Electronic Supplementary Information

Methylations in complex carbapenem biosynthesis are catalyzed by a single cobalamin-dependent radical *S*-adenosylmethionine enzyme

Erica K. Sinner, Michael S. Lichstrahl, Rongfeng Li, Daniel R. Marous, Craig A. Townsend*

Department of Chemistry, Johns Hopkins University, Baltimore MD 21218

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General Methods and Instrumentation

DNA modifying enzymes and cloning reagents were purchased from New England Biolabs (Ipswich, MA) or Thermo Fisher Scientific (Waltham, MA). DNA sequencing was done by the Synthesis and Sequencing Facility at Johns Hopkins University. Protein purification and all methylation assays were carried out in a Coy (Grass Lake, MI) anaerobic chamber. All reagents were used without further purification unless otherwise indicated. Anhydrous solvents were dried using an LC Technology Solutions (Salisbury, MA) SPBT-1 solvent purification system. Silica gel chromatography was performed using Sorbtech Silica Gel (60 Å, 40-75mm particle size) or RediSep Rf disposable flash columns (60 Å, 40-63 μm irregular particle size) on a Teledyne ISCO (Lincoln, NE) CombiFlash EZ Prep. Preparative HPLC was carried out on the same instrument outfitted with a Phenomenex (Torrance, CA) Luna 10μ C18(2) 100 Å column (250 × 21.20 mm ID). All NMR spectra were recorded on a Bruker (Billerica, MA) UltraShield 400 MHz Avance spectrometer. Ultra-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) analyses were done using a Waters (Milford, MA) Acquity/Xevo-G2 at the Johns Hopkins University Department of Chemistry Mass Spectrometry Facility. Chromatographic separations were carried out on a Waters Acquity BEH UPLC column (ethylene-bridged hybrid C-18 stationary phase, 2.1 mm × 350 mm, 1.7 um) with HRMS detection using an electrospray ionization (ESI) ion source in positive or negative mode.

Cloning of *tokK*

TokK was PCR amplified from *Streptomyces tokunonensis* gDNA using the following primers:

5'-GGGCATATGTCCGCCGAACTCGCCAGCCGC-3'

5'-GGGCTCGAGACCTTGAAAATAAAGATTTTCCCACTGTTCGCTGACCACCGGC-3'

The product was digested with NdeI and XhoI, ligated into pET29b (Novagen, Madison, WI), and sequence verified. The reverse primer was designed to append a TEV protease cleavage site between the protein sequence and the *C*-terminal His_6-tag encoded on the vector to facilitate two-step purification of the protein. The resulting pET29b:*tokK*$_{TEV}$ construct and pBAD42-BtuCEDFB were transformed into electrocompetent BL21(DE3) cells harboring pDB1282 so that TokK would be coexpressed with iron-sulfur cluster biosynthetic machinery as well as cobalamin uptake proteins[1].

Cloning of *thnK*

ThnK was amplified from the previously published pET29b:*thnK* plasmid[2] with these primers:

5'-GGGCATATGACCGTCCCCGCCGCGCG-3'

5'-GGGCTCGAGACCTTGAAAATAAAGATTTTCCCGCTGCTCGGTCAGGACGGGCTC3'

The PCR product was treated in the same manner as that described above for *tokK*.

Overexpression of TokK

The three-plasmid construct was selected for and maintained with 50 μg/mL kanamycin (pET29b containing either *thnK* or *tokK*), 50 μg/mL spectinomycin (pBAD42-BtuCEDFB), and 100 μg/mL ampicillin (pDB1282). Starter cultures were grown in LB medium overnight and used to inoculate 6 × 2.5 L expression cultures in M9-ethanolamine medium[3] supplemented with 1.3 μM hydroxocobalamin, (10 mL starter/L of expression culture). These cultures were grown at 37 °C to OD 0.4, and then pDB1282 and pBAD42-BtuCEDFB were induced with arabinose (1g/L), $FeCl_3$ (6.8 mg/L), and cysteine (24 mg/L). The cultures were then grown to OD 0.8 and then cold shocked at 0 °C for 1 h. Arabinose, $FeCl_3$, and cysteine were then added as before, along with IPTG (1 mM final concentration). The cultures were then grown at 18 °C for an additional 20 h before harvesting the cell paste by centrifugation at 4000 × g. Cell paste was flash frozen in liquid N_2 and stored in liquid N_2 until use.

Anaerobic Purification TokK

In a Coy anaerobic chamber, cell paste from 15 L of culture (~60 g wet cell mass) containing over-produced enzyme grown as described above was resuspended in lysis buffer (10% glycerol, 300 mM KCl, 50 mM

HEPES, 10 mM β-mercaptoethanol, 5 mM imidazole, pH 7.5) to a final volume of 160 mL. Lysozyme (160 mg) and DNase (0.1 mg/mL final volume) were added and the mixture was incubated on ice for 1 h, then disrupted by sonication using a Cole-Parmer (Vernon Hills, IL) Ultrasonic Processor (60% amplitude, 9.9 s on/off, ~10 min) on ice. The lysate was clarified by centrifugation (30 min, 40,000 × g, 4 °C) and the supernatant was incubated with Clontech (Mountain View, CA) TALON metal affinity resin (8 mL 50% suspension, pre-equilibrated with 50 mL lysis buffer) on ice for 30 min. The suspension was loaded onto a gravity column and washed with 16 mL lysis buffer. The protein was then eluted with elution buffer (lysis buffer with 250 mM imidazole). Dark colored elution fractions were pooled and concentrated to 3 mL in an Amicon 10 kDa MWCO ultrafiltration device, followed by desalting on an Econo-Pac 10DG column (Bio-Rad) according to the manufacturer's instructions using desalting buffer (lysis buffer with no imidazole). The resulting eluent was then incubated with TEV protease expressed from pRK793 (0.01 mg TEV protease/mg of target protein as measured by the Bradford assay) overnight on ice. The mixture was then incubated with Clontech TALON metal affinity resin (8 mL 50% suspension, pre-equilibrated with desalting buffer) for 30 min on ice. The suspension was loaded onto a gravity column. The flow-through was collected and the resin was washed with desalting buffer (2 × 4 mL). Fractions containing the desired protein by SDS-PAGE analysis were pooled and concentrated in an Amicon 10 kDa MWCO ultrafiltration device, and the resulting pure protein (~5 mg) was kept on ice until use.

Expression and purification of ThnK

ThnK was expressed and purified in a similar manner to that described above for TokK. Characterization data matched that previously reported[2].

Methylation Assays

Assays were conducted anaerobically in the dark at room temperature and contained 100 mM HEPES pH 7.5, 200 mM KCl, 1 mM SAM, 1 mM methyl viologen, 2 mM NADPH, 0.5 mM methyl cobalamin, 100 μM substrate **6a**, and 100 μM enzyme. SAM was omitted in control reactions. At each time point, a 30 μL aliquot was diluted 5× with water and filtered through a 10 kDa MWCO Amicon ultrafiltration device. The filtrate was analyzed for product formation by UPLC-HRMS. Mobile phase (contained 0.1% formic acid, flow rate 0.3 mL/min): 0-1 min 100% water, 1-7.5 min gradient from 0-80% ACN, 7.5-8.4 min isocratic 80% ACN, 8.4-10 min 100% water.

Quantitation of SAM coproducts and determination of reaction stoichiometry

Standard solutions containing SAH and 5'-dA were prepared as previously described[4] with a concentration range of 0.4-100 μM for each molecule in 20 mM HEPES pH 7.5, 40 mM KCl, and 100 μM phenylalanine (internal standard). These solutions were analyzed by UPLC-HRMS (mobile phase described above) to generate a standard curve, which was then used to determine the concentration of SAH and 5'-dA in 30-min fixed time assays set up as described above, except containing 1 mM substrate **6a** and 100 μM phenylalanine. Under these conditions, only methylated product **7** was produced, and its concentration was estimated by relative peak areas of substrate and product with respect to initial substrate concentration. Stoichiometry was determined after subtraction of a no substrate control.

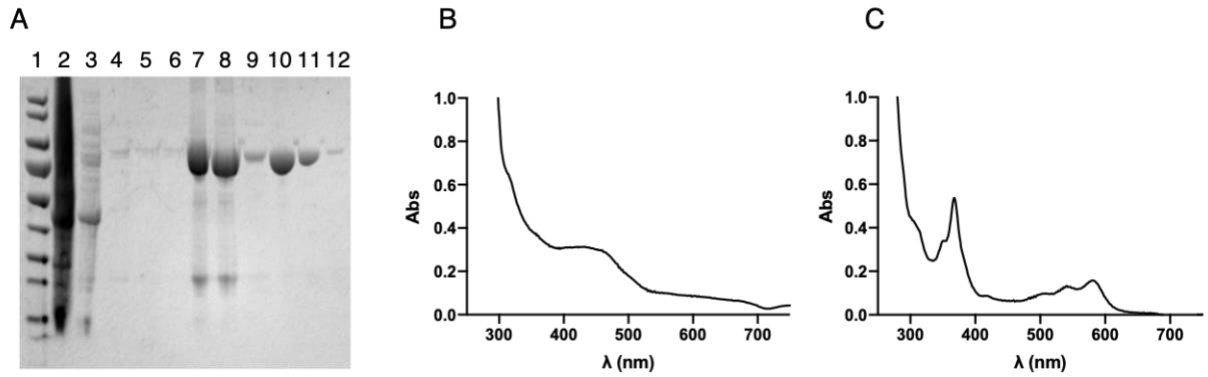


Fig. S1 A. Purification of TokK. 1: PageRuler Protein Ladder (Thermo Scientific, Waltham, MA), 2: flow through, 3-4: wash fractions (lysis buffer), 5-9: elution fractions (1.5 mL each, elution buffer), 10-12: flow through and washes (desalting buffer) from post-TEV cleavage column. B. UV-Visible spectrum of TokK. The 420 nm shoulder indicates a bound iron-sulfur cluster. C. UV-Visible spectrum of TokK after treatment with 10 mM KCN and incubation at 100 °C for 5 min. Peak at 367 nm is due to bound cobalamin being released from the enzyme as dicyanocobalamin[1].

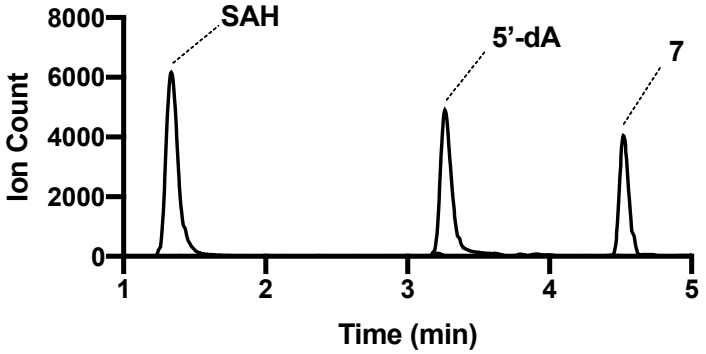
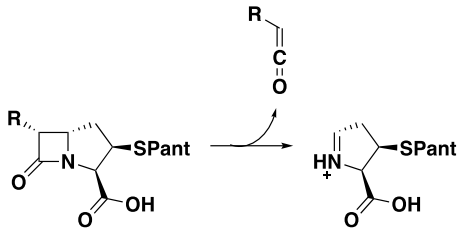


Fig. S2 Overlaid extracted-ion chromatograms (EICs) from 30-min fixed time assay with TokK for determination of reaction stoichiometry. EICs are as follows: SAH *m/z* = 385.13 ± 0.05, 5'dA *m/z* = 252.11 ± 0.05, methylated product **7** *m/z* = 446.20 ± 0.05. SAH, 5'-dA, and methylated product **7** were determined to be produced in a 1.0 :1.1: 0.9 ratio.



Compound Number	Compound Formula	Parent Mass (Calculated)	Parent Mass (Found)	Formal Retro-[2+2] Fragment Calculated: 390.1693
6 (R = H)	$C_{18}H_{29}N_3O_7S$	432.1799	432.1794	390.1685
7 (R = Me)	$C_{19}H_{31}N_3O_7S$	446.1955	446.1954	390.1689
8 (R = Et)	$C_{20}H_{33}N_3O_7S$	460.2112	460.2116	390.1688
9 (R = iPr)	$C_{21}H_{35}N_3O_7S$	474.2268	474.2265	390.1700

Fig. S3 Masses of substrate **6** and methylated intermediates and product detected in TokK assays. Each additional methylation shows a parent mass increase of one carbon and two protons, but the formal retro-[2+2] fragment is identical for all compounds, indicating that carbons are being added onto the azetidinone half of the molecule at C6.

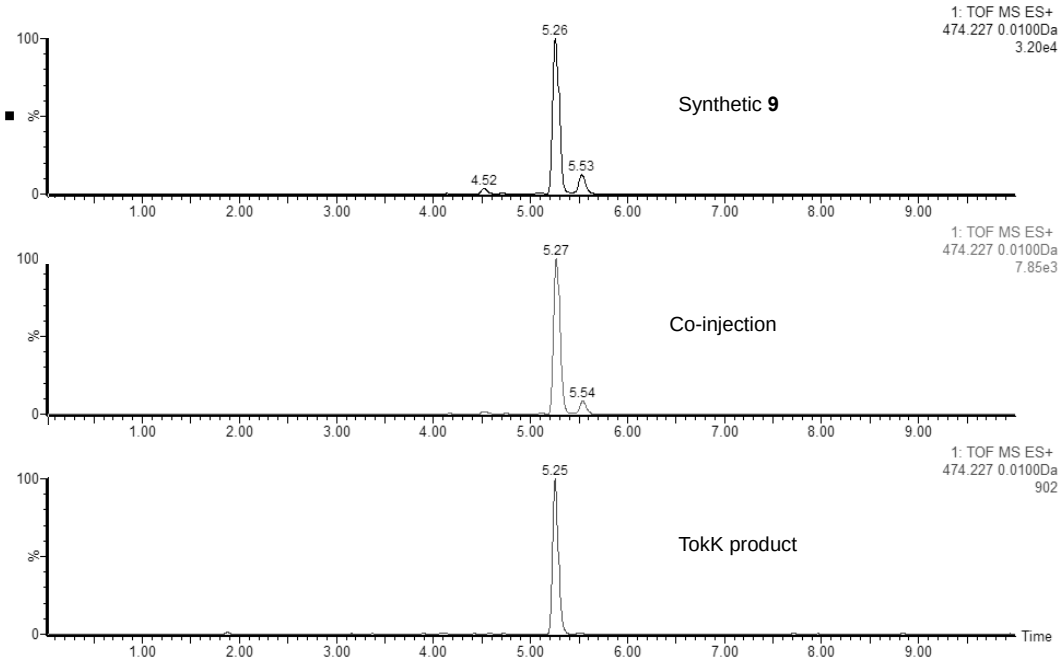


Fig. S4 Extracted-ion chromatogram comparisons of enzymatic and synthetic isopropyl carbapenam **9**. Positive mode ESI: *m/z* = 474.23 ± 0.01. Mobile phase: (0.1% formic acid, flow rate 0.3 mL/min): 0-1 min 100% water, 1-7.5 min gradient from 0-80% ACN, 7.5-8.4 min isocratic 80% ACN, 8.4-10 min 100% water.

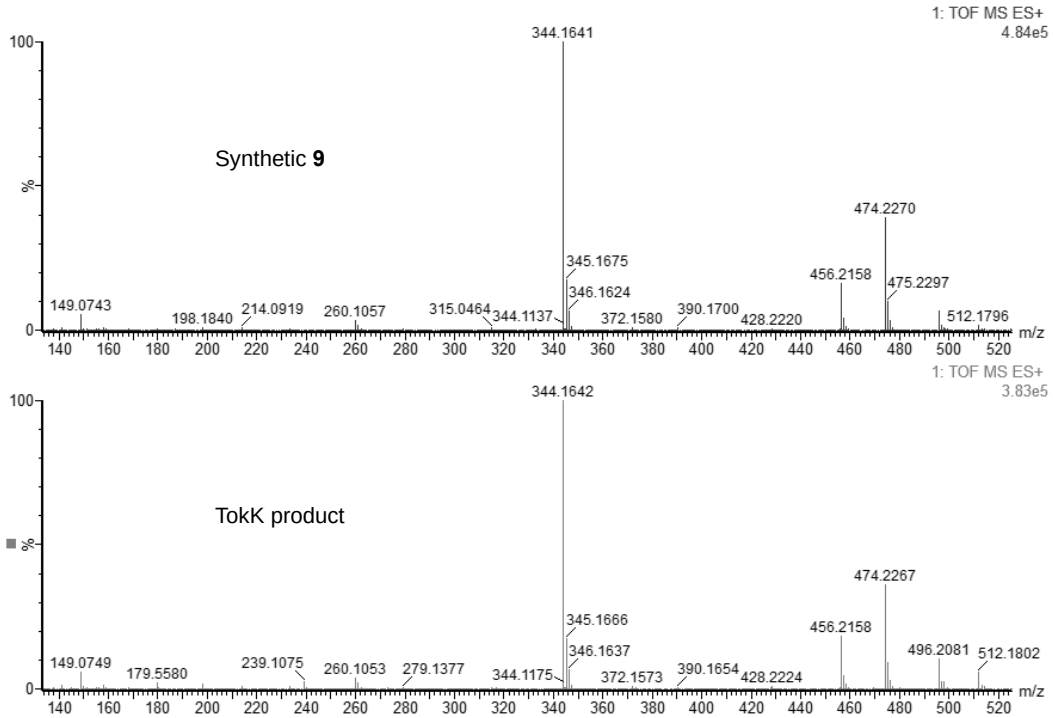


Fig. S5 ESI (+) mass spectra of synthetic **9** and TokK isopropyl carbapenam product; *m/z*: $[M+H]_+$ calculated for $C_{21}H_{35}N_3O_7S$ 474.2268.

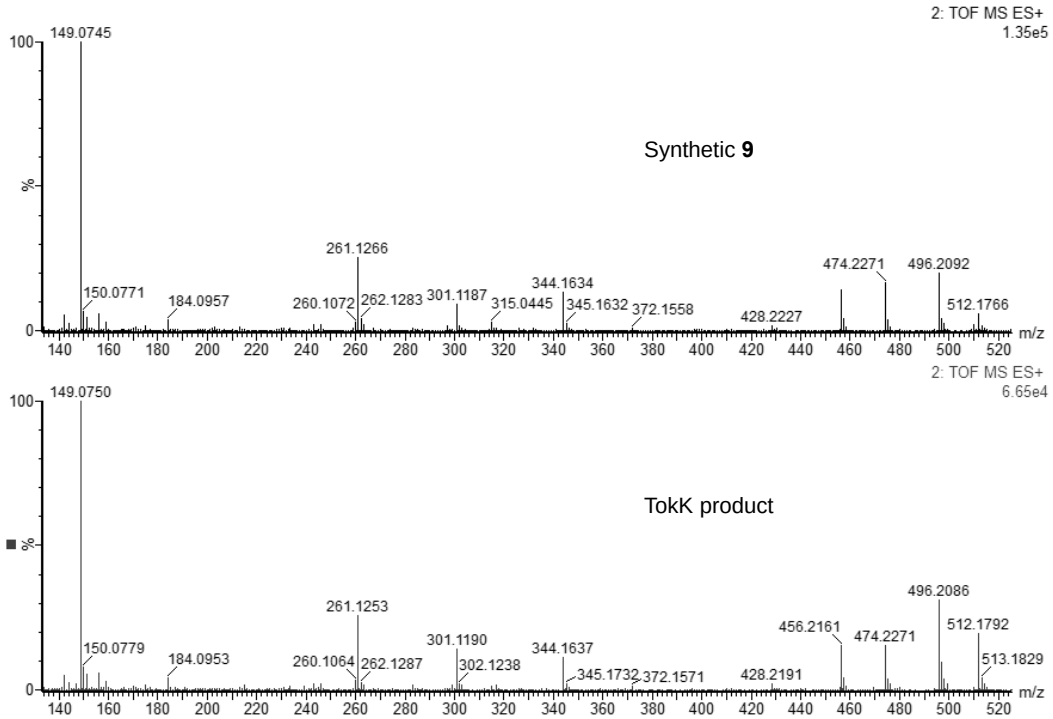


Fig. S6 ESI (+) MS/MS spectra of synthetic **9** and TokK isopropyl carbapenam product.

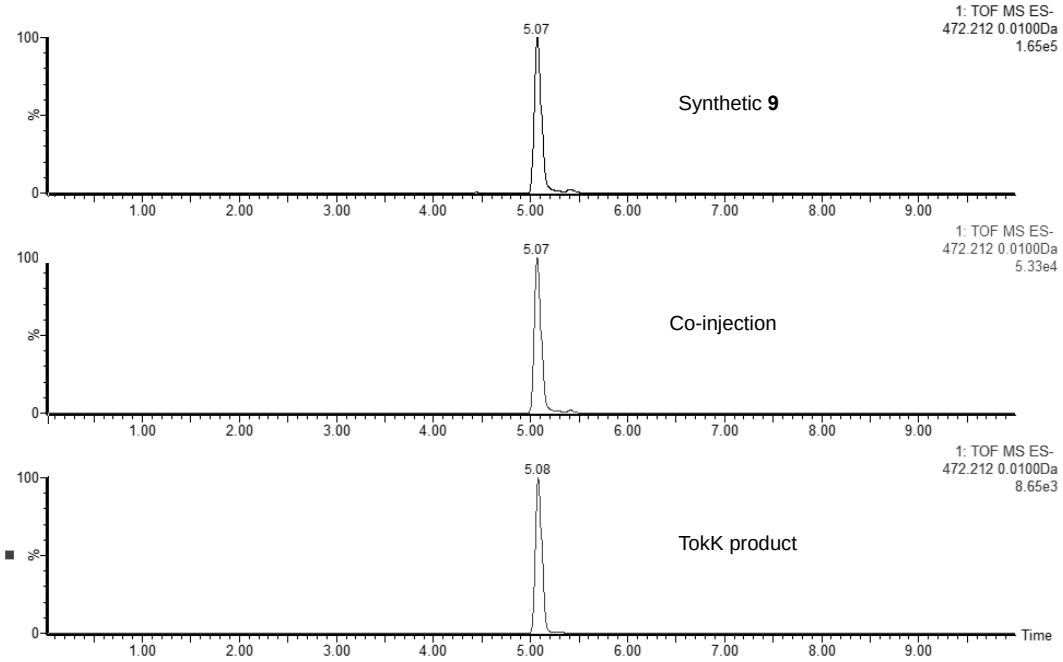


Fig. S7 Extracted-ion chromatogram comparisons of enzymatic and synthetic isopropyl carbapenam **9**. Negative mode ESI: *m/z* = 472.21 ± 0.01. Mobile phase: (flow rate 0.3 mL/min): 0-1 min 100% water, 1-7.5 min gradient from 0-80% ACN, 7.5-8.4 min isocratic 80% ACN, 8.4-10 min 100% water.

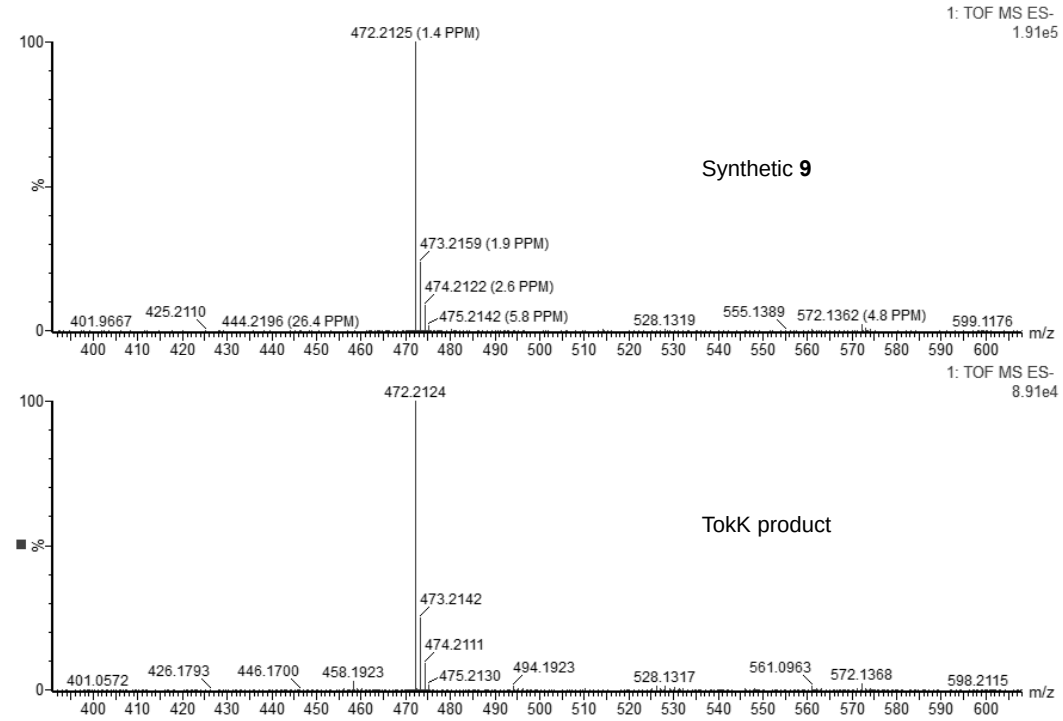


Fig. S8 ESI (-) mass spectra of synthetic **9** and TokK isopropyl carbapenam product; *m/z*: [M-H]- calculated for $C_{21}H_{35}N_3O_7S$ 472.2123.

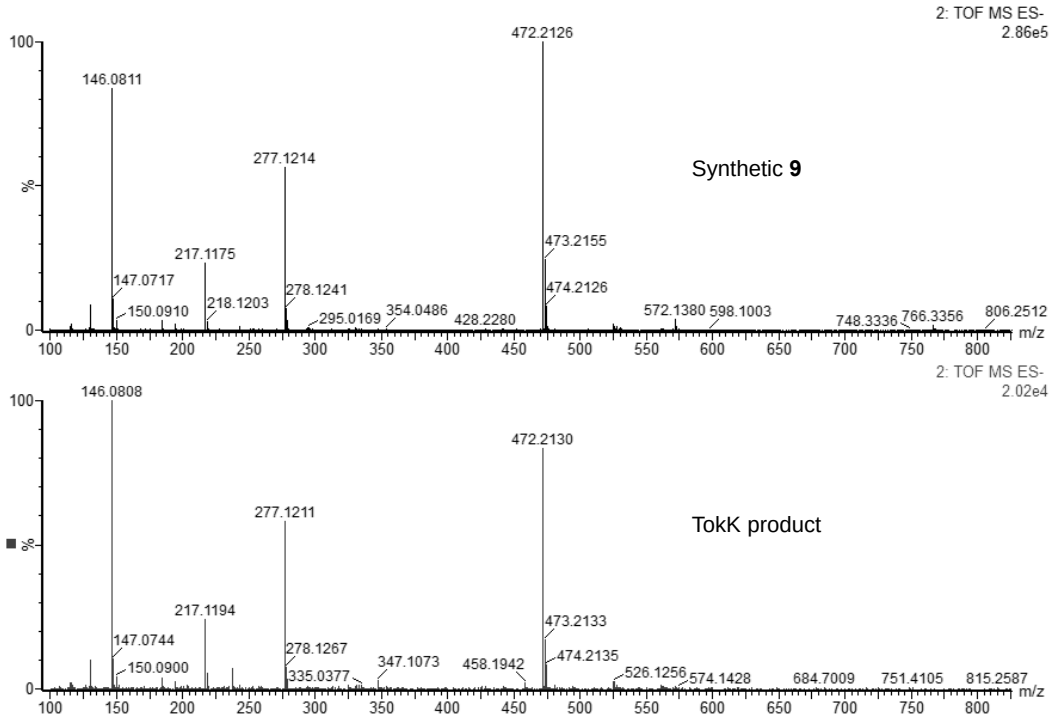


Fig. S9 ESI (-) MS/MS spectra of synthetic **9** and TokK isopropyl carbapenam product.

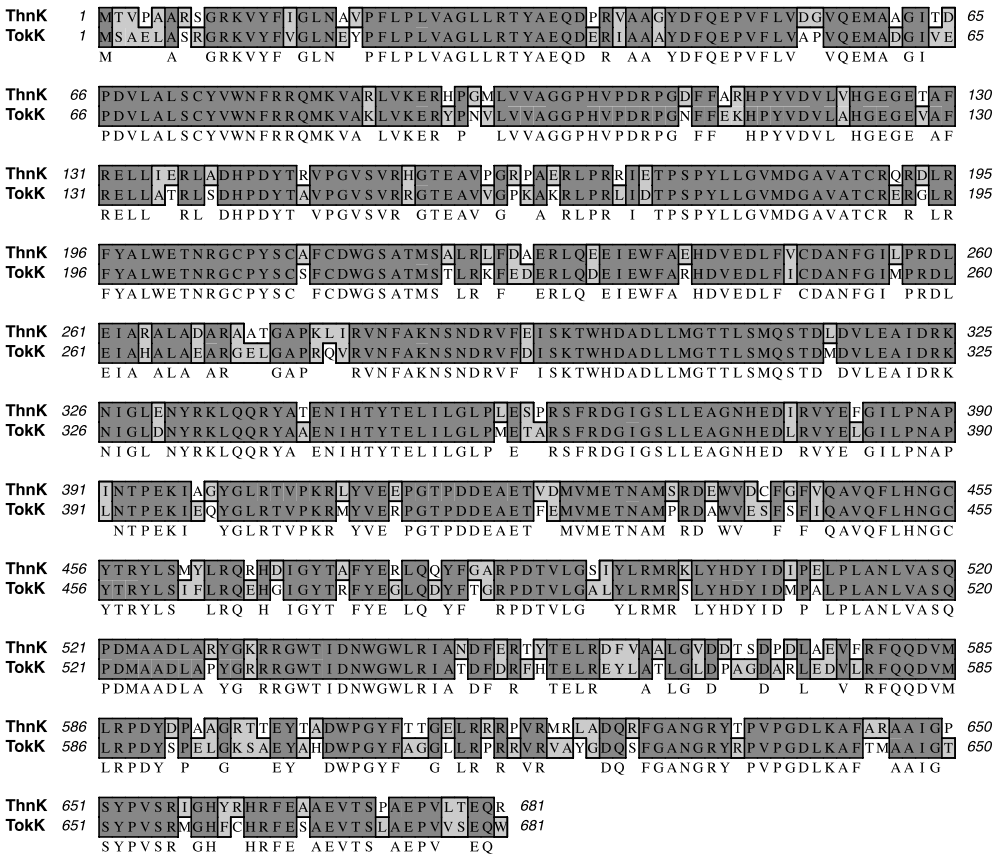
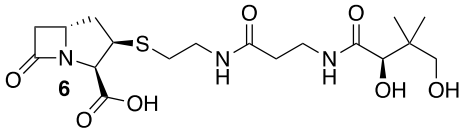
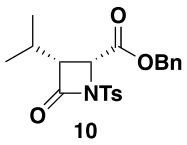


Fig. S10 ThnK *vs.* TokK ClustalW Alignment. Aligned Length = 681, Gaps = 0, Identities = 540 (79%), Similarities = 62 (9%). Identities are shaded in dark gray, while similarities are shaded in light gray. The consensus sequence is listed below the two protein sequences.

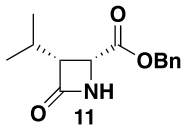
Synthetic Procedures



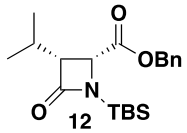
(2*R*,3*R*,5*R*)-3-((2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)thio)-7-oxo-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6). Synthesized as described by Marous, *et al*[2]. Characterization matched reported data.



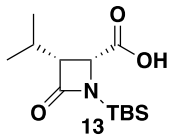
Benzyl (2*R*,3*R*)-3-isopropyl-4-oxo-1-tosylazetidine-2-carboxylate (10). Prepared according to the procedure of Bodner *et al*[5]. The characterization data was consistent with that reported.



Benzyl (2*R*,3*R*)-3-isopropyl-4-oxoazetidine-2-carboxylate (11). A solution of SmI_2 (5.5 mmol) in 55 mL THF was prepared according to the method of Imamoto as described by Szostak *et al*[6]. To this solution was added iPrOH (20 eq) followed by **10** (2.2g, 5.5 mmol) in 20 mL THF. The reaction mixture was stirred for 1 h at room temperature, diluted with 50 mL Et_2O, then filtered through a pad of Celite and washed 2 × 50 mL Et_2O. The filtrate was washed 2 × 100 mL saturated brine solution, then the combined aqueous layer was extracted 2 × 50 mL Et_2O (emulsion). The combined organic extracts were dried over anhydrous Na_2SO_4, solvent removed *in vacuo*, and the residue purified by silica gel flash chromatography (60-70% EtOAc/Hexanes) to yield **11** (838 mg, 60%) as a white solid. The product could be further purified by recrystallization from Et_2O/Hexanes to afford fine, white crystals. **^{1}H NMR** (400 MHz; $CHCl_3$): δ 7.36 (m, 5H), 6.06 (br s, 1H), 5.18 (ABq, *J* = 12.0 Hz, 2H) 4.54 (d, *J* = 5.7 Hz, 1H), 3.26 (ddd, *J* = 0.7, 5.8, 9.5 Hz, 1H), 1.93 (m, 1H), 1.04 (d, *J* = 6.7 Hz, 3H), 0.83 (d, *J* =6.7 Hz, 3H); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 170.5, 169.0, 134.7, 128.9, 128.8, 128.7, 67.4, 62.9, 52.2, 26.3, 21.5, 19.7; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{18}NO_3$ 248.1281, found: 248.1281.

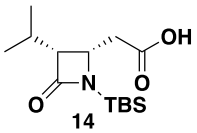


Benzyl (2*R*,3*R*)-1-(*tert*-butyldimethylsilyl)-3-isopropyl-4-oxoazetidine-2-carboxylate (12). Compound **11** (500 mg, 2.03 mmol) was dissolved in 30 mL DCM. DIPEA (0.460 mL, 2.64 mmol) was added to the solution, followed by TBSOTf (0.600 mL, 2.64 mmol). The reaction mixture was stirred at room temperature for 1 h, then concentrated *in vacuo* and purified directly by silica gel flash chromatography (90-100% EtOAc/Hexanes) to yield **12** (675 mg, 92%) as a clear oil. **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.35 (m, 5H), 5.16 (ABq, *J* = 12.0 Hz, 2H), 4.15 (d, *J* = 6.3 Hz, 1H) 3.20 (d, *J* = 6.1, 11.0 Hz, 1H), 1.94 (m, 1H), 1.06 (d, *J* = 6.5 Hz, 3H), 0.90 (s, 9H), 0.71 (d, *J* = 6.5 Hz, 3H), 0.23 (s, 3H), -0.01 (s, 3H); **^{13}C NMR** (100 MHz; CDCl3): δ 173.9, 171.5, 134.8, 129.0, 128.7, 128.6, 67.2, 62.9, 54.1, 26.2, 26.1, 21.3, 20.3, 18.5, -5.8, -6.4; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{20}H_{32}NO_3Si$ 362.2146, found: 362.2149.



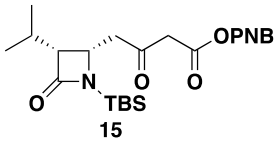
13

(2*R*,3*R*)-1-(*tert*-butyldimethylsilyl)-3-isopropyl-4-oxoazetidine-2-carboxylic acid (13). Compound **12** (1.006 g, 2.79 mmol)) was dissolved in 50 mL THF. Pd/C (295 mg, 0.279 mmol) was added and the suspension was stirred overnight at room temperature under balloon pressure of H_2. The mixture was filtered through Celite, washed 2 × 50 mL EtOAc and concentrated to afford a white solid. The crude material was then recrystallized from DCM/Hexanes to yield **13** (626 mg, 83%) as fine, white crystals. **$_1$H NMR** (400 MHz; $CDCl_3$): δ 4.18 (d, *J* = 6.3 Hz, 1H), 3.30 (dd, *J* = 6.3, 10.8 Hz, 1H), 2.05 (m, 1H), 1.12 (d, *J* = 6.5 Hz, 3H), 0.94 (s, 9H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.29 (s, 3H), 0.10 (s, 3H); **$_{13}$C NMR** (100 MHz; $CDCl_3$): δ 177.0, 174.1, 63.2, 54.1, 26.6, 26.4, 21.6, 20.6, 18.8, -5.6, -6.1; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{13}H_{26}NO_3Si$ 272.1676, found: 272.1677.



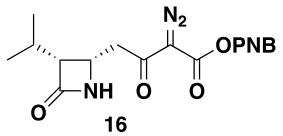
14

2-((2*S*,3*R*)-1-(*tert*-butyldimethylsilyl)-3-isopropyl-4-oxoazetidin-2-yl)acetic acid (14). Compound **13** (300 mg, 1.1 mmol) was subjected to an Arndt-Eistert homologation following the procedure described by Bodner *et al*[5]. The crude product of the homologation was purified by flash chromatography (90% Hexanes/EtOAc 1% Acetic Acid) to afford **14** (134 mg, 42%) as a white solid, which could be further purified by recrystallization from isooctane to afford white plates. **$_1$H NMR** (400 MHz; $CDCl_3$): δ 4.14 (m, 1H), 3.10 (dd, *J* = 5.8, 9.5 Hz, 1H), 2.72 (ABX, J_{AB} = 17.3 Hz, J_{AX} = 8.0 Hz, J_{BX} = 4.7 Hz, 2H), 1.94 (m, 1H), 1.15 (d, *J* = 6.5 Hz, 3H), 0.93 (s, 9H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.22 (s, 3H), 0.19 (s, 3H); **$_{13}$C NMR** (100 MHz; $CDCl_3$): δ 175.8, 175.3, 60.9, 50.1, 36.2, 26.5, 25.6, 22.6, 20.7, 18.7, -5.2, -5.3; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{14}H_{28}NO_3Si$ 286.1833, found: 286.1840.

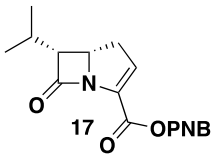


15

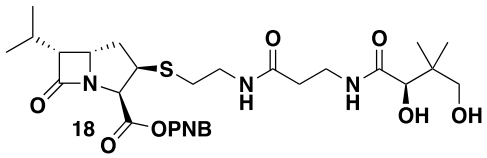
4-Nitrobenzyl 4-((2*S*,3*R*)-1-(*tert*-butyldimethylsilyl)-3-isopropyl-4-oxoazetidin-2-yl)-3-oxobutanoate (15). To azetidinone **14** (120 mg, 0.42 mmol) dissolved in anhydrous acetonitrile (4.2 mL) was added carbonyldiimidazole (82.5 mg, 0.5 mmol). The mixture was allowed to stir at room temperature for 1 h. Mg(*mono*-PNB malonate)$_2$ was then added and the reaction was heated to 55 °C for 18 h. After cooling to room temperature, the reaction mixture was diluted with 10 mL EtOAc, and washed with 10 mL water followed by 10 mL brine. The organic fraction was dried over anhydrous Na_2SO_4, concentrated under vacuum, and purified by silica gel flash chromatography (0-30% EtOAc/Hexanes) to a colorless oil (100 mg, 48%). **$_1$H NMR** (400 MHz; $CDCl_3$): δ 8.22 (d, *J* = 8.8 Hz, 2H), 7.51 (d, *J* = 8.8 Hz, 2H), 5.25 (s, 2H), 4.23 (m, 1H), 3.55 (s, 2H), 3.09 (m, 1H), 2.92 (ABX, J_{AB} = 19.0 Hz, J_{AX} = 8.1 Hz, J_{BX} = 4.0 Hz, 2H), 1.81 (m, 1H), 1.12 (d, *J* = 6.6 Hz, 3H), 0.91 (s, 9H), 0.78 (d, *J* = 6.6 Hz, 3H), 0.18 (s, 3H), 0.132 (s, 3H); **$_{13}$C NMR** (100 MHz; $CDCl_3$): δ 200.0, 175.2, 166.4, 148.1, 142.5, 128.8, 124.1, 65.9, 60.7, 49.6, 48.5, 45.0, 26.5, 25.7, 22.6, 20.6, 18.7, -5.2, -5.3. **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{23}H_{34}N_2O_6Si$ 463.2259, found 463.2260.



4-Nitrobenzyl 2-diazo-4-((2*S*,3*R*)-3-isopropyl-4-oxoazetidin-2-yl)-3-oxobutanoate (16). Compound **15** (100 mg, 0.2 mmol) was dissolved in 7 mL anhydrous THF followed by the addition of TBAF (0.22 mL, 1 M in THF, 0.22 mmol). The mixture was allowed to stir at room temperature for 30 min before being diluted into 20 mL EtOAc and then quenched with 10 mL 0.2 N HCl. The phases were separated, and the aqueous fraction was washed with 3 × 10 mL EtOAc. The combined organic extracts were then washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The resulting oil was run through a plug of silica and eluted with 50% EtOAc/Hexanes. The solvent was removed under reduced pressure, and the residue was diazotized by the method of Taber *et al*[7]. The crude product was purified by silica gel flash chromatography (20-100% EtOAc/Hexanes) to give a white solid (27 mg, 36%). **^{1}H NMR** (400 MHz; $CDCl_3$): δ 8.23 (d, *J* = 8.7 Hz, 2H), 7.51 (d, *J* = 8.7 Hz, 2H), 6.08 (s, 1H), 5.34 (s, 2H), 4.03 (m, 1H), 3.34 (**A**BX, J_{AB} = 18, J_{AX} = 2.8, 1H), 3.07-2.92 (m, 2H), 1.98 (m, 1H), 1.15 (d, *J* = 6.5 Hz, 3H), 0.09 (d, *J* = 6.5 Hz, 3H);**^{13}C NMR** (100 MHz; $CDCl_3$): δ 190.7, 170.2, 160.8, 148.3, 142.1, 129.0, 124.2, 76.3, 65.8, 60.5, 47.6, 41.9, 25.8, 22.6, 20.7; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{17}H_{18}N_4O_6$ 375.1299, found 375.1292.

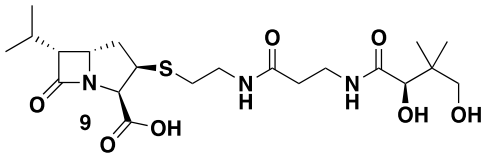


4-Nitrobenzyl(5*S*,6*R*)-6-isopropyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (17). Compound **16** was subjected to ring closure using $Rh_2(OAc)_4$ as described by Salzmann *et al*[8]. The resulting tautomeric 2-oxocarbapenam was converted to carbapenem **17** by the method of Shibuya[9] as described by Freeman et al[10]. The crude product was purified by silica gel flash chromatography (EtOAc/Hexanes, grad. 0-50% EtOAc) to give a white solid (11.6 mg, 48%). **^{1}H NMR** (400 MHz; $CDCl_3$): δ 8.21 (d, *J* = 8.75 Hz, 2H), 7.60 (d, *J* = 8.75 Hz, 2H), 6.62 (bt, *J* = 2.8 Hz, 1H), 5.34 (ABq, *J* = 13.7 Hz, 2H), 4.31 (m, 1H), 3.25 (dd, *J* = 11.6, 6.0 Hz, 1H), 2.79 (m, 2H), 1.97 (m, 1H), 1.18 (d, *J* = 6.6 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H) ;**^{13}C NMR** (100 MHz; $CDCl_3$): δ 179.6, 160.4, 147.9, 142.9, 135.2, 133.9, 128.4, 124.0, 65.6, 59.9, 31.8, 26.2, 21.2, 20.5. ; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{17}H_{18}N_2O_5$ 331.1288, found 331.1292.



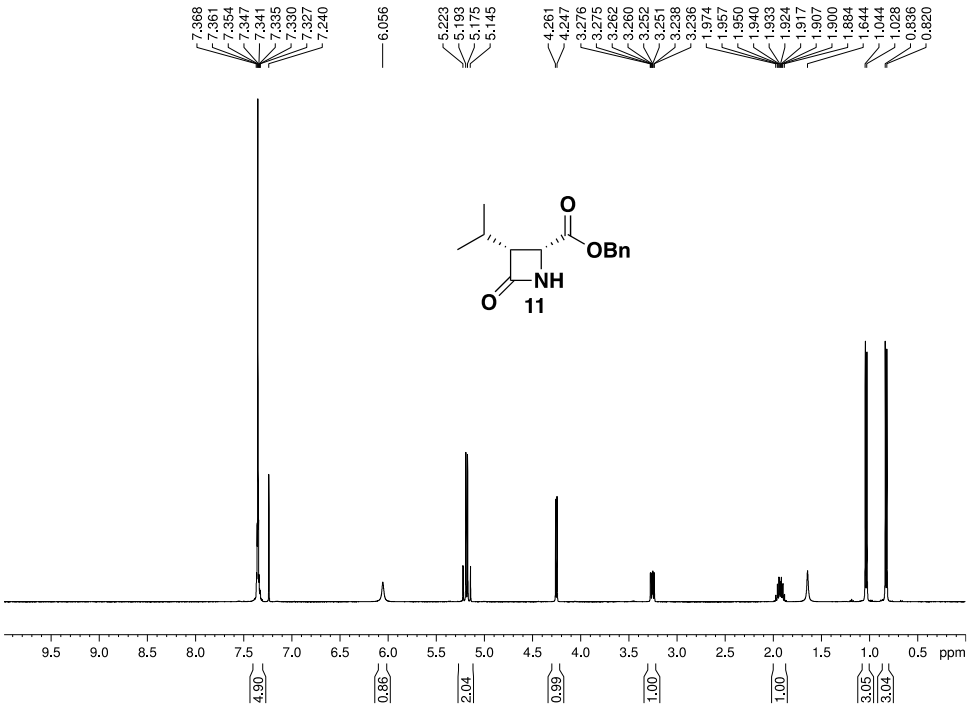
4-Nitrobenzyl(2*R*,3*R*,5*S*,6*R*)-3-((2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido) propanamido)ethyl)thio)-6-isopropyl-7-oxo-1-azabicyclo[3.2.0]heptane-2-carboxylate (18). Pantetheine addition to carbapenem **17** was carried out as described by Marous *et al*.[2] to give a ~1:1 mixture of C2 diastereomers, which was separated by preparative HPLC using a mobile phase of MeOH and H_2O containing 10 mM potassium phosphate (pH 6.65), 21 mL/min, gradient from 30-70% MeOH over 15 min followed by 15 min of 70% MeOH/H_2O isocratic. The C2-C3 *cis* diastereomer elutes second, as determined by the chemical shift of the C3 doublet[2]. Fractions containing the desired product were combined, and the MeOH was removed *in vacuo*. The remaining buffered aqueous solution was extracted 3 × 5 mL EtOAC. The combined organic fractions were dried over anhydrous Na_2SO_4 and concentrated to give a white powder (5.6 mg, 26%) **^{1}H NMR** (400 MHz; $CDCl_3$): δ 8.21 (d, *J* = 8.8 Hz, 2H), 7.54 (d, *J* = 8.8 Hz, 2H), 7.40 (br s, 1H), 6.64 (br s, 1H), 5.26 (s, 2H), 4.72 (d, *J* = 7.2 Hz, 1H), 4.10 (m, 1 H), 3.97 (s, 1H), 3.56-3.38 (m, 6H), 3.30 (m, 1H), 3.08 (dd, *J* = 12, 5.8 Hz, 1H), 2.69 (m, 2H), 2.42 (bt, *J* = 5.8 Hz, 2H), 2.25 (m, 1H), 2.01

(m, 1H), 1.77 (m, 1H), 1.22 (m, 2H), 1.12 (d, *J* = 6.4, 3H), 0.95 (s, 3H), 0.88 (m, 6H); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 179.6, 174.0, 171.8, 168.6, 148.1, 142.2, 129.0, 124.1, 71.0, 65.8, 64.2, 59.8, 56.6, 49.6, 39.5, 39.0, 36.0, 35.5, 33.0, 31.8, 29.9, 26.1, 21.5, 21.1, 20.7, 20.6; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{28}H_{40}N_4O_9S$ 609.2589, found 609.2591.

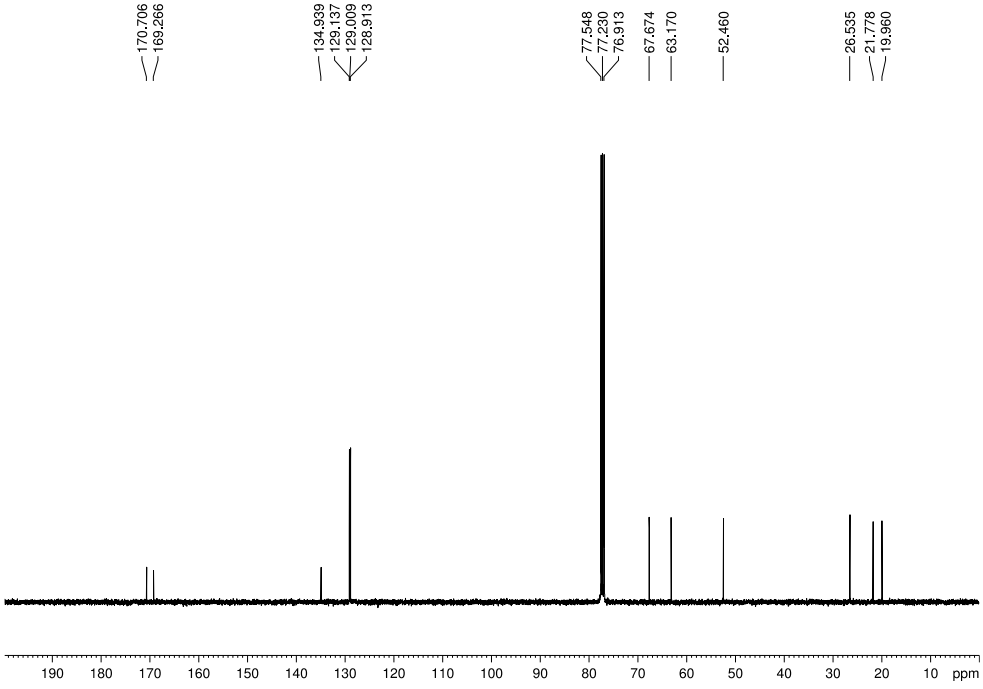


(2*R*,3*R*,5*S*,6*R*)-3-((2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)thio)-6-isopropyl-7-oxo-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (9). Compound **18** (5.6 mg) was dissolved in 1.5 mL 1:1 THF/aqueous potassium phosphate (100 mM, pH 7) in a pressure tube. 10% Pd/C (1 mg) was added and the suspension was shaken on a Parr apparatus under 30 psi H_2 for 1 h followed by filtration over Celite. The solvent was removed under reduced pressure, and NMR analysis was carried out by redissolving the buffer/product mixture in D_2O since removing the buffer results in rapid degradation of the carbapenam. **^{1}H NMR** (400 MHz; D_2O): δ 4.41 (d, *J* = 6.7 Hz, 1H), 4.14 (m, 1H), 3.97 (s, 1H), 3.84 (m, 1H), 3.50 (m, 3H), 3.34 (m, 3H), 3.15 (dd, *J* = 12.1, 5.2 Hz, 1H), 2.77 (br t, *J* = 6.5 Hz, 2H), 2.49 (br t, *J* = 6.2 Hz, 2H), 2.17 (m, 2H), 1.87 (m, 1H), 1.05 (d, *J* = 6.5 Hz, 3H), 0.88 (m, 9H); **^{13}C NMR** (100 MHz; D_2O): δ 184.0, 175.6, 175.1, 173.9, 75.7, 68.3, 65.7, 56.8, 56.3, 50.7, 38.7, 38.6, 35.5, 35.3, 33.5, 31.5, 25.2, 20.5, 20.0, 19.9, 19.1; **HRMS** (ESI) *m/z*: $[M+H]^+$ calculated for $C_{21}H_{35}N_3O_7S$ 474.2268, found 474.2262.

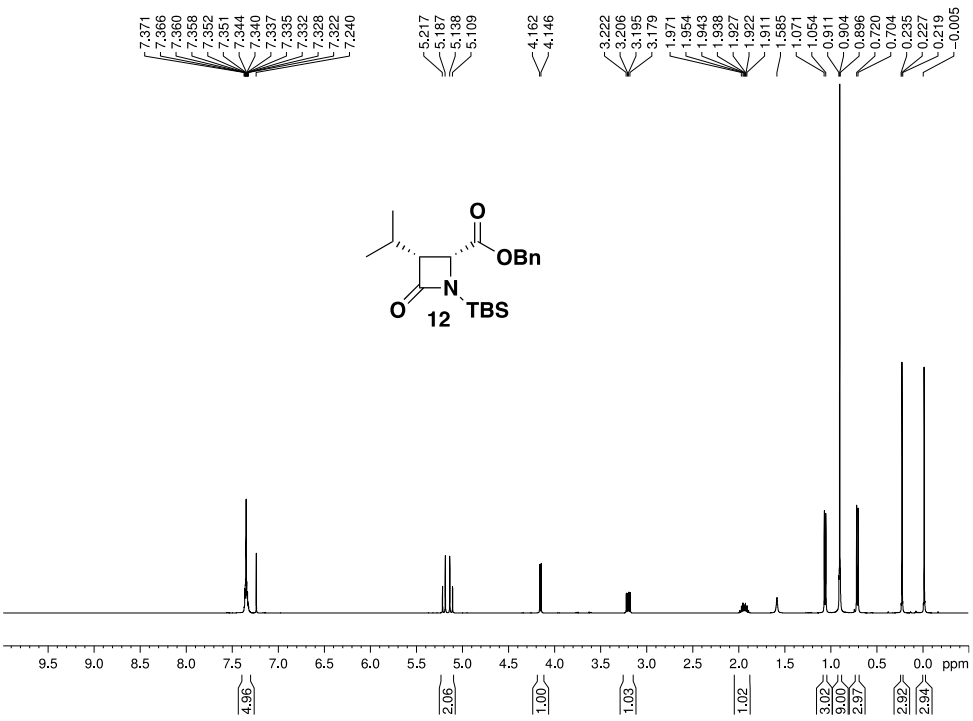
NMR Spectra



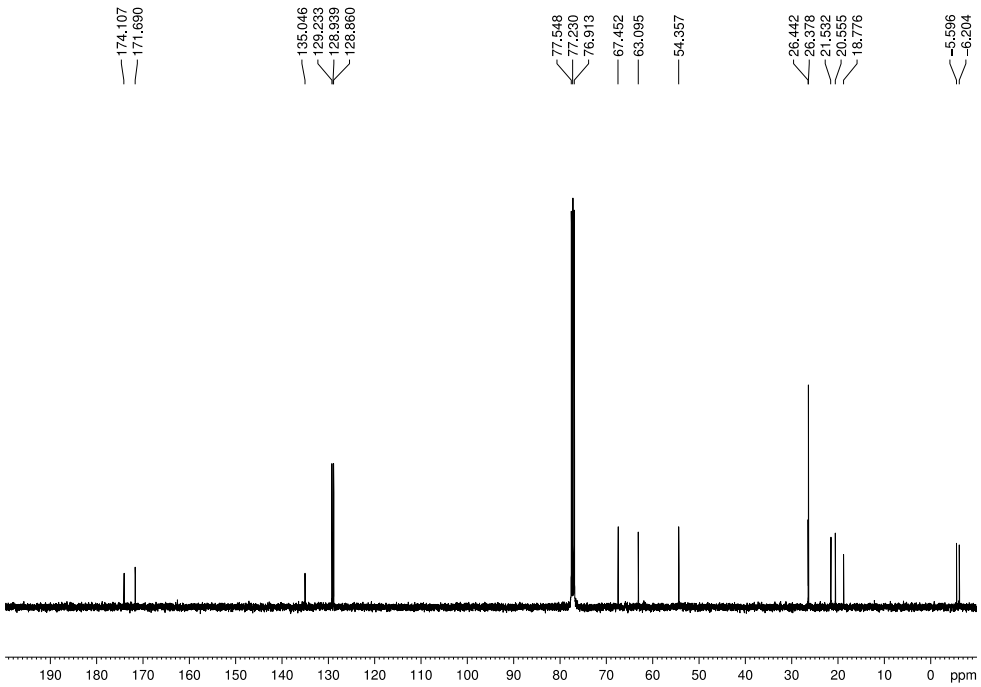
$_{1}$H NMR spectrum of **11** ($CDCl_3$).



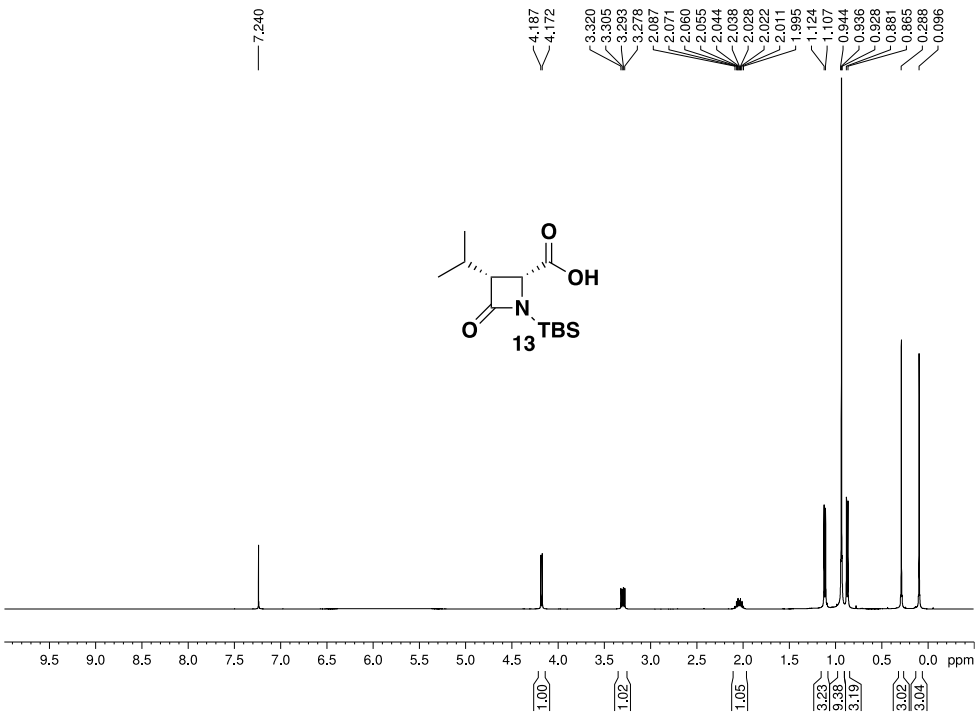
$_{13}$C NMR spectrum of **11** ($CDCl_3$).



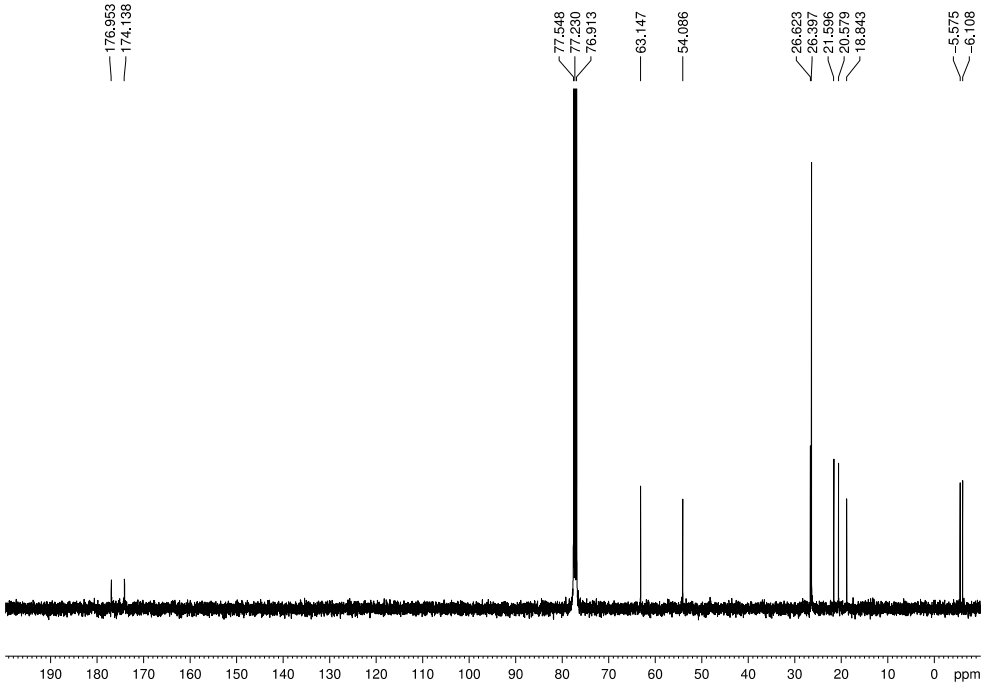
$_{1}$H NMR spectrum of **12** ($CDCl_3$).



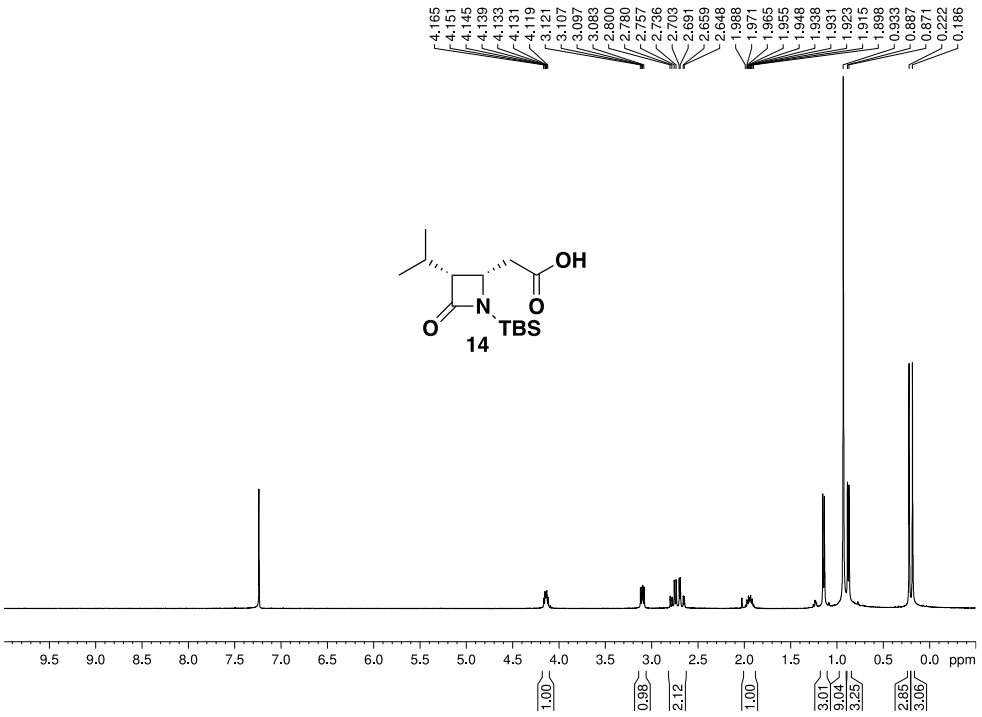
$_{13}$C NMR spectrum of **12** ($CDCl_3$).



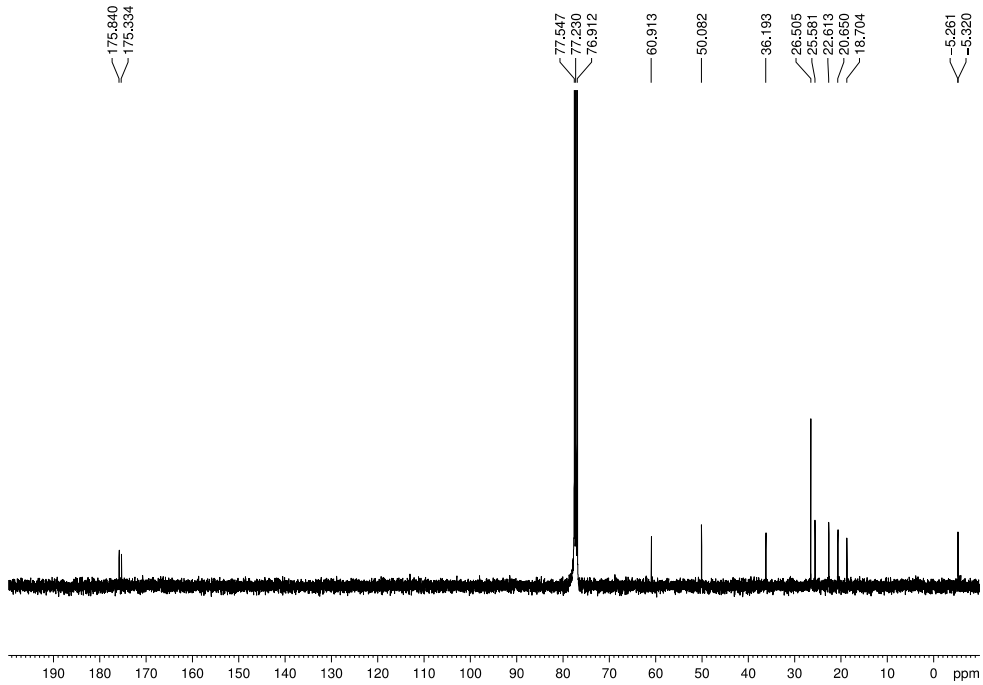
$_{1}$H NMR spectrum of **13** ($CDCl_3$).



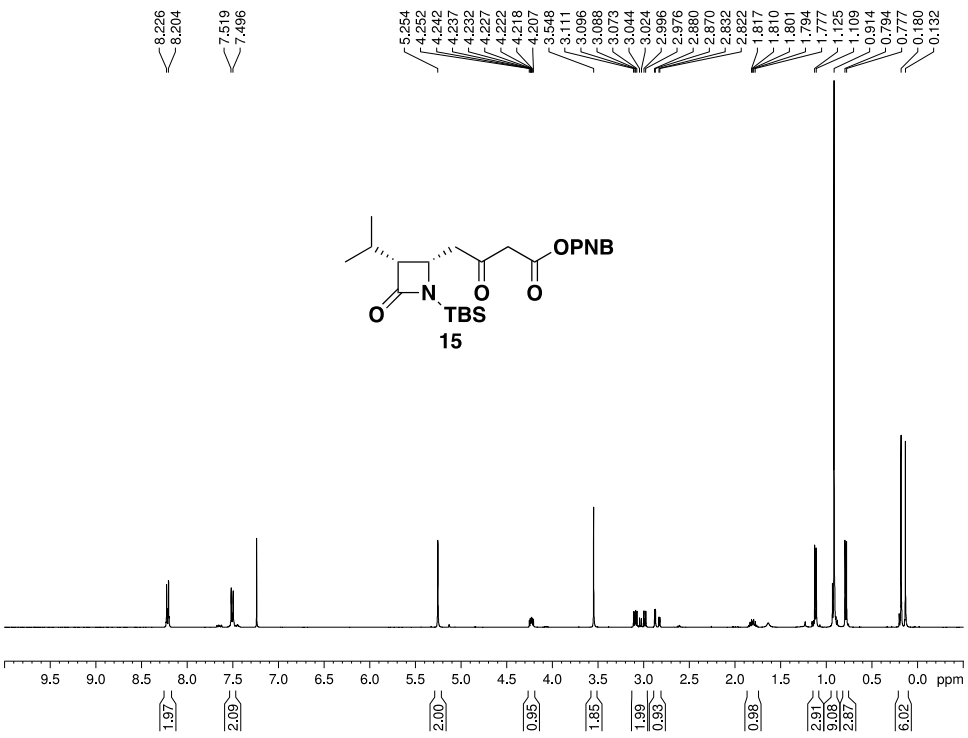
$_{13}$C NMR spectrum of **13** ($CDCl_3$).



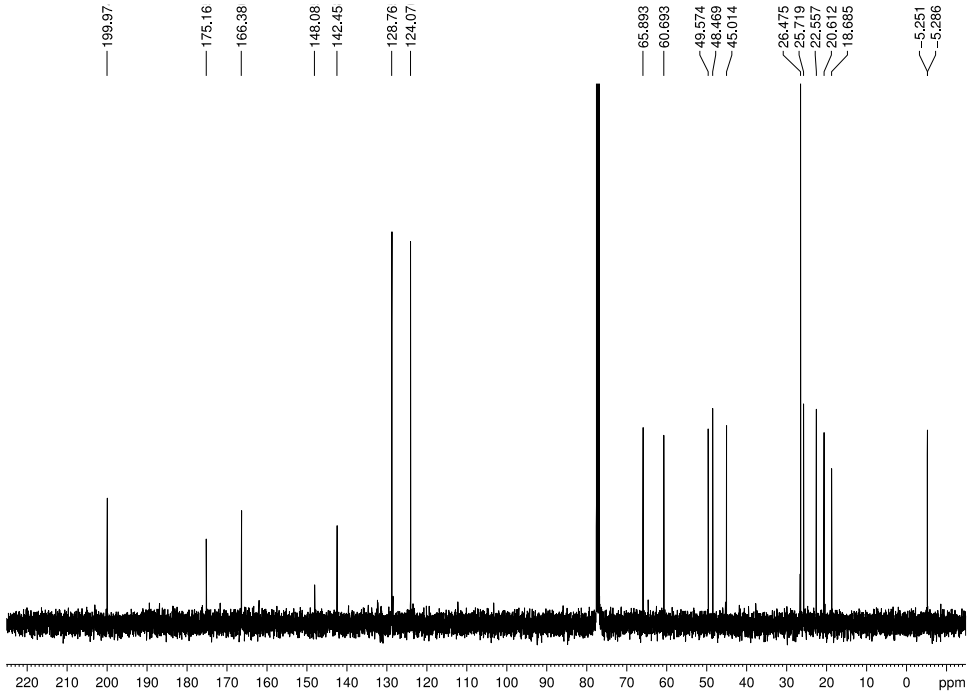
$_{1}$H NMR spectrum of **14** ($CDCl_3$).



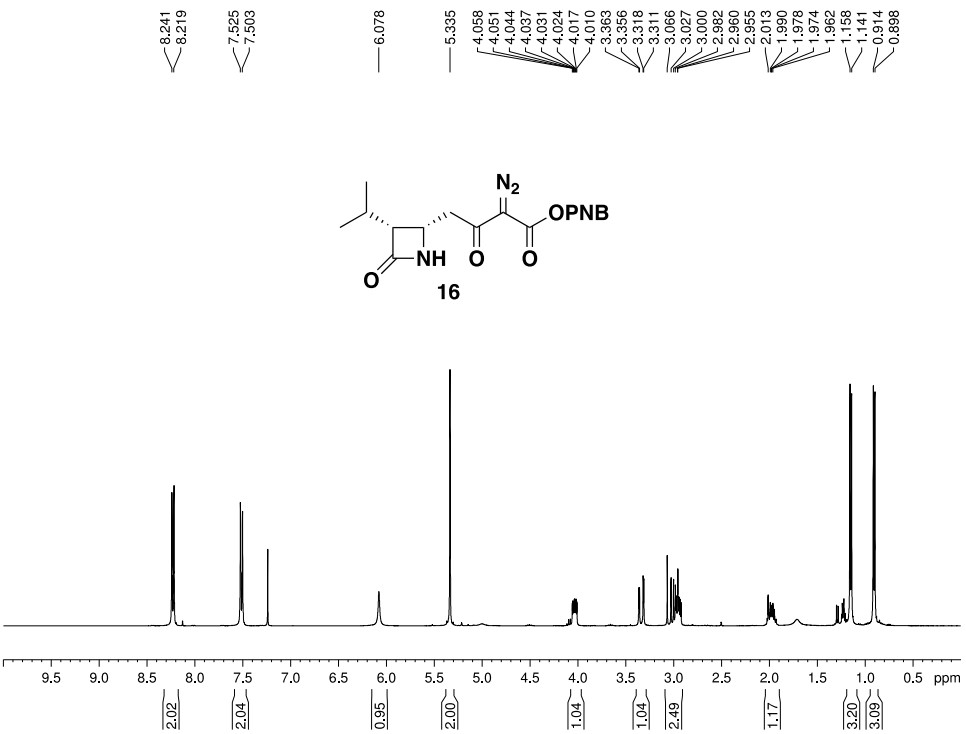
$_{13}$C NMR spectrum of **14** ($CDCl_3$).



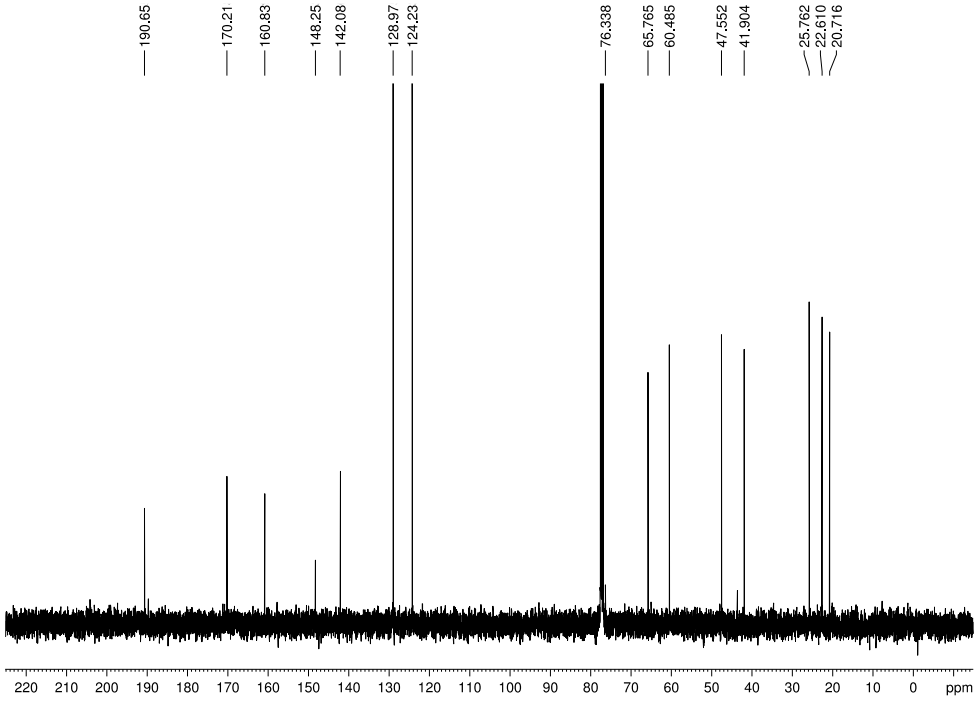
$_{1}$H NMR spectrum of **15** ($CDCl_3$).



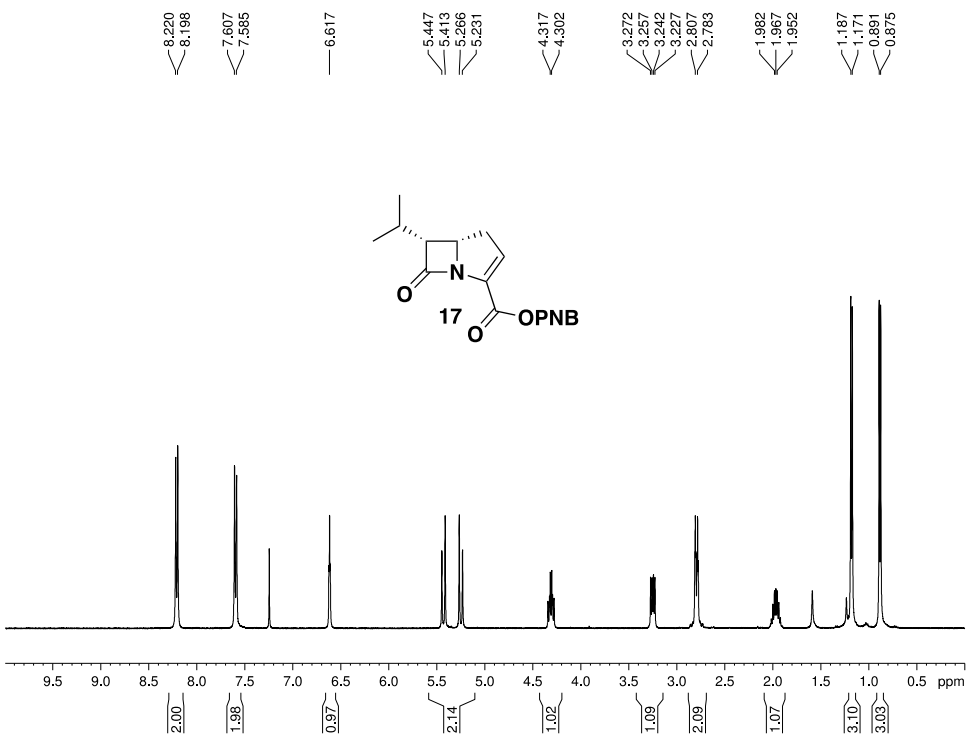
$_{13}$C NMR spectrum of **15** ($CDCl_3$).



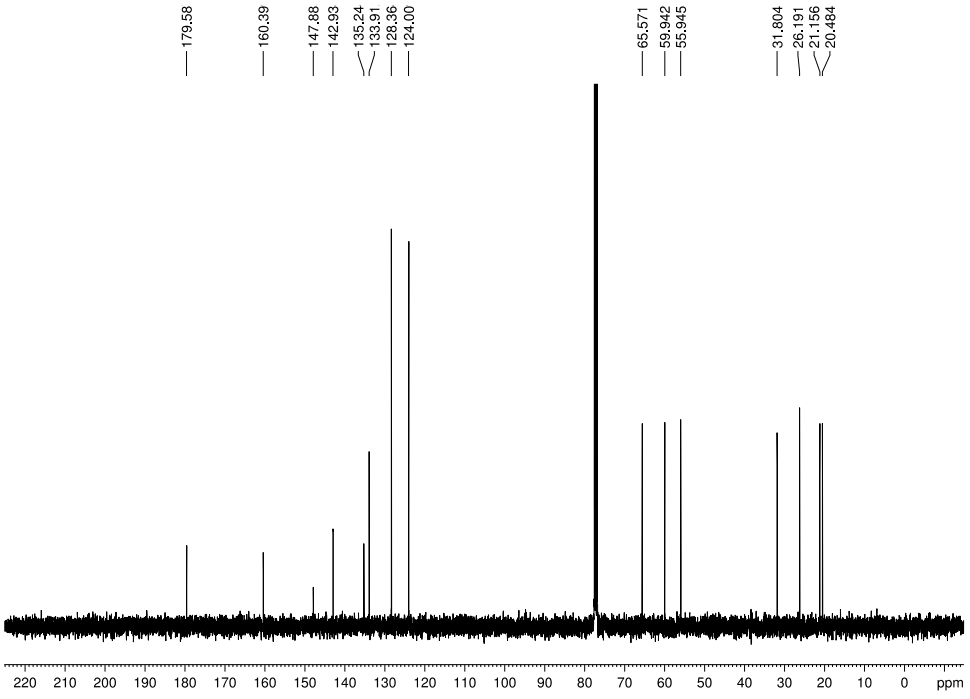
$_1$H NMR spectrum of **16** ($CDCl_3$).



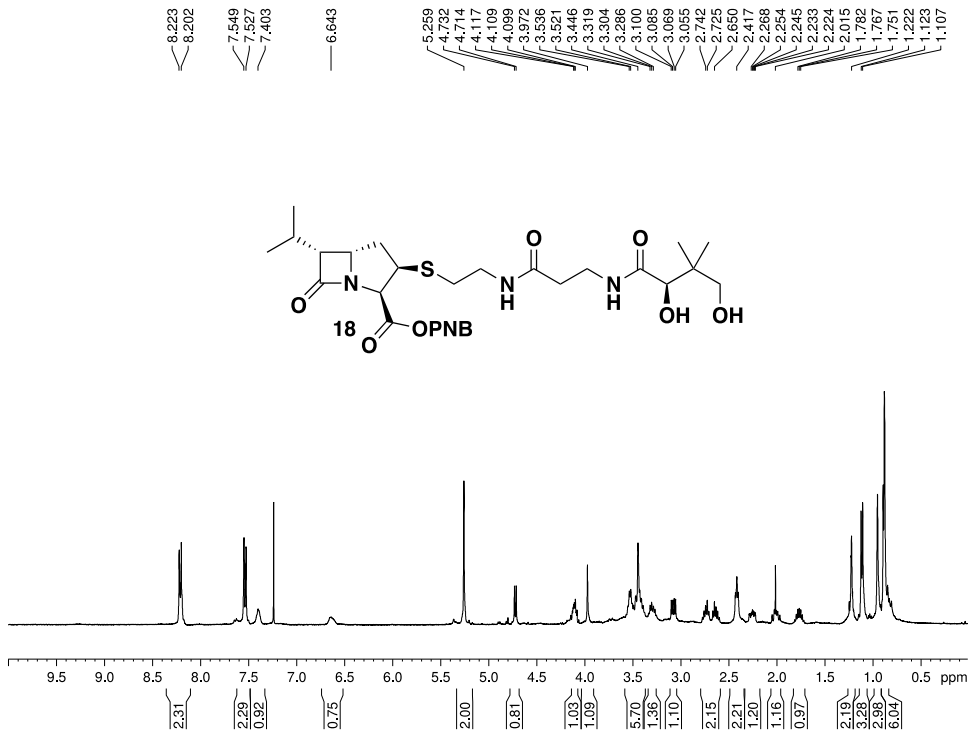
$_{13}$C NMR of **16** ($CDCl_3$).



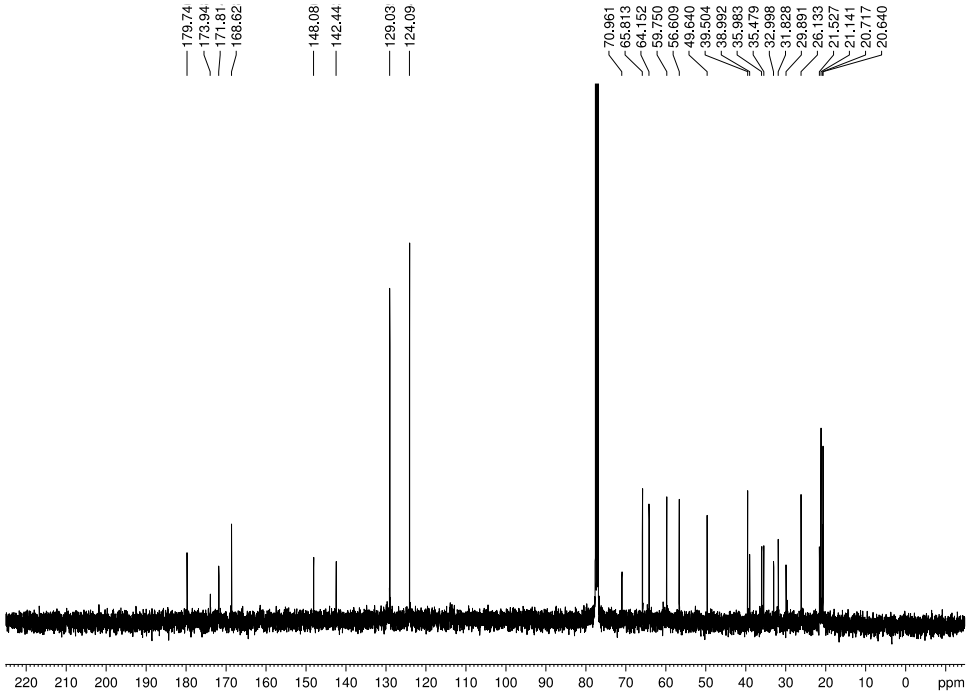
$_{1}$H NMR spectrum of **17** ($CDCl_3$).



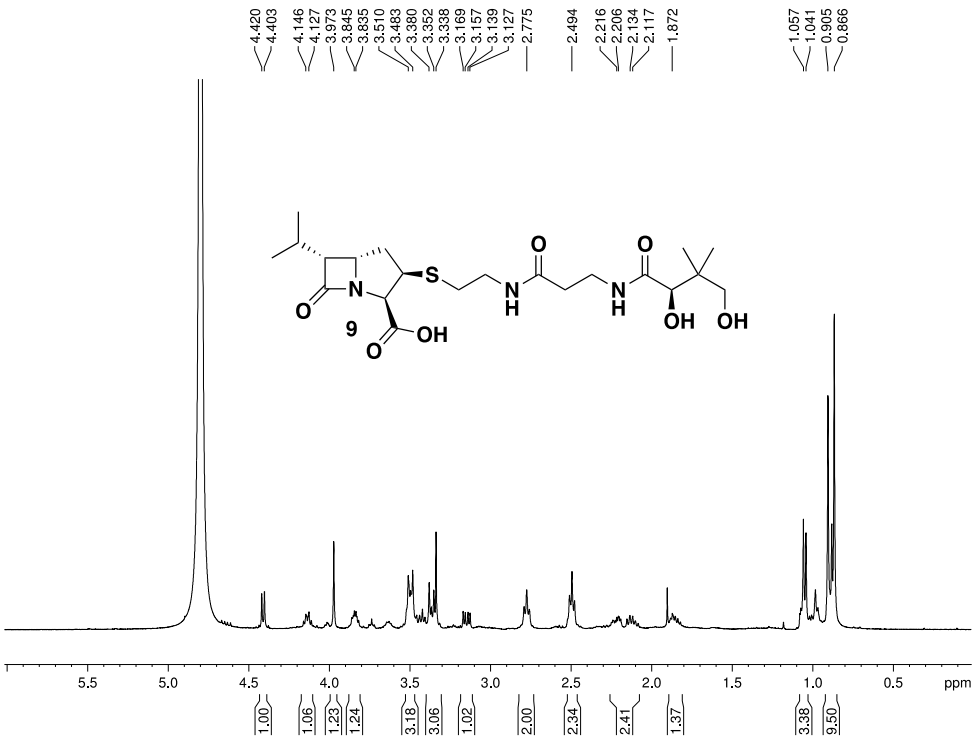
$_{13}$C NMR spectrum of **17** ($CDCl_3$).



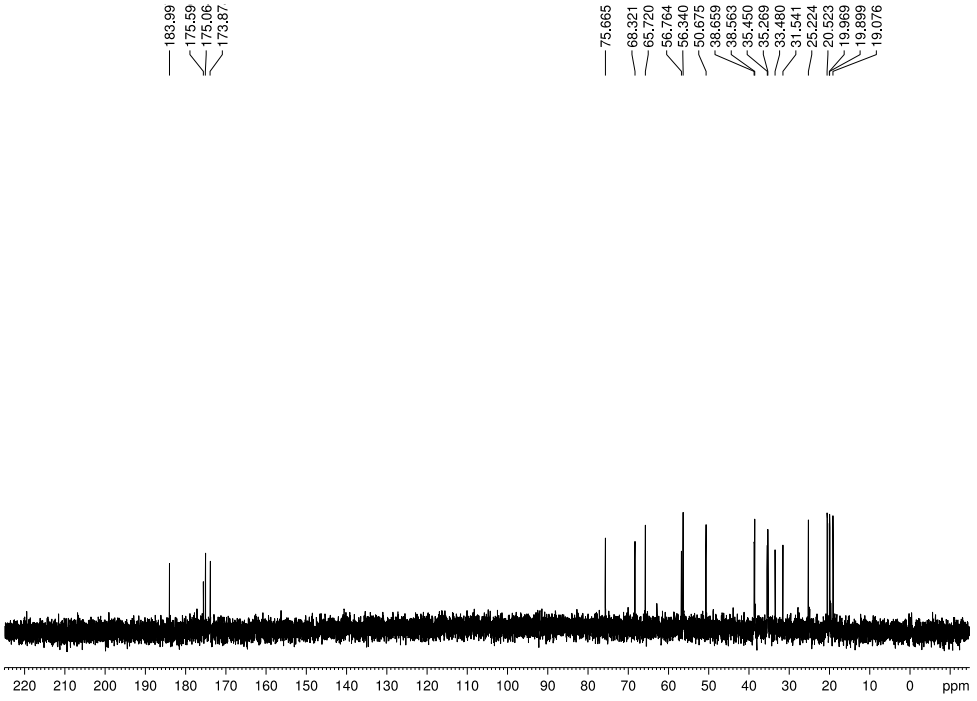
$_1$H NMR spectrum of **18** ($CDCl_3$).



$_{13}$C NMR spectrum of **18** ($CDCl_3$).



$_{1}$H NMR spectrum of **9** (D_2O).



$_{13}$C NMR spectrum of **9** (D_2O).

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