Structure-activity relationship study of splicing modulators on Hsh155/SF3B1 through chemical synthesis and yeast genetics

Jacob P. Beard[1], Sierra L. Love[2,3], John C. Schmitz[4,5], Aaron A. Hoskins[2,6], and Kazunori Koide[1,5]*

[1]Department of Chemistry, University of Pittsburgh

219 Parkman Avenue, Pittsburgh, Pennsylvania 15260, United States

[2]Department of Biochemistry, University of Wisconsin-Madison

433 Babcock Drive, Madison, Wisconsin 53706, United States

[3]Genetics Training Program, University of Wisconsin-Madison

425 Henry Mall, Madison, Wisconsin 53706, United States

[4]Division of Hematology-Oncology, Department of Medicine, University of Pittsburgh School of Medicine

5150 Centre Avenue, Pittsburgh, Pennsylvania 15232, United States

[5]Cancer Therapeutics Program, UPMC Hillman Cancer Center

5117 Centre Ave, Pittsburgh, Pennsylvania 15232, United States

[6]Department of Chemistry, University of Wisconsin-Madison

1101 University Avenue, Madison, Wisconsin 53706, United States

Abstract

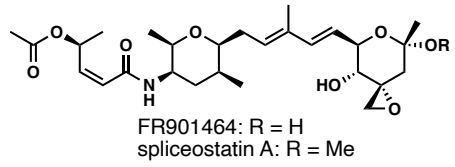
Meayamycins are synthetic analogs of the natural product FR901464 and exhibit potent anticancer activity against human cancers. They bind SF3B1 and PHF5A, components of the human spliceosome, and alter pre-mRNA splicing. Detailed analysis of the active site led us to investigate a narrow pocket within the binding site which surrounds the α,β-unsaturated amide portion of meayamycin. We describe the synthesis and biological activity of two new analogs bearing a methyl substituent on the α or β position of the amide. With these analogs, we investigated the discrete interactions within the narrow region of SF3B1 using a human/yeast chimeric SF3B1 protein and found the V1078 residue of SF3B1 affects compound binding at the amide moiety.

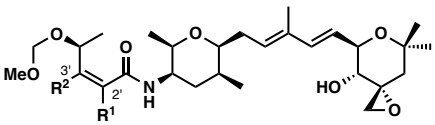
Introduction

FR901464 was isolated from *Burkholderia* sp. FERM BP-3421 as an anticancer agent with half-maximal growth inhibition (GI_{50}) values of 1–2 nM against human cancer cells.[1-4] The molecule binds to human splicing factor 3B subunit 1 (SF3B1), a component of the human spliceosome, to inhibit precursor mRNA splicing.[5] Pladienolide B,[6-8] herboxidiene,[9-13] and other similar natural products[14, 15] were also discovered from natural sources and found to bind to SF3B1 and inhibit splicing.[16, 17] The isolations and biological activities of these natural products have sparked a broad interest in the development of therapeutically useful pre-mRNA splicing inhibitors. FR901464 and closely related analogs have been synthesized and biologically evaluated by many groups.[18-27] The Kitahara group synthetically prepared spliceostatin A

(SSA), a more stable 1-methoxy derivative of FR901464 (Figure 1).[20] After the initial discovery of SF3B1 as the relevant target of FR901464, the Pena group reported the cryo-EM structure of an SSA-bound SF3B complex, a large protein assembly that contains SF3B1 and other SF3B subunits. This structure revealed how the majority of the SSA molecule is bound by SF3B1 near the protein's interface with another SF3B subunit, plant homeodomain-finger domain 5A (PHF5A).[28] The structure showed that SSA forms a covalent adduct between the epoxide of SSA and C26 of PHF5A.

Structure-activity relationship (SAR) studies of FR901464 have primarily focused on the two tetrahydropyran rings, diene moiety, and the C4' position.[18, 25-27, 29-37] In the cryo-EM structure the SSA-SF3B1 complex, the enamide occupied the narrow neck region of the protein pocket (Figure 2). To gain additional insights, we decided to study the SAR around the C2' and C3' positions of FR901464 with our more metabolically stable analog, meayamycin D.[38] The region between L1066 and V1078 residues of human SF3B1 appear to interact with the C2' and C3' positions of SSA. We previously compared the *Z*-enamide (naturally occurring) to the *E*-enamide and the C2'-C3' saturated equivalent.[39] The latter two compounds were found to be significantly less potent, indicating a possible steric constraint on the C3' position as well as preference for a rigid C2'-C3' bond.





	R^1	R^2
meayamycin D (MAMD)	H	H
2'-Me-MAMD	Me	H
3'-Me-MAMD	H	Me

Figure 1. Structures of FR901464 (natural product) and synthetic analogs.

Herein, we report the synthesis and biological evaluation of two new analogs with an additional methyl group at the C2' or C3' position. The analogs were evaluated for their in vivo cytotoxicity, splicing activity, and in vitro plasma stability. We reveal that the C3' substitution is tolerated and leads to a compound with comparable activity to meayamycin D. Lastly, we introduced several mutations into the SF3B1 binding site to understand the discrete interactions at the C2' and C3' site that may be required for compound activity.

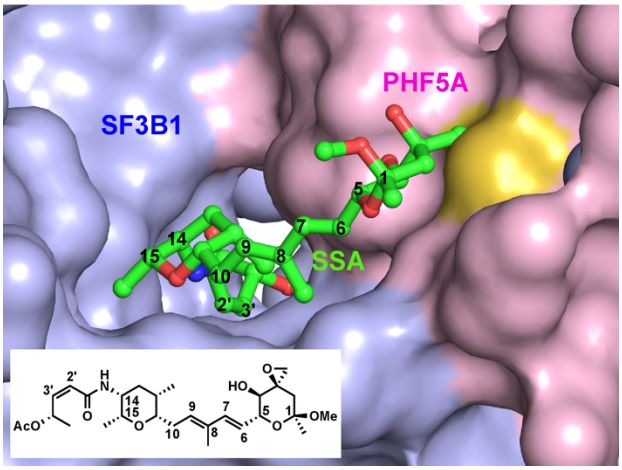
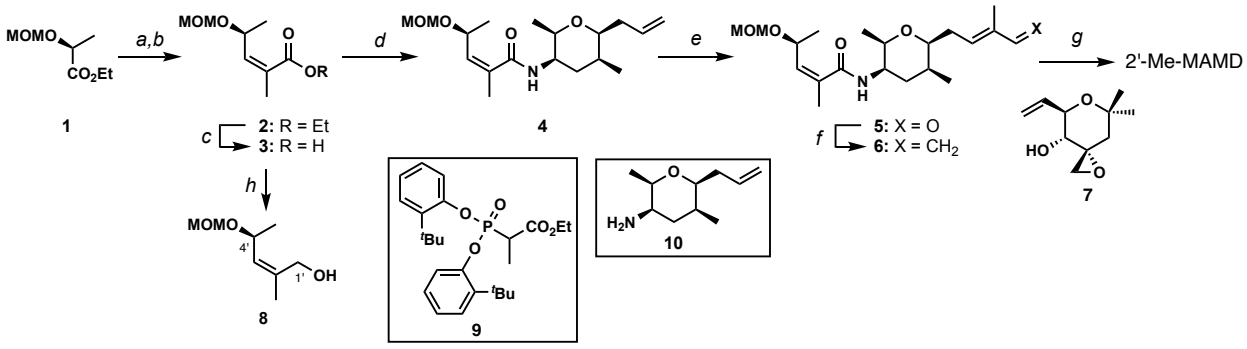


Figure 2. SF3B1 binding pocket occupied by SSA (PDB: 7B9C).

Results and Discussion

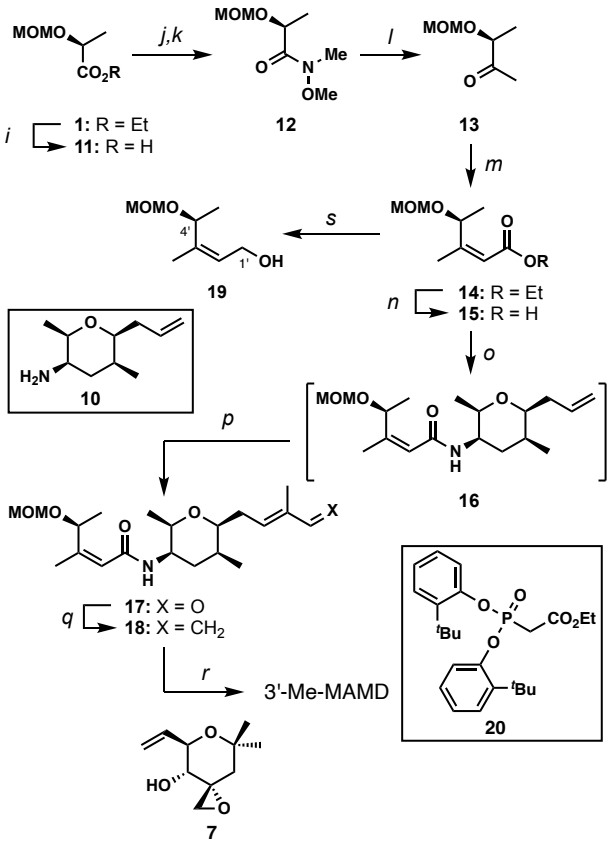
The synthesis of 2'-Me meayamycin D began with **1**, which was prepared in a single step from commercially available ethyl-(*S*)-lactate (Scheme 1).[38] Sequentially, lactate **1** was reduced with diisobutylaluminum hydride (DIBALH), and the in-situ-generated aldehyde was submitted to an Ando-Horner-Wadsworth-Emmons reaction[40] with phosphonate **9**[41] to yield the α-methylated enoate **2** in 52% yield, with an E/Z ratio of 6:94. Enoate **2** was hydrolyzed to acid **3** in quantitative yield. To confirm the correct configuration, we reduced enoate **2** using DIBALH to the allylic alcohol **8** in 68% yield. One-dimensional nuclear Overhauser effect (NOE) signals were detected for the C1' and C4' positions (Figure S1), confirming the Z-olefin geometry. Acid **3** was coupled with amine **10**[42] to afford amide **4** as an inseparable mixture of isomers. The desired compound could be purified after olefin cross-metathesis with methacrolein, using nitro-Grela catalyst, to give aldehyde **5** in 60% yield. Wittig olefination of aldehyde **5** with $Ph_3P{=}CH_2$ gave diene **6** in 78% yield. Finally, this diene was united with fragment **7** to afford 2'-Me meayamycin D in 8% yield.



Scheme 1. Synthesis of 2'-Me meayamycin D. Conditions: (a) diisobutylaluminum hydride (DIBALH), dichloromethane (DCM), -78 °C, 2 h; then (b) **9**, KO^{t}Bu, tetrahydrofuran (THF), -78 °C to rt, 20 h, 52% (E:Z = 6:94); (c) NaOH, MeOH, 0 °C to rt, 16 h,

quant.; (d) **10**, HATU, diisopropylethylamine, DCM, 0 °C to rt, 42 h, inseparable mixture; (e) methacrolein, nitro-Grela catalyst, 50 °C, 20 h, 39% for 2 steps; (f) Ph_3PCH_3Br, KO^{t}Bu, THF, 0 °C to rt, 18 h, 78%; (g) **7**, nitro-Grela catalyst, dichloroethane (DCE), 50 °C, 8 h, 8%; (h) DIBALH, THF, -78 °C, 1.5 h, 68%.

The synthesis of 3'-Me meayamycin D started with the hydrolysis of **1** to acid **11** in 84% yield (Scheme 2). Acid **11** was treated with trimethylacetyl chloride followed by *N,O*-dimethylhydroxylamine to give Weinreb amide **12** in 83% yield. Attempts to directly convert ester **1** to amide **12** failed. Grignard addition of in-situ-generated MeMgI to amide **12** gave ketone **13** in 88% yield, which was directly subjected to an Ando-Horner-Wadsworth-Emmons olefination with phosphonate **20** in the presence of KO^{t}Bu to afford the β-methylated enoate **14** in 50% yield, with an E/Z ratio of 15:85. The olefin geometry of enoate **14** was confirmed using the same method as enoate **2**. Reduction of enoate **14** using DIBALH gave allylic alcohol **19** in 79% yield. Correlating NOE signals were observed between C1' and C4', indicating a Z-olefin geometry (Figure S2). Next, enoate **14** was hydrolyzed to acid **15** quantitatively, which was coupled with amine fragment **10** to afford amide **16** as an inseparable mixture of isomers. In a similar fashion, the desired compound was separated after olefin cross-metathesis with methacrolein, using nitro-Grela catalyst, to afford aldehyde **17** in 32% yield over two steps. Wittig olefination of aldehyde **17** with $Ph_3P{=}CH_2$ gave diene **18** in 82% yield. Cross olefin metathesis of diene **18** with the right fragment **7** gave 3'-Me meayamycin D in 8% yield.



Scheme 2. Synthesis of 3'-Me meayamycin D. Conditions: (i) LiOH, MeOH, H_2O, 3 h, 0 °C, 84%; (j) trimethylacetyl chloride, triethylamine, DCM, 0 °C, 1.5 h; then (k) *N*,*O*-dimethylhydroxylamine hydrochloride, triethylamine, DCM, 0 °C to rt, 20 h, 83%; (l) Mg, MeI, Et_2O, 0 °C, 2.5 h, 88%; (m) **20**, KO^{t}Bu, THF, 0 °C to rt, 18 h, 50% (E:Z = 15:85); (n) NaOH, MeOH, 0 °C, 3.5 h, quant.; (o) **10**, HATU, diisopropylamine, DCM, 0 °C to rt, 40 h, inseparable mixture; (p) methacrolein, nitro-Grela catalyst, 50 °C, 18 h, 32% for 2 steps; (q) Ph_3PCH_3Br, KO^{t}Bu, THF, 0 °C, 1.5 h, 82%; (r) **7**, nitro-Grela catalyst, DCE, 45 °C, 14 h, 8%; (s) DIBALH, THF, -78 °C, 2 h, 79%.

With 2'-Me meayamycin D and 3'-Me meayamycin D in hand, we evaluated the cytotoxicity of the compounds in several human cancer cell lines using meayamycin A and meayamycin D as a comparison (Table 1 and Figure S3). 2'-Me meayamycin D was approximately sixty-fold less potent than meayamycin D. During this work, the Arisawa group reported the synthesis and activity of a similar 2'-methylpentenamide derivative.[43] In their study, they reported that the 2'-methylpentenamide derivative gave less inhibitory activity against androgen receptor splice variant 7 (AR-V7) expression, as compared to SSA. As mentioned above, the crystallographic data suggest that this position lies in a relatively narrow space within the protein binding site. Given this, it is possible that the lower cytotoxicity is due to steric clash between the C2'-methyl and the protein binding pocket of SF3B1. 3'-Me meayamycin D, however, is comparable to meayamycin D (GI_{50} = 2.0–3.9 nM). This suggests that methylation at the C3' position is tolerated while methylation at the C2' position results in a significant loss in activity.

	GI_{50} (nM)			
Cell lines	meayamycin A[a]	meayamycin D[a]	2'-Me meayamycin D	3'-Me meayamycin D
HCT116	0.7 ± 0.1	2.0 ± 0.3	129 ± 14	4.8 ± 0.9
SW48	0.9 ± 0.1	2.5 ± 0.4	127 ± 15	4.6 ± 0.7
A549	2.7 ± 0.9	3.9 ± 1.3	240 ± 48	7.2 ± 2.1
DMS53	0.5 ± 0.1	2.7 ± 0.6	169 ± 23	5.9 ± 1.0
DMS114	0.4 ± 0.1	2.3 ± 0.6	153 ± 20	5.5 ± 1.3

Table 1. Cytotoxicity of meayamycin A, meayamycin D, 2'-Me meayamycin D, and 3'-Me meayamycin D in various human cancer cell lines. [a]The data for meayamycin A and meayamycin D are the same as previously reported since these controls were tested in the same experiment as the previous report.[38]

We evaluated the ability of 2'-Me meayamycin D and 3'-Me meayamycin D to decrease the abundance of proteins whose expression is dependent on splicing of their respective pre-mRNAs (Figure 3). 3'-Me meayamycin D (GI_{50} = 5 nM) showed a comparable decrease in myeloid cell leukemia 1 (MCL-1) protein abundance to meayamycin D (GI_{50} = 2 nM), while 2'-Me meayamycin D showed only small changes in protein levels at concentrations up to 1 μM. We have previously demonstrated that the protein and mRNA levels of MCL-1 are correlated;[44, 45] therefore, these analogs likely directly alter the alternative splicing of *MCL-1*, leading to the observed changes in protein abundance. These results corroborate the cytotoxicity results and may serve as one explanation for the lower cytotoxicity of 2'-Me meayamycin D. Interestingly, we observed a nearly negligible increase in a proteoform of p27 generated by alternative splicing of the coding pre-mRNA, as compared to meayamycin D. All compounds also lead to a decrease in SF3B1 phosphorylation, consistent with disruption of the splicing process.[46] Next, we investigated the stability of 2'-Me meayamycin D and 3'-Me meayamycin D in mouse CD1 plasma (Figure S4). 3'-Me meayamycin D has comparable stability in plasma compared to meayamycin D ($t_{1/2}$ = 13 h)[38] with a half-life of 16 h. Meanwhile, 2'-Me meayamycin D has a higher half-life of 30 h, which may be attributed to steric shielding of the amide bond.

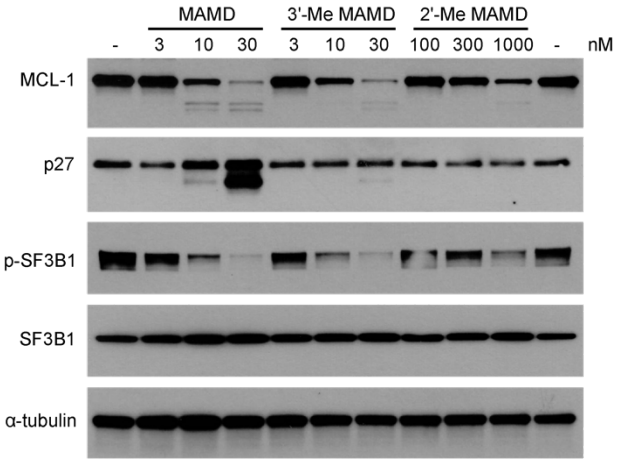


Figure 3. Western blot analysis of HCT116 cells treated with meayamycin D (MAMD), 3'-Me MAMD, and 2'-Me MAMD.

To better understand the binding pocket for meayamycin D on SF3B1, we analyzed the crystal structure[28] and identified four residues that are near the C2' and C3' methyl group: L1066, R1074, T1077, and V1078 (Figure 4). We wondered whether the replacement of these residues with less bulky amino acids would improve the potency of the C2'-methylated analog. Using previously established methods,[47, 48] we generated a human/yeast chimeric SF3B1 protein in *S. cerevisiae*. The chimeric protein has HEAT domains 5–16 of the wild-type yeast SF3B1 (Hsh155) replaced with the human SF3B1 sequence (denoted as Hs5-16). We have previously shown these domains comprise the binding site for SSA and other small molecule splicing inhibitors and are responsible for the observed splicing effect of such compounds.[48] This chimera model provides a genetically tractable and facile way to detect splicing inhibition since pre-mRNA splicing is essential in yeast. With this model in hand, we replaced the residues (L1066, R1074, T1077, V1078) with either alanine or glycine and compared the growth inhibition between meayamycin D, 2'-Me meayamycin D, 3'-Me meayamycin D, and herboxidiene (control) in *S. cerevisiae* with Hs5-16 (Figure S5 and S6). Meayamycin D has an approximate GI_{50} of 108 nM in unmodified Hs5-16, while 2'-Me meayamycin D does not inhibit growth at concentrations up to 1 μM (Figure S5, black curve). 3'-Me meayamycin D has a GI_{50} of 405 nM in Hs5-16, which is a similar trend to the observed activity of these compounds in human cancer cells. Both the L1066A and T1077A mutants were still inhibited by meayamycin D, albeit with less potency. Additionally, none of the selected mutations improved the compound activity of 2'-Me meayamycin D. One possibility is that the steric clash of the 2'-methyl may occur primarily between the protein backbone rather than the specific amino acids. In the case of L1066G, this residue lies close to the beginning of the α-helical fold. Therefore, replacement with the more flexible glycine may destabilize the α-helix and lead to decreased compound activity (although yeast can tolerate this substitution in an essential protein). This flexibility may also explain the observed weaker toxicity in the T1077G mutant as well for meayamycin D and 3'-Me meayamycin D. Notably, however, herboxidiene did not lose toxicity against the

T1077G mutation (Figure S6). Finally, the V1078A mutant showed enhanced effect with 3'-Me meayamycin D with a GI_{50} of 80 nM (Hs5-16 GI_{50} = 405 nM; Figure 5), indicating that the valine at this position may be closer in proximity to the added 3'-methyl group.

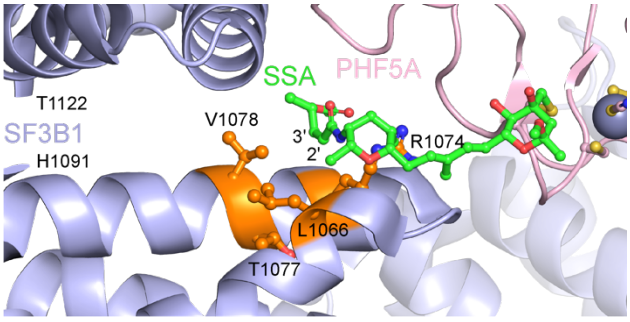


Figure 4. Proximal residues to the C2' and C3' position in the SSA-SF3B1 crystal structure (PDB: 7B9C, residues H1091–T1122 omitted for clarity)

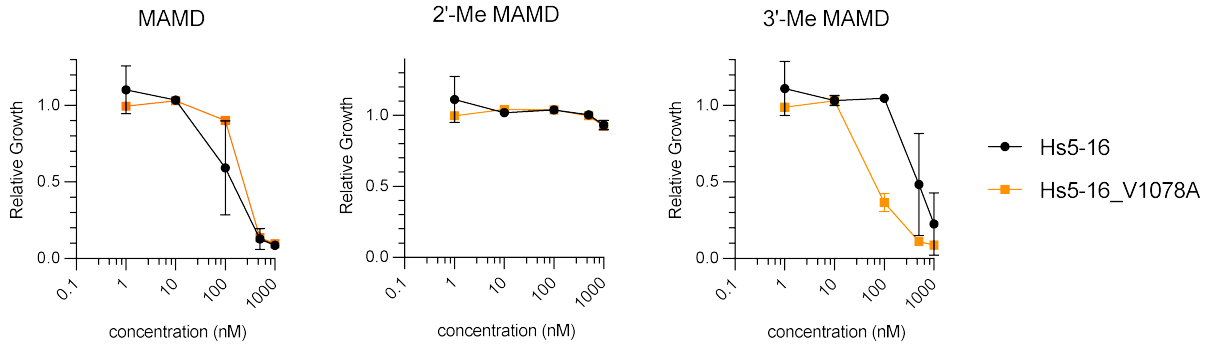


Figure 5. Growth inhibition of *S. cerevisiae* with chimeric Hsh5-16 mutation V1078A in the presence of meayamycin D (MAMD), 2'-Me MAMD, and 3'-Me MAMD. Each point represents the average of n = 3 biological replicates, ± SD.

Conclusions

We designed and synthesized two new analogs bearing methyl groups on the C2' and C3' positions. Our biological evaluation revealed that the C2' position is not tolerated for substitution. In contrast, the C3' substitution still retains modest activity compared to meayamycin D. 3'-Me meayamycin D inhibited the alternative splicing of *MCL-1* similar to meayamycin D, indicating these compounds likely behave similarly to affect cancer cell growth. Additionally, we investigated interactions within the SF3B1 binding pocket using a chimeric SF3B1 protein in yeast to understand the binding of these substituted analogs. None of the mutants tested improved the ability of 2'-Me meayamycin D to inhibit growth, consistent with the intolerability for substitution at this position. Meanwhile, a V1078A mutant was identified to have enhanced activity with 3'-Me meayamycin D in comparison to both meayamycin D and the unmutated chimeric protein. The 3'-Me meayamycin D analog highlights a new position on FR901464-based compounds that

is suitable for single carbon or single atom substitutions without a significant loss in potency and justifies further exploration of the binding pocket through structure-activity relationship studies.

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Experimental Section

Chemistry. All reactions were carried out with freshly distilled solvents under anhydrous conditions unless otherwise noted. All flasks used for carrying out reactions were dried in an oven at 80 °C prior to use. Unless otherwise stated, all reactions that required heating used an oil bath as the heating source, with a thermometer submerged in the bath to monitor the temperature. Unless specifically stated, the temperature of a water bath during the evaporation of organic solvents using a rotary evaporator was about 35 ± 5 °C. THF was distilled over Na metal and benzophenone. DCM was distilled over calcium hydride. MeCN was distilled over calcium hydride and stored over 3 Å molecular sieves. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials unless otherwise stated. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm Merck silica gel plates (60F-254) using UV light (254 nm) for visualization or anisaldehyde in ethanol or 0.2% ninhydrin in ethanol as developing agents and heat for visualization. Silica gel (230−400 mesh) was used for flash column chromatography. NMR spectra were recorded on a Bruker ADVANCE spectrometer at 300, 400, 500, 600, or 700 MHz. The chemical shifts are given in parts per million (ppm) on a delta (δ) scale. The solvent peak was used as a reference value for ^{1}H NMR: C*H*Cl$_3$ = 7.26 ppm, C*H*$_2$Cl$_2$ = 5.32 ppm, for ^{13}C{^{1}H} NMR: CDCl$_3$ = 77.16 ppm, CD$_2$Cl$_2$ = 53.84 ppm. The following abbreviations are used to indicate the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High-resolution mass spectra were recorded on a Thermo Scientific Q Exactive Orbitrap. Infrared (IR) spectra were collected on a PerkinElmer FT-IR Spectrum Two UATR spectrometer. Optical rotation was obtained on a Jasco P-2000 Digital Polarimeter, and $[\alpha]_D^T$ values are given in deg×cm^3×g^{-1}×dm^{-1}; concentrations, *c*, are listed in g×100×mL^{-1}.

*Ethyl (S,Z)-4-(methoxymethoxy)-2-methylpent-2-enoate (**2**)*. A 250-mL round-bottom flask equipped with an addition funnel and ethyl (*S*)-2-(methoxymethoxy)propanoate **1** (2.50 g, 15.4 mmol) was purged with nitrogen gas three times and then charged with DCM (38 mL). The mixture was cooled to -78 °C, and DIBALH (1.0 M in hexanes, 23 mL, 1.5 equiv) was added dropwise down the side of the flask over 30 min at -78 °C with the addition funnel. After stirring for 1.5 h at -78 °C, a separate 100-mL round-bottom flask with ethyl 2-(bis(2-(*tert*-butyl)phenoxy)phosphoryl)propanoate **9** (8.95 g, 20.0 mmol, 1.3 equiv) was purged with nitrogen gas three times and then charged with THF (25 mL) and cooled to 0 °C. Potassium *tert*-butoxide (1.94 g, 17.0 mmol, 1.1 equiv) was added in one portion to the flask with ethyl 2-(bis(2-(*tert*-butyl)phenoxy)phosphoryl)propanoate **9** at 0 °C. After 45 min at 0 °C, the solution of ethyl 2-(bis(2-(*tert*-butyl)phenoxy)phosphoryl)propanoate **9** and potassium *tert*-butoxide was added to the addition funnel of the flask with ethyl (*S*)-2-(methoxymethoxy)propanoate **1** via cannula and added dropwise at -78 °C. The reaction mixture was slowly warmed to 23 °C. After stirring for 20 h at the same temperature, the reaction mixture was quenched with aqueous 1 M sodium citrate (70 mL) and stirred. After 16 h, the organic solvent was removed under reduced pressure, and the residue was extracted with EtOAc/hexanes (1:4, 2 × 50 mL) and washed with brine (1 × 50 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (2 to 10% EtOAc in hexanes) on silica gel (175 mL) to afford ethyl (*S*,*Z*)-4-(methoxymethoxy)-2-methylpent-2-enoate **2** (1.62 g, 52% yield, 6:94 E:Z) as a colorless oil. R_f = 0.30 (10% EtOAc in hexanes); IR (neat): ν_{max} = 2981, 2932, 1714, 1650, 1450, 1370, 1212, 1157, 1096, 1028, 920 cm^{-1}; $[\alpha]_D^{25}$ -89.7 (*c* 1.0, CH_2Cl_2); ^1H NMR (500 MHz, 296K, $CDCl_3$) δ 5.85 (dq, *J* = 8.3, 1.5 Hz, 1H), 5.02 (m, 1H), 4.64 (d, *J* = 6.7 Hz, 1H), 4.57 (d, *J* = 6.7 Hz, 1H), 4.20 (q, *J* = 7.2 Hz, 2H), 3.35 (s, 3H), 1.92 (d, *J* = 1.5 Hz, 3H), 1.30 (t, *J* = 7.2 Hz, 3H), 1.28 (d, *J* = 5.1 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ 167.5, 144.1, 128.3, 94.8, 70.0, 60.6, 55.4, 21.0, 20.5, 14.3; HRMS (ESI+) calcd. for $C_{10}H_{18}O_4Na$ $[M+Na]^+$ 225.1097, found 225.1087.

*(S,Z)-4-(Methoxymethoxy)-2-methylpent-2-enoic acid (**3**)*. A 50-mL round-bottom flask with ethyl (*S*,*Z*)-4-(methoxymethoxy)-2-methylpent-2-enoate **2** (1.09 g, 5.43 mmol, 6:94 E:Z), open to air, in methanol (2.9 mL) was cooled to 0 °C. Aqueous 1.0 M NaOH (13.6 mL, 2.5 equiv) was added dropwise at 0 °C. The resulting mixture was warmed to 23 °C. After stirring for 16 h at the same temperature, the mixture was concentrated under reduced pressure to remove excess methanol, and then acidified with aqueous 4 M HCl to approximately pH 4. The resulting solution was extracted with EtOAc (4 × 20 mL) and washed with brine (1 × 20 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford (*S*,*Z*)-4-(methoxymethoxy)-2-

methylpent-2-enoic acid **3** (1.09 g, quantitative) as a colorless oil. R_f = 0.15 (30% EtOAc in hexanes); IR (neat): ν_{max} = 2934, 1718, 1692, 1646, 1453, 1371, 1214, 1157, 1095, 1027, 921 cm^{-1}; $[\alpha]_D^{25}$ -85.3 (*c* 1.0, CH_2Cl_2); ^1H NMR (300 MHz, 296K, 1% CD_3OD in $CDCl_3$) δ 6.03 (dq, *J* = 8.3, 1.4 Hz, 1H), 5.09 (m, 1H), 4.67 (d, *J* = 6.8 Hz, 1H), 4.62 (d, *J* = 6.8 Hz, 1H), 3.38 (s, 3H), 1.94 (d, *J* = 1.4 Hz, 3H), 1.30 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, 296K, 1% CD_3OD $CDCl_3$) δ 172.3, 147.2, 127.2, 95.0, 70.4, 55.5, 20.9, 20.4. HRMS (ESI-) calcd. for $C_8H_{13}O_4$ $[M-H]^-$ 173.0808, found 173.0804.

*(S,Z)-N-((2R,3R,5S,6S)-6-Allyl-2,5-dimethyltetrahydro-2H-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide (**4**)*. A 50-mL round-bottom flask with (2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-amine **10** (288 mg, 1.70 mmol) was purged with nitrogen and then charged with DCM (5.7 mL), (*S*,*Z*)-4-(methoxymethoxy)-2-methylpent-2-enoic acid **3** (445 mg, 2.55 mmol, 1.5 equiv), and diisopropylethylamine (1.63 mL, 8.94 mmol, 3.5 equiv) at 23 °C. The resulting solution was cooled to 0 °C, and HATU (991 mg, 2.55 mmol, 1.5 equiv) was added at 0 °C. The mixture was warmed to 23 °C. After 42 h, the reaction mixture was quenched with aqueous satd. NH_4Cl (10 mL), extracted with EtOAc (2 × 10 mL), and washed with brine (1 × 10 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (10 to 25% EtOAc in hexanes) on silica gel (70 mL) to afford (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **4** (351 mg) as an inseparable mixture of isomers as a yellow oil. The mixture of isomers was used in the next step without further purification. A small portion of the mixture was subjected to HPLC purification for spectroscopic analysis. R_f = 0.29 (40% EtOAc in hexanes); ^1H NMR (500 MHz, 296K, $CDCl_3$) δ 5.85 (d, *J* = 9.0 Hz, 1H), 5.84–5.74 (m, 1H), 5.52 (dq, *J* = 8.9, 1.5 Hz, 1H), 5.11 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.05 (br d, *J* = 10.0 Hz, 1H), 4.78 (dq, *J* = 8.9, 6.3 Hz, 1H), 4.67 (d, *J* = 6.7 Hz, 1H), 4.56 (d, *J* = 6.7 Hz, 1H), 3.99–3.94 (m, 1H), 3.68 (qd, *J* = 6.4, 2.1 Hz, 1H), 3.54 (ddd, *J* = 7.2, 7.2, 2.7 Hz, 1H), 3.35 (s, 3H), 2.37–2.29 (m, 1H), 2.18–2.08 (m, 1H), 1.97–1.93 (m, 5H), 1.83–1.76 (m, 1H), 1.29 (d, *J* = 6.3 Hz, 3H), 1.16 (d, *J* = 6.5 Hz, 3H), 1.03 (d, *J* = 7.4 Hz, 3H); HRMS (ESI+) calcd. for $C_{18}H_{32}NO_4$ $[M+H]^+$ 326.2326, found 326.2311.

*(S,Z)-N-((2R,3R,5S,6S)-2,5-Dimethyl-6-((E)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide (**5**)*. A 5-mL sealed tube with the mixture of (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **4** (216 mg, 665 mmol) was placed under a flow of argon gas and then charged with methacrolein (1.65 mL, 15.9 mmol, 24 equiv) and nitro-Grela catalyst (22 mg, 0.033 mmol, 5 mol%). The sealed tube was capped, and the reaction was heated to 50 °C (external temperature). After 20 h at 50 °C, the reaction

was cooled to 23 °C. The crude contents were transferred to a separate 10-mL pear-shaped flask, concentrated under reduced pressure, and purified by flash chromatography (20 to 60% EtOAc in hexanes) on silica gel (30 mL) to afford (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **5** (147 mg, 39% yield, over two steps) as a yellow-brown oil. R_f = 0.31 (60% EtOAc in hexanes); IR (neat): ν_{max} = 3450, 3343, 2968, 2925, 1683, 1640, 1504, 1467, 1446, 1372, 1215, 1156, 1096, 1065, 1028, 918 cm^{-1}; $[\alpha]_D^{25}$ -91.4 (*c* 1.0, CH_2Cl_2); ^1H NMR (500 MHz, 296K, $CDCl_3$) δ 9.42 (s, 1H), 6.57–6.51 (m, 1H), 5.94 (d, *J* = 8.6 Hz, 1H), 5.53 (dq, *J* = 8.9, 1.5 Hz, 1H), 4.78 (dq, *J* = 8.9, 6.4 Hz, 1H), 4.68 (d, *J* = 6.7 Hz, 1H), 4.57 (d, *J* = 6.7 Hz, 1H), 4.02–3.97 (m, 1H), 3.71 (qd, *J* = 6.4, 2.3 Hz, 1H), 3.66 (ddd, *J* = 8.4, 5.3, 2.8 Hz, 1H), 3.35 (s, 3H), 2.61–2.53 (m, 1H), 2.45–2.38 (m, 1H), 2.00 (app t, *J* = 3.6 Hz, 2H), 1.96 (d, *J* = 1.4 Hz, 3H), 1.87–1.79 (m, 1H), 1.76 (br s, 3H), 1.20 (d, *J* = 6.4 Hz, 3H), 1.17 (d, *J* = 6.5 Hz, 3H), 1.07 (d, *J* = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, 296K, $CDCl_3$) δ 195.2, 168.6, 150.4, 140.7, 136.8, 132.6, 94.5, 79.9, 76.3, 69.7, 55.4, 47.0, 35.9, 32.9, 29.6, 21.5, 21.2, 17.9, 15.3, 9.6; HRMS (ESI+) calcd. for $C_{20}H_{34}NO_5$ $[M+H]^+$ 368.2432, found 368.2429.

*(S,Z)-N-((2R,3R,5S,6S)-2,5-Dimethyl-6-((E)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2H-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide (**6**)*. A 2-dram vial with methyltriphenylphosphonium bromide (139 mg, 0.382 mmol, 3.5 equiv) was purged with nitrogen gas three times, charged with THF (1 mL), and cooled to 0 °C. Potassium *tert*-butoxide (29 mg, 0.35 mmol, 3.2 equiv) was added at 0 °C. After 30 min at 0 °C, (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **5** (29.2 mg, 0.109 mmol) in THF (0.5 mL) was added, rinsed with THF (0.5 mL), and the mixture was warmed to 23 °C. After stirring for 18 h at the same temperature, the reaction was quenched with aqueous satd. NH_4Cl (1 mL). The mixture was separated, and the aqueous layer was extracted with Et_2O (2 × 2 mL) using a separatory funnel. The combined organic layers were washed with brine (1 × 2 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (10 to 25% EtOAc in hexanes) on silica gel (7 mL) to afford (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **6** (23 mg, 78% yield) as a colorless oil. R_f = 0.37 (40% EtOAc in hexanes); IR (neat): ν_{max} = 3453, 3335, 2973, 2931, 1669, 1638, 1500, 1469, 1445, 1372, 1215, 1157, 1097, 1062, 1031, 918 cm^{-1}; $[\alpha]_D^{25}$ -79.8 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, 296K, $CDCl_3$) δ 6.36 (dd, *J* = 17.3, 10.7 Hz, 1H), 5.92 (d, *J* = 8.7 Hz, 1H), 5.51 (dq, *J* = 8.9, 1.5 Hz, 1H), 5.54 (app t, *J* = 7.0 Hz, 1H), 5.11 (d, *J* = 17.3 Hz, 1H), 4.95 (d, *J* = 10.7 Hz, 1H), 4.78 (dq, *J* = 8.9, 6.4 Hz, 1H), 4.67 (d, *J* = 6.6 Hz, 1H), 4.56 (d, *J* = 6.6 Hz, 1H), 4.00–3.93 (m, 1H), 3.68 (qd, *J* = 6.4, 2.3 Hz, 1H), 3.54 (ddd, *J* = 7.3, 7.3, 2.7 Hz, 1H), 3.34 (s, 3H), 2.44–2.34 (m, 1H), 2.30–2.20 (m, 1H), 1.97–

1.91 (m, 5H), 1.84–1.77 (m, 1H), 1.75 (br s, 3H), 1.29 (d, J = 6.4 Hz, 3H), 1.16 (d, J = 6.5 Hz, 3H), 1.02 (d, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ 168.5, 141.3, 136.7, 135.8, 132.6, 128.2, 111.3, 94.4, 80.8, 76.0, 69.7, 55.4, 47.2, 36.0, 32.0, 28.9, 21.5, 21.1, 18.0, 15.1, 12.1; HRMS (ESI+) calcd. for $C_{21}H_{36}NO_4$ $[M+H]^+$ 366.2639, found 366.2644.

2'-Me meayamycin D. A 2-mL sealed tube was treated with (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **6** (59 mg, 0.16 mmol) in DCE (315 µL). (3*R*,4*R*,5*R*)-7,7-Dimethyl-5-vinyl-1,6-dioxaspiro[2.5]octan-4-ol **7** in DCE (148 µL, 100 mg/mL solution, 0.5 equiv) and nitro-Grela catalyst (5 mg, 7 µmol, 5 mol%) were added to the sealed tube at 23 °C, and the sealed tube was purged with argon. The sealed tube was heated to 50 °C. After 2 h at 50 °C, additional right-hand fragment **7** in DCE (148 µL, 100 mg/mL solution, 0.5 equiv) was added. After an additional 2 h at 50 °C, right-hand fragment **7** in DCE (148 µL, 100 mg/mL, 0.5 equiv) and nitro-Grela catalyst (5 mg, 7 µmol, 5 mol%) were added. After an additional 4 h at 50 °C, the reaction was cooled to 23 °C and concentrated under reduced pressure. The crude material was purified by flash chromatography (20 to 70% EtOAc in hexanes) on silica gel (30 mL) to afford a complex mixture, which was further purified by preparative TLC (60% EtOAc in hexanes). Unreacted (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-2-methylpent-2-enamide **6** and right-hand fragment **7** were resubmitted to the reaction conditions and purified by preparative TLC (60% EtOAc in hexanes). The product mixtures were combined, dissolved in DCM (5 mL), and charcoal (1.5 g, 50× by weight) was added. After 3 h, the mixture was filtered through Celite® and the filtrate was concentrated under reduced pressure, and further purified by preparative TLC (60% EtOAc in hexanes) to afford 2'-Me meayamycin D (7 mg, 8% yield) as a tan oil. The resulting oil was further purified by semi-preparative HPLC for biological studies. R_f = 0.21 (60% EtOAc in hexanes); IR (neat): ν_{max} = 3446, 3348, 2968, 2925, 1666, 1637, 1501, 1450, 1381, 1336, 1216, 1156, 1114, 1096, 1059, 1031, 973 cm^{-1}; $[\alpha]_D^{25}$ +18.9 (*c* 0.1, CH_2Cl_2); ^{1}H NMR (500 MHz, 296K, CD_2Cl_2) δ 6.34 (d, J = 15.7 Hz, 1H), 5.89 (d, J = 8.8 Hz, 1H), 5.64 (dd, J = 15.7, 6.6 Hz, 1H), 5.52 (app t, J = 7.0 Hz, 1H), 5.46 (dq, J = 8.9, 1.5 Hz, 1H), 4.69 (dq, J = 8.9, 6.4 Hz, 1H), 4.62 (d, J = 6.7 Hz, 1H), 4.51 (d, J = 6.7 Hz, 1H), 3.96 (dd, J = 9.3, 6.7 Hz, 1H), 3.93–3.87 (m, 1H), 3.67 (qd, J = 6.4, 2.3 Hz, 1H), 3.53 (ddd, J = 7.8, 6.7, 2.7 Hz, 1H), 3.48 (dd, J = 10.1, 10.1 Hz, 1H), 3.31 (s, 3H), 2.96 (d, J = 4.7 Hz, 1H), 2.46 (d, J = 4.7 Hz, 1H), 2.40–2.32 (m, 1H), 2.26–2.18 (m, 1H), 2.17 (d, J = 14.3 Hz, 1H) 1.96–1.91 (m, 5H), 1.81–1.74 (m, 4H), 1.61 (d, J = 10.4 Hz, 1H), 1.39 (d, J = 14.3 Hz, 1H), 1.36 (s, 3H), 1.25–1.22 (overlapping d + s, 6H), 1.12 (d, J = 6.4 Hz, 3H), 1.02 (d, J = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, 296K, CD_2Cl_2) δ 168.7, 137.8, 136.1, 135.0, 133.6, 129.5, 125.9, 94.5, 81.2, 76.2, 74.9, 73.0, 69.7, 68.6, 57.8, 55.4, 47.8, 47.5, 43.1,

36.2, 32.4, 31.1, 29.5, 23.7, 21.6, 21.1, 18.0, 15.3, 12.8; HRMS (ESI+) calcd. for $C_{29}H_{48}NO_7$ $[M+H]^+$ 522.3425, found 522.3418.

*(S)-2-(Methoxymethoxy)propanoic acid (**11**)*. The preparation of (*S*)-2-(methoxymethoxy)propanoic acid **11** followed the reported procedure.[49] We note that a 5% MeOH in DCM solution or a 25% ^{i}PrOH in $CHCl_3$ solution may be used as suitable replacements for extraction when the product remains in the aqueous layer.

*(S)-N-Methoxy-2-(methoxymethoxy)-N-methylpropanamide (**12**)*. A 500-mL round-bottom flask with (*S*)-2-(methoxymethoxy)propanoic acid **11** (6.68 g, 49.8 mmol) was purged with nitrogen and then charged with DCM (165 mL) and trimethylacetyl chloride (6.81 mL, 54.8 mmol, 1.1 equiv) at 23 °C. The resulting solution was cooled to 0 °C, and triethylamine (7.71 mL, 54.8 mmol, 1.1 equiv) was added at 0 °C. After 1.5 h at 0 °C, *N*,*O*-dimethylhydroxylamine hydrochloride (5.45 g, 5.48 mmol, 1.1 equiv) and triethylamine (9.82 mL, 69.7 mmol, 1.4 equiv) were added at 0 °C. The mixture was warmed to 23 °C. After stirring for 20 h at the same temperature, the mixture was diluted with EtOAc (100 mL) and washed with 1M HCl (100 mL) using a separatory funnel. The organic layer was washed with aqueous satd. $NaHCO_3$ (100 mL), and then washed with brine (100 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (40 to 70% EtOAc in hexanes) on silica gel (500 mL) to afford (*S*)-*N*-methoxy-2-(methoxymethoxy)-*N*-methylpropanamide **12** (7.31 g, 83% yield) as a pale oil. R_f = 0.20 (60% EtOAc in hexanes); IR (neat): ν_{max} = 2941, 1671, 1461, 1390, 1162, 1105, 1043, 920 cm^{-1}; $[\alpha]_D^{25}$ -102.0 (*c* 1.0, CH_2Cl_2); ^{1}H NMR (300 MHz, 296K, $CDCl_3$) δ 4.68 (d, *J* = 7.0 Hz, 1H), 4.65 (d, *J* = 7.0 Hz, 1H), 4.69–4.58 (m, 1H), 3.72 (s, 3H), 3.39 (s, 3H), 3.21 (s, 3H), 1.40 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, 296K, $CDCl_3$) δ 173.7, 95.6, 68.9, 61.5, 55.9, 32.4, 18.3; HRMS (ESI+) calcd. for $C_7H_{16}NO_4$ $[M+H]^+$ 178.1074, found 178.1066.

*(S)-3-(Methoxymethoxy)butan-2-one (**13**)*. A 250-mL round-bottom flask equipped with a reflux condenser, with magnesium granules (1.24 g, 51.2 mmol, 3.2 equiv) was purged with nitrogen gas three times, charged with Et_2O (25 mL), and cooled to 0 °C. Iodomethane (1.5 mL, 48 mmol, 3.0 equiv) was added at 0 °C. After 30 min at 0 °C, (*S*)-*N*-methoxy-2-(methoxymethoxy)-*N*-methylpropanamide **12** (2.84 g, 16.0 mmol) in Et_2O (25 mL) was added, and stirred at 0 °C. After 2.5 h at 0 °C, the reaction was quenched with aqueous satd. NH_4Cl (50 mL). The mixture was separated, and the aqueous layer was extracted with Et_2O (2 × 25 mL) using a separatory funnel. The combined organic layers were washed with brine (1 × 25 mL), dried over anhydrous Na_2SO_4, filtered, and carefully concentrated under reduced pressure to afford (*S*)-3-(methoxymethoxy)butan-2-one **13** (1.86 g, 88% yield) as a yellow oil. R_f = 0.37 (30% EtOAc in hexanes); ν_{max} = 2939, 2894, 1718, 1356, 1154, 1111, 1026, 943, 920 cm^{-1}; $[\alpha]_D^{25}$ -13.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400

MHz, 296K, $CDCl_3$) δ 4.68 (d, *J* = 6.8 Hz, 1H), 4.63 (d, *J* = 6.8 Hz, 1H), 4.09 (q, *J* = 6.9 Hz, 1H), 3.37 (s, 3H), 2.18 (s, 3H), 1.32 (d, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ 210.2, 96.0, 78.6, 56.0, 25.6, 17.4. GCMS (EI+) m/z: 117 (M – CH_3), 105, 89 (M – C_2H_3O), 74 (M – C_2H_3O – CH_3), 45 (C_2H_5O) (Figure S7).

*Ethyl (S,Z)-4-(methoxymethoxy)-3-methylpent-2-enoate (**14**)*. A 50-mL round-bottom flask with ethyl 2-(bis(2-(*tert*-butyl)phenoxy)phosphoryl)acetate **20** (2.46 g, 5.68 mmol, 1.3 equiv) was purged with nitrogen gas three times and then charged with THF (6 mL) and cooled to 0 °C. Potassium *tert*-butoxide (600 mg, 5.24 mmol, 1.2 equiv) was added in one portion to the flask at 0 °C. After 30 min at 0 °C, (*S*)-3-(methoxymethoxy)butan-2-one **13** (578 mg, 4.37 mmol) in THF (4 mL) was added at 0 °C. The mixture was warmed to 23 °C. After stirring for 18 h at the same temperature, the reaction mixture was quenched with aqueous satd. NH_4Cl (8 mL). The organic solvent was removed under reduced pressure, and the residue was extracted with EtOAc (2 × 10 mL) and washed with brine (1 × 50 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (1.5 to 5% EtOAc in hexanes) on silica gel (60 mL) to afford ethyl (*S*,*Z*)-4-(methoxymethoxy)-3-methylpent-2-enoate **14** (445 mg, 50% yield, 15:85 E:Z) as a colorless oil. R_f = 0.30 (10% EtOAc in hexanes); ν_{max} = 2985, 2935, 1713, 1647, 1445, 1376, 1232, 1149, 1096, 1031, 920, 861 cm^{-1}; $[\alpha]_D^{25}$ -35.8 (*c* 1.0, CH_2Cl_2); ^{1}H NMR (300 MHz, 296K, $CDCl_3$) δ 5.71 (br s, 1H), 5.59 (q, *J* = 6.5 Hz, 1H), 4.57 (d, *J* = 6.6 Hz, 1H), 4.54 (d, *J* = 6.6 Hz, 1H), 4.14 (q, *J* = 7.1 Hz, 1H), 3.36 (s, 3H), 1.88 (br s, 3H), 1.29 (d, *J* = 6.5 Hz, 3H), 1.27 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ 165.8, 160.2, 117.5, 95.2, 70.4, 60.0, 55.7, 19.9, 18.6, 14.4; HRMS (ESI+) calcd. for $C_{10}H_{18}O_4Na$ $[M+Na]^+$ 225.1097, found 225.1087.

*(S,Z)-4-(Methoxymethoxy)-3-methylpent-2-enoic acid (**15**)*. A 25-mL round-bottom flask with ethyl (*S*,*Z*)-4-(methoxymethoxy)-3-methylpent-2-enoate **14** (261 mg, 1.29 mmol), open to air, in methanol (670 µL) was cooled to 0 °C. Aqueous 1.0 M NaOH (3.2 mL, 2.5 equiv) was added dropwise at 0 °C. After 3.5 h at 0 °C, the mixture was concentrated under reduced pressure to remove excess methanol, and then acidified with aqueous 4 M HCl to approximately pH 4. The resulting solution was extracted with DCM (4 × 5 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford (*S*,*Z*)-4-(methoxymethoxy)-3-methylpent-2-enoic acid **15** (227 mg, quantitative) as a yellow oil. R_f = 0.16 (30% EtOAc in hexanes); ν_{max} = 2982, 2935, 2896, 1689, 1643, 1445, 1374, 1156, 1097, 1030, 920 cm^{-1}; $[\alpha]_D^{25}$ -83.7 (*c* 1.0, CH_2Cl_2); ^{1}H NMR (400 MHz, 296K, 1% CD_3OD in $CDCl_3$) δ 5.74 (br s, 1H), 5.54 (q, *J* = 6.5 Hz, 1H), 4.60 (d, *J* = 6.6 Hz, 1H), 4.56 (d, *J* = 6.6 Hz, 1H), 3.37 (s, 3H), 1.93 (d, *J* = 1.1 Hz, 3H), 1.31 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ

170.2, 163.1, 116.8, 95.3, 70.7, 55.7, 19.9, 19.0; HRMS (ESI-) calcd. for $C_8H_{13}O_4$ $[M-H]^-$ 173.0808, found 173.0803.

*(S,Z)-N-((2R,3R,5S,6S)-2,5-Dimethyl-6-((E)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2H-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide (**17**)*. A 50-mL round-bottom flask with (2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-amine **10** (382 mg, 2.25 mmol) was purged with nitrogen, charged with DCM (7.5 mL), and cooled to 0 °C. (*S*,*Z*)-4-(Methoxymethoxy)-3-methylpent-2-enoic acid **15** (450 mg, 2.58 mmol, 1.1 equiv) and diisopropylethylamine (1.20 mL, 6.76 mmol, 3.0 equiv) were added at 0 °C. After 10 min at 0 °C, HATU (1.03 g, 2.58 mmol, 1.1 equiv) was added at 0 °C. The mixture was warmed to 23 °C. After 40 h, the reaction mixture was quenched with aqueous satd. NH_4Cl (15 mL), extracted with EtOAc (2 × 15 mL), and washed with brine (1 × 15 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (5 to 20% EtOAc in hexanes) on silica gel (30 mL) to afford an inseparable mixture of isomers of (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **16** (459 mg) as a yellow oil. The mixture of isomers was used in the next step without further purification.

A 10-mL sealed tube with the mixture of (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-6-allyl-2,5-dimethyltetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **16** (439 mg) was placed under a flow of argon gas and then charged with methacrolein (3.50 mL, 33.8 mmol, 24 equiv) and nitro-Grela catalyst (47 mg, 0.070 mmol, 5 mol%). The sealed tube was capped, and the reaction was heated to 50 °C (external temperature). After 18 h at 50 °C, the reaction mixture was cooled to 23 °C. The crude contents were transferred to a separate 10-mL pear-shaped flask, concentrated under reduced pressure, and purified by flash chromatography (20 to 60% EtOAc in hexanes) on silica gel (70 mL) to afford (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **17** (269 mg, 32% yield, over two steps) as a yellow-brown oil. R_f = 0.25 (60% EtOAc in hexanes); IR (neat): ν_{max} = 3348, 2976, 2932, 1684, 1664, 1640, 1509, 1469, 1444, 1376, 1216, 1157, 1097, 1069, 1033, 919 cm^{-1}; $[\alpha]_D^{25}$ -33.3 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, 296K, $CDCl_3$) δ 9.42 (s, 1H), 6.58–6.49 (m, 1H), 5.71 (d, *J* = 8.9 Hz, 1H), 5.63 (br s, 1H), 5.61 (q, *J* = 6.5 Hz, 1H), 4.58 (app s, 2H), 3.97–3.90 (m, 1H), 3.72–3.60 (m, 2H), 3.36 (s, 3H), 2.62–2.51 (m, 1H), 2.46–2.35 (m, 1H), 2.00–1.94 (m, 2H), 1.84 (d, *J* = 1.0 Hz, 3H), 1.84–1.78 (m, 1H), 1.76 (br s, 3H), 1.32 (d, *J* = 6.5 Hz, 3H), 1.14 (d, *J* = 6.4 Hz, 3H), 1.06 (d, *J* = 7.4 Hz, 3H); ^{13}C NMR (125 MHz, 296K, $CDCl_3$) δ 195.2, 165.5, 154.7, 150.5, 140.7, 120.3, 95.1, 79.9, 76.4, 70.4, 55.7, 46.9, 35.9, 33.0, 29.7, 20.0, 18.3, 17.9, 15.4, 9.6; HRMS (ESI+) calcd. for $C_{20}H_{34}NO_5$ $[M+H]^+$ 368.2431, found 368.2427.

(S,Z)-N-((2R,3R,5S,6S)-2,5-Dimethyl-6-((E)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2H-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide ***(18)***. A 10-mL round-bottom flask with methyltriphenylphosphonium bromide (798 mg, 2.19 mmol, 3.5 equiv) was purged with nitrogen gas three times, charged with THF (2 mL), and cooled to 0 °C. Potassium *tert*-butoxide (229 mg, 2.00 mmol, 3.2 equiv) was added at 0 °C. After 30 min at 0 °C, (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methyl-4-oxobut-2-en-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **17** (230 mg, 0.62 mmol) in THF (1 mL) was added, rinsed with THF (1 mL), and stirred 0 °C. After stirring for 1.5 h at 0 °C, the reaction was quenched with aqueous satd. NH_4Cl (5 mL). The mixture was separated, and the aqueous layer was extracted with EtOAc (2 × 5 mL) using a separatory funnel. The combined organic layers were washed with brine (1 × 5 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (10 to 30% EtOAc in hexanes) on silica gel (30 mL) to afford (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **18** (188 mg, 82% yield) as a colorless oil. R_f = 0.18 (30% EtOAc in hexanes); IR (neat): ν_{max} = 3345, 2975, 2930, 1663, 1635, 1503, 1468, 1444, 1376, 1217, 1158, 1096, 1072, 1063, 1033, 919 cm^{-1}; $[\alpha]_D^{25}$ -77.4 (*c* 1.0, CH_2Cl_2); ^1H NMR (500 MHz, 296K, $CDCl_3$) δ 6.37 (dd, *J* = 17.4, 10.7 Hz, 1H), 5.72 (d, *J* = 9.0 Hz, 1H), 5.62 (q, *J* = 6.6 Hz, 1H), 5.61 (br s, 1H), 5.46 (app t, *J* = 7.1 Hz, 1H), 5.11 (d, *J* = 17.4 Hz, 1H), 4.95 (d, *J* = 10.7 Hz, 1H), 4.58 (app s, 2H), 3.94–3.88 (m, 1H), 3.65 (qd, *J* = 6.5, 2.2 Hz, 1H), 3.53 (ddd, *J* = 7.3, 7.3, 2.8 Hz, 1H), 3.36 (s, 3H), 2.43–2.34 (m, 1H), 2.29–2.20 (m, 1H), 1.95–1.90 (m, 2H), 1.84 (d, *J* = 1.3 Hz, 1H), 1.81–1.76 (m, 1H), 1.76 (br s, 3H), 1.31 (d, *J* = 6.6 Hz, 3H), 1.13 (d, *J* = 6.5 Hz, 3H), 1.02 (d, *J* = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, 296K, $CDCl_3$) δ 165.5, 154.4, 141.4, 135.8, 128.3, 120.4, 111.3, 95.1, 80.9, 76.2, 70.4, 55.7, 47.1, 36.1, 32.1, 29.1, 20.0, 18.3, 18.0, 15.3, 12.1; HRMS (ESI+) calcd. for $C_{21}H_{36}NO_4$ $[M+H]^+$ 366.2639, found 366.2624.

3'-Me meayamycin D. A 5-mL sealed tube was treated with (*S*,*Z*)-*N*-((2*R*,3*R*,5*S*,6*S*)-2,5-dimethyl-6-((*E*)-3-methylpenta-2,4-dien-1-yl)tetrahydro-2*H*-pyran-3-yl)-4-(methoxymethoxy)-3-methylpent-2-enamide **18** (80 mg, 0.22 mmol) in DCE (429 µL). (3*R*,4*R*,5*R*)-7,7-Dimethyl-5-vinyl-1,6-dioxaspiro[2.5]octan-4-ol **7** in DCE (203 µL, 100 mg/mL solution, 0.5 equiv) and nitro-Grela catalyst (10 mg, 15 µmol, 6.7 mol%) were added to the sealed tube at 23 °C, and the sealed tube was purged with argon. The sealed tube was heated to 45 °C. After 1 h at 45 °C, additional right-hand fragment **7** in DCE (203 µL, 100 mg/mL solution, 0.5 equiv) and nitro-Grela catalyst (10 mg, 15 µmol, 6.7 mol%) were added. After an additional 1 h at 45 °C, the final portion of right-hand fragment **7** in DCE (203 µL, 100 mg/mL, 0.5 equiv) and nitro-Grela catalyst (10 mg, 7 µmol, 6.7 mol%) was added. After 12 h at 45 °C, the reaction was cooled to 23 °C and concentrated under reduced pressure. The crude material was filtered through a plug of silica and rinsed

with 80% EtOAc in hexanes. Charcoal (1.7 g, 10× by weight) was added to the mixture and the resulting mixture was heated to 45 °C and stirred at that temperature. After 3 h at 45 °C, the mixture was cooled to 23 °C, filtered through Celite®, and concentrated under reduced pressure. The crude material was purified by flash chromatography (20 to 70% EtOAc in hexanes) on silica gel (20 mL) to afford a mixture, which was further purified by preparative TLC (60% EtOAc in hexanes) to afford 3'-Me meayamycin D (9 mg, 8% yield) as a tan solid. The resulting solid was further purified by semi-preparative HPLC for biological studies. R_f = 0.20 (60% EtOAc in hexanes); IR (neat): ν_{max} = 3368, 2974, 2926, 1663, 1637, 1506, 1468, 1444, 1381, 1216, 1158, 1114, 1096, 1060, 1035, 973 cm^{-1}; $[\alpha]_D^{25}$ -35.1 (*c* 0.25, CH_2Cl_2); ^1H NMR (600 MHz, 296K, CD_2Cl_2) δ 6.34 (d, *J* = 15.7 Hz, 1H), 5.71 (d, *J* = 9.0 Hz, 1H), 5.64 (dd, *J* = 15.6, 6.6 Hz, 1H), 5.63 (br s, 1H), 5.59 (q, *J* = 6.5 Hz, 1H), 5.52 (app t, *J* = 7.1 Hz, 1H), 4.52 (app s, 2H), 3.96 (dd, *J* = 9.4, 6.7 Hz, 1H), 3.88–3.82 (m, 1H), 3.64 (qd, *J* = 6.4, 2.2 Hz, 1H), 3.52 (ddd, *J* = 7.9, 6.6, 2.8 Hz, 1H), 3.48 (dd, *J* = 10.0, 10.0 Hz, 1H), 3.33 (s, 3H), 2.95 (d, *J* = 4.7 Hz, 1H), 2.46 (d, *J* = 4.7 Hz, 1H), 2.39–2.32 (m, 1H), 2.25–2.19 (m, 1H), 2.17 (d, *J* = 14.3 Hz, 1H) 1.92–1.88 (m, 2H), 1.81 (d, *J* = 1.3 Hz, 3H), 1.78 (br s, 3H), 1.78–1.73 (m, 1H), 1.61 (d, *J* = 10.4 Hz, 1H), 1.39 (d, *J* = 14.3 Hz, 1H), 1.36 (s, 3H), 1.26 (d, *J* = 6.5 Hz, 3H), 1.23 (s, 3H), 1.09 (d, *J* = 6.4 Hz, 3H), 1.01 (d, *J* = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, 296K, CD_2Cl_2) δ 165.5, 154.3, 137.8, 134.9, 129.6, 125.8, 120.8, 95.1, 81.2, 76.4, 74.9, 73.0, 70.3, 68.6, 57.8, 55.6, 47.8, 47.3, 43.1, 36.3, 32.4, 31.1, 29.6, 23.7, 20.0, 18.2, 18.0, 15.3, 12.8; HRMS (ESI+) calcd. for $C_{29}H_{48}NO_7$ $[M+H]^+$ 522.3425, found 522.3430.

*(S,Z)-4-(Methoxymethoxy)-2-methylpent-2-en-1-ol (**8**)*. A 10-mL round-bottom flask with ethyl (*S*,*Z*)-4-(methoxymethoxy)-2-methylpent-2-enoate **2** (29 mg, 14 mmol, 13:87 E:Z) was purged with nitrogen and charged with THF (520 µL). The mixture was cooled to -78 °C, and DIBALH (1.0 M in hexanes, 472 µL, 3.3 equiv) was added at -78 °C. After stirring for 1.5 h at -78 °C, the reaction mixture was quenched with aqueous satd. NH_4Cl (3 mL) and concentrated under reduced pressure to remove THF. The remaining mixture was extracted with EtOAc (3 × 3 mL) and washed with brine (1 × 3 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (10 to 40% EtOAc in hexanes) on silica gel (10 mL) to afford (*S*,*Z*)-4-(methoxymethoxy)-2-methylpent-2-en-1-ol **8** (17 mg, 68% yield) as a colorless oil. R_f = 0.30 (40% EtOAc in hexanes); ν_{max} = 3416, 2973, 2932, 1449, 1374, 1157, 1095, 1027, 918 cm^{-1}; $[\alpha]_D^{25}$ -174.0 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, 296K, 1% CD_3OD in $CDCl_3$) δ 5.13 (br d, *J* = 9.6 Hz, 1H), 4.73 (d, *J* = 6.9 Hz, 1H), 4.55 (dq, *J* = 9.7, 6.3 Hz, 1H), 4.51 (d, *J* = 6.9 Hz, 1H), 4.31 (d, *J* = 12.1 Hz, 1H), 3.80 (d, *J* = 12.1 Hz, 1H), 3.37 (s, 3H), 1.84 (d, *J* = 1.3 Hz, 3H), 1.23 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, 296K, 1% CD_3OD in $CDCl_3$) δ 139.8, 128.8, 93.1, 67.1, 61.5, 55.2, 22.0, 21.7; HRMS (ESI+) calcd. for $C_8H_{16}O_3Na$ $[M+Na]^+$ 183.0992, found 183.0984.

*(S,Z)-4-(Methoxymethoxy)-3-methylpent-2-en-1-ol (**19**)*. A 10-mL round-bottom flask with ethyl (*S*,*Z*)-4-(methoxymethoxy)-3-methylpent-2-enoate **14** (30 mg, 15 mmol, 39:61 E:Z) was purged with nitrogen and charged with THF (494 µL). The mixture was cooled to -78 °C, and DIBALH (1.0 M in hexanes, 445 µL, 3.0 equiv) was added at -78 °C. After stirring for 2 h at -78 °C, the reaction mixture was quenched with aqueous satd. NH_4Cl (3 mL) and concentrated under reduced pressure to remove THF. The remaining mixture was extracted with EtOAc (2 × 3 mL) and washed with brine (1 × 3 mL) using a separatory funnel. The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (7.5 to 30% EtOAc in hexanes) on silica gel (10 mL) to afford (*S*,*Z*)-4-(methoxymethoxy)-3-methylpent-2-en-1-ol **19** (19 mg, 79% yield, 38:62 E:Z) as a colorless oil. A small portion of the mixture was repurified by flash chromatography for spectroscopic analysis. Data: R_f = 0.24 (40% EtOAc in hexanes); v_{max} = 3408, 2980, 2933, 1447, 1378, 1157, 1096, 1031, 919 cm^{-1}; $[\alpha]_D^{25}$ -126.9 (*c* 1.0, CH_2Cl_2); ^1H NMR (400 MHz, 296K, $CDCl_3$) δ 5.68 (app t, *J* = 7.9 Hz, 1H), 4.69 (q, *J* = 6.6 Hz, 1H), 4.59 (d, *J* = 6.8 Hz, 1H), 4.50 (d, *J* = 6.8 Hz, 1H), 4.28 (dd, *J* = 12.4, 8.6 Hz, 1H), 3.90 (dd, *J* = 12.4, 6.4 Hz, 1H), 3.37 (s, 3H), 1.68 (br s, 3H), 1.26 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (100 MHz, 296K, $CDCl_3$) δ 138.9, 128.3, 93.3, 68.2, 57.5, 55.3, 19.3, 17.3; HRMS (ESI+) calcd. for $C_8H_{16}O_3Na$ $[M+Na]^+$ 183.0992, found 183.0983.

Growth Inhibition Assay

All cell lines were obtained from ATCC (Manassas, VA) and maintained in RPMI-1640 media, Waymouth media (DMS53 and DMS114 cells) + 10% (v/v) fetal bovine serum. Cells were mycoplasma-free as determined by the e-Myco PLUS mycoplasma PCR detection kit (Bulldog Bio, Portsmouth, NH). Cells were plated in 96-well plates at an initial density of 1500 or 5000 cells per well in culture media (100 µL) and were incubated for 24 h prior to compound addition. The compounds were prepared separately as 10 mM in 100% DMSO or 10 µM in 100% DMSO. Serial dilution in sterile water gave 10× dilutions that were added directly to the cells as 100-fold dilutions to give the desired concentration of compound, 0.1 nM – 30 µM meayamycin A, meayamycin D, 2'-Me meayamycin D or 3'-Me meayamycin D, in 0.1–0.3% (v/v) DMSO. The cells were then incubated for an additional 72 h. Cell proliferation was measured by using the commercial 3-(4,5-dimethylthiazol-2-yl)-5-(3-car-boxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) dye or 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene sulfonate (WST-1) dye reduction assay. The absorbance at 490 nm (MTS) or 450 nm (WST-1) was measured with a Modulus II Microplate Multimode Reader (Promega) or Tecan Infinite M1000 PRO Multimode Reader. Evaluation of the compounds was performed in duplicate at each concentration. GraphPad Prism 9.4.0 was used to construct dose-response curves and calculate the GI_{50} values.

Immunoblot Analysis

HCT116 cells were treated with various concentrations of meayamycin D analogs for 8 h and then lysed using RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) IGEPAL, 0.5% (w/v) sodium deoxycholate) containing phosphatase and protease inhibitors. Approximately 20 µg protein from each cell lysate was resolved on SDS-PAGE gels (Cat#5671084; Bio-Rad). Proteins were transferred onto nitrocellulose membranes followed by 1 h incubation at room temperature in blocking solution (1× TBS, 0.1% (v/v) Tween-20, 5% (w/v) milk powder). Membranes were incubated overnight at 4°C with the following antibodies: anti-phospho-SF3B1(#25009; Cell Signaling), anti-SF3B1 (#14434; Cell Signaling), anti-MCL-1 (#5453; Cell Signaling), anti-p27 (#3686; Cell Signaling), and anti-α-tubulin (#2125; Cell Signaling). Proteins were detected using SuperSignal West Pico substrate (Pierce; Rockford, IL).

In Vitro Plasma Stability

Mouse CD1 plasma K2 EDTA (Innovative Research) was prepared in a 2-mL microcentrifuge tube. The compounds were prepared separately as 1 mM solutions in 10% (v/v) DMSO and added to the plasma as 100× dilutions to give 700 µL at a concentration of compound, 10 µM procaine, meayamycin A, 2'-Me meayamycin D, or 3'-Me meayamycin D in 0.1% (v/v) DMSO. The mixture was vortexed for 10 sec, capped, and placed in a shaking incubator (Corning) for 48 h at 125 rpm at 37 °C. At the indicated times an aliquot (70 µL) of the mixture was taken and added to an equal volume of ice-cold MeCN and centrifuged for 15 min at 14000 relative centrifugal force (RCF) at 4 °C. The supernatant was collected and frozen at -80 °C until sample analysis. The samples were analyzed by LC-MS using Fmoc-L-phenylalanine as an internal standard at a concentration of 25 µM. A standard curve was prepared separately in a matrix-matched solution by 2-fold serial dilution from 10 µM. The decomposition was determined by comparing the ratio of analyte to Fmoc-L-phenylalanine with the ratio of the analyte to Fmoc-L-phenylalanine in the first data point.

Preparation of Yeast Strains

Parental *Saccharomyces cerevisiae* strains and plasmids are as described in previous reports.[47, 48] Hs5-16 point mutations were generated via site-directed mutagenesis and validated through DNA sequencing. Plasmids containing the mutant Hs5-16 protein genes were then introduced into yeast by plasmid shuffling and loss of the wild-type Hsh155 plasmid selected for using 5-FOA.[50] In strains containing the SF3B1 chimeras, the chimera is the only source of functional Hsh155/SF3B1 protein. Standard yeast growth media and conditions were employed unless stated otherwise.

Microplate Yeast Growth Assay

Yeast growth was done as previously described.[48] Strains were grown overnight in -tryptophan dropout media with 1% (v/v) DMSO at 30 °C while shaking (220 rpm). Cells were then diluted to an OD_{600} = 0.1 in -tryptophan dropout media. An aliquot of the diluted cultures (100 µL) was plated in a Corning Costar 96-well clear round-bottom cell culture plate. The compounds were added directly as 10-fold dilutions to give the desired concentration of compound, 1 nM – 1 µM meayamycin D, 2'-Me meayamycin D, or 3'-Me meayamycin D, in 1% (v/v) DMSO at OD_{600} = 0.1. The plates were covered with Breathe-Easy® plate sealing membranes to minimize evaporation and placed in a Tecan Infinite® 200 PRO plate reader set at 30 °C while shaking (220 rpm) for 24 h. OD_{600} measurements were read every 15 min. Absorbance values were corrected using measurements from wells that contained only media.

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