Stereochemical Assignment of Intermediates in the Rifamycin Polyketide Synthase Pathway by Precursor-directed Biosynthesis

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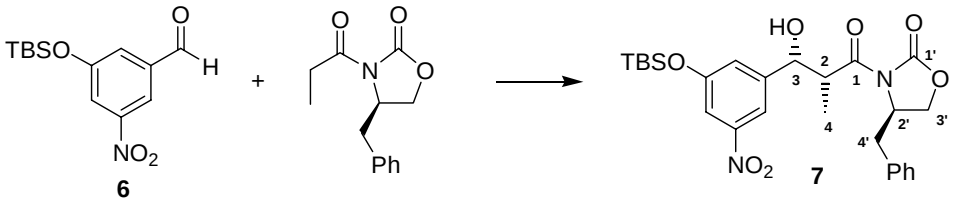
Supporting Information

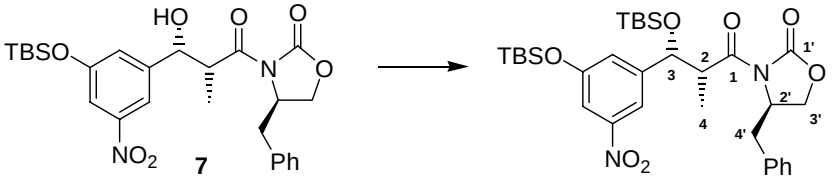
Syn-Series



91 mg (*R*)-(+)-4-benzyl-3-propionyl-2-oxazolidinone (0.391 mmol, 1.1 equiv.; 99% ee, Aldrich Chemical Company) were dissolved in 1.3 mL dry Et_2O (0.3 M), cooled with an ice-salt bath and treated with 450 µL Bu_2BOTf solution (1.0 M in CH_2Cl_2; 0.45 mmol, 1.27 equiv.). After 5 min at this temperature 89 µL Hünig's base (0.51 mmol, 1.43 equiv.) were added and stirring at 0 °C was continued for 45 min. The mixture was cooled to -78 °C and treated with a solution of 100 mg aldehyde **6** (0.36 mmol, 1.0 equiv.) in 0.4 mL dry Et_2O. After 2h at -78 °C and 1h at 0 °C the reaction was quenched by addition of pH7 phosphate buffer and extracted with ether (4x), dried over Na_2SO_4 and evaporated to dryness. The residue was redissolved in 5 mL MeOH, cooled to 0 °C and treated with 1.3 mL 30% H_2O_2. Stirring was continued for one hour, followed by evaporation to dryness, work-up with ether/1 N HCl, ether extraction (3x), drying with Na_2SO_4 and evaporation. Flash column chromatography (hexane/EtOAc 10:1, 5:1 [P]) yielded 197 mg **7** as a colorless foam (containing residual amount of the imide; yield calculated based on nmr integration to be 97% from aldehyde **6**; d.r. > 25:1).

	MW 514.65 $C_{26}H_{34}N_2O_7Si$
^{1}H-NMR	7.85-7.86 (m, 1H; Ar); 7.55 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.24-7.35 (m, 3H; Ph); 7.21-
500 MHz	7.22 (m, 1H; Ar); 7.18-7.20 (m, 2H; Ph); 5.18 (d, *J* = 2.7 Hz, 1H; H-3); 4.69-4.75 (m,

$CDCl_3$	1H; H-2'); 4.19-4.27 (m, 2H; H-3'); 3.98 (dq, *J* = 7.1, 2.7 Hz, 1H; H-2); 3.24 (dd, *J* = 13.4, 3.4 Hz, 1H; H-4'a); 2.80 (dd, *J* = 13.4, 9.4 Hz, 1H; H-4'b); 1.11 (d, *J* = 7.0 Hz, 3H; H-4); 0.98 (s, 9H; TBS); 0.23 (s, 6H; TBS).
^{13}C-NMR	176.82 (C_q; C-1); 156.41 (C_q; Ar); 152.94 (C_q; C-1'); 149.12 (C_q; Ar); 144.33 (C_q; Ar);
125 MHz	134.84 (C_q; Ph); 129.49 (CH; Ph); 129.10 (CH; Ph); 127.63 (CH; Ph); 124.16 (CH;
$CDCl_3$	Ar); 114.16 (CH; Ar); 114.03 (CH; Ar); 72.03 (CH; C-3); 66.44 (CH_2; C-3'); 55.15 (CH; C-2'); 44.17 (CH; C-2); 37.81 (CH_2; C-4'); 25.64 (CH_3; TBS); 18.28 (C_q; TBS); 10.05 (CH_3; C-4); -4.40 (CH_3; TBS).
ESI-MS	1051.7 (2M + Na; 10); 539.4 (10); 538.4 (35); 537.4 (M + Na; 100).

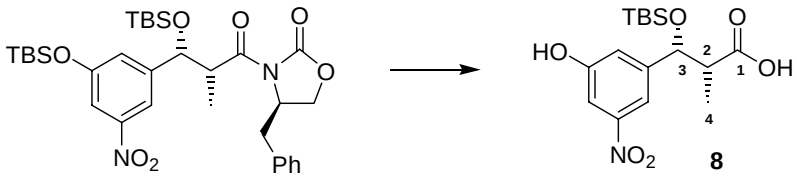


267.1 mg of the Evans-aldol product **7** (0.52 mmol, 1.0 equiv.) were dissolved in 2.6 mL dry CH_2Cl_2 (0.2 M), cooled to 0 °C, treated with 91 µL 2,6-lutidine (0.78 mmol, 1.5 equiv.) and subsequently by dropwise addition with 155 µL TBSOTf (0.68 mmol, 1.3 equiv.). The reaction continued for 16h at rt, was quenched with saturated NH_4Cl solution, extracted with EtOAc (4x), dried with Na_2SO_4 and evaporated to dryness. Flash column chromatography (hexane; hexane/EtOAc 10:1) yielded 318 mg silyl ether as a white solid (97%).

	MW 628.91 $C_{32}H_{48}N_2O_7Si_2$
^{1}H-NMR	7.86 (t, *J* = 1.5-2.0 Hz, 1H; Ar); 7.51 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.16-7.32 (m, 6H; Ar
500 MHz	+ Ph); 5.03 (d, *J* = 5.5 Hz, 1H; H-3); 4.46-4.51 (m, 1H; H-2'); 4.12 (dd, *J* = 9.1, 2.2
$CDCl_3$	Hz, 1H; H-3'a); 4.01 (t, *J* = 8.1-8.9 Hz, 1H; H-3'b); 3.98 (dq, *J* = 6.8, 5.5 Hz, 1H; H-2); 3.23 (dd, *J* = 13.4, 3.2 Hz, 1H; H-4'a); 2.74 (dd, *J* = 13.4, 9.6 Hz, 1H; H-4'b); 1.17 (d, *J* = 7.0 Hz, 3H; H-4); 0.96 (s, 9H; TBS); 0.88 (s, 9H; TBS); 0.21 (s, 6H; TBS); -0.01 (d, 3H; TBS); -0.21 (s, 3H; TBS).
^{13}C-NMR	173.60 (C_q; C-1); 156.21 (C_q; Ar); 153.08 (C_q; C-1'); 148.87 (C_q; Ar); 146.36 (C_q; Ar);
125 MHz	135.16 (C_q; Ph); 129.50 (CH; Ph); 129.05 (CH; Ph); 127.50 (CH; Ph); 124.39 (CH;
$CDCl_3$	Ar); 114.65 (CH; Ar); 114.18 (CH; Ar); 74.02 (CH; C-3); 66.16 (CH_2; C-3'); 55.64 (CH; C-2'); 46.74 (CH; C-2); 37.63 (CH_2; C-4'); 25.77 (CH_3; TBS); 25.62 (CH_3; TBS); 18.26 (C_q; TBS); 18.15 (C_q; TBS); 11.66 (CH_3; C-4); -4.40 (CH_3; TBS); -4.46 (CH_3; TBS); -5.32 (CH_3; TBS).
ESI-MS	653.1 (15); 652.2 (40); 651.1 (M + Na; 100).

HR-MS $C_{32}H_{48}N_2O_7Si_2 = [M + Na]^+$

calculated: 651.2898 found: 651.2910 [+ 1.9 ppm]

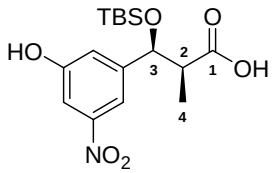


315 mg of the bis-TBS ether (0.5 mmol, 1.0 equiv.) were dissolved in 5 mL THF (0.1 M), cooled to 0 °C and treated subsequently with 0.80 mL H_2O_2 (30%; 7.0 mmol, 14.0 equiv.) and 147 mg $LiOH•H_2O$ (3.5 mmol, 7.0 equiv.). The instantly formed yellowish-orange solution was warmed gradually to rt and quenched after 16h reaction time with 1N HCl, extracted with EtOAc (4x), the combined organic extract was washed with Na_2SO_3 solution, dried with Na_2SO_4 and evaporated to dryness. Flash chromatography (hexane/EtOAc 10:1, 5:1 [P], 2:1) provided pure product **8** along with some fractions of impure product which were re-chromatographed to yield a total of 162 mg free acid **8** (91%).

MW 355.40 $C_{16}H_{25}NO_6Si$

^{1}H-NMR 500 MHz d_6-acetone: 7.76-7.77 (m, 1H; Ar); 7.56 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.30-7.32 (m, 1H; Ar); 5.24 (d, *J* = 5.1 Hz, 1H; H-3); 2.72 (dq, *J* = 7.0, 5.3 Hz, 1H; H-2); 1.11 (d, *J* = 7.0 Hz, 3H; H-4); 0.92 (s, 9H; TBS); 0.11 (s, 3H; TBS); -0.11 (s, 3H, TBS).

^{13}C-NMR 125 MHz d_6-acetone: 174.00 (C_q; C-1); 157.91 (C_q; Ar); 149.02 (C_q; Ar); 147.05 (C_q; Ar); 119.85 (CH; Ar); 112.49 (CH; Ar); 108.84 (CH; Ar); 74.60 (CH; C-3); 47.89 (CH; C-2); 25.31 (CH_3; TBS); 17.85 (C_q; TBS); 10.55 (CH_3; C-4); -5.28 (CH_3; TBS); -5.90 (CH_3; TBS).



***ent*-8**

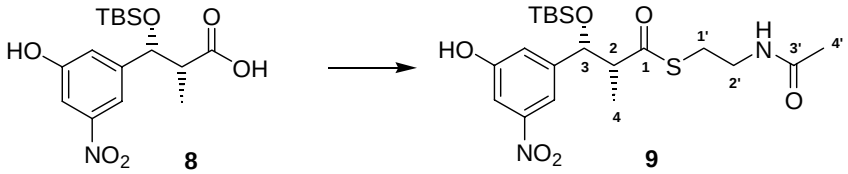
MW 355.40 $C_{16}H_{25}NO_6Si$

^{1}H-NMR 500 MHz: 7.77-7.76 (m, 1H; Ar); 7.56 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.30-7.31 (m, 1H; Ar); 5.23 (d, *J* = 5.1 Hz, 1H; H-3); 2.72 (dq, *J* = 7.0, 5.2 Hz, 1H; H-2); 1.11 (d, *J* = 7.0 Hz, 3H; H-

d_6-acetone	4); 0.91 (s, 9H; TBS); 0.10 (s, 3H; TBS); -0.11 (s, 3H, TBS).
^{13}C-NMR	174.05 (C_q; C-1); 157.88 (C_q; Ar); 148.99 (C_q; Ar); 147.00 (C_q; Ar); 119.82 (CH; Ar);
125 MHz	112.45 (CH; Ar); 108.82 (CH; Ar); 74.57 (CH; C-3); 47.87 (CH; C-2); 25.29 (CH_3;
d_6-acetone	TBS); 17.82 (C_q; TBS); 10.52 (CH_3; C-4); -5.30 (CH_3; TBS); -5.91 (CH_3; TBS).
ESI-MS (-)	731.2 (2M + Na - 2H; 15); 709 (2M - H; 10); 354.8 (15); 354 (M - H; 100).

HR-MS $C_{16}H_{24}NO_6Si = [M - H]^-$

calculated: 354.1373 found: 354.1365 [-2.2 ppm].



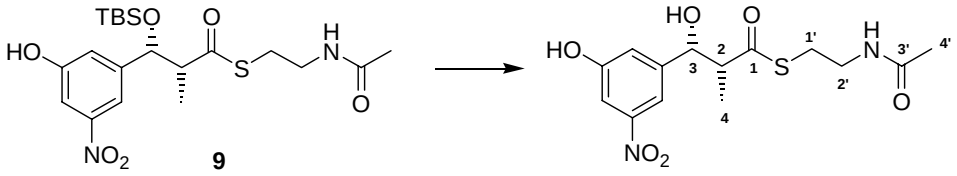
160 mg of the carboxylic acid **8** (0.45 mmol, 1.0 equiv.) and 153 mg CDI (0.95 mmol, 2.1 equiv.) were dissolved in 9 mL dry DMF (0.05 M) and were stirred at rt for 3h followed by administration of 191 µL HSNAC (neat; 1.80 mmol, 4.0 equiv.). After 1h 55 mg DMAP (0.45 mmol, 1.0 equiv.) were added and stirring was continued for 16h upon which additional 96 µL HSNAC (0.9 mmol, 2.0 equiv.) were added and stirring was continued for 24h (TLC showed only trace amounts of starting material). The reaction mixture was quenched with 0.5 N HCl and extracted with EtOAc (4x). The combined organic extracts were thoroughly washed with brine (2x) and dried with Na_2SO_4. Flash column chromatography ($CuSO_4$-impregnated silica on top of a regular silica gel column; hexane/EtOAc 5:1 [traces SM], 1:1, EtOAc pure [P], $CHCl_3$/MeOH 10:1) yielded 8.8 mg recovered starting material (6%) and 171.3 mg of the SNAC ester **9** (83%) as a yellowish oil, which crystallized upon storage.

MW 456.63 $C_{20}H_{32}N_2O_6SiS$

^{1}H-NMR	9.70 (br, 1H; O*H*); 7.70-7.72 (m, 1H; Ar); 7.56 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.39 (br,
500 MHz	1H; N*H*Ac); 7.25-7.26 (m, 1H; Ar); 5.09 (d, *J* = 5.9 Hz, 1H; H-3); 3.24-3.34 (m, 2H;
d_6-acetone	H-2'); 2.87-2.99 (m, 3H; H-2 + H-1'); 1.89 (s, 3H; H-4'); 1.20 (d, *J* = 6.8 Hz, 3H; H-4); 0.92 (s, 9H; TBS); 0.10 (s, 3H; TBS); -0.13 (s, 3H, TBS).
^{13}C-NMR	199.92 (C_q: C-1); 169.88 (C_q; C-3'); 158.14 (C_q; Ar); 149.03 (C_q; Ar); 146.22 (C_q;
125 MHz	Ar); 120.01 (CH; Ar); 112.36 (CH; Ar); 109.15 (CH; Ar); 75.11 (CH; C-3); 56.87
d_6-acetone	(CH; C-2); 38.74 (CH_2; C-2'); 28.24 (CH_2; C-1'); 25.30 (CH_3; TBS); 22.00 (CH_3; C-4'); 17.84 (C_q; TBS); 12.15 (CH_3; C-4); -5.33 (CH_3; TBS); -5.82 (CH_3; TBS).
ESI-MS (+)	481.1 (5); 480.1 (13); 479.1 (M + Na; 46); 457.0 (M + H; 10).
ESI-MS (-)	910.7 (2M - H; 10); 455.1 (M - H; 100).

HR-MS $C_{20}H_{32}N_2O_6NaSiS = M^+$

calculated: 479.1648 found: 479.1637 [-2.3 ppm].



125.7 mg of the TBS-protected SNAC ester **9** (0.275 mmol, 1.0 equiv.) were dissolved in 5.5 mL acetonitril (0.05 M) and treated at 0 °C with 1.1 mL HF (30%). The mixture was gradually warmed to rt and stirring was continued until TLC analysis showed complete turnover (44h). The mixture was quenched with pH 7.0 phosphate buffer and brine, extracted with EtOAc (4x) and dried with Na_2SO_4. Flash column chromatography (hexane/EtOAc 1:1, EtOAc pure, $CHCl_3$/MeOH 10:1 [P]) yielded 88.4 mg of the free alcohol (94%) as a white solid.

MW 342.27 $C_{14}H_{18}N_2O_6S$

^{1}H-NMR 9.54 (br, 1H; O*H*); 7.74-7.76 (m, 1H; Ar); 7.54 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.40 (br, 1H; N*H*Ac); 7.26-7.27 (m, 1H; Ar); 5.08 (d, *J* = 5.4 Hz, 1H; H-3); 5.02 (br, 1H; O*H*); 3.21-3.36 (m, 2H; H-2'); 2.87-3.03 (m, 3H; H-2 + H-1'); 1.89 (s, 3H; H-4'); 1.19 (d, *J* = 6.9 Hz, 3H; H-4).

500 MHz d_6-acetone

^{13}C-NMR 200.50 (C_q: C-1); 170.22 (C_q; C-3'); 158.05 (C_q; Ar); 149.15 (C_q; Ar); 146.76 (C_q; Ar); 119.86 (CH; Ar); 112.16 (CH; Ar); 108.78 (CH; Ar); 73.29 (CH; C-3); 55.91 (CH; C-2); 38.52 (CH_2; C-2'); 28.40 (CH_2; C-1'); 22.00 (CH_3; C-4'); 11.76 (CH_3; C-4).

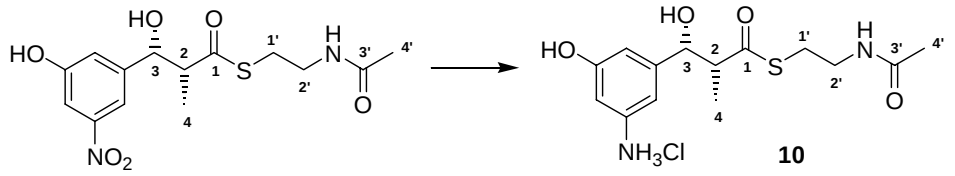
125 MHz d_6-acetone

ESI-MS (+) 365.0 (M + Na; 50); 343.0 (M + H; 100).

ESI-MS (-) 341.0 (M - H; 100).

HR-MS $C_{14}H_{18}N_2O_6NaS = [M + Na]^+$

calculated: 365.0783 found: 365.0787 [+ 1.0 ppm].

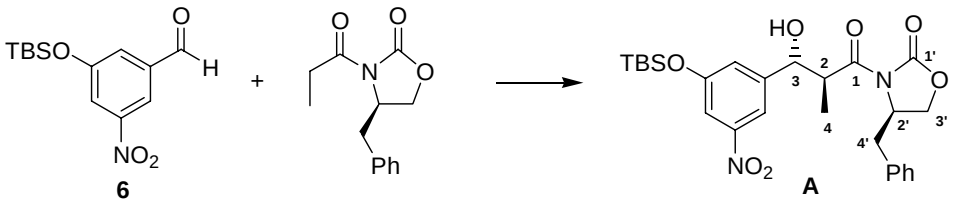


82.4 mg of the nitro-SNAC ester (0.241 mmol) were dissolved in 10 mL ethanol. This solution was added to 170 mg Pd/C (10%), followed by addition of 1 mL 1 N HCl. The air in the reaction flask was replaced by hydrogen and the slurry was vigorously stirred for 2 ½ h and then filtered

through a plug of celite. The celite was washed with additional ethanol. The ethanol solution was concentrated, toluene was added to the concentrated solution and evaporation was continued to dryness yielding the hydrochloride as a white-brownish solid in quantitative yield.

	MW 348.85 $C_{14}H_{21}N_2O_4SCl$
^{1}H-NMR	6.87 (br, 1H; Ar); 6.86 (br, 1H; Ar); 6.75 (br, 1H; Ar); 4.86 (d, *J* = 6.7 Hz, 1H; H-3);
500 MHz	3.26-3.36 (m, 2H; H-2'); 2.92-3.02 (m, 3H; H-1' + H-2); 2.08 (s, 3H; H-4'); 1.24 (d, *J*
CD_3OD	= 6.8 Hz, 3H; H-4).
^{13}C-NMR	202.22 (C_q; C-1); 174.52 (C_q; C-3'); 159.93 (C_q; Ar); 148.10 (C_q; Ar); 132.46 (C_q;
125 MHz	Ar); 115.11 (CH; Ar); 112.76 (CH; Ar); 110.06 (CH; Ar); 75.01 (CH; C-3); 57.26
CD_3OD	(CH; C-2); 40.66 (CH_2; C-2'); 28.49 (CH_2; C-1'); 21.94 (CH_3; C-4'); 13.27 (CH_3; C-4).

Anti-Series



59 mg of the Evans imide (0.254 mmol, 1.1 equiv.) were dissolved in 0.8 mL of dry ether (0.3 M) and treated at 0 °C with 508 µL Bu_2BOTf solution (1.0 M in CH_2Cl_2; 0.508 mmol, 2.2 equiv.) and after 5 min with 51 µL DIPEA (0.292 mmol, 1.27 equiv.). After 45 min at 0 °C the mixture was cooled to -78 °C and a solution of 65 mg aldeyhde **6** (0.231 mmol, 1.0 equiv.) in 0.5 mL Et_2O were added dropwise. After 2h at -78 °C the mixture was quenched with 1M $NaHSO_4$ solution, extracted with hexane/EtOAc 1:1 (4x), dried with brine and evaporated to dryness. The residue was taken up in 2.5 mL Et_2O and 0.6 mL pH7 buffer and treated with 0.6 mL H_2O_2 (30%) at 0 °C for 90 min. Watery work-up, EtOAc extraction, drying with Na_2SO_4 and flash column chromatography (hexane/EtOAc 10:1, 5:1 [Evans imide], 2:1 [P]) gave 93 mg of the aldol product **A** (78%) as a 3.6 : 1.0 diastereomeric mixture (*anti*: non-Evans-*syn*).

	MW 514.65 $C_{26}H_{34}N_2O_7Si$
^{1}H-NMR	7.86 (t, *J* = 1.6 Hz, 1H; Ar); 7.56 (t, *J* = 2.2 Hz, 1H; Ar); 7.12-7.33 (m, 6H; Ar + Ph);
500 MHz	4.84 (d, *J* = 7.6 Hz, 1H; H-3); 4.64-4.70 (m, 1H; H-2'); 4.13-4.27 (m, 3H; H-2 + H-3');
$CDCl_3$	3.18 (dd, *J* = 13.6, 3.3 Hz, 1H; H-4'b); 2.66 (dd. *J* = 13.6, 9.4 Hz, 1H; h-4'b); 1.15 (d,

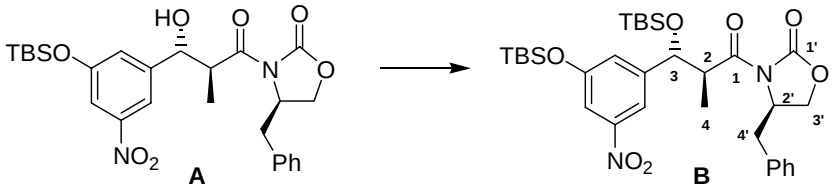
J = 7.0 Hz, 3H; H-4); 0.97 (s, 9H; TBS); 0.23 (s, 6H; TBS).

^{13}C-NMR 125 MHz $CDCl_3$ 176.03 (C_q; C-1); 156.67 (C_q; Ar); 153.46 (C_q; C-1'); 149.16 (C_q; Ar); 145.30 (C_q; Ar); 134.95 (C_q; Ph); 129.45 (CH; Ph); 129.05 (CH; Ph); 127.50 (CH; Ph); 124.57 (CH; Ar); 114.58 (CH; Ar); 114.48 (CH; Ar); 76.29 (CH; C-3); 66.19 (CH_2; C-3'); 55.47 (CH; C-2'); 44.30 (CH; C-2); 37.71 (CH_2; C-4'); 25.60 (CH_3; TBS); 18.25 (C_q; TBS); 14.94 (CH_3; C-4); -4.40 (CH_3; TBS).

ESI-MS 1053.8 (10); 1052.8 (20); 1051.8 (2M + Na; 25); 539.4 (15); 538.4 (50); 537.4 (M + Na; 100); 497.4 (M -H_2O + H; 90).

HR-MS $C_{26}H_{34}N_2O_7NaSi = [M + Na]^+$

calculated: 537.2033 found: 537.2024 [-1.7 ppm].



267 mg of the Evans-aldol product **A** (0.52 mmol, 1.0 equiv.) were dissolved in 2.6 mL dry CH_2Cl_2 (0.2 M), cooled to 0 °C, treated with 91 µL 2,6-lutidine (0.78 mmol, 1.5 equiv.) and subsequently by dropwise addition with 156 µL TBSOTf (0.68 mmol, 1.3 equiv.). The reaction continued for 16h at rt, was quenched with saturated NH_4Cl solution, extracted with EtOAc (4x), dried with Na_2SO_4 and evaporated to dryness. Flash column chromatography (hexane; hexane/EtOAc 10:1) yielded 301 mg **B** as a white solid (92%; d.r. 3.0:1.0).

MW 628.91 $C_{32}H_{48}N_2O_7Si_2$

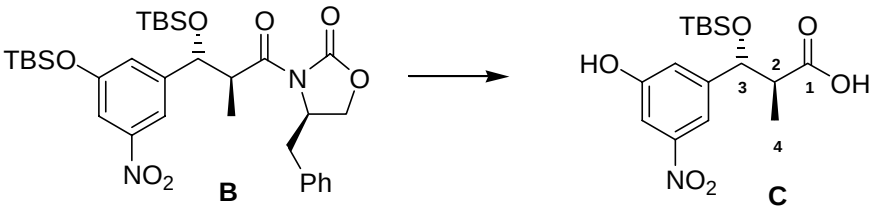
^{1}H-NMR 500 MHz $CDCl_3$ 7.80 (br, 1H; Ar); 7.57-7.59 (m, 1H; Ar); 7.20-7.36 (m, 6H; Ar + Ph); 5.00 (d, *J* = 9.0 Hz, 1H; H-3); 4.65-4.71 (m, 1H; H-2'); 4.03-4.21 (m, 3H; H-2 + H-3'); 3.44 (dd, *J* = 13.3, 3.3 Hz, 1H; H-4'a); 2.69 (dd, *J* = 13.3, 10.4 Hz, 1H; H-4'b); 0.98 (s, 3H; TBS); 0.97 (d, 3H; H-4); 0.80 (s, 9H; TBS); 0.24 (s, 6H; TBS); 0.04 (s, 3H; TBS); -0.24 (s, 3 H; TBS).

^{13}C-NMR 125 MHz $CDCl_3$ 174.91 (C_q; C-1); 156.48 (C_q; Ar); 153.19 (C_q; C-1'); 148.83 (C_q; Ar); 145.76 (C_q; Ar); 135.54 (C_q; Ph); 129.44 (CH; Ph); 129.11 (CH; Ph); 127.44 (CH; Ph); 125.28 (CH; Ar); 115.34 (CH; Ar); 114.70 (CH; Ar); 76.36 (CH; C-3); 66.04 (CH_2; C-3'); 55.62 (CH; C-2'); 46.33 (CH; C-2); 38.43 (CH_2; C-4'); 25.80 (CH_3; TBS); 25.62 (CH_3: TBS); 18.28 (C_q; TBS); 18.04 (C_q; TBS); 14.33 (CH_3; C-4); -4.32 (CH_3; TBS); -4.38 (CH_3; TBS); -4.49 (CH_3; TBS); -4.89 (CH_3; TBS).

ESI-MS 1280 (2M + Na; 10); 653.5 (15); 652.5 (50); 651.5 (M + Na; 100).

HR-MS $C_{32}H_{48}N_2O_7Si_2Na = [M + Na]^+$

calculated: 651.2898 found: 651.2894 [-0.6 ppm]



280 mg of the imide **B** (0.445 mmol, 1.0 equiv.; 3.0:1.0 diastereomeric mixture) were dissolved in 4.5 mL THF (0.1 M), cooled to 0 °C and treated subsequently with 0.71 mL H_2O_2 (30%; 6.23 mmol, 14.0 equiv.) and 131 mg $LiOH•H_2O$ (3.12 mmol, 7.0 equiv.). The instantly formed yellowish-orange solution was warmed gradually to rt and quenched after 16h reaction time with 1N HCl, extracted with EtOAc (4x), the combined organic extract was washed with Na_2SO_3 solution, dried with Na_2SO_4 and evaporated to dryness. Flash chromatography (hexane/EtOAc 10:1, 5:1 [P], 2:1 [P] provided 120.1 mg pure product **C** (76%, d.r. 7.6:1.0; 89% relative to major diastereomer). Additional fractions containing mainly the second diastereomer were not isolated.

MW 355.46 $C_{16}H_{25}NO_6Si$

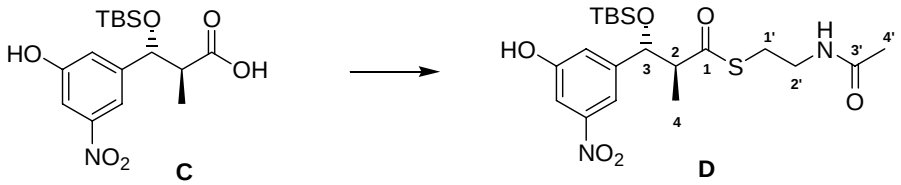
^{1}H-NMR 500 MHz d_6-acetone: 7.76-7.77 (m, 1H; Ar); 7.60 (t, *J* = 2.2-2.3 Hz, 1H; Ar); 7.32-7.33 (m, 1H; Ar); 4.96 (d, *J* = 8.4 Hz, 1H; H-3); 2.74 (dq, *J* = 8.4, 7.1 Hz, 1H; H-2); 0.92 (d, *J* = 7.1 Hz, 3H; H-4); 0.85 (s, 9H; TBS); 0.07 (s, 3H; TBS); -0.16 (s, 3H; TBS).

^{13}C-NMR 125 MHz d_6-acetone: 174.65 (C_q; C-1); 158.03 (C_q; Ar); 149.12 (C_q; Ar); 146.09 (C_q; Ar); 120.44 (CH; Ar); 112.96 (CH; Ar); 109.20 (CH; Ar); 76.24 (CH; C-3); 48.54 (CH; C-2); 25.19 (CH_3: TBS); 17.75 (C_q; TBS); 12.85 (CH_3; C-4); -5.37 (CH_3; TBS); -5.95 (CH_3; TBS).

ESI-MS 380.3 (10); 379.4 (35); 378.4 (M + Na; 100); 356.4 (M + H; 8).

HR-MS $C_{16}H_{25}NO_6NaSi = M + Na$

calculated: 378.1349 found: 378.1357 [+ 2.2 ppm]



120 mg of the carboxylic acid **C** (0.338 mmol, 1.0 equiv.) and 115 mg CDI (0.71 mmol, 2.1 equiv.) were dissolved in 6.8 mL dry DMF (0.05 M) and were stirred at rt for 3h followed by administration of 144 µL HSNAC (1.35 mmol, 4.0 equiv.). After 1h 46 mg DMAP (0.37 mmol, 1.0 equiv.) were added and stirring was continued for 16h upon which additional 72 µL HSNAC (0.68 mmol, 2.0 equiv.) were added and stirring was continued for 24h (TLC still showed some starting material). The reaction mixture was quenched with 0.5 N HCl and extracted with EtOAc (4x). The combined organic extracts were thoroughly washed with brine (2x) and dried with Na_2SO_4. Flash column chromatography ($CuSO_4$-impregnated silica on top of a regular silica gel column; hexane/EtOAc 5:1 [SM], 1:1, EtOAc pure [P], $CHCl_3$/MeOH 10:1) yielded 24.4 mg recovered starting material (20%) and 102 mg of the SNAC ester **D** (66%, d.r. 6.6:1.0; 79% based on recovered starting material) as a yellowish solid.

MW 456.63 $C_{20}H_{32}N_2O_6SiS$

^{1}H-NMR 9.80 (br, 1H; OH); 7.76 (t, *J* = 1.5-1.8 Hz, 1H; Ar); 7.59 (t, *J* = 3.2-3.3 Hz, 1H; Ar); 7.49 (br, 1H; N*H*Ac); 7.31 (dd, *J* = 1.6, 2.0 Hz, 1H; Ar); 4.99 (d, *J* = 8.3 Hz, 1H; H-3); 3.32-3.45 (m, 2H; H-2'); 2.94-3.12 (m, 3H; H-2 + H-1'); 1.91 (s, 3H; H-4'); 0.92 (d, *J* = 7.1 Hz, 3H; H-4); 0.85 (s, 9H; TBS); 0.04 (s, 3H; TBS); -0.20 (s, 3H; TBS).

500 MHz, d_6-acetone

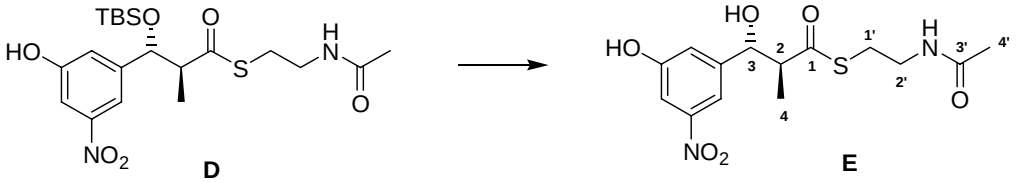
^{13}C-NMR 200.82 (C_q; C-1); 170.22 (C_q; C-3'); 158.48 (C_q; Ar); 149.40 (C_q; Ar) ; 146.00 (C_q; Ar); 120.53 (CH; Ar); 112.96 (CH; Ar); 109.61 (CH; Ar); 76.32 (CH; C-3); 57.04 (CH; C-2); 39.06 (CH_2; C-2'); 28.58 (CH_2: C-1'); 25.42 (CH_3: TBS); 22.25 (CH_3; C-4'); 17.99 (C_q; TBS); 13.94 (CH_3; C-4); -5.13 (CH_3; TBS); -5.80 (CH_3; TBS).

125 MHz, d_6-acetone

ESI-MS 935.7 (2M + Na; 20); 481.5 (20); 480.4 (35); 479.4 (M +Na; 100); 457.4 (M + H; 10).

HR-MS $C_{20}H_{32}N_2O_6SiS = [M + Na]^+$

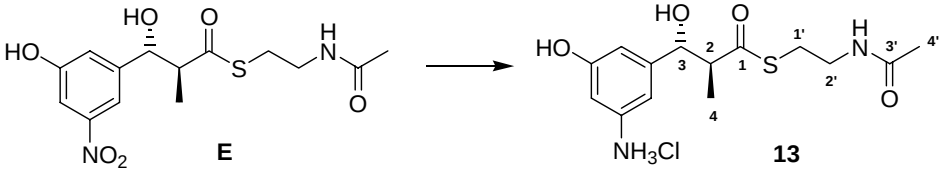
calculated: 479.1648 found: 479.1634 [- 2.9 ppm].



105 mg of the TBS-protected SNAC ester **D** (0.230 mmol, 1.0 equiv.) were dissolved in 4.6 mL acetonitril (0.05 M) and treated at 0 °C with 1.9 mL HF (30%). The mixture was gradually warmed to rt and stirring was continued until TLC analysis showed complete turnover (44h). The mixture was quenched with pH 7.0 phosphate buffer and brine, extracted with EtOAc (4x) and dried with Na_2SO_4. Flash column chromatography (hexane/EtOAc 1:1, EtOAc pure, $CHCl_3$/MeOH 10:1 [P]) yielded 75.9 mg of the free alcohol **E** (96%) as a white solid.

MW 342.37 $C_{14}H_{18}N_2O_6S$

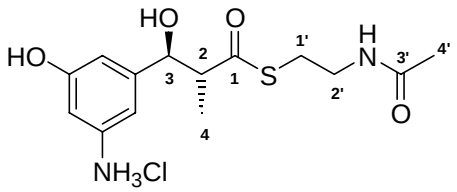
^{1}H-NMR	7.76 (t, *J* = 1.6-1.7 Hz, 1H; Ar); 7.56 (t, *J* = 2.2-2.3 Hz, 1 H; Ar); 7.50 (br, 1H, N*H*Ac);
500 MHz	7.28 (dd, *J* = 2.1, 1.5 Hz, 1H, Ar); 4.97 (d, *J* = 8.1 Hz, 1H, H-3); 3.38 (q, *J* = 6.1-6.6
d_6-acetone	Hz, 2H; H-2'); 2.95-3.05 (m, 3H, H-1' + H-4); 1.91 (s. 3H; H-4'); 0.96 (d, *J* = 7.1 Hz).
^{13}C-NMR	200.78 (C_q; C-1); 170.40 (C_q; C-3'); 158.16 (C_q; Ar); 149.17 (C_q; Ar) ; 146.16 (C_q;
125 MHz	Ar); 120.29 (CH; Ar); 112.57 (CH; Ar); 109.09 (CH; Ar); 74.65 (CH; C-3); 55.71
d_6-acetone	(CH; C-2); 38.75 (CH_2; C-2'); 28.34 (CH_2: C-1'); 22.00 (CH_3; C-4'); 13.66 (CH_3; C-4).
ESI-MS	707.2 (2M + Na; 25); 366.3 (20); 365.3 (M + Na;100).
HR-MS	$C_{14}H_{18}N_2O_6NaS = [M+Na]^+$
	calculated: 365.0783 found: 365.0768 [- 4.2 ppm].



72.9 mg of the nitro-SNAC ester **E** (0.213 mmol) were dissolved in 6.6 mL ethanol. This solution was added to 145 mg Pd/C (10%), followed by addition of 0.66 mL 1 N HCl. The air in the reaction flask was replaced by hydrogen and the slurry was vigorously stirred for 2 ½ h and then filtered through a plug of celite. The celite was washed with additional ethanol. The ethanol solution was concentrated, toluene was added to the concentrated solution and evaporation was continued to dryness yielding the hydrochloride **13** as a white-brownish solid in quantitative yield.

	MW 348.85 $C_{14}H_{21}N_2O_4SCl$
^{1}H-NMR	6.89 (br. s, 1H; Ar); 6.86 (br. s, 1H; Ar); 6.78 (br. s, 1H; Ar); 4.76 (d, *J* = 8.7 Hz, 1H;
500 MHz	H-3); 3.36-3.42 (m, 2H; H-2'); 3.02-3.10 (m, 2H; H-1'); 2.90-2.98 (m, 1H; H-2); 1.99
CD_3OD	(s, 3H; H-4'); 0.93 (d, *J* = 6.8 Hz, 3H; H-4).
^{13}C-NMR	202.92 (C_q; C-1); 174.03 (C_q; C-3'); 160.14 (C_q ; Ar); 147.56 (C_q ; Ar); 132.70 (C_q ;
125 MHz	Ar); 115.56 (CH; Ar); 113.05 (CH; Ar); 110.46 (CH; Ar); 76.36 (CH; C-3); 56.84
CD_3OD	(CH; C-2); 40.36 (CH_2; C-2'); 28.98 (CH_2; C-1'); 22.29 (CH_3; C-4'); 15.21 (CH_3; C-4).
ESI-MS	347.3 (M - H; 100).
HR-MS	$C_{14}H_{20}N_2O_4NaS = [M - HCl + Na]^+$

calculated: 335.1041 found: 335.1028



12

	MW 348.85 $C_{14}H_{21}N_2O_4SCl$
^{1}H-NMR	6.87-6.89 (m, 1H; Ar); 6.83-6.84 (m, 1H; Ar); 6.74 (t, *J* = 2.1-2.2 Hz, 1H;
500 MHz	Ar); 4.76 (d, *J* = 8.7 Hz, 1H; H-3); 3.38 (dt, *J* = 6.8, 1.6-2.1 Hz,, 2H; H-2');
CD_3OD	3.05 (dt, *J* = 6.7, 1.1-1.7 Hz, 2H; H-1'); 2.93 (dq, J = 8.6, 7.1 Hz, 1H; H-2);
	1.94 (s, 3H; H-4'); 0.94 (d, *J* = 7.1 Hz, 3H; H-4).
ESI-MS (+)	336.4 (20); 335.3 (M - HCl + Na; 95); 295.4 (100).
ESI-MS (-)	349.3 (40); 347.4 (M - H; 100).
HR-MS	$C_{14}H_{20}N_2O_4SCl = [M - H]^-$
	calculated: 347.0832 found: 347.0825 [- 2.1 ppm].

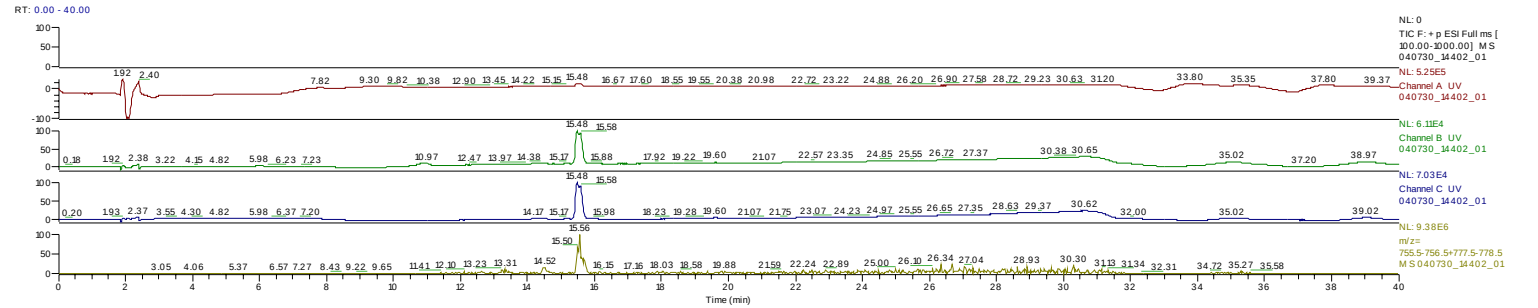
Typical Fermentation Procedure

Amycolatopsis mediterranei HGF003 was grown on 40 mL YMG agar plates at 30 °C for three days prior to substrate addition. The hydrochlorides **10-13** (d.r. for syn diastereomers > 15:1; d.r. for **12** 4.8:1.0; d.r. for **13** 6.0:1.0 based on nmr integration; synthesized from 99% ee commercial grade Evans oxazolidinone) were dissolved in 20% DMSO/water (15-20 mg substrate per plate, 1.5 mL volume per plate). After pH adjustment to 7.2-7.3, substrate solutions were administered to plates by sterile filtration. After seven days of additional growth, homogenization was followed by extraction with EtOAc (containing 1% AcOH). The combined extracts were dried over Na_2SO_4, filtered and evaporated to dryness. For HPLC and LCMS analysis the crude extraction residue was redissolved in AcCN/iPrOH 1:1 and filtered.

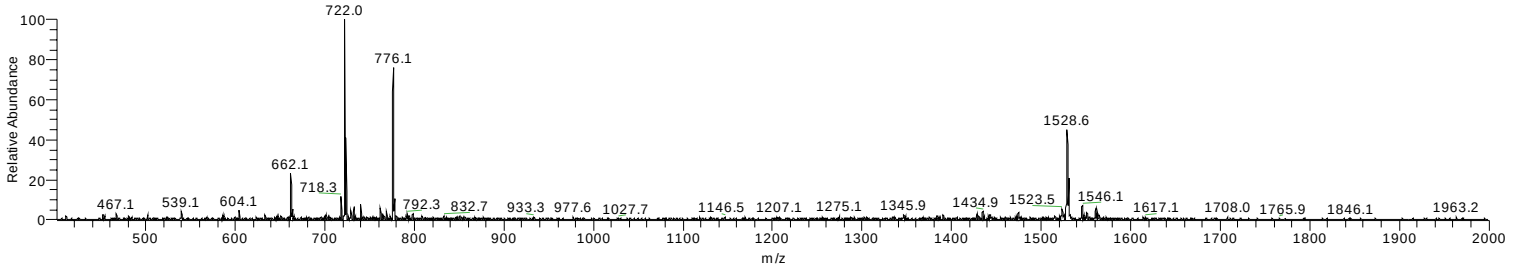
Representative LCMS Traces

Reference: Rifamycin B (commercial standard)

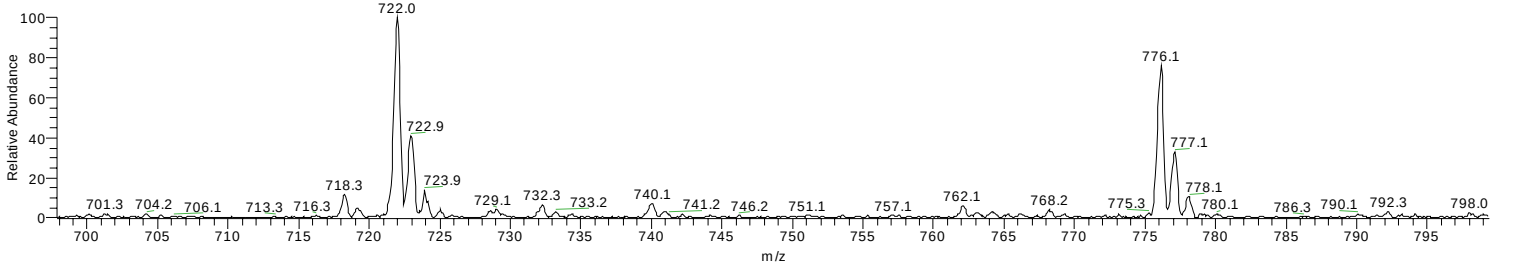
H:\Khosla\Hartung_Ingo\040730_14402_01 08/02/2004 02:08:27 PM IH Ref. RifB



040730_14402_01 #558-597 RT: 14.98-16.04 AV: 40 NL: 1.67E6
T: + p ESI Full ms [400.00-2000.00]

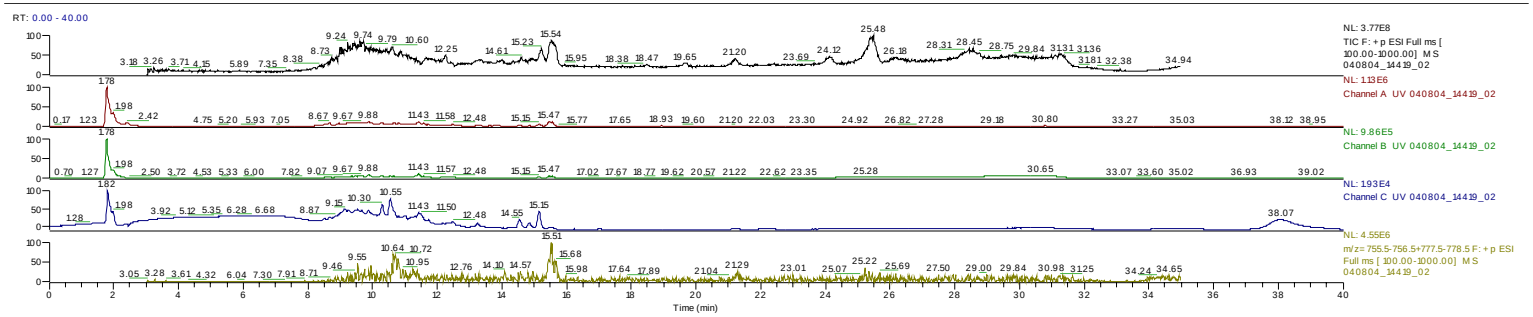


040730_14402_01 #557-595 RT: 14.96-15.99 AV: 39 NL: 1.71E6
T: + p ESI Full ms [400.00-2000.00]

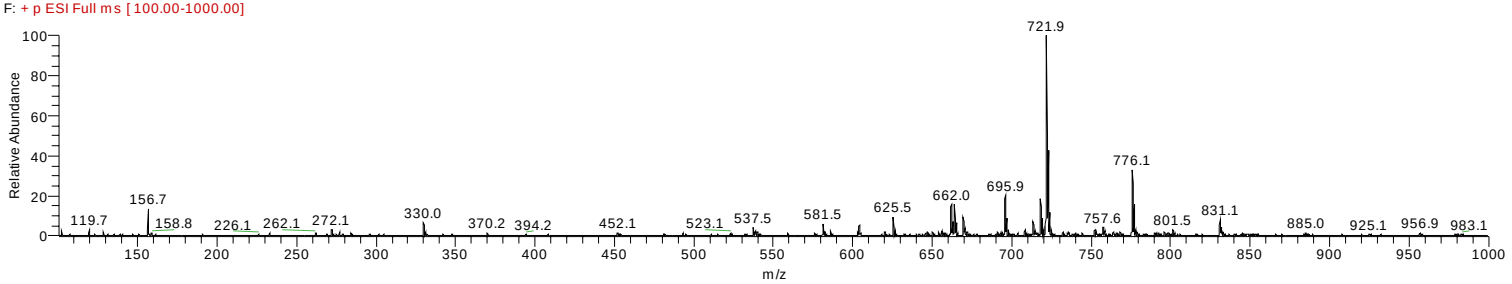


Positive Control: Supplementation with AHBA (10 mg per plate)

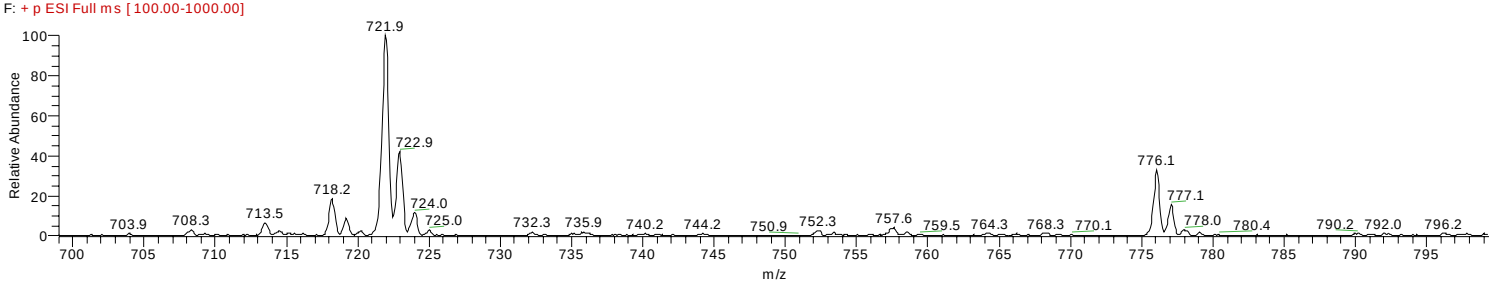
H:\Khosla\Hartung_Ingo\040804_14419_02 08/04/2004 07:05:42 PM IHB05-AHBA



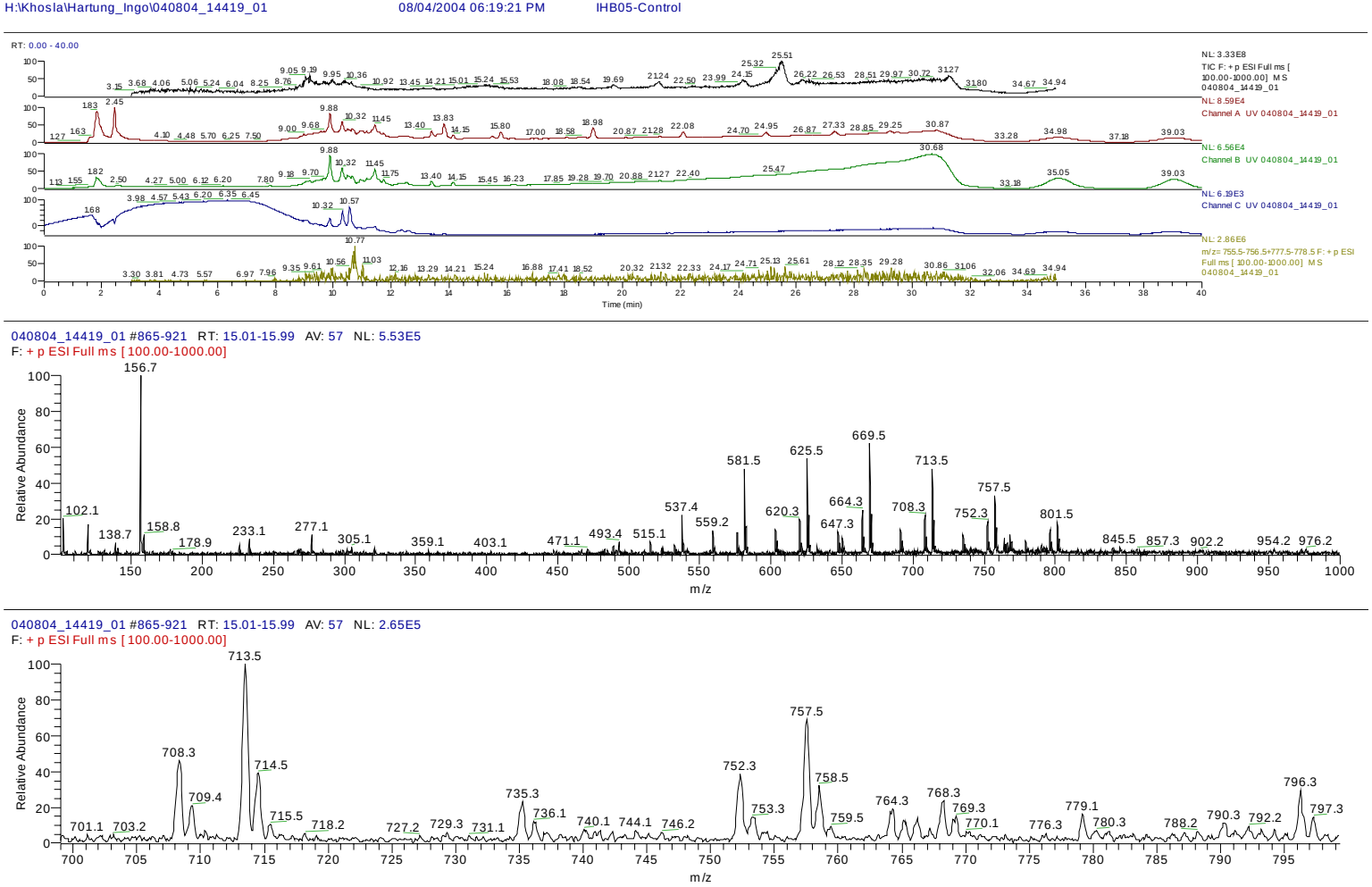
040804_14419_02 #860-925 RT: 14.94-16.07 AV: 66 NL: 2.71E6
F: + p ESI Full ms [100.00-1000.00]



040804_14419_02 #864-921 RT: 15.01-16.00 AV: 58 NL: 3.06E6
F: + p ESI Full ms [100.00-1000.00]



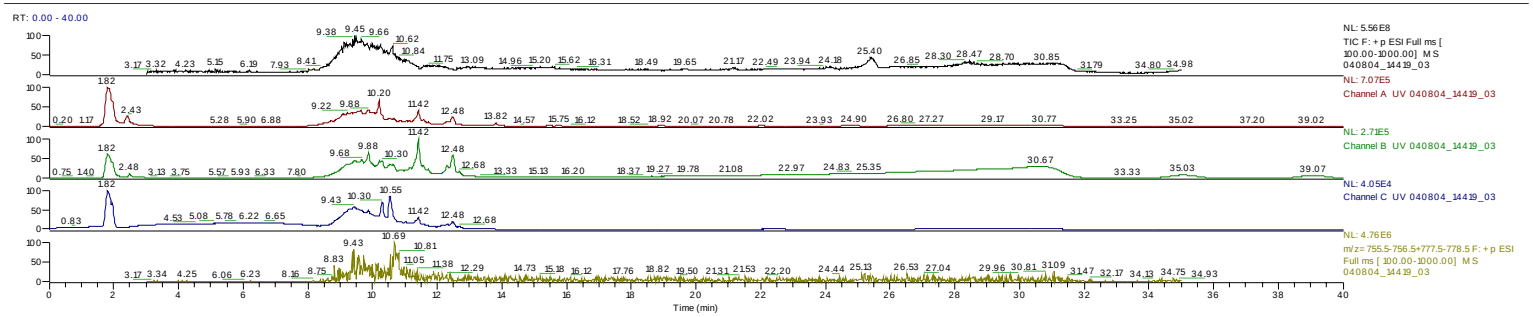
Negative Control: Mock Supplementation

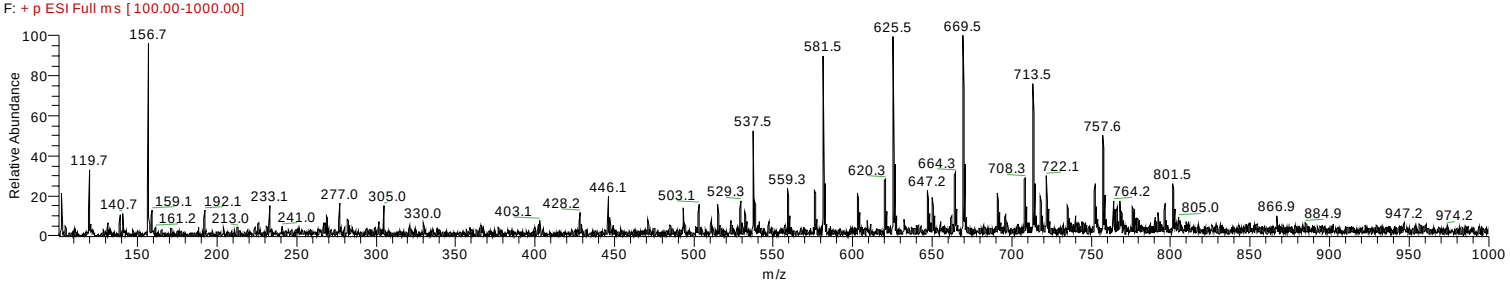


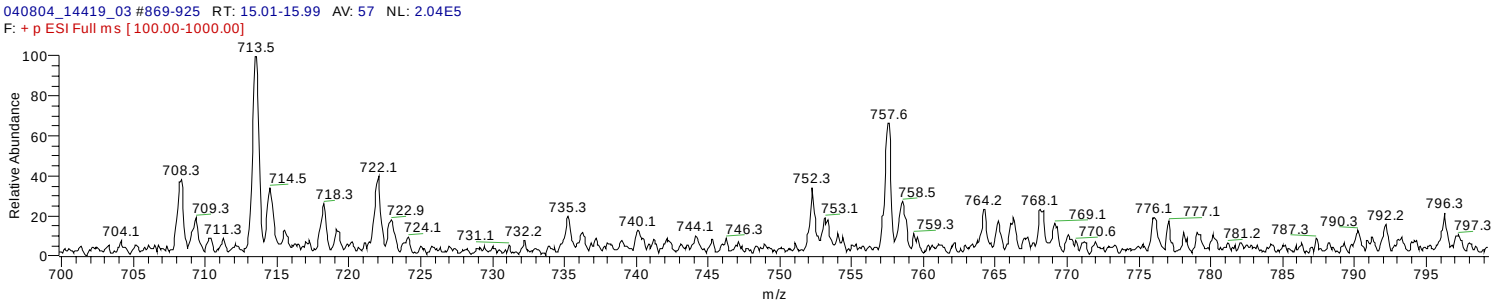
Ladder of Δ = 44 mass units results from PEG residue (filtration steps).

Supplementation with Substrate 12

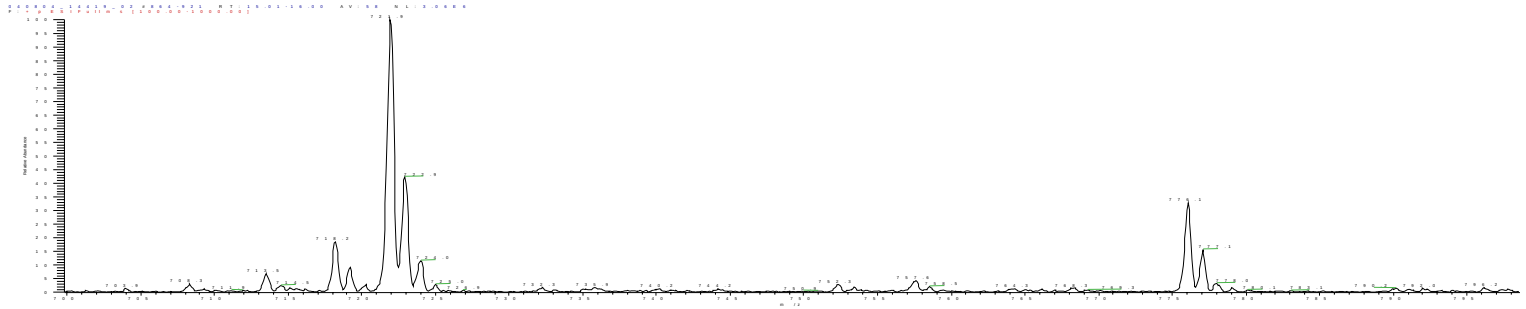
H:\Khosla\Hartung_Ingo\040804_14419_03 08/04/2004 07:52:00 PM IHB07IH190



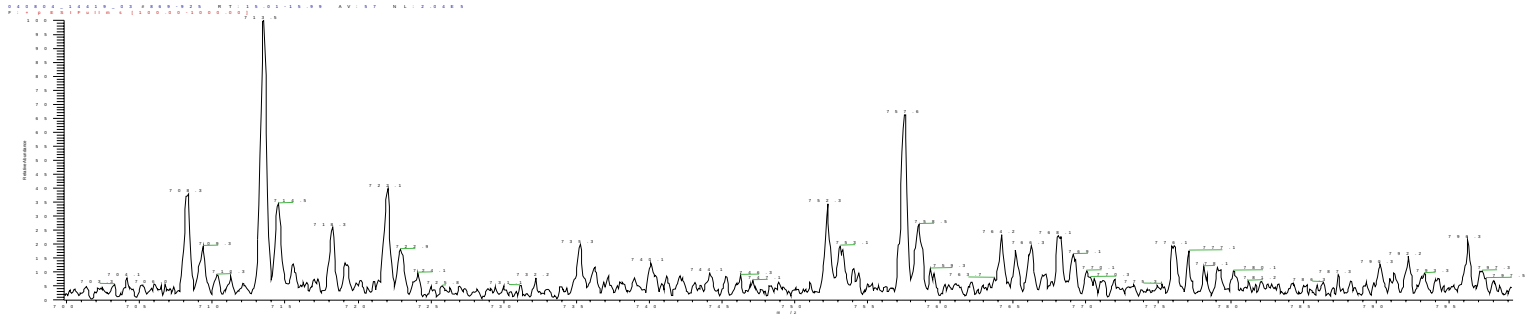




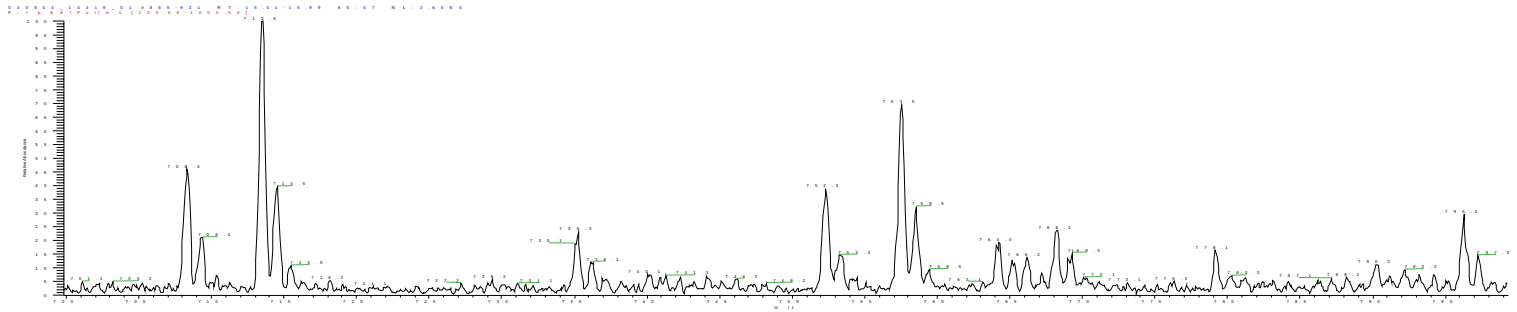
Supplementation with AHBA; 700-800 mass units



Supplementation with Substrate 12; 700-800 mass units



Mock Supplementation; 700-800 mass units



Prediction of the Stereoselectivity of the Ketoreductase Domain of RifM1

Reid Signature Sequence:

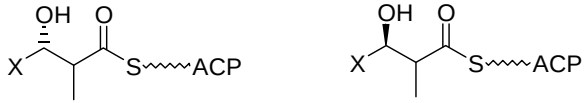
Reid, R.; Piagentini, M.; Rodriguez, E.; Ashley, G.; Viswanathan, N.; Carney, J.; Santi, D. V.; Hutchinson, C. R.; McDaniel, R. *Biochemistry* **2003**, *42*, 72.

Caffrey Signature Sequence:

Caffrey, P. *ChemBioChem* **2003**, *4*, 649.

Catalytic triad coloured in green, predictive residues in grey.

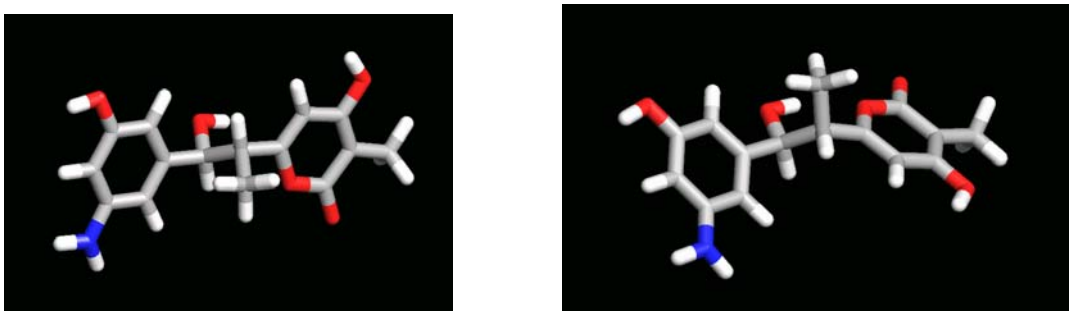
L-configured ***D-configured***



Amino Acid Position	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	113
Caffrey L																	
Caffrey D						**L**	**D**	**D**									
Reid L																	**K**
Reid D								**D**									**K**
Rif M1 KR	H	T	A	G	V	**L**	**D**	**D**	G	V	V	T	E	L	T	P	**K**
DEBS M1 KR (D)	H	A	A	A	T	**L**	**D**	**D**	G	T	V	D	T	L	T	G	**K**
DEBS M2 KR (L)	H	A	A	G	L	**P**	**Q**	**Q**	V	A	I	N	D	M	D	E	**K**

	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153
Caffrey L								**W**												
Caffrey D											**P**				**N**					
Reid L			**S**													**Y**				**N**
Reid D			**S**													**Y**				**N**
Rif KR1	F	S	**S**	A	A	G	V	**L**	G	N	**P**	G	Q	A	**G**	**Y**	A	A	A	**N**
DEBS KR1	F	S	**S**	F	A	S	A	**F**	G	A	**P**	G	L	G	**G**	**Y**	A	P	G	**N**
DEBS KR2	F	S	**S**	G	A	G	V	**W**	G	S	**A**	R	Q	G	**A**	**Y**	A	A	G	**N**

Energetically minimized conformations of P8/1-OG lactones



***Anti*-2** (left) and ***syn*-2** (right) using MacroModel 7.2 (MMFFs force field, solvent model water). The dihedral angle between protons H6 and H7 were deduced to be 173.86° for ***anti*-2** and 76.76° for ***syn*-2**.

Construction of RM2 Expression Vector

PCR was performed to clone the RM2 gene fragment from the *RifA* gene using the primers N-terminal primer 5'-GATCGTCGCGATGGCGTGCC-3' and the C-terminal primer 5'-CCGGCCGTCCTCGCGGACCAACTAGT-3' where a SpeI site (underlined in the primer sequence) was added to the end of the gene fragment. The PCR amplified fragment was then ligated into a linearized pCR-Blunt vector (Invitrogen) producing pFL457. The 2.9 kbp BsaBI-SpeI fragment was ligated into pST164 to yield pKW106. pST164 is a pUC19 vector that contains the 6-deoxyerythronolide B N-terminal linker region of eryM3 (M3N) flanked with a 5'-NdeI site and a 3'-BsaBI site and the 6-deoxyerythronolide B C-terminal linker region of eryM2 (M2C) flanked with a 5'-SpeI site and a 3'-EcoRI site[1]. The two linker regions borrowed from the 6-deoxyerthryonolide system aided in protein expression (data not shown). Next the 3.2 kbp NdeI-EcoRI fragment was ligated into pET28b(+) (Novagen) to yield pKW184. pET28 provided RM2 with both N-terminal and C-terminal poly-His tags.

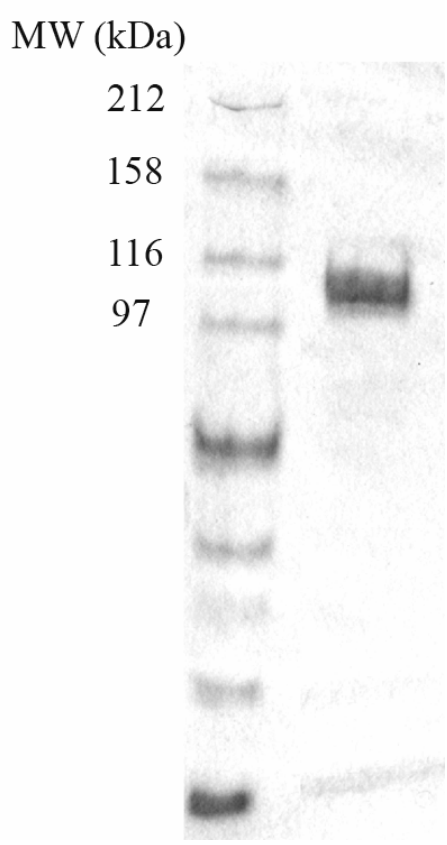
RM2 Expression and Purification

Escherchia coli BL21 (DE3) (Novagen) was used as a host to express the RM2 construct. A single transformant was used to start 25 ml of LB media cultures with kanamycin (50 mg/L) at 37°C and 200 rpm. The starter culture was grown overnight and used to inoculate 5L of LB medium containing the same concentration of kanamycin. The culture was grown at 37°C and 200 rpm until an OD of 0.6-0.8 at which point the cultures were placed on ice for 15 minutes.

[1] (a) Tsuji, S.Y.; Cane, D.E.; Khosla, C. *Biochemistry* **2001,** 40, 2326. (b) Wu, N.; Tsuji, S.Y.; Cane, D.E.; Khosla, C. *J. Am. Chem. Soc.* **2001**, 123, 6465. (c) Wu, N.; Cane, D.E.; Khosla, C. *Biochemistry* **2002**, 41, 5056.

Next the cultures were induced with 100 μM of ispopropyl-β-D-thiogalactopyranoside and incubated at 13°C and 200 rpm for 20 hrs.

All purification procedures were performed on ice or at 4°C. RM2 was isolated by first spinning the cell culture at 2,500 x g for 20 min and resuspending the cell pellet in disruption buffer (200 mM sodium phosphate, pH 7.2/200 mM sodium chloride/0.2 mM DTT/1.5 mM benzamine/2 mg/L pepstatin/ 2mg/L leupeptin/30% glycerol). Resuspended cells were lysed using sonication and then clarified at 40,000 x g for 60 min. The lysate was then equilibrated with 5 ml of Ni-NTA resin (Qiagen) for 1 hr. The resin was first rinsed with 10 mM imidazole in a pH 7.2 100 mM sodium phosphate buffer, and then the protein was eluted off the resin with 200 mM imidazole in the same buffer. Additional purification was carried out using anion-exchange chromatography. The protein solution was loaded onto a HiTrap Q 5 ml column (Amersham). A gradient of 0-1 M sodium chloride in 100 mM sodium phosphate (pH 7.2), 2.0 mM DTT, and 10% vol glycerol was run at 3 ml/min for 15 column volumes. Three ml fractions were collected, and those fractions containing RM2 were pooled, concentrated, and buffer exchanged into 100 mM sodium phosphate (pH 7.2), 2.0 mM DTT, 20% vol glycerol using an Amcion Ultra protein concentrator (Waters). Purified protein was analyzed on a 7.5% polyacrylamide gel (Bio-Rad) stained with Coomassie brilliant blue stain. This protein purification yielded 1 mg/L of purified RM2.



Lane 1 contains a protein molecular weight marker and lane 2 contains purified RM2 with an expected molecular weight of 110 kDa

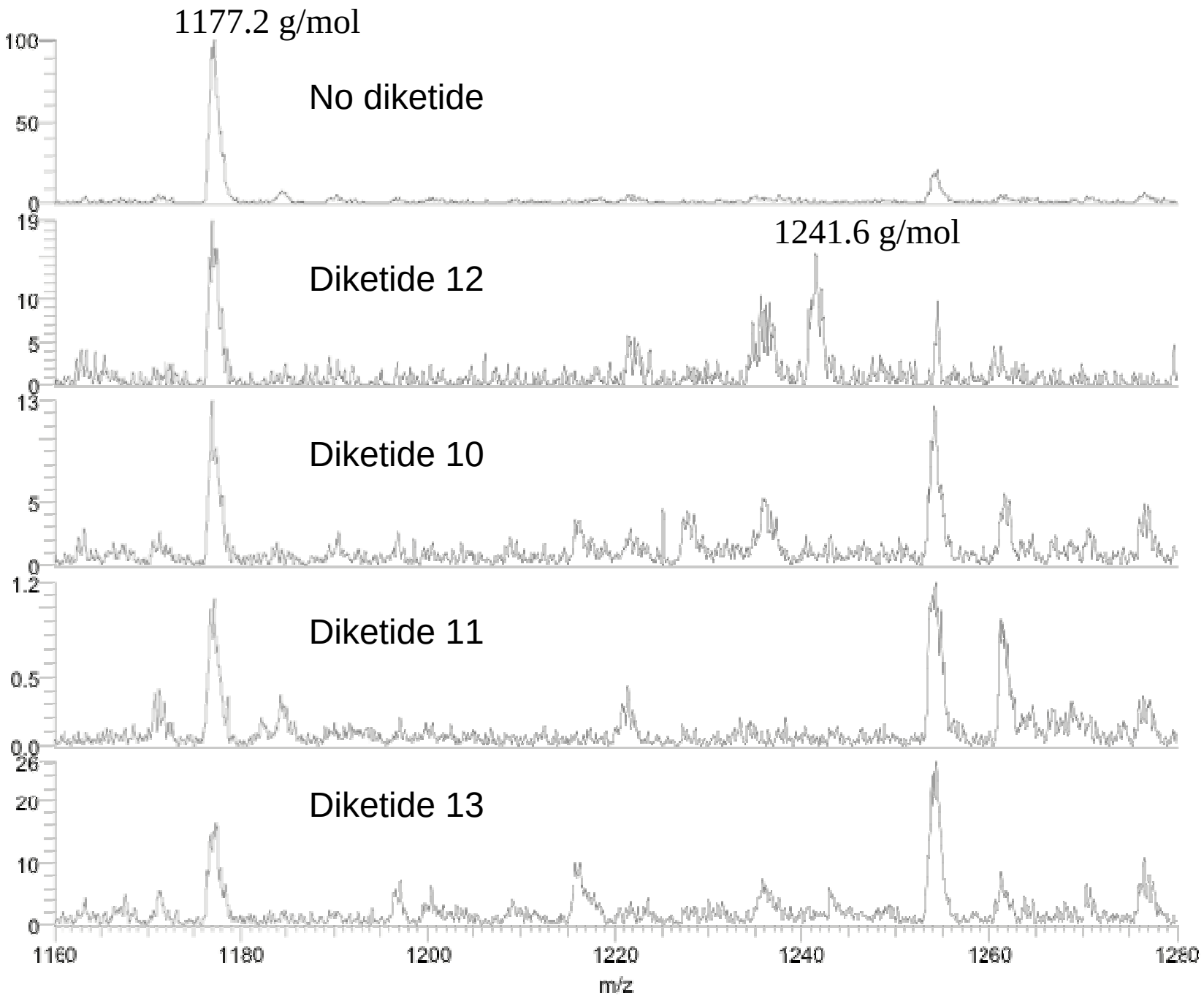
RM2 *in vitro* Diketide Labelling and LC/MS Analysis

To determine if the KS domain of RM2 provides a selectivity barrier to diketide incorporation, purified RM2 was incubated with individual diketides **10-13**, digested with trypsin, and the digest was analyzed using LC/MS to see if the KS active site cysteine was covalently bound to any of the diketides.

Trypsin proteoloysis of RM2. The incubation reaction was carried out with 100 mM sodium phosphate (pH 7.2), 12.5 μM Rm2, and 10 mM of **10**, **11**, **12**, **13**, or no diketide for 60 minutes at room temperature at a 50 μl scale. Next, 1.25 μl of a 100 μM trypsin solution was added to each diketide reaction and incubated at 30°C for 60 min. The reactions were quenched with 50 μl of a 10% formic acid solution.

LC-MS analysis. 50 μl of each quenched reaction were analyzed using a Surveyor HPLC system (ThermoFinnigan) equipped with a Vydac C18 reverse phase polymer column. Mass spectra were collected on an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometer equipped with an electrospray ion source operating in positive ion mode. The sheath gas was set to 60 (arbitrary units), spray voltage to 4.5 kV, and capillary temperature to 200°C. A linear gradient between buffer A (water, 0.1% formic acid) and buffer B (acetonitrile, 0.1% formic acid) from 5% to 95% over 60 minutes at a flow rate of 0.2 ml/min was used.

LC-MS results. The expected molecular mass of the trypsin digest fragment containing the active site cysteine is 3527.8 g/mol. The m/2 mass is 1763.0 g/mol (data not shown) and the m/3 mass is 1175.9 g/mol (see figure below). The m/3 peak was significantly larger then the m/2 peak. Diketides **10-13** have the same molecular mass and when bound to the KS active site cysteine yield a trypsin digest fragment with a mass of 3721 g/mol. The m/3 mass of this fragment is 1240.3 g/mol. In the reaction with no diketide a peptide eluted at 22.3-22.5 min with a mass of 1177.2 g/mol and 1765.3 g/mol corresponding to the m/2 and m/3 mass of the peptide fragment with the active site cysteine. The 1177.2 g/mol peak was observed in all the reactions. The reaction with diketide **12** was the only reaction to produce a fragment at 1241.6 g/mol corresponding to the mass of the diketide covalently bound to the active site cysteine fragment. This result demonstrates that the stereochemistry of **12** is the only stereochemistry accepted by the RM2 KS domain at a measurable rate.


1177.2 g/mol
No diketide
1241.6 g/mol
Diketide 12
Diketide 10
Diketide 11
Diketide 13
100
50
0
15
10
5
0
13
5
0
1.2
0.5
0.0
25
20
10
0
1160
1180
1200
1220
1240
1260
1280
m/z