# Synthetic mRNA Splicing Modulator Compounds with in Vivo Antitumor Activity

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We report our progress on the development of new synthetic anticancer lead compounds that modulate the splicing of mRNA. We also report the synthesis and evaluation of new biologically active ester and carbamate analogues. Further, we describe initial animal studies demonstrating the antitumor efficacy of compound **5** in vivo. Additionally, we report the enantioselective and diastereospecific synthesis of a new 1,3-dioxane series of active analogues. We confirm that compound **5** inhibits the splicing of mRNA in cell-free nuclear extracts and in a cell-based dual-reporter mRNA splicing assay. In summary, we have developed totally synthetic novel spliceosome modulators as therapeutic lead compounds for a number of highly aggressive cancers. Future efforts will be directed toward the more complete optimization of these compounds as potential human therapeutics.

## Introduction

The spliceosome is a critical component of the cellular machinery in higher organisms and has recently emerged as an important target for the discovery of selective antitumor agents.<sup>1</sup> The complexity of the spliceosome rivals that of the ribosome; it is the part of the eukaryotic cell machinery responsible for the splicing of Pre-mRNA to mRNA and consists of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles and over 150 associated proteins.<sup>2</sup> An intricate arrangement of small nuclear RNAs and associated spliceosomal proteins interact with the pre-mRNA during spliceosome assembly, leading ultimately to intron removal, ligation of exons, and the release of correctly processed mRNA. Two natural products have been recently reported to target the SF3b subunit of the spliceosome.<sup>3,4</sup> These naturally occurring compounds are pladienolide<sup>4</sup> (PD,<sup>a</sup> actually a set of closely related compounds that have been designated pladienolides A–G, isolated from Streptomyces platensis)<sup>5</sup> and 1(FR901464)<sup>6</sup> isolated from *Pseudomonas* sp. No. 2663.<sup>6–8</sup>

The first of these natural products to be discovered (1) demonstrated significant efficacy in in vivo cancer models but was chemically unstable and showed a relatively narrow therapeutic window.<sup>6</sup> This prompted development of several types of partially stabilized compounds by modification of the hemiketal function of 1.<sup>9,10</sup> PD-based compounds (first reported in 2004) show a very promising combination of dramatic efficacy and very broad therapeutic window.<sup>4</sup> Of seven naturally occurring PDs, pladienolide B has the most selective

anticancer activity in vivo.<sup>5</sup> Recently, a pladienolide B derivative  $2(E7107)^4$  entered clinical trials as an anticancer agent (see Figure 1).<sup>4</sup> The natural products 1 and PD both show cytotoxicity with IC50 values in the low nanomolar range against several tumor cell lines and have been reported to cause a similar distinctive effect on the cell cycle in mammalian cell lines; cell cycle arrest that can occur at both the G1 and G2/M phases.<sup>5,6</sup> The interaction of these compounds with the spliceosome leads to what has been described as the inhibition of mRNA splicing, the inhibition of the nuclear retention of premRNA, and the translation of unspliced mRNA, which ultimately result in the observed effects on the cell cvcle.<sup>3,4</sup> The identification of the spliceosome as the target of 1 analogues was made possible through the application of a biotinylated derivative of 1 that was found to interact with the SAP155, SAP145, and SAP130 subunits of SF3b.<sup>3</sup> The binding of these subunits to streptavidin beads loaded with the biotinylated 1 derivative was reversible. This result indicates that no irreversible covalent bond is formed via reaction of the epoxide group with these proteins, under these conditions, despite the natural initial expectation to the contrary.<sup>3</sup> In the case of the elucidation of the mechanism of action of PD and related analogues, a slightly different approach was used: a doubly labeled affinity probe that cross-linked specifically to the SAP130 subunit was prepared, and from additional experiments it was concluded that SAP145 was required for the binding of the probe to SAP130.4

Partially stabilized analogues of **1**, such as spliceostatin (Figure 1), have been used to elucidate the effects of inhibition of the SF3b subunit on cell function.<sup>3</sup> This work led to the observation that inhibition of SF3b by **1** analogues, or knockdown of SF3b by siRNA, is associated with the same phenotypic changes in cells and results in export from the nucleus to the cytoplasm and translation of "unspliced" or "incompletely spliced" pre-mRNAs. One of the proteins aberrantly

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<sup>&</sup>lt;sup>*a*</sup> Abbreviations: PD, pladienolide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide); DIAD, diisopropyl azodicarboxylate; DI-BAL H, diisobutylaluminum hydride; TBAF, tetra-*n*-butylammonium fluoride; DMAP, 4-dimethylaminopyridine.



**Figure 1.** Published structures of pladienolide B, **1** (FR901464), **2** (E7107), and spliceostatin.



**Figure 2.** Overview of some of the representative published data on SAR for 1 analogues.<sup>3,10,12,13</sup> Activity is reported relative to that of 1.

translated as a result of compound treatment (or siRNA knockdown of SF3b) is p27, resulting in accumulation of a truncated p27 in treated cells. The truncated p27 retains its activity as a cell cycle inhibitor but is not targeted for degradation by the proteasome because of deletion of part of its C-terminus that contains a phosphorylation site at threonine-187, which normally targets the protein for degradation once phosphorylated.<sup>11</sup> Accumulation of this truncated, function-ally intact but degradation-deficient p27 in tumor cells contributes to their cell cycle arrest and potentially to the anticancer effects of SF3b modulation. It should be emphasized that these alterations of p27 represent only one of undoubtedly many (yet-to-be characterized) effects of spliceosome modulation on the expression of oncoproteins, tumor suppressors, and other proteins that contribute to oncogenesis.

Previous work identified some of the structural requirements for activity in 1 and PD analogues, either by modification of the natural products,<sup>4</sup> or by the total synthesis of some modified compounds (Figure 2).<sup>3,10,12,13</sup> Prior studies have shown the orientation of the acetyl group and the presence of the epoxide to be important in conferring activity to 1 analogues<sup>10,13</sup> and that the glycosidic hydroxyl group can be replaced by an alkoxy<sup>14</sup> or a methyl group<sup>9</sup> to give more stable compounds, or as in the latter case more stable *and* more active compounds<sup>9</sup> (Figure 2). Active analogues of PD also contain functionality similar to that found in 1,<sup>4,5</sup> though less structure–activity information has been reported on these more recently discovered compounds.

We have recently published the design and enantioselective synthesis of active concise analogues of 1.<sup>15</sup> Our analogue design was founded on the recognition that 1 and PD have an apparently identical mechanism of action and present a similar pharmacophore.<sup>15</sup> Molecular overlays of 1 and PD revealed that despite the gross dissimilarities between the two, both molecules share certain common key features (known to be critical to their activity) that can overlap in low energy conformations in three dimensions (Figure 3).<sup>15</sup> Our published 3D pharmacophore model prompted our proposal that compounds of the general structures **3a**, **3b**, and **3c** (see Figure 4) should share a common pharmacophore with



**Figure 3.** A 3D representation of an example of an overlay of the presumed key interaction groups in a low energy conformation of pladienolide B (shown in yellow; some atoms have been removed for clarity) and compound **3a**, showing that the epoxy group and the carbonyloxy groups are the same distance in both molecules. This figure includes a calculated van der Waals interaction surface with the following color coding: purple = hydrogen-bonding, green = hydrophobic, blue = mildly polar.<sup>16</sup> This alignment represents the best *S* value (*S* = 167.18) for molecules matching the hypothetical pharmacophore and the second best overall of 69 alignments from 500 iterations. The alignment was prepared using the Molecular Operating System (MOE 2007.09, Chemical Computing Group, Inc.) using the Flexible Alignment function with both molecules, following a conformational minimization using MOE default settings.<sup>17,18</sup>



**Figure 4.** Synthetic targets that matched a proposed spliceosome modulation pharmacophore.<sup>15</sup>

**1** and PD and therefore might serve as starting points for development of both tool and lead compounds with potential application in human cancer therapy.<sup>15</sup> The proposed compound **3a** contains the unchanged methylpenta-2,4-diene and 5-oxopent-3-en-2-yl acetate groups along with the modified deoxyspiroepoxide group, with the central pyran ring replaced with a cis-1,4-substituted cyclohexyl group, whereas compounds **3b** and **3c** include additional polar modifications to the methylpenta-2,4-diene group that are very synthetically accessible. These changes (particularly for compound **3a**) were expected to impart similar conformational presentation of the linker, the epoxy, and the carbonyloxy groups to that found in **1**.

There have been numerous synthetic approaches to 1;<sup>12,14,19–22</sup> however, since these approaches target the much more complex natural product, they were not highly instructive for the synthetic targets **3a**, **3b**, and **3c**. Our approach to these concise analogues provided compounds **3a**, **3b**, and **3c** enantioselectively and diasterospecifically in a highly step-efficient manner.<sup>15</sup> Our recent report included initial data on the antitumor activity of compounds **3a**, **3b**, and **3c** against a small panel of human cancer cell lines.<sup>15</sup> Compound **3a** showed cytotoxic activity against all seven cancer lines in which it was initially assayed. In particular, compound **3a** exhibited a cytotoxic IC<sub>50</sub> of 100 nM against the JeKo-1 mantle cell lymphoma (MCL) line, which was ~50-fold selective for this line compared to the A549 lung cancer cell line. By contrast, compounds **3b** and **3c** demonstrated only modest cytotoxic effects at the highest concentration in

# Scheme 1. Synthesis of Carbamate and Ester Analogues of Compound 3a<sup>a</sup>



<sup>*a*</sup> Reagents and conditions: (a) 4-nitrophenyl chloroformate, Et<sub>3</sub>N, dichloromethane; (b) R<sub>1</sub>-piperazine, 1,2-dichloroethane; (c) isobutyryl chloride, Et<sub>3</sub>N, DMAP, CH<sub>2</sub>Cl<sub>2</sub>; (d) 4-nitrophenyl chloroformate, pyridine, THF/CH<sub>3</sub>CN; (e) (1-cycloheptyl)piperazine, 1,2-dichloroethane.

all cell lines tested (IC<sub>50</sub> > 5  $\mu$ M).<sup>15</sup> In summary, although compound 3a is less active than the natural products PD and 1 (that both possess GI<sub>50</sub> values in the single-digit nanomolar range against several tumor lines)<sup>5,6</sup> against certain cancer cell lines tested in common, compound 3a showed considerable activity (IC<sub>50</sub>  $< 5 \,\mu$ M) against all of the tumor lines examined and was especially selective and potent in the killing of the two MCL lines JeKo-1 and JVM-2.15 Additionally, we have reported that compound 3a has an effect on the cell cycle that is similar to that of the spliceosome modulator compounds PD and 1,<sup>15</sup> which induce an accumulation of cells in  $G_2/M$ , with a simultaneous decrease in the fraction of cells in S phase.<sup>3,5,6</sup> In our current studies herein, we describe the design and synthesis of multiple analogues of compound 3a together with their biological characterization, including an initial demonstration of the in vivo antitumor activity of two of the analogues. Our approach is based on the idea that the availability of new analogues related to our synthetic scaffolds will allow for the discovery of drug leads that may circumvent the liabilities that are present in 1 or PD derivatives.

### **Results and Discussion**

Modification of the ester group of compound 3a yields active novel compounds. In order to advance novel analogues of 1 as potential therapeutics for cancer, we have initiated work directed toward establishing a more robust understanding of the structure-activity relationships (SAR) of compounds in this series. We knew that the diene portion of our scaffold was sensitive to some substitution by heteroatoms (see compounds 3b and 3c, Figure 4).<sup>15</sup> We initiated experiments designed to test the hypothesis that 1 and PD (and compound 3a) share a common pharmacophore consisting of a carbonyloxy group (2 hydrogen-bond acceptors), a rigid diene linker (hydrophobic), and a potentially reactive secondary epoxide (electrophilic hydrogen-bond acceptor) arranged in a specific three-dimensional orientation constrained by the possible overlap of 1, 3a, and PD (see Figure 3).<sup>15</sup> In addition to the acid-sensitive electrophilic epoxide group,

both of the natural products 1 and PD contain an acetyl ester group that would be expected to have limited stability in vivo due to the susceptibility of ester groups to hydrolysis by ubiquitous plasma and cellular enzymes that exhibit ester hydrolase activity.<sup>23</sup> Indeed, this acetyl has been replaced with a basic carbamate in the clinical compound 2 (Figure 1) that has been reported to have better in vivo activity compared to PD.<sup>4</sup> The presence of significant esterase metabolic activity in vivo has been widely studied in the development of prodrug lead compounds<sup>24</sup> and is known to show sig-nificant species variability.<sup>25</sup> Thus, our first experiments were directed toward experimentally testing the requirements for activity of analogues containing modifications of the O-acetyl and epoxide groups of compound 3a, using chemistry shown in Scheme 1. We first chose to replace the acetyl with the more hindered isobutyryl ester (compound 5) using the alcohol intermediate (compound 4) from our published synthesis of acetyl derivative 3a, since no information regarding such modifications have been published and because such hindered esters could be more stable in vivo.<sup>24</sup> We next prepared carbamate derivatives that contain a basic amine group, since it is known that in pladienolide analogues such compounds (e.g., 2, Figure 1) maintain substantial in vivo activity and indeed have entered clinical trials.<sup>4</sup> This type of modification is expected to impart both improved solubility and esterase resistance to target compounds. In addition, good synthetic protocols exist for the preparation of carbamates of this type.<sup>26</sup> The key intermediate in the preparation of these carbamates is carbonate 6, which was converted to basic carbamates 7-10 using the published protocol with minor modifications (Scheme 1).<sup>26</sup> Additionally, it is known that the "epoxide-opened" chloro alcohol 1 derivative<sup>6-8</sup> and related derivatives also show activity in tumor growth inhibition like 1. To confirm the importance of the intact epoxide group for our analogues, we explored the synthesis of the ring-opened compound (11, Scheme 1), which is analogous to the known chloro alcohol analogue of  $1.^{6-8}$ 

cell line <sup>a</sup>	$IC_{50} (\mu M)$								
	3a	4	5	7	8	9	10	11	
JeKo-1	0.10	1.6	0.12	ND	> 0.5	> 0.5	> 0.5	ND	
JVM-2	0.13	1.5	0.19	> 1.0	> 0.5	> 0.5	> 0.5	>10.0	
PC-3	2.1	10	2.2	> 5.0	> 2.5	> 2.5	> 2.5	> 5.0	
WiDr	2.0	10	0.91	4-5	> 1.25	> 1.25	> 1.25	> 1.25	

Table 1. In Vitro Cytotoxicity Screening Data As Determined by XTT for Compounds from Scheme 1, after a 72 h Exposure

<sup>a</sup> JeKo-1 and JVM-2, mantle cell lymphoma; PC-3, prostate adenocarcinoma; WiDr, colorectal adenocarcinoma.

We then screened compounds 4. 5. and 7-11 for in vitro cytotoxicity and obtained the results shown in Table 1. Acylation of the alcohol formed ester 5, which retained potency similar to compound 3a. This compound was prepared with the expectation that it might have a longer halflife in mouse and human plasma, since it would be expected to be more resistant to esterases present in many biological fluids (see the section on chemical stability of compounds 3a and 5 below).<sup>24</sup> It is interesting to note that the alcohol 4shows a dramatic reduction in activity when compared to the esters 3a and 5 (Table 1); this is very relevant to the in vivo activity of these compounds, since it is known that unhindered esters have a very limited half-life in mouse plasma in vivo.<sup>24</sup> The more hindered ester 5 demonstrates an in vitro cytotoxicity profile similar to that of compound **3a** in every tumor line we have examined. Carbamate derivatives 7-10 showed significant reduction in cytotoxic activity by > 5-fold, as in the case of the JVM-2 line (Table 1). The epoxide ring-opened compound (11) shows an additional reduction in activity in this case (see Table 1).<sup>6</sup> For comparison, the literature contains only one report of an active carbamate PD analogue (2), which is 4-fold less active than pladienolide B.<sup>4</sup> It is noted that despite several very high quality total syntheses,<sup>9,13,14,20,22</sup> the complexity of **1** has hindered development of extensive SAR data, and there have been no reports on the activity of corresponding 1 reference compounds to compare to most of the analogues in Scheme 1. For example, there are no reports on the synthesis, or bioactivity, of carbamate analogues of 1 to directly compare to carbamates 7-10. Our recent results are very useful for the design of compounds for evaluation in in vivo efficacy models, since we now know it is relatively easy to prepare many more new ester derivatives that may have significantly different pharmacokinetic behavior and yet still retain reasonably potent tumor cytotoxicity in vitro (see Scheme 2 below).

Increased Chemical Stability of Compounds 3a and 5 Compared to 1 or Meayamycin. One of the goals of our initial animal studies is to modify the various pharmacodynamic properties of our analogues so that we may determine how this affects the efficacy and toxicity of spliceosome modulators in antitumor efficacy studies. We have noted that the biologically active compounds 3a and 5 are chemically stable in physiologic buffer (phosphate buffered saline, pH 7.4;  $t_{1/2} = 1200$  h at 22 °C) and DMSO (no detectable decomposition after 2 weeks at 22 °C) but are rapidly degraded in dilute aqueous acid. This is in marked contrast to the published stability of 1 ( $t_{1/2} = 4.0$  h at 37 °C at pH 7.4)<sup>9</sup> and analogues reported in the literature (the most stable of which has a  $t_{1/2} = 48$  h at 37 °C at pH 7.4).<sup>9</sup>

**Tumor Cell Line Screening Results.** Compound **5** exhibits selective antitumor activity against certain adult and pediatric cancer types. Given our initial SAR data, we sought to explore the sensitivity of tumor lines in the NCI-60 tumor cell

Scheme 2. Synthesis of New Ester Analogues<sup>a</sup>



<sup>*a*</sup> Reagents and conditions. (a) For compound **12** the corresponding acid, EDC, and DMAP were used. In all other cases the conditions were acid chloride/Et<sub>3</sub>N/DMAP. In vitro cytotoxicity screening data as determined by XTT assays are shown for the compounds following a 72 h exposure.

line screen.<sup>27</sup> This screen identified a number of lines that appeared to be particularly sensitive to compound **5** (see Supporting Information Figure S1). Using the NCI-60 cell line screen as a starting point, we have begun to validate and determine the IC<sub>50</sub> of compound **5** for various adult cancer types in addition to the cancer cell lines used in Table 1 (see Table 2, upper panel).

In addition to adult cancers, we were also interested in the response of the pediatric malignancies, which by and large are cancers completely different from those that occur in adult patients, to this class of agents as well. Toward this goal, we selected a representative panel of cell lines derived from the more frequent childhood cancers for examination, with a special focus directed to the inclusion of those tumor types for which the prognosis remains poor, even following current best available clinical management (examples include high-risk neuroblastoma, MLL fusion-positive acute leukemia, and rhabdoid tumor).<sup>28–31</sup> As shown in Table 2, middle panel, the pediatric cancer cell lines were, in general, sensitive to compound 5. Importantly, lines representing several poor-prognosis malignancies such as MLL-AF4positive acute myeloid leukemia (MV-4-11) and high-risk neuroblastoma (NB-1643) were very sensitive to the cytotoxic activity of compound 5, with IC<sub>50</sub> values of  $\sim$ 300 and 230 nM, respectively. Certain other pediatric tumor types that are also very aggressive but for which the prognosis is less dismal, if the patients undergo aggressive therapies that are frequently associated with both short- and long-term morbidity as well as occasional mortality, were quite sensitive. For instance, one example of such a tumor, Burkitt's lymphoma, demonstrated marked sensitivity to compound 5 (cell line Ramos;  $IC_{50} \approx 80$  nM). Although obviously quite preliminary, these data are nonetheless promising and suggest a potential role for spliceosome modulator therapy not

 Table 2. Compound 5 Cytotoxicity Determinations against Various

 Adult and Pediatric Tumor Cell Lines and Cells from Normal Tissues<sup>a</sup>

cell line	cell type	$IC_{50}(\mu M)$	
	Adult Cancer Type		
SK-MEL-2	melanoma	$0.29 \pm 0.04$	
SK-MEL-5	melanoma	$0.64 \pm 0.07$	
SK-MEL-28	melanoma	$0.55 \pm 0.15$	
MIA PaCa-2	pancreatic carcinoma	$1.20 \pm 0.12$	
PANC-1	pancreatic carcinoma	$3.87\pm0.70$	
	Pediatric Cancer Type		
MOLT-4	acute lymphoblastic leukemia	$0.08\pm0.004$	
Ramos	Burkitt's lymphoma	$0.08\pm0.008$	
NB-1643	neuroblastoma	$0.23\pm0.04$	
MV-4-11	acute myeloid leukemia	$0.30\pm0.01$	
Rh41	rhabdomyosarcoma	$0.55\pm0.06$	
NB-EBc1	neuroblastoma	$0.66\pm0.05$	
SUP-B15	B-cell precursor leukemia	$0.67\pm0.14$	
Rh30	rhabdomyosarcoma	$0.69\pm0.12$	
SK-NEP-1	Ewing sarcoma	$0.70\pm0.07$	
SJ-GBM2	glioblastoma	$0.90\pm0.09$	
Karpas-299	anaplastic large-cell lymphoma	$0.92 \pm 0.14$	
BT-14	medulloblastoma	$1.34 \pm 0.23$	
BT-12	rhabdoid tumor	$2.79 \pm 0.12$	
BT-3	medulloblastoma	$4.41\pm0.26$	
	Normal Tissue		
PrEC	normal prostate epithelial cell	$7.01 \pm 1.1$	
HFF	human foreskin fibroblast	>10	
HFF/tert2	human foreskin fibroblast	$9.69 \pm 0.09$	
	(immortalized)		

<sup>*a*</sup> The cells were treated with compound **5** for 72 h, and cytotoxicity was determined by XTT assay. Data represent the mean  $\pm$  SEM (standard error of the mean) from three independent experiments.

only in the management of certain adult cancers but for selected pediatric malignancies as well.

To gain preliminary insight into the toxicity of these compounds in normal tissues, we also investigated the cytotoxicity of compound **5** in normal prostate cells and human foreskin fibroblasts (Table 2, lower panel). We found that in the PrEC (normal prostate epithelial cell) line compound **5** had an IC<sub>50</sub> of  $7.0 \pm 1.1 \,\mu$ M and IC<sub>50</sub> values of  $> 10 \,\mu$ M and  $\sim 10 \,\mu$ M in the normal human foreskin fibroblasts (HFF) and HFF/tert2 (HFF immortalized by expression of telomerase), respectively. These results indicate a significant selectivity of compound **5** for many tumor versus normal cell lines, even under cell culture conditions (e.g., compound **5** shows an IC<sub>50</sub> of 0.08  $\mu$ M for MOLT-4 acute T-cell leukemia and Ramos Burkitt's lymphoma cell lines under identical conditions, indicating a selectivity index of 125 or greater compared to the HFF cells).

Synthesis and in Vitro Cytotoxicity Characterization of Additional Ester Analogues of Compound 3a. To follow up on the promising activity of ester analogue 5, we next prepared a set of diverse ester analogues by acylation of alcohol 4 (Scheme 2). These ester derivatives were expected to give analogues with improved plasma stability or solubility or both. As seen in Scheme 2, though highly polar modifications were not well tolerated, weakly basic groups could be abided with only a modest decrease in cytotoxic activity. Most interesting, additional branching of the hydrophobic group gave compound 19, which showed  $IC_{50}$ values of ~40 and 800 nM for the test cell lines JeKo-1 and PC-3, respectively (Scheme 2, lower right panel). Although we have yet to test the plasma half-life of compound 19, the additional hindrance imparted by the *tert*-butyl group in this compound is expected to convey additional plasma stability, as was seen with isobutyryl ester **5**.

Initial Determination of a Tolerated Dose for Compound 5. As a prelude to the in vivo efficacy and toxicity studies that we plan for our spliceosome modulators, we performed preliminary maximal tolerated dose (MTD) studies using compound 5 at doses ranging from 0 to 50 mg/kg (physicochemical properties that limited the solubility of the compound using the formulation described below precluded the administration of higher doses). For this study, NOD/SCID mice (three per group) were dosed with vehicle, 5, 10, 25, or 50 mg/kg of compound 5 daily for 5 days by both iv and ip routes. Compound 5 was dissolved in 3.0% DMSO, 6.5% Tween-80 in 5% glucose solution, which is a modification of the formulation used by Mizui et al. for the spliceosome modulator pladienolide B.5 To determine compound-related toxicities, mice were monitored daily for weight loss, morbidity, and mortality. Following administration of the last dose of compound 5 on day 5, complete blood counts (CBCs) were determined along with a full diagnostic chemistry panel measuring albumin, alkaline phosphatase, alanine aminotransferase (ALT), amylase, blood urea nitrogen (BUN), calcium, creatinine, globulin, glucose, phosphorus, potassium, sodium, total bilirubin, and total protein. No significant differences in blood cell counts were observed in treated versus vehicle-only mice, indicating that compound 5 is not associated with the induction of anemia, leukopenia, or thrombocytopenia when administered under these conditions; likewise, no abnormalities of the blood chemistries were observed in the animals. The mice were monitored for 1 week following compound administration. No significant weight loss was observed in any group during or after compound administration. There was one fatality in the ip 25 mg/kg and one in the iv 50 mg/kg groups. It is unknown whether these deaths were due to compoundrelated toxicities or technical complications due to injections and/or bleeds for the CBCs and diagnostic panels; however, no fatalities were noted in subsequent efficacy studies employing these same compound 5 doses (see below), suggesting that technical complications were the likely cause. Total necropsies to assess organ histologies of animals from the various treatment and vehicle-only groups in these MTD studies showed no evidence for significant toxicities in the mice.

Initial in Vivo Efficacy Studies. Following the MTD studies described above, we sought to determine whether the doses of compound 5 that we could readily administer were capable of inhibiting tumor growth in mice (Figure 5). For these studies, we transplanted JeKo-1 mantle cell lymphoma tumors (which we had found to be sensitive to spliceosome modulator compounds, as shown in Table 1) to NOD/SCID mice on day 1. Beginning on day 4, the mice received iv injections of vehicle, 5, 10, 25, or 50 mg/kg of compound 5 daily for 5 consecutive days. No fatalities or significant weight loss were observed in any of the groups (data not shown). Administration of 10, 25, or 50 mg/kg of compound 5 each led to statistically significant decreases in tumor volumes compared to vehicle-treated mice (Figure 5). A widely used criterion for determining antitumor activity is tumor growth inhibition (T/C).<sup>32</sup> This value is calculated by dividing the median tumor volumes of treated mice by the median volumes in control mice. According to NCI standards, a  $T/C \leq 42\%$  is the minimum requirement for activity.<sup>32</sup> By performing this calculation, we determined that the 50 mg/kg compound 5 dose achieved a T/C value of 35%. Although preliminary, these data indicate that even a



Figure 5. Compound 5 inhibition of tumor growth in JeKo-1 tumor-bearing mice. JeKo-1 mantle cell lymphoma tumors were transplanted to NOD/SCID mice on day 1, and beginning on day 4, the mice (4 per group) received iv injections of vehicle, 5, 10, 25, or 50 mg/kg of compound 5 daily for 5 consecutive days. Vehicle alone did not inhibit tumor growth compared to saline-treated mice (data not shown). Arrows indicate the dosing schedule. The data are represented as the mean  $\pm$  SEM. Tumor volumes were modeled with treatment, linear and quadratic trends over time, and interaction of treatment with linear and quadratic trends over time in a repeated measure framework using autoregressive 1 AR(1) variance-covariance structure and empirical sandwich error estimates for fixed effect. Significance is reported as the comparison of tumor volumes in vehicle-treated mice to compound-treated animals. All analyses were performed using SAS software (SAS Institute, Cary, NC), Windows, version 9.1: (\*) p = 0.01; (\*\*) p < 0.0001.

first-generation, minimally optimized compound from this series of spliceosome modulators could be administered at the doses necessary to inhibit tumor growth without observable toxicity. As shown below, we have already designed and prepared additional compounds with the goal of further increasing in vivo potency, solubility, and selectivity for the spliceosome, in order to begin to optimize their antitumor potential.

Synthesis of an Active Heteroatom Modified Scaffold Related to Compound 3a, with Improved Solubility. The activity of compounds 5 and 19 was promising, but these compounds showed limited solubility in water or aqueous buffer (e.g., the measured solubility of 5 was less than 1  $\mu$ M in PBS). Since improved solubility is desirable for both the formulation and in vivo distribution of drugs, we considered alternative approaches to the introduction of more polar groups to our lead structure. Since our attempts to obtain more soluble and potent (IC<sub>50</sub> < 100 nM) analogues through the carbamate (Table 1) or polar ester (Scheme 2) approaches were not completely successful in the identification of compounds with both improved solubility and stability, we turned our attention to the introduction of more polar functionality into the scaffold of our lead compounds, without the addition of asymmetric centers. We envisioned (Scheme 3) that compounds such as the cyclic acetal 26, in which the cyclohexane ring is replaced with a 1,3-dioxane ring, would be substantially more soluble. Though the acetal is acid-sensitive, we surmised that this would not be an issue, since intravenous infusion will be the most likely method of dosing in both animal model experiments and the clinical setting.

The azidosulfone intermediate **23** was prepared from commercially available 1,1,2,2-tetramethoxypropane and 2-amino-1,3-propanediol via a selective transketalization reaction to give a known aminoacetal,<sup>33</sup> which is obtained exclusively as the cis-product, as shown in Scheme 3. This amine was





<sup>*a*</sup> Reagents, conditions, and yields: (a) 1*H*-imidazole-1-sulfonylazide·HCl, CuSO<sub>4</sub>·5H<sub>2</sub>O, K<sub>2</sub>CO<sub>3</sub>, MeOH; (b) CF<sub>3</sub>COOH/H<sub>2</sub>O/CHCl<sub>3</sub>; (c) ylide (shown), benzene; (d) (1) DIBAL-H, CH<sub>2</sub>Cl<sub>2</sub>, 64% overall; (2) 2-mercaptobenzothiazole, Ph<sub>3</sub>P, DIAD, THF, 0 °C; (3) (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> tetrahydrate/H<sub>2</sub>O<sub>2</sub>, 78% overall; (e) LiHMDS, then aldehyde **24**, 37%; (f) (1) Ph<sub>3</sub>P, H<sub>2</sub>O, benzene, 45 °C; (2) (*S*,*Z*)-4-(TBSO)pent-2-enoic acid, HBTU, <sup>7</sup>Pr<sub>2</sub>EtN, 69% overall; (3) TBAF, THF, 47%; (4) isobutyric anhydride, Et<sub>3</sub>N, DMAP, 96%; (g) (1) Ph<sub>3</sub>P, H<sub>2</sub>O, benzene, 45 °C; (2) acetic anhydride/pyridine.

converted into the corresponding azide 20,<sup>34</sup> which was then converted to the aldehyde 21 by selective hydrolysis of the dimethyl acetal functionality via TFA/H<sub>2</sub>O (19.5:0.5) in CHCl<sub>3</sub>. This aldehyde was further elaborated with the Wittig ylide (Scheme 3, step c) to give ester 22, which was reduced by DIBAL-H to provide the alcohol derivative that was converted to the corresponding Julia–Kocienski olefination reagent<sup>35</sup> via a sequential Mitsunobu reaction with the 2-mercaptobenzothiazole anion followed by oxidation of the resulting sulfide with ammonium molybdate and hydrogen peroxide to give the sulfone derivative 23 in moderate yields.

The subsequent synthetic steps then utilize and improve upon the chemistry we have published. The azide sulfone 23 intermediate was then treated with an aldehyde 24 (prepared as published<sup>15</sup>) in the presence of LiHMDS (1.1 equiv) to give the key azide diene intermediate 25. The azide group in this diene was then reduced to the corresponding amine using Ph<sub>3</sub>P and H<sub>2</sub>O, and this step was followed by amidation with the protected hydroxyl-cis-pentenoic acid to give the corresponding amide derivative. Cleavage of the TBS group in diene was accomplished with tetrabutylammonium fluoride at 0 °C, and the resulting alcohol was then treated with isobutyric anhydride to afford the final analogue 26. The overall yield of compound 26 using this approach was similar to that obtained for the synthesis of 5 (see Scheme 1). This compound showed in vitro activity similar to that of compound 5 (e.g., JeKo-1 IC<sub>50</sub> = 150 nM); furthermore, an in vivo treatment trial using JeKo-1 tumor-bearing mice revealed compound 26 to possess robust anticancer activity that was comparable to compound 5 (see Supporting Information Figure S2). As we had hoped, the solubility of this 1,3-dioxane derivative was significantly improved (measured solubility =  $47 \,\mu$ M in PBS, which is > 50-fold more soluble than the cyclohexane derivative 5).

Experimental Demonstration of Splicing Modulation by Compounds 5 and 26. As an initial step in determining whether these new compounds affect splicing, we treated HeLa cervical carcinoma cells with 10  $\mu$ M compound 26, the most soluble compound, for 4 h and examined the effects of this drug on the



Figure 6. Compound 26 causes aggregation of the splicing factor SC35 and the SF3b protein SAP155. HeLa cells were incubated for 4 h in culture media containing 10  $\mu$ M compound 26 in DMSO (B and D) or an equal volume of DMSO (A and C) and stained with antibodies recognizing SC35 (A and B) or SAP155 (C and D). The cells were then stained with fluorescent secondary antibodies and examined with a fluorescence microscope using an objective with 60× magnification.



Figure 7. Compounds 5 and 26 selectively inhibit splicing. 293T cells were transiently transfected with a dual-reporter splicing construct (see Experimental Section for details) for 48 h and subsequently treated with or without compound for 5 h. The degree of cell death was negligible at this time at the above tested concentrations. The cells were lysed, and equalized amounts of total cellular proteins were assayed for luciferase and  $\beta$ -galactosidase activity using the Dual-Light system. The data are expressed as the relative ratio of luciferase to  $\beta$ -galactosidase activity compared to a control vehicle (DMSO) treated sample and are presented as the mean  $\pm$  SEM from multiple independent experiments. Note that compound 27 (which lacks cytotoxic activity; data not shown) had no effect in this assay and that compound 4 (which exhibits only minimal cytotoxic effects at these concentrations; see Table 1) altered splicing only very slightly at the highest concentration tested; thus, the cytotoxic effects of our compounds correlate closely with their effects on splicing.

localization of splicing factors in vivo using immunofluorescence analysis and antibodies recognizing the general splicing factor SC35 and SF3b3 (SAP155). These studies revealed that compound **26** caused the splicing factors to form large aggregates, which were similar to those reported in the literature to be observed in cells treated with either PD or **1**  (Figure 6).<sup>3,4</sup> To confirm that our new compounds were functioning as spliceosome modulators in a manner similar to PD and **1**, we tested them in a dual-reporter assay that directly measures the effects on splicing in cells (Figure 7).<sup>36,37</sup> Lastly, as an additional means to conclusively demonstrate that our compounds alter splicing, we also performed an in vitro cell-free splicing assay that demonstrated compound **5** to inhibit the mRNA splicing activity of a HeLa nuclear extract (see Supporting Information Figure S3).<sup>38,39</sup>

### Conclusions

We have reported our progress on the development of new synthetic anticancer lead compounds that are concise analogues of the natural product 1. These novel compounds contain only three chiral centers, which is six less than the natural product, and were designed on the basis of our recognition of a common spliceosome modulatory pharmacophore in both 1 and PD. Our design process integrates pharmacophore information with the simultaneous execution of synthetic step efficiency and the application of enantioselective and diastereospecific synthetic approaches. This type of design approach allows for the "molecular concision" of complex natural products into more approachable synthetic targets. This in turn makes possible a more robust synthetic examination of numerous features of the spliceosome pharmacophore, which have not previously been investigated.<sup>15</sup> We present data that demonstrate these natural product analogues to be substantially more chemically stable than either 1 or the previously reported stabilized active analogue meayamycin.9 We have developed active ester and carbamate analogues of our initial compound 3a, including the equipotent hindered ester 5, that have new diverse steric and physical properties with a range of cytotoxic activity. We have explored the scope of selective antitumor cytotoxic activity of 3a and 5 and discovered a number of sensitive adult and pediatric tumor lines, which include leukemia, lymphoma, melanoma, and pancreatic carcinoma lines, among others. These findings led to some initial antitumor efficacy studies with compound 5 that clearly demonstrate significant in vivo inhibition of JeKo-1 tumor growth in immunocompromised mice following only five single daily iv doses. Additionally, we have reported the enantioselective and diastereospecific synthesis of a new template for active analogues (represented by 26) with significant improvements in measured solubility, which dramatically facilitates the formulation of these compounds for intravenous dosing. In vivo efficacy in a JeKo-1 tumor regression study has also been observed with compound 26 following a minimal 5-day dosing schedule. We also report for the first time that compound 5 inhibits the splicing of mRNA in cell-free nuclear extracts. Data are also presented showing that these compounds selectively inhibit mRNA splicing in a dual-reporter cell-based splicing assay, and there appears to be a close correlation between cytotoxicity and the inhibition of splicing by the compounds tested. In summary, we have developed a totally synthetic novel spliceosome modulator as a lead compound as one step in the development of new treatments for a number of highly aggressive cancers. Future efforts will be directed toward the more complete optimization of these compounds as potential human therapeutics.

# **Experimental Section**

**Chemistry. General Aspects.** All materials and reagents were used as is unless otherwise indicated. Air- or moisture-sensitive

reactions were carried out under a nitrogen atmosphere. THF, toluene, acetonitrile, N,N-dimethylformamide, and CH<sub>2</sub>Cl<sub>2</sub> were distilled before use. Flash column chromatography was performed according to Still's procedure using 100-700 times excess 32-64 mm grade silica gel. TLC analysis was performed using glass TLC plates (0.25 mm, 60 F-254 silica gel). Visualization of the developed plates was accomplished by staining with ethanolic phosphomolybdic acid, ceric ammonium molybdate, or ethanolic ninhydrin followed by heating on a hot plate (120 °C). All tested compounds possessed a purity of >95%as determined by ultrahigh pressure liquid chromatography on a Waters Acquity UPLC/PDA/ELSD/MS system carried out with a BEH C18 2.1 mm  $\times$  50 mm column using gradient elution with stationary phase: BEH C18, 1.7 mm; solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile. NMR spectra were obtained on Bruker Avance II 400 MHz. The values  $\delta_{\rm H}$  7.26 and  $\delta_{\rm C}$  77.0 ppm were used as references for NMR spectroscopy in CDCl<sub>3</sub>. The coupling constants deduced in <sup>1</sup>H NMR data cases were obtained by first-order coupling analysis. Analytical and preparative SFC (supercritical fluid chromatography) systems (AD-H and OD-H columns) were used for analysis and purification. IR spectra were collected using a Nicolet IR 100 (FTIR). FTIR analyses were prepared as neat and neat films on KBr plates, and the data are reported in wavenumbers (cm<sup>-1</sup>) unless specified otherwise. The University of Illinois Mass Spectroscopy Laboratories collected the high resolution mass spectral data.

(S,Z)-5-((1R,4R)-4-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)cyclohexylamino)-5-oxopent-3-en-2-yl Isobutyrate (5). A solution of the alcohol derivative 4 (215 mg, 0.52 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was allowed to stir at 0 °C as Et<sub>3</sub>N (0.36 mL, 2.63 mmol) and DMAP (12 mg, 0.10 mmol) were sequentially added. After 5 min, isobutyryl chloride (0.16 mL, 1.58 mmol) was added dropwise. The resulting solution was allowed to stir for 30 min at this temp. The reaction mixture was then diluted with water (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (30 mL). The organic layer was separated, and the aqueous layer was again extracted with  $CH_2Cl_2$  (2 × 15 mL). The combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (20 mL), brine (20 mL) and then dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated and the crude residue was purified by flash chromatography (30% ethyl acetate in hexane) to give 215 mg (84%) of 5 as a white viscous solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.25 (d, J = 5.2 Hz, 1H), 6.27 (d, J = 15.7 Hz, 1H), 5.78 (d, 11.8 Hz, 1H), 5.78–5.72 (m, 1H), 5.63 (dd, J =11.8, 9.1 Hz, 1H), 5.55 (dd, J = 15.7, 6.7 Hz, 1H), 5.49 (t, J = 7.5Hz, 1H), 4.50–4.42 (m, 1H), 4.16–4.09 (m, 1H), 2.57 (s, 2H), 2.58-2.49 (m, 1H), 2.12-2.04 (d, J = 14.2 Hz, 2H), 2.03-1.86 (m, 2H), 1.72 (s, 3H). 1.72–1.66 (m, 1H), 1.64–1.54 (m, 6H), 1.40 (s, 3H), 1.36 (d, J = 6.4 Hz, 3H), 1.28 (s, 3H), 1.25 - 1.19 (m, 3H)3H) 1.17 (d, J = 7.0 Hz, 6H), 1.14 (d, J = 2.0 Hz, 1H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 177.36, 164.87, 137.59, 136.22, 133.77, 132.06, 126.97, 125.62, 73.04, 69.59, 68.97, 55.69, 51.05, 45.31, 42.46, 38.64, 36.60, 34.44, 34.00, 31.55, 29.58, 29.47, 27.84, 27.54, 23.77, 20.30, 18.98, 18.86, 12.49; IR (neat film) 3314, 2973, 2931, 2856, 1733, 1688, 1628, 1533, 1450, 1368, 1241, 1195, 1047 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for C<sub>29</sub>H<sub>46</sub>NO<sub>5</sub> (M + 1)<sup>+</sup> 488.3376, found 488.3364

(S,Z)-5-((1R,4R)-4-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)cyclohexylamino)-5-oxopent-3-en-2-yl 4-Nitrophenylcarbonate (6). A stirred solution of alcohol 4 (45 mg, 0.108 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was treated with Et<sub>3</sub>N (30  $\mu$ L, 0.21 mmol) and 4-nitrophenyl chloroformate (32.6 mg, 0.16 mmol) at room temperature. The resulting light-yellow suspension was allowed to stir for 4 h at room temperature. This mixture was then diluted with CH<sub>2</sub>Cl<sub>2</sub> (20 mL), washed with aqueous 1 N HCl (8 mL), saturated aqueous NaHCO<sub>3</sub> (8 mL), and brine (8 mL), and dried over Na<sub>2</sub>SO<sub>4</sub>. Concentration and purification of the residue by flash chromatography (30–40% ethyl acetate in hexane) gave 36 mg (57%) of **6** as a liquid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.38–8.19 (m, 2H), 7.49–7.33 (m, 2H), 6.32–6.22 (m, 2H), 6.11 (d, J = 7.5 Hz, 1H), 5.97 (dd, J = 11.6, 8.1 Hz, 1H), 5.85 (dd, J = 11.6, 1.0 Hz, 1H), 5.54 (dd, J = 15.7, 6.7 Hz, 1H), 4.07 (s, 1H), 2.58 (s, 2H), 2.04 (t, J = 6.4 Hz, 2H), 1.98–1.88 (m, 2H), 1.67 (s, 3H), 1.63–1.57 (m, 6H), 1.53 (d, J = 6.5 Hz, 3H), 1.40 (s, 3H), 1.28 (s, 3H), 1.25–1.13 (m, 5H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  164.12, 155.51, 151.90, 145.36, 140.14, 136.10, 133.94, 131.63, 127.19, 125.27, 124.76, 121.75, 74.38, 73.08, 69.59, 55.69, 51.06, 45.58, 42.46, 38.63, 36.38, 34.14, 31.54, 29.39, 29.32, 27.87, 27.71, 23.79, 20.05, 12.50; IR (neat film) 3316, 2974, 2930, 2856, 1766, 1668, 1631, 1526, 1347, 1256, 1215, 1048 cm<sup>-1</sup>; HRMS (ESI) *m*/*z* calcd for C<sub>32</sub>H<sub>43</sub>N<sub>2</sub>O<sub>8</sub> (M + 1)<sup>+</sup> 583.3019, found 583.3028.

(S,Z)-5-((1R,4R)-4-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)cyclohexylamino)-5-oxopent-3-en-2-yl 4-Cycloheptylpiperazine-1-carboxylate (7). A solution of the activated carbonate (6) (4.2 mg, 7.21 µmol) in 1,2-dichloroethane (0.2 mL) was allowed to stir at room temperature as 1-cycloheptylpiperazine (0.21 mL, 0.02 mmol, 0.1 M stock solution) was added. The resulting solution was stirred for 75 min, and the solvent was then removed under vacuum. The crude was purified by flash chromatography (2-3% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to give 1.2 mg (27%) of 7 as a viscous oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.22–8.08 (m, 1H), 6.27 (d, J = 15.7 Hz, 1H), 5.82 (d, J = 11.2 Hz, 1H), 5.64-5.47 (m, 4H), 4.55-4.40 (m, 1H), 4.20-4.07 (m, 1H), 3.46 (s, 3H), 2.57 (s, 2H), 2.49 (s, 3H), 2.08 (s, 2H), 2.10-2.03 (m, 2H), 2.02-1.87 (m, 2H), 1.83-1.76 (m, 2H), 1.77 (s, 3H), 1.65-1.50 (m, 14H), 1.47-1.43 (m, 3H), 1.40 (s, 3H), 1.34 (d, J = 6.0 Hz, 3H), 1.28 (s, 3H), 1.25–1.14 (m, 5H); IR (neat film) 3300, 2926, 2855, 1668, 1535, 1434, 1372, 1244, 1121, 1049 cm<sup>-</sup> HRMS (ESI) m/z calcd for C<sub>37</sub>H<sub>60</sub>N<sub>3</sub>O5 (M + 1)<sup>+</sup> 626.4533, found 626.4546.

(S,Z)-5-((1R,4R)-4-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)cyclohexylamino)-5-oxopent-3-en-2-yl 1-Methylpiperidine-4-carboxylate (12). A solution of alcohol derivative  $4^{15}$  (10 mg, 0.024 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.22 mL) was treated with 1-methylpiperidine-4carboxylic acid (9.3 mg, 0.065 mmol), diisopropylethylamine (13  $\mu$ L, 0.072 mmol), and DMAP (7.3 mg, 0.06 mmol). The resulting solution was cooled to 0 °C and treated with solid EDC (11.4 mg, 0.06 mmol). The resulting suspension was allowed to stir at room temperature overnight. Saturated aqueous NH<sub>4</sub>Cl (1 mL) was added, and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub>  $(2 \times 5 \text{ mL})$ . The combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (3 mL) and brine (3 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. This was concentrated and the residue was purified by flash column (5% MeOH in CHCl3 with 0.2% Et3N) to give 5 mg (39%) of **12** as a viscous liquid. <sup>1</sup>H NMR (400 MHz,  $\overline{CDCl_3}$ )  $\delta$ 7.04 (bd, 1H), 6.27 (d, J = 15.7 Hz, 1H), 5.90–5.74 (m, 2H), 5.71-5.42 (m, 3H), 4.46 (ddd, J = 11.1, 6.7, 1.8 Hz, 1H), 4.12 (m,1H), 2.82 (d, J = 11.5 Hz, 2H), 2.57 (s, 2H), 2.28 (s, 4H), 2.12-1.90 (m, 9H), 1.72 (s, 3H), 1.70-1.66 (m, 2H), 1.64-1.54 (m, 6H), 1.40 (s, 3H), 1.34 (d, 3H), 1.27 (s, 3H), 1.26-1.12 (m, 4H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  175.07, 164.78, 138.02, 136.22, 133.80, 132.02, 127.00, 125.50, 73.05, 69.61, 69.17, 55.70, 54.81, 51.06, 46.28, 45.86, 45.37, 42.47, 38.65, 36.60, 34.42, 31.55, 29.55, 29.44, 28.07, 27.87, 27.60, 23.78, 20.27, 12.52; IR (neat film) 3284, 2927, 2853, 1729, 1666, 1628, 1533, 1449, 1374, 1218, 1182, 1047 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for  $C_{32}H_{51}N_2O_5 (M + 1)^+$  543.3798, found 543.3784.

General Procedure for the Preparation of Ester Derivatives from Alcohol 4<sup>15</sup> Using a Variety of Acid Chlorides. A stirred solution of the alcohol derivative 4<sup>15</sup> (10 mg, 0.52 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (0.5 mL) at 0 °C was sequentially treated with Et<sub>3</sub>N (16.6  $\mu$ L, 0.12 mmol) and DMAP (4.8  $\mu$ L, 0.10 mmol, 0.5 M in CH<sub>2</sub>Cl<sub>2</sub>). After 5 min at 0 °C, the appropriate acid chloride (0.07 mmol) was added dropwise. The resulting solution was then allowed to stir for 1 h at the same temperature. The reaction mixture was then diluted with water (3 mL) and  $CH_2Cl_2$  (6 mL). The aqueous layer was extracted with  $CH_2Cl_2$  (2 × 5 mL). The combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (3 mL) and brine (3 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated, and the crude residues were purified by flash chromatography (ethyl acetate in hexane) to give corresponding ester derivatives.

(S,Z)-5-((1R,4R)-4-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)cyclohexylamino)-5-oxopent-3-en-2-yl Tetrahydro-2H-pyran-4-carboxylate (13). Yield: 8 mg (63%) of 13 as a viscous oil. <sup>1</sup>H NMR (400MHz, CDCl<sub>3</sub>)  $\delta$  7.00 (d, J = 7.7 Hz, 1H), 6.27 (d, J = 15.7 Hz, 1H), 5.93-5.83 (m, 1H), 5.80 (d, J = 11.7 Hz, 1H), 5.65 (dd, J =11.7, 8.9 Hz, 1H, 5.55 (dd, J = 15.7, 6.7 Hz, 1H), 5.48 (t, J = 7.5 Hz, 1H) Hz, 1H), 4.52–4.40 (m, 1H), 4.11 (s, 1H), 3.96 (dt, J = 11.6, 3.6 Hz, 2H), 3.43 (td, J = 11.3, 2.9 Hz, 2H), 2.57 (s, 2H), 2.56–2.47 (m, 1H), 2.10–2.04 (m, 2H), 2.02–1.87 (m, 2H), 1.86–1.74 (m, 4H), 1.72 (s, 3H), 1.71-1.65 (m, 1H), 1.64-1.55 (m, 6H), 1.40 (s, 3H), 1.37 (d, J = 6.5 Hz, 3H), 1.28 (s, 3H), 1.26–1.12 (m, 4H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  174.60, 164.71, 138.11, 136.19, 133.81, 131.97, 127.05, 125.45, 73.05, 69.60, 69.30, 67.04, 67.02, 55.69, 51.05, 45.38, 42.46, 40.14, 38.64, 36.56, 34.42, 31.55, 29.72, 29.54, 28.64, 28.59, 27.88, 27.60, 23.78, 20.25, 12.52; IR (neat film) 3319, 2927, 2853, 1731, 1668, 1629, 1533, 1447, 1378, 1184, 1093, 1046 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for C<sub>31</sub>H<sub>48</sub>NO<sub>6</sub>  $(M + 1)^+$  530.3482, found 530.3481.

(2s,5s)-5-Azido-2-(2,2-dimethoxyethyl)-1,3-dioxane (20). To a solution of (2s,5s)-2-(2,2-dimethoxyethyl)-1,3-dioxan-5amine<sup>33</sup> (59.8 g, 313 mmol) in MeOH (1.5 L) at 0 °C was added K<sub>2</sub>CO<sub>3</sub> (86 g, 625 mmol), CuSO<sub>4</sub>·5H<sub>2</sub>O (780 mg, 3.13 mmol), and 1H-imidazole-1-sulfonylazide ·HCl (65.3 g, 375 mmol). The mixture was warmed to room temperature and stirred overnight. The reaction mixture was concentrated and diluted with water. The resulting mixture was extracted with EtOAc  $(3 \times 350 \text{ mL})$ . The combined organic fractions were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. The crude product was chromatographed using 30% EtOAc/hexane with 0.5% Et<sub>3</sub>N to obtain the azide acetal (20) as a colorless oil. Yield: 33.65 g (50%). <sup>1</sup>H NMR (400 MHz, CDCl3)  $\delta$  4.72 (t, J = 5.5 Hz, 1H), 4.56 (t, J =5.9 Hz, 1H, 4.21 (dd, J = 1.4, 12.5 Hz, 2H), 4.05 - 3.98 (m, 2H),3.33 (d, J = 3.7 Hz, 6H), 3.01 (s, 1H), 1.99 (t, J = 5.7 Hz, 2H);<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  100.90, 99.96, 69.59, 53.38, 53.13, 38.19; IR (neat film) 2950, 2107, 1409, 1123; HRMS (ESI) m/z calcd for C<sub>8</sub>H<sub>15</sub>N<sub>3</sub>O<sub>4</sub> (M + Na) 240.0960, found 240.0953.

(*E*)-Ethyl 4-((2s,5s)-5-Azido-1,3-dioxan-2-yl)-2-methylbut-2enoate (22). Dioxane 20 (33.65 g, 155 mmol) was dissolved in CHCl<sub>3</sub> (1 L) with water (6.25 mL, 347 mmol) and cooled to 0 °C. TFA (250 mL, 3.37 mol) was added to the reaction mixture to give 20% TFA/CHCl<sub>3</sub> which was stirred at 0 °C for 40 min. The reaction was quenched with solid NaHCO<sub>3</sub> (400 g, 4.76 mol) at 0 °C. The reaction mixture was filtered and concentrated. The resulting aldehyde 21 was used in the next step without further purification and contained 20% of the starting material 20 based on <sup>1</sup>H NMR. Data for 21: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.79 (t, J = 2.0 Hz, 1H), 5.10 (t, J = 4.7 Hz, 1H), 4.25 (dd, J = 12.6, 1.4 Hz, 2H), 4.06 (dd, J = 12.6, 1.6 Hz, 2H), 3.05 (s, 1H), 2.76 (dd, J = 4.7, 2.0 Hz, 2H).

(Carbethoxyethyldiene)triphenylphosphorane (51.15 g, 141 mmol) was added to a solution of this crude aldehyde **21** (26.59 g, 155 mmol) dissolved in benzene (333 mL) in an ice bath. The reaction mixture was warmed to room temperature and stirred for 2 h. TLC was done in 20% EtOAc/hexane. The reaction mixture was concentrated and the crude product was chromatographed using 20% EtOAc/hexane with 0.5% Et<sub>3</sub>N to give **22** with **20** as an oil. Yield: 28.61 g. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.78 (td, J = 7.2, 1.5 Hz, 1H), 4.74 (t, J = 5.1 Hz, 1H), 4.24 (dd, J = 12.5, 1.4 Hz, 2H), 4.18 (q, J = 7.1, 5.2, 1.0 Hz, 2H), 1.84 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.4 Hz, 3H); <sup>13</sup>C NMR (101 MHz,

CDCl<sub>3</sub>)  $\delta$  167.80, 134.53, 130.54, 101.12, 69.76, 60.58, 53.12, 34.44, 14.28, 12.71; IR (neat film) 2981, 2860, 2105, 1707, 1654, 1451, 1254, 885; HRMS (ESI) *m*/*z* calcd for C<sub>11</sub>H<sub>17</sub>N<sub>3</sub>O<sub>4</sub> (M + Na) 278.1117, found 278.1106.

(*E*)-4-((2*s*,5*s*)-5-Azido-1,3-dioxan-2-yl)-2-methylbut-2-en-1-ol. A solution of DIBAL-H (280 mL, 1 M in hexanes, 280 mmol) was added dropwise to a well stirred solution of ester 22 (28.61 g, 112 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (500 mL) that had been precooled to -78 °C in an acetone/dry ice bath for 1 h. After this time the reaction was quenched successively with MeOH (200 mL) at -78 °C and saturated aqueous potassium sodium tartrate tetrahydrate (200 mL). The reaction mixture was then filtered and extracted with  $CH_2Cl_2$  (3 × 300 mL). The combined organic fractions were dried over MgSO4 and concentrated. The crude product was chromatographed using 30% EtOAc/hexane to give 17.32 g (52%, in three steps) of pure alcohol along with 4 g (12%) of **20**. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.50 (s, 1H), 4.67 (t, J = 4.9 Hz, 1H), 4.24 (d, J = 11.1 Hz, 2H), 4.05-3.99 (m, J = 11.1 Hz, 3.01 (m, J = 11.1 Hz, 3.01 (m, J = 11.1 Hz), 4.05-3.99 (m, J =4H), 2.96 (s, 1H), 2.49-2.41 (m, 2H), 1.67 (s, 3H), 1.46 (bs, 1H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 137.97, 118.29, 102.03, 69.82, 68.56, 53.20, 33.36, 13.94; IR (neat film) 3397, 2917, 2861, 2107, 1393, 1289, 1243, 1142, 1043, 1007; HRMS (ESI) m/z calcd for  $C_9H_{15}N_3O_3$  (M + Na) 236.1011, found 236.1004.

2-((E)-4-((2s,5s)-5-Azido-1,3-dioxan-2-yl)-2-methylbut-2-enylthio)benzo[d]thiazole. A solution of 2-mecaptobenzothiazole (16.1 g, 97 mmol) and Ph<sub>3</sub>P (24.3 g, 93 mmol) in anhydrous THF (350 mL) was stirred at 0 °C as DIAD (20.2 mL, 97 mmol) in toluene (40 mL) was added slowly over 30 min. The resulting yellow suspension was allowed to stir for 15 min as the alcohol from above (17.2 g, 81 mmol) in anhydrous THF (80 mL) was added dropwise over 20 min. The resulting yellow suspension was allowed to stir for 30 min at 0 °C. The reaction mixture was then diluted with hexane (200 mL) and ethyl acetate (200 mL), and the resulting solids were filtered and washed with ethyl acetate (100 mL). This mixture was concentrated, then diluted with ethyl acetate (400 mL) and washed with saturated aqueous NaHCO3 (100 mL) and water (100 mL), then dried over Na<sub>2</sub>SO<sub>4</sub>. This solution was concentrated and purified by flash chromatography (30% ethyl acetate in hexane) to give 25 g (84%) of the sulfide as a viscous oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.87 (d, J = 8.2 Hz, 1H), 7.75 (dd, J = 8.0, 0.6 Hz, 1H), 7.45 - 7.37 (m, 1H), 7.33 - 7.27(m, 1H), 5.62 (t, J = 7.1 Hz, 1H), 4.56 (t, J = 5.1 Hz, 1H), 4.18 (d, J = 5.1 Hz, 1H), 4.18 (dJ = 11.6 Hz, 2H), 4.00 (s, 2H), 3.93 (dd, J = 12.4 Hz, 2H), 2.95 (s, 1H), 2.48–2.37 (m, 2H), 1.80 (s, 3H); <sup>13</sup>C NMR (101 MHz, 2H), 1.80 (s, 2H), 1.80 CDCl<sub>3</sub>) & 166.84, 153.22, 135.42, 132.62, 126.00, 124.22, 123.40, 121.62, 120.92, 101.87, 69.64, 53.25, 42.96, 33.97, 15.60; IR (neat film) 2975, 2917, 2856, 2104, 1457, 1426, 1291, 1239, 1140, 1044, 1004 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for C<sub>16</sub>H<sub>19</sub>N<sub>4</sub>O<sub>2</sub>S<sub>2</sub> (M + 1)<sup>+</sup> 363.0949, found 363.0942.

2-((E)-4-((2s,5s)-5-Azido-1,3-dioxan-2-yl)-2-methylbut-2-enylsulfonyl)benzo[d]thiazole (23). A solution of sulfide derivative from above (0.3 g, 0.82 mmol) in EtOH (6 mL) was treated with a mixture of ammonium molybdate · 4H<sub>2</sub>O (0.2 g, 0.16 mmol) and 30% H<sub>2</sub>O<sub>2</sub> (1 mL, 8.28 mmol), and the pH was adjusted to 4-5 with 7.0 buffer. This solution was allowed to stir for 4 h at room temperature and then diluted with water (15 mL) and extracted with  $CHCl_3$  (2 × 20 mL). The combined organic phase was washed with brine (25 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was then removed under reduced pressure and the residue was purified by flash chromatography (40-65% ethyl acetate in hexane) to give 23 (254 mg, 78%) as a viscous oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.29-8.21 (m, 1H), 8.05-7.98 (m, 1H), 7.68-7.55 (m, 2H), 5.37 (t, J = 7.1 Hz, 1H), 4.30 (t, J = 5.3 Hz, 1H), 4.17 (s, 2H), 4.03-3.95 (m, 2H), 3.77-3.68 (m, 2H), 2.84 (s, 1H), 2.36–2.28 (m, 2H), 1.86 (s, 3H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  165.57, 152.78, 137.02, 130.65, 127.90, 127.57, 125.60, 125.03, 122.29, 101.14, 69.48, 64.41, 53.01, 34.05, 17.12; IR (neat film) 2974, 2922, 2857, 2105, 1471, 1422, 1327, 1291, 1239, 1136, 1021 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for  $C_{16}H_{19}N_4O_4S_2 (M + 1)^+$  395.0848, found 395.0848.

(3R,7S)-7-((1E,3E)-5-((2s,5R)-5-Azido-1,3-dioxan-2-yl)-3-methylpenta-1,3-dienyl)-5,5-dimethyl-1,6-dioxaspiro[2.5]octane (25). A solution of the sulfone derivative 23 (215 mg, 0.55 mmol) in anhydrous THF (8 mL) was allowed to stir at -78 °C, while a solution of lithium bis(trimethylsilyl)amide (0.62 mL, 0.64 mmol, 1.0 M in THF) was added dropwise. After the mixture was stirred for 30 min at -78 °C, aldehyde  $24^{15}$  (93 mg, 0.54 mmol) in anhydrous THF (5 mL) was added dropwise via syringe. The resulting suspension was stirred at -78 °C for 1.5 h, allowed to warm to room temperature, and stirred for 12 h. The mixture was then poured into a saturated aqueous NH<sub>4</sub>Cl solution (10 mL), and the product was extracted with ethyl acetate  $(3 \times 25 \text{ mL})$ . The combined extracts were washed with saturated aqueous NaHCO3 (20 mL) and brine (20 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent and purification by flash chromatography (20% ethyl acetate in hexane) gave 70 mg (37%) of diene 25 (88:12 E/Z) as a viscous liquid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.27 (d, J = 15.8 Hz, 1H), 5.60 (dd, *J* = 15.7, 6.6 Hz, 1H), 5.51 (t, *J* = 7.3 Hz, 1H), 4.62 (t, J = 5.2 Hz, 1H), 4.46 (ddd, J = 11.1, 6.7, 1.8 Hz, 1H), 4.27-4.19 (m, 2H), 4.00 (dd, J = 12.4, 2.1 Hz, 2H), 2.98 (s, 1H), 2.59–2.55 (m, 2H), 2.54–2.49 (m, 2H), 2.04–1.85 (m, 2H), 1.74 (s, 3H), 1.40 (s, 3H), 1.28 (s, 3H), 1.25–1.11 (m, 2H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 135.75, 135.68, 127.95, 125.51, 102.09, 73.03, 69.72, 69.49, 55.68, 53.30, 51.06, 42.46, 38.57, 34.08, 31.54, 23.77, 12.61; IR (neat film) 2974, 2915, 2861, 2105, 1447, 1328, 1289, 1141, 1045 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for  $C_{18}H_{28}N_3O_4 (M + 1)^+$  350.2080, found 350.2067.

(S,Z)-4-(*tert*-Butyldimethylsilyloxy)-*N*-((2R,5R)-2-((2E,4E)-5-((3R,5S)-7,7-dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)-1,3-dioxan-5-yl)pent-2-enamide. A solution of the azide derivative 25 (2 g, 5.72 mmol) in benzene (40 mL) at room temperature under N<sub>2</sub> was allowed to stir as Ph<sub>3</sub>P (4.50 g, 17.17 mmol) was added. The resulting solution was then heated at 45 °C for 2.5 h, by which time LC/MS showed that the starting material had been consumed. Water (1 mL) was added and the mixture was heated at 45 °C for an additional 2 h, by which time LC/MS indicated that the formation of free amine was complete. The reaction mixture was cooled to room temperature, diluted with CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and ether (20 mL), and then dried over Na<sub>2</sub>SO<sub>4</sub>. This mixture was then filtered and concentrated. The residue was dissolved in anhydrous acetonitrile (6 mL) and used in the next step without further purification.

A stirred solution of (S,Z)-4-(TBSO)pent-2-enoic acid<sup>40</sup> (2.6 g, 11.44 mmol) in anhydrous CH<sub>3</sub>CN (30 mL) at 0 °C was treated with 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4.1 g, 10.87) and diisopropylethylamine (6.12 mL, 34.3 mmol) sequentially. The above reaction solution was added to a solution containing the amine from above in CH<sub>3</sub>CN (30 mL) at 0 °C. The resulting solution was then stirred for another 2 h at room temperature at which time LC/MC showed the formation of product and disappearance of all of the starting material. Saturated aqueous NaHCO<sub>3</sub> (70 mL) and ethyl acetate (400 mL) were added. The organic layer was separated and the aqueous layer was extracted with ethyl acetate  $(2 \times 100 \text{ mL})$  and washed with brine (100 mL). Evaporation and purification of the crude by flash chromatography (25% ethyl acetate in hexane) gave 2.1 g (69%) of the title compound as a liquid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.41 (d, J = 8.8 Hz, 1H), 6.28 (d, J = 15.7 Hz, 12H), 6.06 (dd, J = 11.6, 7.8 Hz, 1H),5.64-5.52 (m, 2H), 5.48 (t, J = 7.1 Hz, 1H), 4.59 (t, J = 5.2 Hz, 1H), 4.51-4.42 (m, 1H), 3.98-3.92 (m, 5H), 2.59-2.55 (m, 2H), 2.50-2.46 (m, 2H), 2.04-1.86 (m, 2H), 1.75 (s, 3H), 1.40 (s, 3H), 1.29-1.24 (m, 6H), 1.23-1.14 (m, 2H), 0.88 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  164.98, 151.41, 135.75, 135.55, 128.11, 125.41, 119.06, 102.29, 73.07, 70.44, 70.32, 69.46, 65.43, 55.66, 51.06, 43.52, 42.46, 38.59, 34.17, 31.54, 25.90, 23.78, 18.20, 12.65, -4.68, -4.74; IR (neat film) 3332, 2955, 2930, 2857, 1758, 1715, 1665, 1526, 1471, 1373, 1254, 1117, 1077, 1003 cm<sup>-1</sup>; HRMS (ESI) m/z calcd for C<sub>29</sub>H<sub>50</sub>NO<sub>6-</sub> Si  $(M + 1)^+$  536.3407, found 536.3397.

(S,Z)-N-((2R,5R)-2-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)-1,3-dioxan-5yl)-4-hydroxypent-2-enamide. A solution of TBS protected amide from above (2.1 g, 3.92 mmol) in 20 mL of THF at -10 °C was stirred as TBAF (5.4 mL, 5.49 mmol, 1.0 M in THF) was added. The light-yellow solution was allowed to stir for 1.5 h at 0 °C and allowed to stir at room temperature for 2 h, by which time TLC and LC/MS showed the starting material had been consumed. THF was evaporated and diluted with CHCl<sub>3</sub> (100 mL) and water (20 mL) and saturated aqueous NaHCO<sub>3</sub> (30 mL). The CHCl<sub>3</sub> layer was separated, and the aqueous layer was extracted with  $CHCl_3$  (2 × 75 mL). The combined extracts were washed with brine (50 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The residue was purified by Prep-SFC (OD-H column, 10% MeOH) to give 780 mg (47%) of the title alcohol as a liquid. <sup>1</sup>H NMR  $(400 \text{ MHz}, \text{CDCl}_3) \delta 6.67 \text{ (d}, J = 7.9 \text{ Hz}, 1\text{H}), 6.28 \text{ (d}, J = 15.7$ Hz, 1H), 6.21 (dd, J = 11.9, 5.6 Hz, 1H), 5.82 (dd, J = 12.0, 1.6 Hz, 1H), 5.61 (dd, J = 15.7, 6.6 Hz, 1H), 5.47 (t, J = 7.2 Hz, 1H), 5.25 (s, 1H), 4.80 (s, 1H), 4.60 (t, J = 5.2 Hz, 1H), 4.46 (ddd, J = 10.9, 6.6, 1.8 Hz, 1H), 4.02-3.90 (m, 5H), 2.57 (s, 2H), 2.53-2.44 (m, 2H), 2.02-1.85 (m, 2H), 1.74 (s, 3H), 1.40 (s, 3H), 1.35 (d, J = 6.7 Hz, 3H), 1.27 (s, 3H), 1.23–1.13 (m, 2H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 165.89, 150.80, 135.82, 135.52, 128.17, 125.28, 122.49, 102.34, 73.08, 70.13, 69.46, 64.48, 55.66, 51.06, 43.87, 42.44, 38.58, 34.11, 31.53, 23.77, 22.67, 12.66; IR (neat film) 3322, 2973, 2912, 2856, 1656, 1627, 1526, 1451, 1377, 1254, 1117, 1077, 1003 cm<sup>-1</sup>; HRMS (ESI) *m/z* calcd for  $C_{23}H_{36}NO_6 (M + 1)^+ 422.2543$ , found 422.2534.

(S,Z)-5-((2R,5R)-2-((2E,4E)-5-((3R,5S)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)-1,3-dioxan-5-ylamino)-5-oxopent-3-en-2-yl Isobutyrate (26). A solution of alcohol from above (690 mg, 1.63 mmol) in CH2Cl2 (14 mL) at 0 °C was stirred as Et<sub>3</sub>N (8.18 mmol, 1.1 mL) and DMAP (30 mg, 0.246 mmol) were sequentially added. After 5 min, isobutyric anhydride (0.9 mL, 5.40 mmol) was added. The solution was allowed to stir for 1 h at the same temperature, by which time TLC indicated that starting material has completely been consumed. The reaction mixture was diluted with water (10 mL) and CH<sub>2</sub>Cl<sub>2</sub> (30 mL). The aqueous layer was extracted with  $CH_2Cl_2$  (2  $\times$  50 mL). The organic layer was washed with saturated aqueous NaHCO<sub>3</sub> (20 mL) and brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated and the residue was purified by flash column (40-50% ethyl acetate in hexane) to give 770 mg (96%) of **26** as a viscous oil. <sup>1</sup>H NMR (400 MHz,  $CDCl_3$ )  $\delta$  7.16 (d, J = 8.2 Hz, 1H), 6.21 (d, J = 15.7 Hz, 1H), 6.10-5.99 (m, 1H), 5.81-5.72 (m, 2H), 5.53 (dd, J = 15.7, 6.6Hz, 1H), 5.42 (t, J = 7.1 Hz, 1H), 4.52 (t, J = 5.3 Hz, 1H), 4.45-4.33 (m, 1H), 3.97-3.80 (m, 5H), 2.50 (s, 2H), 2.49-2.39 (m, 3H), 1.97–1.74 (m, 2H), 1.67 (s, 3H), 1.33 (s, 3H), 1.31 (d, J = 6.7 Hz, 3H, 1.21 (s, 3H), 1.17–1.07 (m, 8H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 176.71, 164.82, 142.32, 135.64, 135.62, 127.99, 125.65, 123.25, 102.19, 73.05, 70.23, 70.20, 69.47, 68.55, 55.66, 51.06, 43.73, 42.46, 38.59, 34.16, 34.02, 31.54, 23.77, 20.04, 18.98, 18.90, 12.63; IR (neat film) 3332, 2974, 2936, 2871, 1730, 1669, 1638, 1525, 1415, 1373, 1266, 1195, 1130  $\text{cm}^{-1}$ ; HRMS (ESI) m/z calcd for C<sub>27</sub>H<sub>42</sub>NO<sub>7</sub> (M + 1)<sup>+</sup> 492.2961, found 492.2949.

*N*-((2*s*,5*R*)-2-((2*E*,4*E*)-5-((3*R*,5*S*)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)-1,3-dioxan-5-yl)acetamide (27). (2*s*,5*R*)-2-((2*E*,4*E*)-5-((3*R*,5*S*)-7,7-Dimethyl-1,6-dioxaspiro[2.5]octan-5-yl)-3-methylpenta-2,4-dienyl)-1,3dioxan-5-amine (9 mg, 0.029 mmol) which had been dried azeotropically in CH<sub>3</sub>CN was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1.8 mL). diisopropylethylamine (51  $\mu$ L, 0.294 mmol) was added to the reaction mixture followed by Ac<sub>2</sub>O (14  $\mu$ L, 0.147 mmol). The mixture was stirred at room temperature for 28 h. The reaction mixture was concentrated and chromatographed using 5% MeOH/CHCl<sub>3</sub> to give 1.2 mg (12%) of acetamide **27** as a viscous liquid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.39 (d, *J* = 8.0 Hz, 1H), 6.28 (d, *J* = 15.8 Hz, 1H), 5.61 (dd, *J* = 15.7, 6.6 Hz, 1H), 5.48 (t, J = 7.3 Hz, 1H), 4.58 (t, J = 5.3 Hz, 1H), 4.51–4.42 (m, 1H), 3.99–3.87 (m, 5H), 2.57 (s, 2H), 2.52–2.45 (m, 2H), 2.05 (s, 3H), 1.99 (d, J = 14.2 Hz, 1H), 1.91 (dd, J = 13.7, 11.7 Hz, 1H), 1.75 (d, J = 1.0 Hz, 3H), 1.40 (s, 3H), 1.28 (s, 3H), 1.24–1.18 (m, 1H), 1.16 (dd, J = 13.8, 2.0 Hz, 1H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  170.25, 136.41, 136.20, 128.77, 126.03, 102.93, 73.72, 71.05, 70.12, 56.31, 51.71, 44.40, 43.10, 39.24, 34.82, 32.18, 24.42, 24.12, 13.31; HRMS (ESI) m/z calcd for C<sub>20</sub>H<sub>32</sub>NO<sub>5</sub> (M + 1)<sup>+</sup> 366.2280, found 366.2284.

**Biology Experimental Section. Cell Culture.** The cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) or the DSMZ (Brunswick, Germany) and were maintained in growth media as recommended by the vendor. The PrEC normal prostate epithelial cells were purchased from Lonza (Basel, Switzerland) and maintained in prostate epithelial cell growth medium (Lonza). Human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. All cells were grown under standard culture conditions at 37 °C and 5% CO<sub>2</sub> in a humidified environment.

In Vitro Cytotoxicity Assay. Cell viabilities were determined by measuring the cleavage of the tetrazolium salt XTT to a water-soluble orange formazan by mitochondrial dehydrogenase, following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, cell lines were seeded at various densities depending on the cell type. The adherent cell lines were allowed to adhere overnight before spliceosome modulator compound addition, whereas suspension cell lines were seeded the day of compound addition. Stock concentrations of the compounds were made in DMSO, diluted in culture media, and subsequently added to the plates at various final concentrations, then incubated for 72 h. After the incubation period, the XTT labeling reagent, containing the electroncoupling reagent, was added to each well and incubated for 4 h. The absorbance of each well was then determined at 490 nm with a reference wavelength of 690 nm using a VersaMax microplate reader (Sunnyvale, CA). The data are expressed as the percentage of growth compared to vehicle-treated cells, as calculated from absorbance and corrected for background absorbance. The  $IC_{50}$  is defined as the drug concentration that inhibits growth to 50% of the vehicle-treated control; IC<sub>50</sub> values were calculated from sigmoidal analysis of the dose-response curves using Origin, version 7.5, software (Northampton, MA).

In Vivo Tumor Growth Inhibition. JeKo-1 tumors were transplanted to female NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME) on day 1, and beginning on day 4, the mice (4 per group) received iv injections of vehicle (3.0% DMSO and 6.5% Tween-80 in 5% glucose solution), 5, 10, 25, or 50 mg/kg of compound 5 daily for 5 consecutive days. Tumor volume and mouse weights were measured three times a week. Tumor volumes were determined on the basis of the following equation: volume = [length  $\times$  (width)<sup>2</sup>]/2.

**Dual-Reporter Cell-Based Splicing Assay.** 293T cells were transiently transfected using FuGENE 6 (Roche Applied Science), as described by the manufacturer, with the pTN24 dual-reporter splicing construct (gracious gift from Dr. Talat Nasim, Guy's Hospital, London, U.K.) for 48 h and subsequently treated with or without spliceosome modulator compounds for 5 h.<sup>37,38</sup> The degree of cell death was negligible at this time (data not shown). The cells were lysed, and equal amounts of protein for each compound concentration were assayed for luciferase and  $\beta$ -galactosidase activity using the Dual-Light assay system (Applied Biosystems, Foster City, CA). Each entire assay was conducted in a single 96-well plate. The pTN24 dual-reporter construct contains  $\beta$ -galactosidase and luciferase coding sequences separated by an intronic linker containing a stop codon.<sup>37,38</sup> In the presence of splicing, the intronic sequence containing the stop codon is removed and

both  $\beta$ -galactosidase and luciferase are expressed. If splicing is inhibited, the intron remains and only  $\beta$ -galactosidase is expressed. Assay data are expressed as the relative ratio of luciferase to  $\beta$ -galactosidase activity compared to control vehicle-treated samples.

In Vitro Splicing Assay. In vitro splicing reactions were performed as previously described.<sup>38</sup> Briefly, standard reactions contained HeLa nuclear extract (Promega, Madison, WI), 13% (w/v) polyvinyl alcohol, <sup>33</sup>P-labeled  $\beta$ -globin pre-mRNA substrate, 80 mM MgCl<sub>2</sub>, 12.5 mM ATP/0.5 M creatine phosphate, and Dignam's buffer D.<sup>39</sup> Reactions were incubated at 30 °C for 1 h with or without spliceosome modulator compound and then terminated and the RNA products analyzed by autoradiography following electrophoresis on a 6% polyacrylamide TBE–urea gel.

**Immunofluorescence Analysis.** HeLa cells were grown on glass coverslips for 24 h, fixed with cold methanol at -20 °C for 10 min, and rinsed with PBS prior to incubation with SC35 (Sigma) or SF3B1 (SAP155, MBL International) primary antibodies ( $^{1}/_{100}$  dilution with PBS) for 1 h at 37 °C in PBS. After the samples were washed with PBS, the coverslips were incubated with an antimouse fluorescent secondary antibody ( $^{1}/_{200}$  dilution) labeled with Alexa Fluor 488 (Molecular Probes) as described above, washed, mounted in Vectashield (Vector Laboratories), and viewed on an Olympus BX50 fluorescence microscope equipped with a Cool Snap digital camera using a 60× magnification objective.

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Supporting Information Available: Procedures for the synthesis of compounds 8-11 and 14-19; supplemental Figures S1-S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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- Lagisetti et al.
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