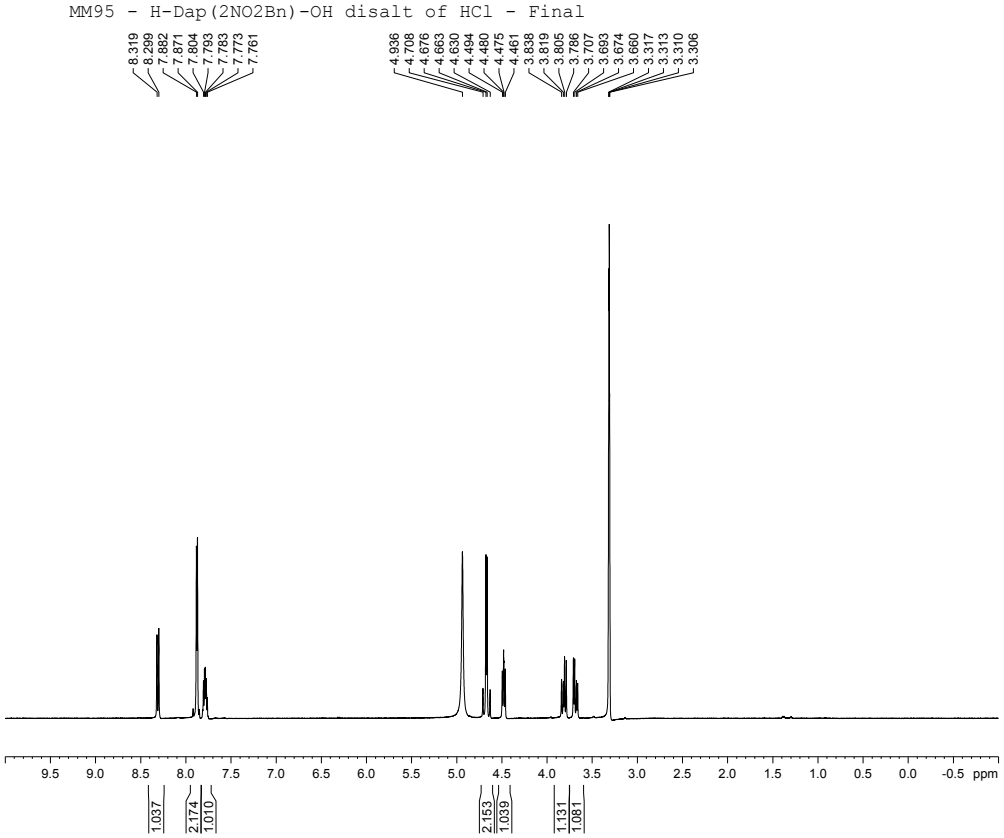
^{1}H NMR Spectrum of 2

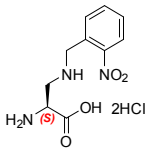


^{13}C{1H}, DEPT135 and DEPT90 NMR Spectra of 2



^{1}H NMR Spectrum of 3





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PROCNO 1

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FIDRES 0.146344 Hz
AQ 3.4166102 sec
RG 144
DW 52.133 usec
DE 8.65 usec
TE 291.6 K
D1 1.00000000 sec

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P1 13.50 usec
PLW1 15.00000000 W
SFO1 400.1324710 MHz

F2 - Processing parameters
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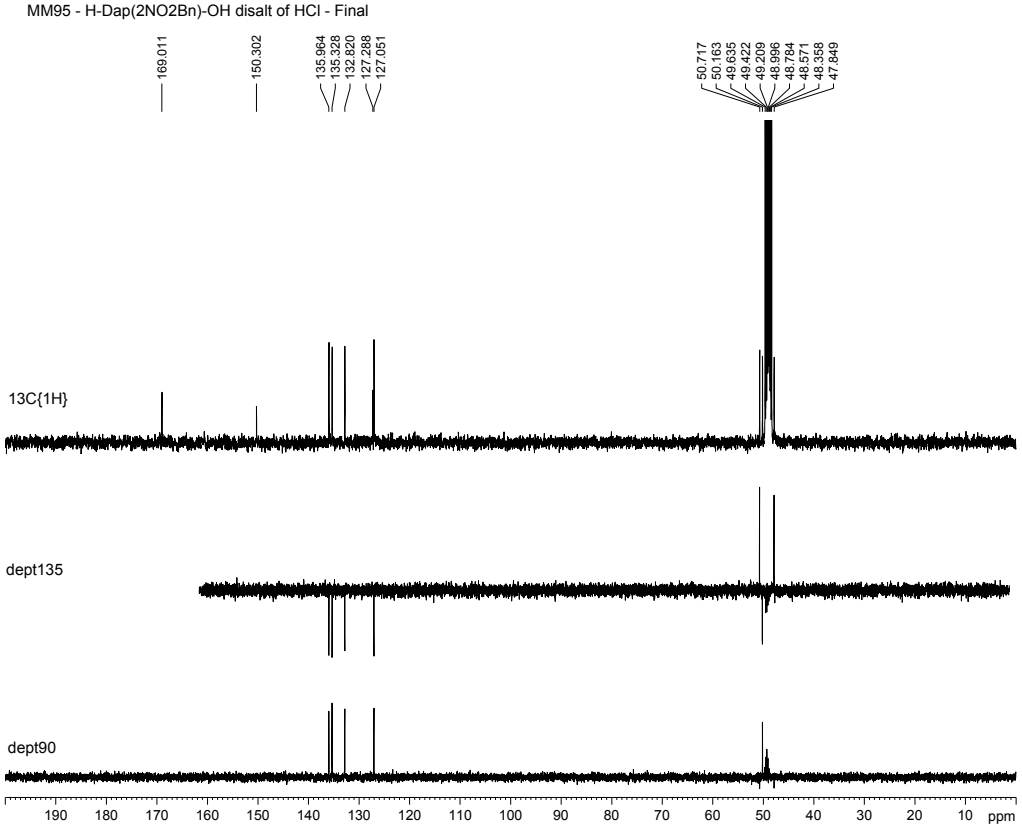
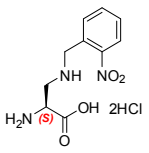
13C{1H}, DEPT135 and DEPT90 NMR Spectra of 3





3. 2HCl

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PROCNO 1

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FIDRES 0.366798 Hz
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RG 1820
DW 20.800 usec
DE 8.25 usec
TE 292.6 K
D1 2.00000000 sec
D11 0.03000000 sec

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P1 8.70 usec
PLW1 70.00000000 W
SFO1 100.6228293 MHz

======== CHANNEL f2 ========
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PLW2 15.00000000 W
PLW12 0.33750001 W
PLW13 0.16900000 W
SFO2 400.1316005 MHz

F2 - Processing parameters
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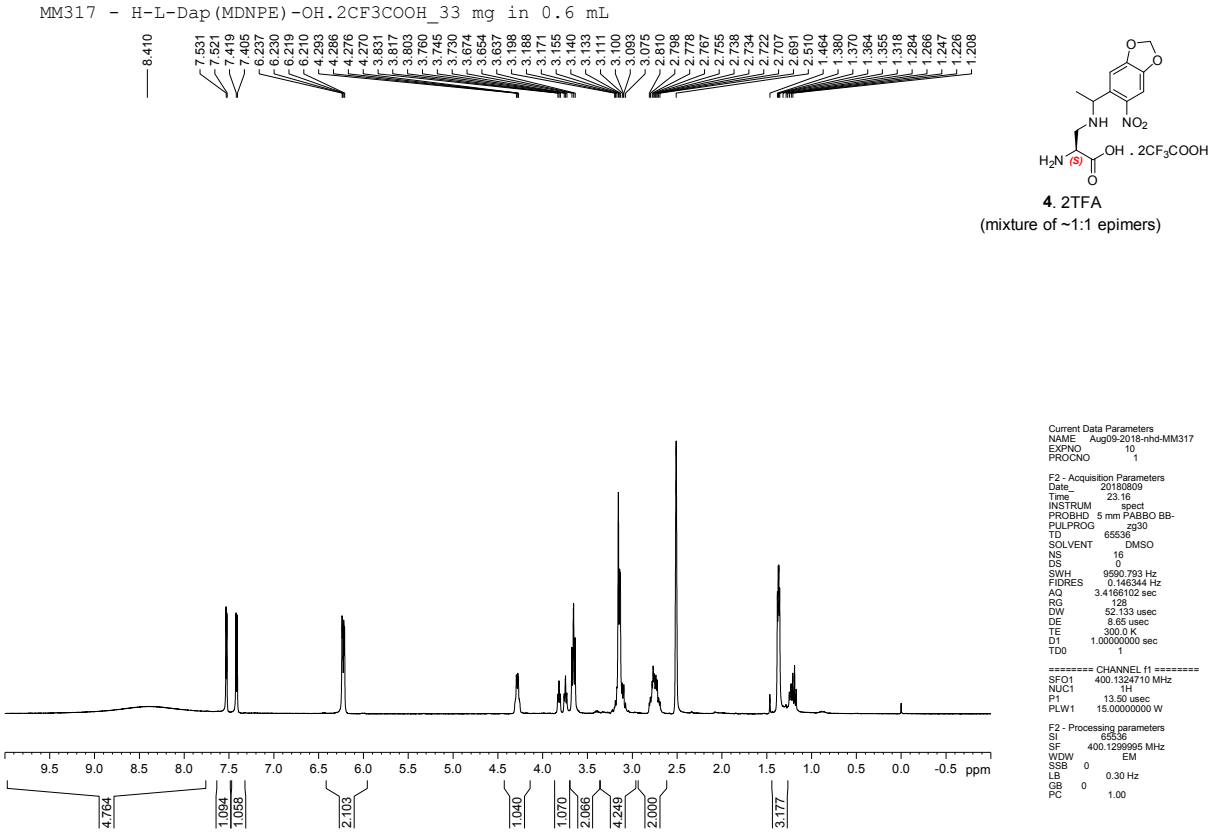
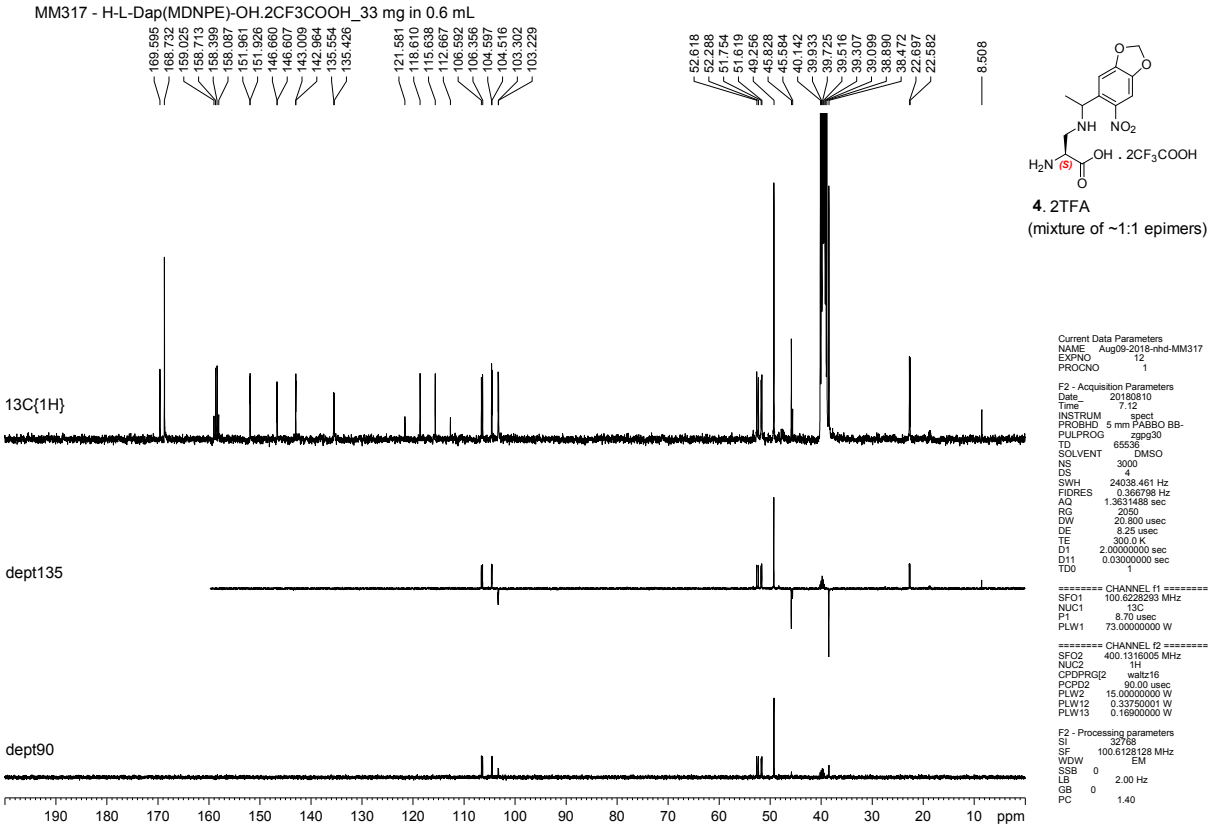
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.40

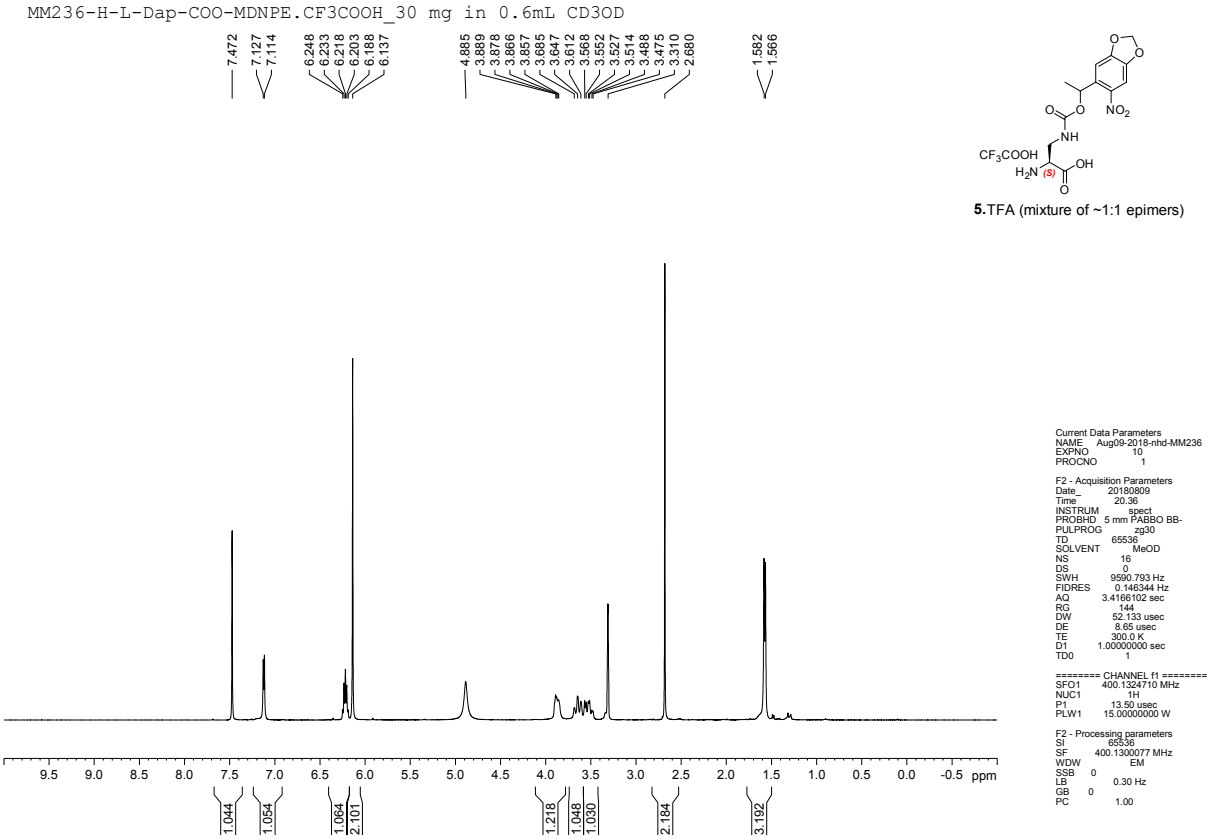
^{1}H NMR Spectrum of 4



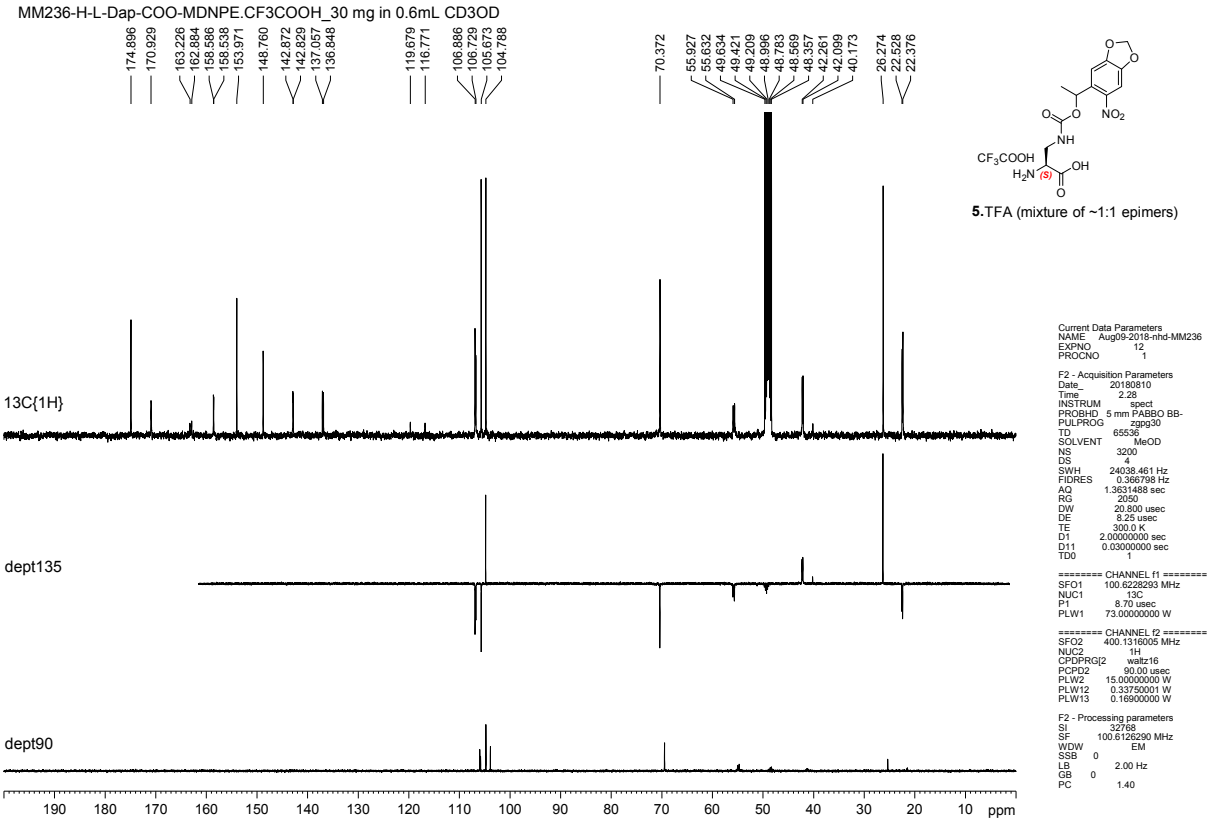
13C{1H}, DEPT135 and DEPT90 NMR Spectra of 4



^{1}H NMR Spectrum of 5

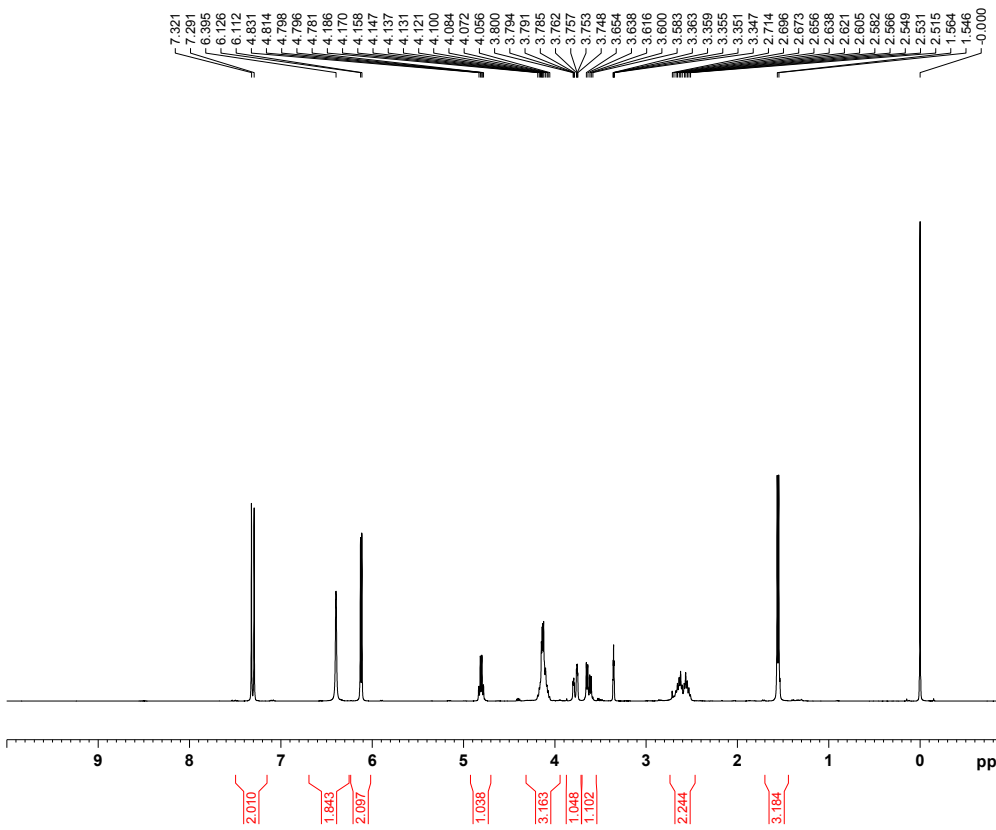


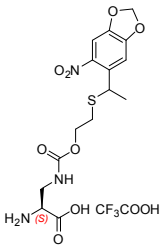
13C{1H}, DEPT135 and DEPT90 NMR Spectra of 5



^{1}H NMR Spectrum of 6

MM550 - H-L-Dap(COO-CH2CH2S-MDNPE)-OH.CF3COOH_50 mg in 0.5 mL CD3OD + 0.1 mL CF3COOD (CD3OD contains 1% v/v TMS int. std.)





6 (Mixture of ~1:1 Epimers)

Current Data Parameters
NAME Jul24-2017-nhd
EXPNO 10
PROCNO 1

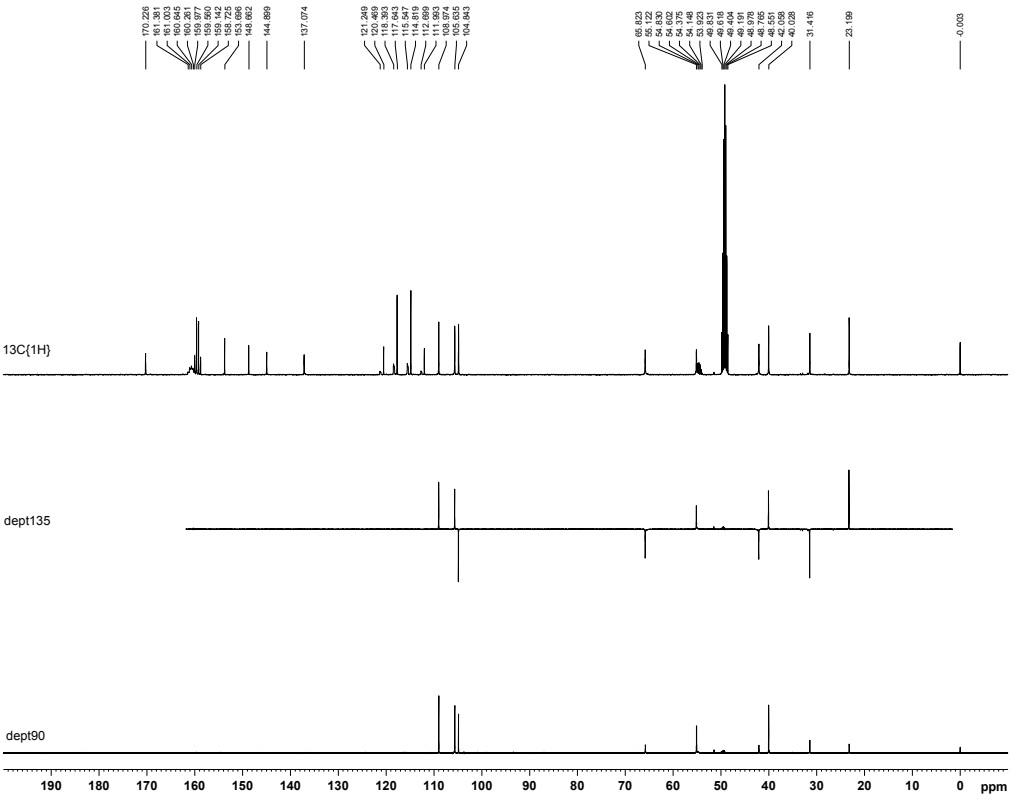
F2 - Acquisition Parameters
Date_ 20170724
Time 21.09
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT MeOD
NS 16
DS 0
SWH 9590.793 Hz
FIDRES 0.146344 Hz
AQ 3.4166102 sec
RG 90.5
DW 52.133 usec
DE 8.65 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1

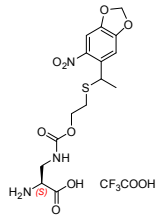
======== CHANNEL f1 ========
SFO1 400.1324710 MHz
NUC1 1H
P1 13.50 usec
PLW1 15.00000000 W

F2 - Processing parameters
SI 65536
SF 400.1299894 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

13C{1H}, DEPT135 and DEPT90 NMR Spectra of 6

MM550 - H-L-Dap(COO-CH2CH2S-MDNPE)-OH.CF3COOH_50 mg in 0.5 mL CD3OD + 0.1 mL CF3COOD (CD3OD contains 1% v/v TMS int. std.)





6 (Mixture of ~1:1 Epimers)

Current Data Parameters
NAME Jul24-2017-nhd
EXPNO 13
PROCNO 1

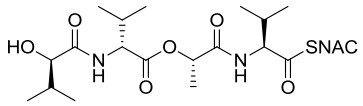
F2 - Acquisition Parameters
Date_ 20170725
Time 7.52
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT MeOD
NS 11000
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631488 sec
RG 2050
DW 20.800 usec
DE 8.25 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 100.6228293 MHz
NUC1 13C
P1 8.70 usec
PLW1 73.00000000 W

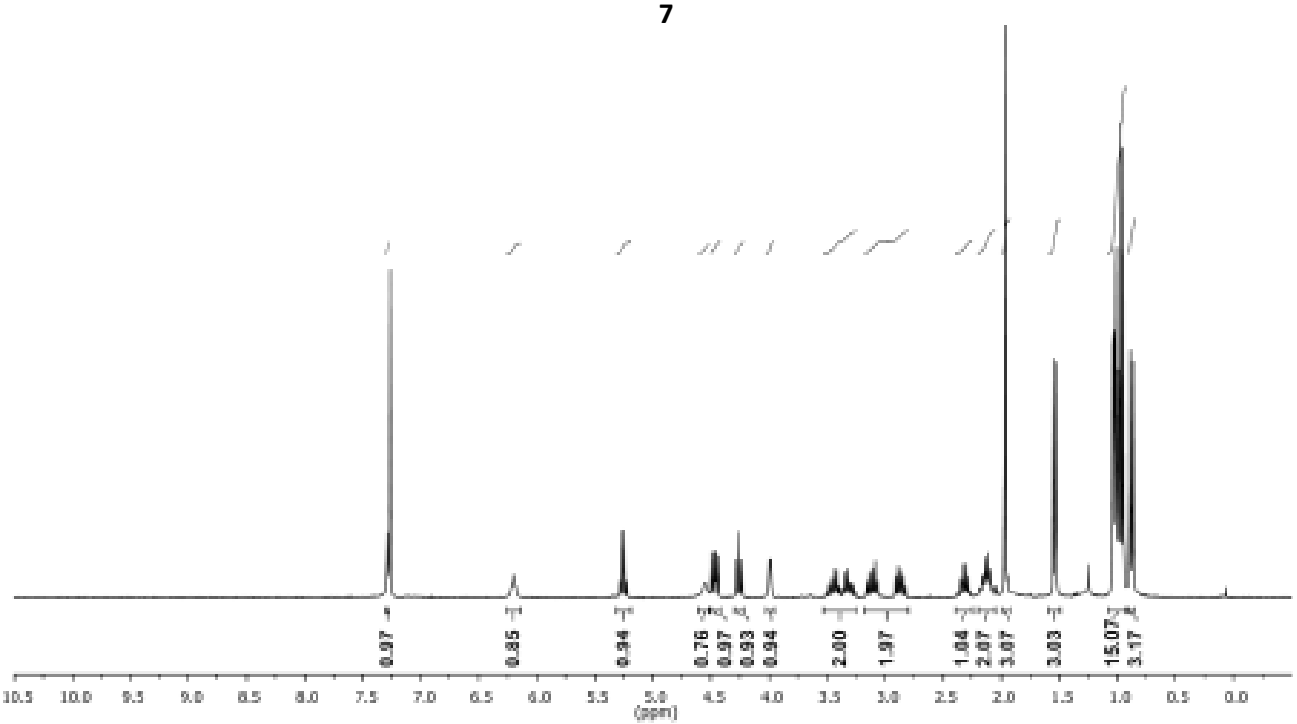
======== CHANNEL f2 ========
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 15.00000000 W
PLW12 0.33750001 W
PLW13 0.16900000 W

F2 - Processing parameters
SI 32768
SF 100.6126016 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.40

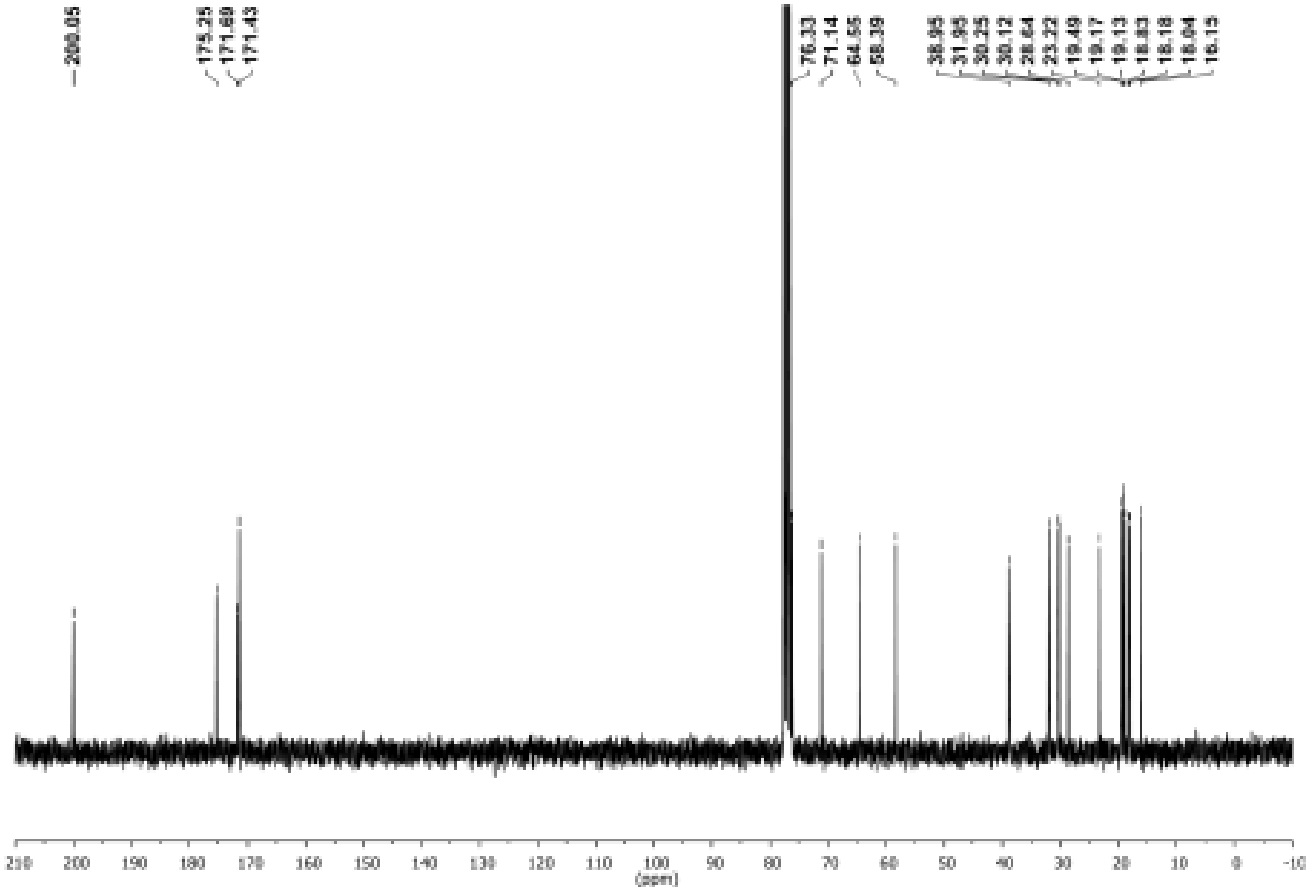
^{1}H NMR Spectrum of 7



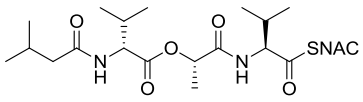
7



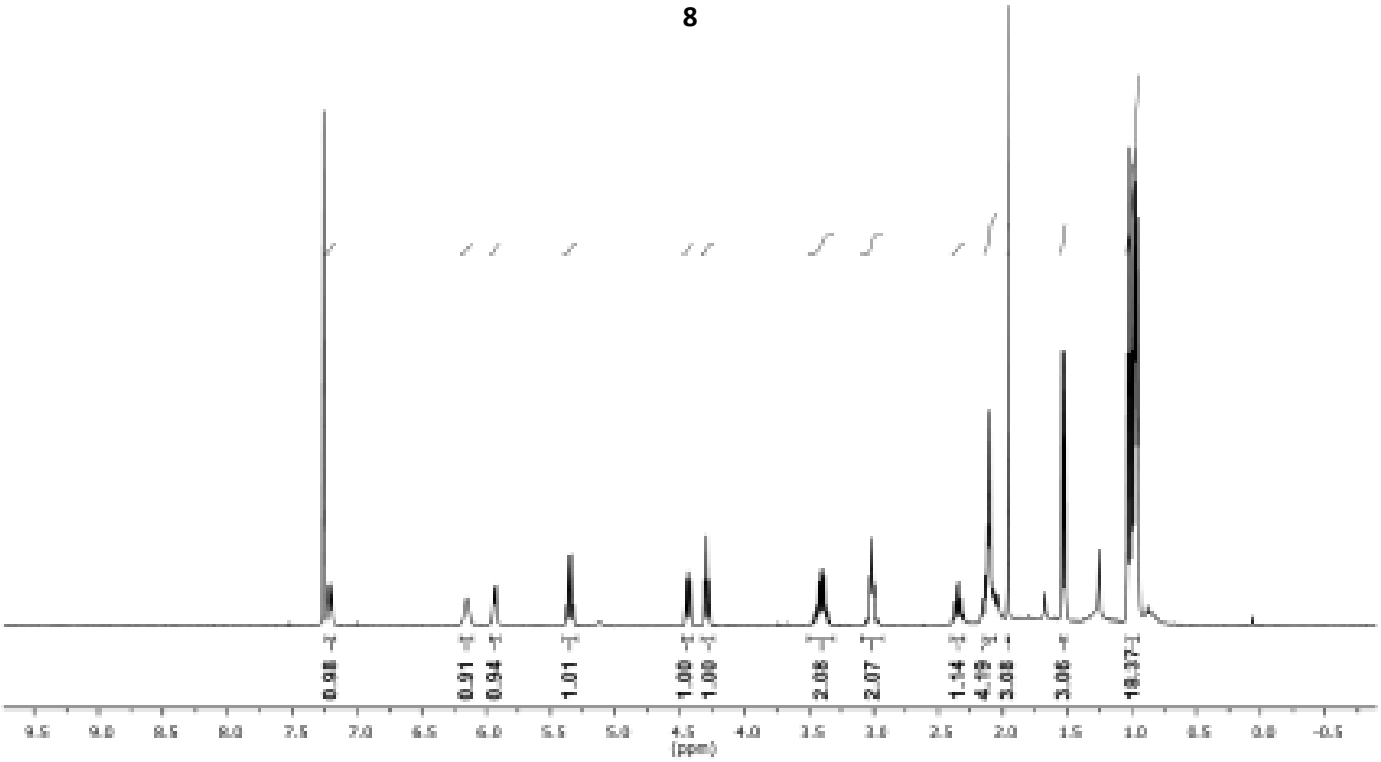
^{13}C NMR Spectrum of 7



^{1}H NMR Spectrum of 8



8



^{13}C NMR Spectrum of 8