Utilization of Alternate Substrates by the First Three Modules of the Epothilone Synthetase Assembly Line

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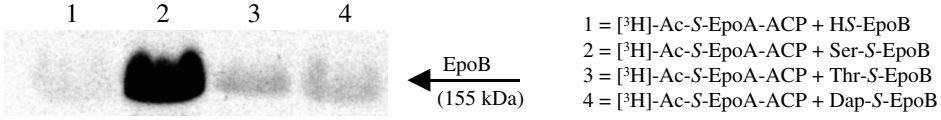
Purification of all proteins was performed as previously described.[1,2] Assay conditions for reactions among the EpoA-ACP/B/C proteins were also performed as described.[1] All radiolabeled compounds were purchased from New England Nuclear.

Transfer of [^{3}H]-acetyl from EpoA-ACP to EpoB to EpoC: Analysis of covalently linked products

Apo-EpoA-ACP was primed with [acetyl-^{3}H]-S-CoA. EpoA-ACP (0.45 nmol) in buffer (50 mM Tris, pH 7, 5 mM $MgCl_2$, 5 mM tris-2-carboxyethylphosphine (TCEP), 20 mM NaCl) was incubated with [^{3}H]-acetyl-S-CoA (2 nmol, 28.3 Ci/mmol). Loading of the EpoA-ACP domain was initiated by the addition of the phosphopantetheinyl transferase Sfp (5 μM) and allowed to incubate at ambient temperature for 30 minutes. EpoB (0.15 nmol) in buffer (50 mM Tris, pH 7, 5 mM $MgCl_2$, 5 mM TCEP, 20 mM NaCl, 5 mM ATP) was primed with HS-CoA (5 nmol) and then loaded with cysteine, serine, threonine, or 2,3-diaminopropionate (5 nmol). When EpoC was included in the reaction mixture, the holo-ACP domain of EpoC (0.5 nmol) was loaded with methylmalonyl-CoA in buffer (50 mM Tris, pH 7, 5mM $MgCl_2$, 5 mM TCEP, 1 mM NADPH). The three proteins were then combined and allowed to incubate at room temperature for 60 minutes. Non-reducing protein sample buffer was then added to the solution and the sample was loaded onto a 5% SDS-PAGE gel. The gel was stained with Coomasie, destained, soaked in the fluorographic reagent Amplify (Amersham) for 30 minutes and then dried. The gel was exposed to film or a phosphorimager for 48 hours at -80˚C.



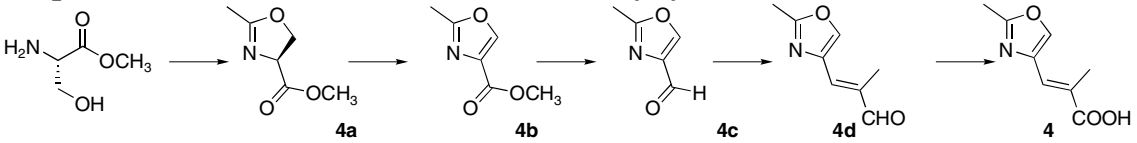
Autoradiogram of the reaction of [^{3}H]-acetyl-*S*-EpoA-ACP with EpoB alone. Transfer of [^{3}H]-acetyl from EpoA-ACP (16.5 kDa) to EpoB (155 kDa) occurs above background levels (holo-EpoB alone, lane 1) when EpoB is loaded with serine (lane 2), threonine (lane 3), and 2,3-diaminopropionate (lane 4). Additional experiments indicated that EpoB could be covalently modified with [^{3}H]-L-serine to 48% while modification with [^{14}C]-L-threonine was only detected to 30%, perhaps explaining the difference in degree of acetyl transfer seen above for the two amino acids.

Transfer of acetyl from EpoA-ACP to EpoB to EpoC: Radio-HPLC analysis of hydrolyzed products

Acetyl-*S*-EpoA-ACP, cysteinyl-*S*-EpoB and methylmalonyl-*S*-EpoC were incubated together as described above. The radiolabel was incorporated either as the [^{3}H]-acetyl-*S*-EpoA-ACP from [acetyl-^{3}H]-CoA (28.3 Ci/mmol), [^{35}S]-cysteinyl-*S*-EpoB from [^{35}S]-L-cysteine (2.6 Ci/mmol), [^{3}H]-seryl-*S*-EpoB from L-[^{3}H(G)]-serine (23 Ci/mmol) (G= generally radiolabeled), or as [^{14}C]-methylmalonyl-*S*-EpoC from D,L-[2-

methyl-^{14}C]-malonyl-CoA (60 Ci/mol). The reaction was quenched after 1 h by the addition of 10% trichloroacetic acid (TCA), and precipitated protein was pelleted by centrifugation and was then washed twice with 10% TCA. The protein pellet was then dissolved in KOH (0.1 M, 100 μL) and heated to 65˚C for 5 minutes. Trifluoroacetic acid (TFA) (50%, 5 μL) was then added, and the solution was centrifuged to remove precipitated proteins. A chemically synthesized standard was added to the radioactive enzymatic reaction, and injected directly onto the HPLC (Vydac C18 reverse phase column for all but 2B). Dual on-line UV (254 nm) and radioisotope detectors (tuned for ^{3}H, ^{14}C or ^{35}S) were used to monitor the retention time of standards and radioactive enzymatic products, respectively. All traces but 2B were a gradient of Buffer A (0.1% TFA in water) and Buffer B (acetonitrile) over a period of 25 minutes at a flow rate of 1 mL/min. In Figure 2A and 2C, the gradient was 0-70% Buffer B in Buffer A. Figure 2B, an isocrat of 5 mM potassium phosphate, pH 3.0 was used on a Zorbax 300-SCX strong cation exchange column. In Figure 2D and 2E, the gradient was 0-15% Buffer B in Buffer A.

Preparation and characterization of chemically synthesized standards



*Methyl oxazole methyl acrylate carboxylic acid (**4**).*

*2-Methyloxazoline-4-carboxylic acid methyl ester[3] (**4a**):* Preparation followed literature protocol for the preparation of very similar compounds[3] to give a yellow oil (540 mg, 63%) in greater than 95% purity. ^{1}HNMR (200 MHz, $CDCl_3$): 4.64-4.75 (m, 1H, $OCH_2C(COOCH_3)\underline{H}$); 4.32-4.50 (m, 2H, $OC\underline{H}_2C(COOCH_3)H$); 3.76 (s, 3H, $OC\underline{H}_3$); 2.00 (d, 3H, $CC\underline{H}_3$).

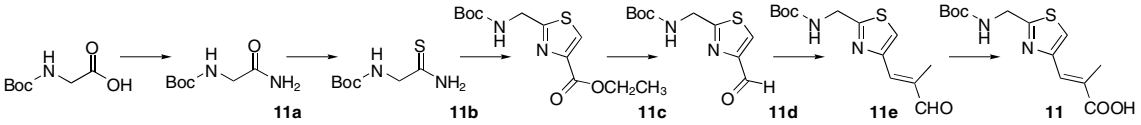
*2-Methyloxazole-4-carboxylic acid methyl ester[4] (**4b**)***:*** Oxidation of **4a** followed established protocols for the oxidation of oxazolines,[4] yielding a white powder (230 mg, 56%). ^{1}HNMR (200 MHz, $CDCl_3$): 8.08 (s, 1H, $OC(=C)\underline{H}$); 3.84 (s, 3H, $OC\underline{H}_3$); 2.45 (s, 3H, $CC\underline{H}_3$).

*2-Methyloxazole-4-carbaldehyde[1] (**4c**)*: Preparation of **4c, 4d,** and **4** was performed following literature protocols for analogous thiazole-containing molecules.[1] The final reaction mixture from **4c** was found to be a mixture of product aldehyde and starting material ester (1:2) by ^{1}H NMR analysis. The mixture was used without further purification for the preparation of **4d**. ^{1}HNMR for product **4c** (200 MHz, $CDCl_3$): 9.81 (s, 1H, $C\underline{H}O$); 8.12 (s, 1H, $OC(=C)\underline{H}$); 2.44 (s, 3H, $CC\underline{H}_3$).

*2-Methyloxazole-4-methyl acrylate aldehyde (**4d**)*: Product **4d** was a white powder (36.5 mg, 15% overall for conversion of **4b** to **4d**). ^{1}HNMR (200 MHz, $CDCl_3$): 9.52 (s, 1H, $CHC(CH_3)C\underline{H}O$); 7.81 (s, 1H, $OC(=C)\underline{H}$); 7.05 (s, 1H, $C\underline{H}C(CH_3)CHO$); 2.49 (s, 3H, $CC\underline{H}_3$); 2.07 (d, 3H, $CHC(C\underline{H}_3)CHO$).

*Methyl oxazole methyl acrylate carboxylic acid (**4**)*: Aldehyde **4d** (36.5 mg, 0.242 mmol) was dissolved in isobutanol (5 mL) and 2-methyl-2-butene (2M in THF, 1.34 mL, 2.69

mmol). $NaClO_2$ (230 mg, 2.57 mmol) and NaH_2PO_4 (227 mg, 1.89 mmol) in water (2.5 mL) were added. After 4 hours at 25 ˚C, the mixture was diluted with water (5 mL), brine (5 mL), and ethyl acetate-hexanes (1:1, 10 mL). The organic layer was collected, and the aqueous layer was extracted with ethyl acetate-hexanes (1:1, 10 mL). The combined organic layers were dried over sodium sulfate and concentrated to yield the carboxylic acid as a white solid (25.7 mg, 64%). Analysis by ^{1}H NMR revealed that the product was greater than 95% pure and the material was used without further purification. ^{1}HNMR (200 MHz, $CDCl_3$): 7.72 (s, 1H, OC(=C)H); 7.54 (q, 1H, CHC(CH_3)CHO); 2.50 (s, 3H, CCH_3); 2.23 (d, 3H, CHC(CH_3)CHO). $[M+H]^+$: obsvd. 168.3, expect. 168.2.



*Ethyl 2-[N-(t-Butoxycarbonyl)aminomethyl]thiazole methyl acrylate carboxylic acid (**11**)*[5]

*N-(t-Butoxycarbonyl)glycinamide (**11a**):* Ethyl chloroformate (1.09 mL, 11.4 mmol) was added dropwise to a stirring solution of L-*N*-Boc-Glycine (2g, 11.4 mmol) and triethylamine (1.59 mL, 11.4 mmol) in anhydrous THF at -10˚C. The mixture was stirred under nitrogen for 30 minutes and NH_4OH (2.9 mL, 30%) was then added. After stirring for an additional 45 minutes, the reaction was partitioned between ethyl acetate (20 mL) and water (20 mL). The organic layer was reserved and the aqueous layer was extracted again with ethyl acetate (20 mL). The combined ethyl acetate layers were washed with aqueous sodium hydrogen carbonate (20 mL), brine (20 mL), dried with sodium sulfate, and concentrated. The product (1.4 g, 67%) was used without further purification. ^{1}HNMR (200 MHz, $CDCl_3$): 6.76 (b, 1H, CONHH); 6.59 (b, 1H, CONHH); 5.86 (b, 1H, CH_2NH); 3.62 (d, 2H, CH_2NH); 1.31 (s, 9H, C(CH_3)$_3$).

*N-(t-Butoxycarbonyl)thioglycinamide (**11b**):* Lawesson's reagent (1.70 g, 4.21 mmol) was added to compound **11a** (1.40 g, 8.08 mmol) dissolved in anhydrous CH_2Cl_2 (75 mL). After 15 h of stirring under nitrogen atmosphere at 25˚C, the reaction was washed with 0.5% NaOH (4 x 20 mL), brine (2 x 30 mL) and concentrated, and the residue dissolved in CH_2Cl_2 and purified by flash chromatography on silica gel (ethyl acetate:hexanes, 1:9). (0.56 g, 37%); ^{1}HNMR (200 MHz, $CDCl_3$): 8.21 (b, 1H, (CSNHH); 8.10 (b, 1H, (CSNHH); 5.86 (b, 1H, (CH_2NH); 4.05 (d, 2H, (CH_2NH); 1.42 (s, 9H, C(CH_3)$_3$).

*Ethyl 2-[N-(t-Butoxycarbonyl)aminomethyl]thiazole-4-carboxylate (**11c**):* To a mixture of compound **11b** (0.56 g, 2.95 mmol) and $KHCO_3$ (2.36 g, 23.6 mmol) was added anhydrous ethylene glycol dimethyl ether (DME) (4 mL) and ethyl bromopyruvate (1.12 mL, 8.89 mmol). The solution turned bright yellow, and the reaction was allowed to stir under nitrogen at 25˚C for 2 h. The mixture was then cooled to 0˚C, and a solution of trifluoroacetic anhydride (1.69 mL, 12.0 mmol) and pyridine (2.04 mL, 25.2 mmol) in DME (2 mL) was added. The reaction mixture turned orange in color and was warmed to ambient temperature and was stirred under nitrogen for 1 hour. The reaction was then concentrated, and the residue dissolved in $CHCl_3$ (10 mL), extracted with brine, dried

with anhydrous sodium sulfate and concentrated. The residue was purified by flash column chromatography on silica gel (ethyl acetate:hexanes, 3:7). (0.40 g, 48%); ^{1}HNMR (200 MHz, $CDCl_3$): 8.10 (s, 1H, SC(=C)H); 5.30 (b, 1H, CH_2NH); 4.62 (d, 2H, CH_2NH); 4.42 (quart, 2H, $COCH_2CH_3$); 1.45 (s, 9H, $C(CH_3)_3$); 1.35 (t, 3H, CH_3CH_2CS).

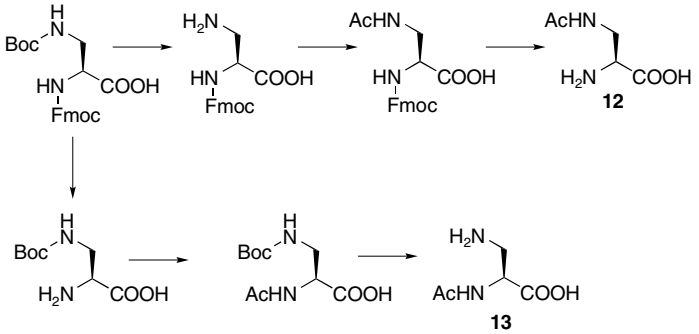
*Ethyl 2-[N-(t-Butoxycarbonyl)aminomethyl]thiazole-4-aldehyde (**11d**)*: A solution of DIBAl-H in toluene (1 M, 1.22 mL, 1.22 mmol, 1.2 equiv) was added to a dry solution of compound **11c** (0.29 g, 1.01 mmol, 1 equiv) in toluene at -78˚C. After 1 hour, excess aluminum hydride was quenched by the addition of methanol (3 mL) at -78˚C. After 5 minutes, the reaction mixture was allowed to warm to room temperature, partitioned between ether and an aqueous solution of Rochelle's salt (2 M, 8 mL) and the solution was stirred for 1.5 hours to provide a clear bilayer. The aqueous layer was extracted once with ether (10 mL) and the combined organic layers were washed with brine, were dried with anhydrous sodium sulfate and were concentrated. The resulting oil was found to be a mixture of product aldehyde and starting material ester (3:1) by ^{1}HNMR analysis. The mixture was used without further purification. ^{1}HNMR (for product **11d**) (200 MHz, $CDCl_3$): 9.91 (s, 1H, CHO); 8.08 (s, 1H, SC(=C)H); 5.70 (b, 1H, CH_2NH); 4.59 (d, 2H, CH_2NH); 1.42 (s, 9H, $C(CH_3)_3$).

*Ethyl 2-[N-(t-Butoxycarbonyl)aminomethyl]thiazole methyl acrylate aldehyde (**11e**):* To a solution of **11d** (0.24 g, 1.00 mmol) dissolved in anhydrous benzene (4 mL) was added Ph_3P=C(Me)CHO (0.38 g, 1.20 mmol). The mixture was allowed to reflux at 80˚C under nitrogen for 2.5 hours. The solution was then cooled to room temperature and diluted with ethyl acetate:hexanes (1:1, 20 mL). The resulting mixture was extracted with a saturated aqueous solution of sodium bicarbonate, brine, dried over anhydrous sodium sulfate and concentrated. Purification of the residue by flash column chromatography on silica gel (eluant: ethyl acetate-hexanes, 4:3), provided aldehyde **10** as a solid (0.046 g, 39% for two steps with recovery of starting material). ^{1}HNMR (200 MHz, $CDCl_3$): 9.55 (s, 1H, CHO); 7.52 (s, 1H, SC(=C)H); 7.23 (s, 1H, CHC(CH_3)CHO); 5.35 (b, 1H, CH_2NH); 4.63 (d, 2H, CH_2NH); 2.18 (d, 3H, CHC(CH_3)CHO); 1.46 (s, 9H, $C(CH_3)_3$).

*Ethyl 2-[N-(t-Butoxycarbonyl)aminomethyl]thiazole methyl acrylate carboxylic acid (**11**):* Compound **10** (46 mg, 163 μmol) was dissolved in *t*-BuOH (1.5 mL) and 2-methyl-2-butene (2M in THF, 0.91 mL, 1.81 mmol). $NaClO_2$ (156 mg, 1.73 mmol) and NaH_2PO_4 (198 mg, 1.27 mmol) in water (0.75 mL) was added. After 1.5 hours at 25˚C, the mixture was diluted with water (10 mL), a saturated solution of sodium chloride (10 mL), and ethyl acetate:hexanes (1:1, 20 mL). The organic layer was collected, and the aqueous layer was extracted with ethyl acetate:hexanes (1:1, 20 mL). The combined organic layers were dried over anhydrous sodium sulfate, and were concentrated to provide the carboxylic acid as a solid (50 mg, 99%). Analysis by ^{1}HNMR revealed that the product was greater than 95% pure and was used without further purification. ^{1}HNMR (200 MHz, $CDCl_3$): 7.70 (s, 1H, SC(=C)H); 7.41 (s, 1H, CHC(CH_3)COOH); 5.38 (b, 1H, CH_2NH); 4.63 (d, 2H, CH_2NH); 2.34 (s, 3H, CHC(CH_3)COOH); 1.46 (s, 9H, $C(CH_3)_3$). $[M+H]^+$: obsvd. 299.11, expect. 299.10.

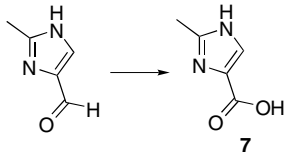
*Ethyl 2-aminomethylthiazole methyl acrylate carboxylic acid (**9**):*

Compound **11** (4 mg, 13 μmol) was dissolved in methylene chloride:trifluroacetic acid (1:1) and allowed to stir at room temperature for 30 minutes. The reaction was concentrated and used without further purification (2.6 mg, quant.). ^{1}HNMR (200 MHz, D_2O): 7.60 (s, 1H, SC(=C)H); 7.42 (s, 1H, CHC(CH_3)CHO); 4.35 (s, 2H, CH_2NH_2); 2.00 (s, 3H, CHC(CH_3)COOH.



*L-N-3-Acetyl-2-aminopropionic acid (**12**):* L-*N*-3-Boc-*N*-2-Fmoc-propionic acid (100 mg, 0.234 mmol, Novabiochem) was stirred in 1 mL 50% TFA/dichloromethane for 1 hour. The solvent was removed under vacuum and the crude product was carried on without further purification. L-3-Amino-*N*-2-Fmoc-propionic acid (76 mg, 0.234 mmol) was dissolved in 0.5 mL pyridine. Acetic anhydride (55 μL, 0.585 mmol, 2.5 equiv) was added to the reaction mixture. After 1 hour at 25 ˚C, solvent was removed under vacuum. The crude product was carried on without further purification. Crude L-*N*-3-Acetyl-*N*-2-Fmoc-aminopropionic acid was dissolved in 0.5 mL of 20% piperdine in DMF. The reaction mixture was stirred at 25 ˚C for 45 minutes and the solvent was then removed under vacuum, and preparative reverse phase HPLC (Vydac C18) yielded the purified product (18 mg, 52%). ^{1}HNMR (200 MHz, D_2O): 4.00-3.96 (m, 1H, α-H); 3.76-3.52 (m, 2H, β-H); 1.92 (s, 3H, NC(=O)CH_3). $[M+H]^+$: obsvd. 147.20, expect. 147.07.

*L-N-2-Acetyl-3-aminopropionic acid (**13**)* was prepared similarly to **12**, also from L-*N*-3-Boc-*N*-2-Fmoc-propionic acid and with the expected different order of deprotection. ^{1}HNMR (200 MHz, D_2O): 3.39-3.29 (m, 1H, α-H); 3.17-3.06 (m, 2H, β-H), 1.91 (s, 3H, NC(=O)CH_3). $[M+H]^+$: obsvd. 147.34, expect. 147.07.



*2-Methyl-1H-imidazole-4-carboxylic acid (**7**)*: 2-Methyl-1*H*-imidazole-4-carbaldehyde (Maybridge) was oxidized to give carboxylic acid **7** as described above for the conversion of aldehyde **4d** to carboxylic acid **4**. In this case, ^{1}HNMR revealed that the product was greater than 95% pure and was simply isolated from remaining salts using preparative HPLC (isocrat of Buffer A over 15 minutes). ^{1}HNMR (200 MHz, D_2O): 7.46 (s, 1H, NC(=C)H); 2.45 (s, 3H, CCH_3).

Mass spectrometry

All mass spectrometry was MALDI-TOF (matrix assisted laser desorption ionization–time of flight) spectrometry performed at a Voyager-DE STR BioSpectrometry Workstation (PerSeptive Biosystems) with predicted accuracy to 0.1%. All samples were prepared using 2,5-dihydroxybenzoic acid as matrix.

References

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