Title

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Journal

Journal of Medicinal Chemistry, 56(17)

ISSN

0022-2623

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Publication Date

2013-09-12

DOI

10.1021/jm400861t

Peer reviewed

Published in final edited form as:
J Med Chem. 2013 September 12; 56(17): . doi:10.1021/jm400861t.

Stabilized Cyclopropane Analogs of the Splicing Inhibitor FD-895

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Abstract

Targeting the spliceosome with small molecule inhibitors provides a new avenue to target cancer by intercepting alternate splicing pathways. Although our understanding of alternate mRNA splicing remains poorly understood, it provides an escape pathway for many cancers resistant to current therapeutics. These findings have encouraged recent academic and industrial efforts to develop natural product spliceosome inhibitors, including FD-895 (**1a**), pladienolide B (**1b**) and pladienolide D (**1c**), into next-generation anticancer drugs. The present study describes the application of semi-synthesis and total synthesis to reveal key structure activity relationships (SARs) for the spliceosome inhibition by **1a**. This information is applied to deliver new analogs with improved stability and potent activity at inhibiting splicing in patient derived cell lines.

INTRODUCTION

Given the successful clinical advance of polyketide natural product analogs for treatment of diverse cancers,[1] FD-895 (**1a**)[2] and related pladienolides B (**1b**) and D (**1c**) [3–4] have been the subject of considerable synthetic,[5–11] biosynthetic,[12–14] and mode of action (MOA) studies. The latter MOA studies identified[15] and validated[16] splicing factor SF3b as the primary target of **1b**.[17] In parallel efforts, SF3b has also been shown to be targeted by spliceostatin A (**2**, Fig. 1c), an analog of the natural product FD901464.[18–20]

In 2006, Eisai Co. Ltd. launched clinical trials (NCT00459823) with a carbamate analog of **1b**, E7101 (**1d**, Fig. 1), for application in solid tumors.[15,17] Concomitantly, we focused our efforts on a combination of semi-synthetic and total synthetic methods to identify next-generation leads for further clinical examination. Since this effort, ongoing studies in our[2,3] and other laboratories[11,19–20] have confirmed the importance of small molecule regulators of splicing and the need for next-generation synthetic derivatives for clinical evaluation.[21–22]

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SUPPORTING INFORMATION AVAILABLE

Selected copies of ^{1}H NMR, ^{13}C NMR and ^{1}H-^{1}H gCOSY NMR spectra for compounds **7a, 7b**, **9**, **10**, **13a** and **13b** as well as NCI-60 data collected from analyses of **5a**, **6a**, and **6c**. This material is available free of charge via the Internet at http://pubs.acs.org.

RESULTS AND DISCUSSION

Our efforts began with a detailed evaluation of the stability of **1a**. As shown in Fig. 2, we identified that **1a** undergoes hydrolysis under neutral conditions to afford a mixture of acids **3a**–**3c**.[6] As determined by NMR monitoring (Fig. 3), this reaction occurs with the formation of **3a**, **3b** and **3c** in an approximated 3:2:5 ratio (Fig. 3 and Fig. 4a), suggesting the hydrolysis occurs via non-selective addition of water to an incipient allylic cation. While not unexpected, decomposition begins within 7 h in aqueous media. This process would be expected to be much faster in serum or tissue where the combination of pH changes and enzymatic activity could accelerate the process. The hydrolyzed products collected at 216 h, a mixture of **3a**–**3c** demonstrate significant loss of activity, with an IC_{50} value of 11.2 ± 1.4 μM (Table 1), over 1000-fold less active, in HCT-116 cells.[23–24] Based on this observation, we turned our efforts to identify analogs with improved hydrolytic stability.

While synthetic access has been established,[5–11] a detailed understanding of structure activity relationship (SAR) within the family of macrolides encompassing **1a**–**1c** has yet to be completed. The first piece of SAR data was derived from probe development efforts associated with the MOA investigation.[15] In these studies, linkers applied at the C7 ester led to >100 fold loss in activity, suggesting that modifications at C7, as in E7107 (**1d**), could prove problematic.

A second set of SAR data can be derived from analogs prepared in our synthetic campaign. Early efforts in our laboratories provided access to core modified analogs **4a**-**4d** (Fig. 5).[5] All four of these analogs proved inactive, returning IC_{50} values > 50 μM when screened against the HCT-116 cell line using the MTT assay (Table 1).[23–24] This observation was further confirmed by recent studies from the Webb laboratory, which demonstrated that analogs such as **4e** lack activity.[11] These studies also identified a TBS ether **4f** that partially rescued activity, implying a need for a hydrophobic group at C21.

Additional activity data was also obtained directly from **1a**. Per-acetylation of **1a** afforded **5a** (Fig. 6) in excellent yield. In addition, **1a** was selectively converted to the **C3** triethylsilyl ether, which then could be converted to bis-acetate **5d** in a stepwise fashion. From this effort, we found that acetates **5a** and **5d** displayed reduced activity (see Table 1 and NCI-60 data provided within the Supporting Information).

Recently, we reported the total synthesis[6] of **1a** and applied these methods to access analogs that were not readily prepared from **1a**. Using the same intermediates used in our preparation of **1a**, we were able to apply the allenylstannane chemistry of Marshall[25] to prepare each of the C16-C17 isomers **6a**–**6c** (Fig. 7). Cytotoxicity analyses via the MTT assay[23–24] indicated that all four isomers **1a** and **6a**–**6c** displayed high pM to low nM activity when screened against the HCT-116 tumor cell line (see Table 1). Isomers with the natural *R*-configuration at C16 were less active than those with *S*. Additional activity was also observed when C17 was left in its natural *R* configuration. Isomer **6c** was nearly 25 times more active than **1a**. Most importantly, application of the hydrolysis conditions applied to **1a** (as shown in Fig. 2) indicated that **6c** was more resistant to hydrolysis, with lack of the corresponding acid products appearing under the same conditions (data from sampling at 143 h is provided in Fig. 4b).

To further address stability, we then turned to examine the replacement of the epoxide moiety, which is commonly associated with toxicity, off-target reactivity and instability.[26] We chose to prepare cyclopropanes **7a** and **7b**, which share the stereochemical assignment at C16 and C17 with **6c** and **6b**, respectively (Fig. 7). Synthesis of these materials began with allyl alcohol **8** (Scheme 1), a central component prepared on the multigram scale for

the synthesis of **1a**.[6] Using a Charette cyclopropanation,[27] we were able to obtain cyclopropanol **9** as a single diasteromer. Oxidation with Dess-Martin periodinane afforded aldehyde **10** in 66% yield from **8**. Analysis of the ^{1}H–NMR spectrum of **10** confirmed the stereochemistry as shown by coupling constants of $J_{H18\text{-}H19}$ = 4.4 Hz (syn), $J_{H19\text{-}H20}$ = 9.3 Hz (anti), $J_{H20\text{-}H21}$ = 4.1 Hz (syn).

Aldehyde **10** served as a central building block to intersect our established synthetic route to **1a**.[6] Using methods developed by Marshall,[25] addition of allenylstannane **12** to aldehyde **10** afforded major isomer **13a** along with minor isomer **13b**. Fortunately, these materials were chromatographically separable. We then converted **13a** and **13b** to their respective analogs **7a** and **7b** of **1a** using a rapid two-step process. As shown in Scheme 1, alkyne **13a** was converted to vinystannane **14a**, which was quickly purified and coupled directly to core **15** (compound **14a** was stable, however, we found improved yields when conducting a rapid flash purification and coupling this material directly to **15**), delivering **7a** in 23% overall yield from **8**. Comparable methods provide isomer **7b** in 16% from **8**.

We then turned to evaluate the biological activity of synthesized compounds. When interpreting our NCI-60 cell screening data of select analogs of **1a** (see example data set provided in the Supporting Information), we identified enhanced potency of several analogs in leukemia cell lines. We then focused our program on leads that delivered maximum potency in patient-derived Chronic Lymphocytic Leukemia (CLL) cells.[28–29] Using the induction of apoptosis as a marker, we determined that **1a** displayed an IC_{50} value of 2.1 ± 0.1 nM, respectively (Table 1). The C16-C17 analog **6b** lacked activity (IC_{50} value > 50 μM) while **6a** displayed reduced activity with an IC_{50} value of 108.0 ± 0.2 nM. Most importantly, the cyclopropane derivative **7a** retained activity delivering an IC_{50} value of 102.9 ± 0.1 nM (Table 1).

We then implemented an RT-PCR gel-shift assay to confirm their splicing inhibitory activity. As shown in Fig. 8, we could correlate the splicing activity of **1a** with solely the non-hydrolyzed natural product, as isolated hydrolyzed material proved inactive in this assay. (See Fig. 8a, 3a–3c vs. **1a**). We also learned that other analogs also inhibited the splicing of *DNAJB1*, DnaJ (Hsp40) homolog subfamily B member 1, but not by F-Ara-A (negative drug control) or glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, a control housekeeping gene). Subsequent analyses (Fig. 8b) indicated that while **7a** inhibited splicing, the activity of **7a** activity was lower than that of **1a** and **1b**. This finding, however, is quite remarkable. All known spliceosome inhibitors in the pladienolide and spliceostatin families, including hybrid analogs, have all contained epoxide moieties considered essential for activity.[11,19–20] However, analog **7a** indicates that the epoxide moiety is not essential for spliceosome activity in **1a**, offering the promise of improved pharmacophores for potential therapeutic use.

CONCLUSION

We have investigated the instability of **1a**, which leads to 10^3 fold decrease in activity, followed by a SAR study to identify stabilized, active analogs. This included synthesis of cyclopropane derivatives of **1a**, compounds **7a** and **7b**. Compound **7a** displayed improved stability and effectively mimicked the apoptotic and splicing activity of **1a**. While the activity of **7a** was less than **1a**, its increase in stability provides a key next step in advancing stable materials for *in vivo* applications. Furthermore, this study demonstrates that labile functional groups such as the C18-C19 epoxide can be removed, opening new avenues for analog design. Efforts are now underway to further evaluate **7a** as a preclinical entry for applications to leukemia, with a particular emphasis for CLL patients that display resistance to conventional treatments such as fludarabine.

EXPERIMENTAL SECTION

Materials and Methods

Chemical reagents were purchased from Acros, Fluka, Sigma–Aldrich, or TCI. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. All oligonucleotides were custom synthesized by Integrated DNA Technologies (Skokie, IL, USA). Fludarabine phosphate was purchased from Sigma-Aldrich. All chemical reactions were conducted with rigorously dried anhydrous solvents that were obtained by passing through a solvent column composed of activated A1 alumina. Reactions were conducted under positive pressure of Ar in oven–dried glassware sealed with septa, with stirring from a Teflon coated stir bars using an IKAMAG RCT–basic mechanical stirrer (IKA GmbH). Solutions were heated using either a sand or silicon oil bath. Analytical Thin Layer Chromatography (TLC) was performed on Silica Gel 60 F254 precoated glass plates (EM Sciences). Preparative TLC (pTLC) was conducted on Silica Gel 60 plates (EM Sciences). Visualization was achieved with UV light and/or an appropriate stain (I_2 on SiO_2, $KMnO_4$, bromocresol green, dinitrophenylhydrazine, ninhydrin, and ceric ammonium molybdate). Flash chromatography was carried out Geduran Silica Gel 60 (40–63 mesh) from EM Biosciences. Yields and characterization data correspond to isolated, chromatographically and spectroscopically homogeneous materials. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian VX500 spectrometer equipped with an Xsens Cold probe. Chemical shift δ values for ^{1}H and ^{13}C spectra are reported in parts per million (ppm) relative to these referenced values, and multiplicities are abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. All ^{13}C NMR spectra were recorded with complete proton decoupling. FID files were processed using MestraNova 8.0.0 (MestreLab Research). Electrospray (ESI) mass spectrometric analyses were performed using a ThermoFinnigan LCQ Deca spectrometer, and high–resolution analyses were conducted using a ThermoFinnigan MAT900XL mass spectrometer with electron impact (EI) ionization. A Thermo Scientific LTQ Orbitrap XL mass spectrometer was used for high–resolution electrospray ionization mass spectrometry analysis (HR–ESI–MS). All tested compounds possessed a purity of not less than 98% as confirmed by LC-MS analyses prior to use.

((1S,2S)-2-((2S,3S)-3-Methoxypentan-2-yl)cyclopropyl)-methanol (9)—A flame dried 100 mL round-bottom flask was charged with CH_2Cl_2 (5 mL) and dimethoxyethane (766 uL, 7.36 mmol) and cooled to −10 °C. Et_2Zn (13.4 mL, 7.36 mmol) was added in one portion followed by the addition of CH_2I_2 (3.94 g, 14.72 mmol) in a drop wise fashion over 20 min. After stirring for an additional 15 min, a solution of dioxaborolane ligand **11** (426 mg, 4.3 mmol) in CH_2Cl_2 (2 mL) was added drop wise over 5 min. After 10 min, this was followed by the addition of allylic alcohol **8** (292.0 mg, 1.84 mmol) in CH_2Cl_2 (5 mL) over 5 min. The reaction was allowed to warm to rt over 8 h, at which point it was quenched by the addition of satd. NH_4Cl (3 mL) and 10% HCl (10 mL). The mixture was diluted with Et_2O (10 mL), the layers were separated and the aqueous layer was extracted further with Et_2O (10 mL). The combined organic layers were transferred to an Erlenmeyer flask and a solution containing 2N NaOH (15 mL) and 30% aqueous H_2O_2 (3 mL). The resulting biphasic mixture was stirred vigorously for 5 min. The layers were separated and the organic layer was washed sequentially with 10% HCl (5 mL), satd. $NaHCO_3$ (5 mL), H_2O (5 mL), and brine (5 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product. Purification by flash chromatography with 8:1 hexanes/EtOAc afforded cyclopropanol **9** (263 mg, 83%), as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.37 (dd, *J* = 6.9, 11.2 Hz, 1H), 3.32 (dd, *J* = 7.0, 11.2 Hz, 1H), 3.30 (s, 3H), 2.92 (qt, *J* = 1.9, 5.4 Hz, 1H), 1.51 (td, *J* = 7.3, 13.9 Hz, 1H), 1.41 (dqd, *J* = 5.4, 7.4, 14.9 Hz, 1H), 0.86 (m, 3H), 0.80 (t, *J* = 7.4 Hz, 3H), 0.75 (m, 1H), 0.52 (m, 1H), 0.33 (m, 2H). ^{13}C NMR (125 MHz,

$CDCl_3$) δ 128.3, 86.8, 66.8, 58.0, 40.0, 23.9, 21.0, 20.2, 14.6, 10.3, 10.0. HR–ESI–MS *m/z* calcd. for $C_{10}H_{20}O_2Na$ $[M]^+$: 195.1356, found 195.1341.

(1S,2R)-2-((2S,3S)-3-Methoxypentan-2-yl)cyclopropane-1-carbaldehyde (10)— Dess-Martin periodinane (634.0 mg, 1.50 mmol) was added to a solution of cyclopropanol **9** (198.0 mg, 1.15 mmol) in CH_2Cl_2 (20 mL) cooled to 0 °C. After addition, the mixture was warmed to rt. The reaction went to complete after an additional 15 min at rt. At this point, satd. $NaHCO_3$ (4 mL) and satd. $Na_2S_2O_3$ (4 mL) were added and the mixture was stirred for 1 h. The layers were separated and the aqueous layer was further extracted with CH_2Cl_2 (10 mL). The combined organic layers were washed with satd. $NaHCO_3$ (5 mL), H_2O (5 mL), brine (5 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification with flash column chromatography using 2:1 hexanes/EtOAc provided pure aldehyde **10** (159.0 mg, 80%), as a clear oil. ^1H NMR (500 MHz, $CDCl_3$) δ 8.99 (d, *J* = 5.6 Hz, 1H), 3.37 (s, 3H), 2.99 (ddd, *J* = 4.1, 5.5, 7.2 Hz, 1H), 1.62 (tdd, *J* = 4.3, 5.6, 8.3 Hz, 1H), 1.57 (td, *J* = 7.3, 13.9 Hz, 1H), 1.48 (m, 2H), 1.30 (td, *J* = 4.6, 9.1 Hz, 1H), 1.10 (dqd, *J* = 4.1, 6.8, 9.3 Hz, 1H), 1.03 (ddd, *J* = 4.7, 6.7, 8.0 Hz, 1H), 0.94 (d, *J* = 6.9, 3H), 0.88 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ 201.2, 86.5, 58.3, 39.7, 29.7, 26.6, 23.9, 14.7, 14.5, 10.4. HR–ESI–MS *m/z* calcd. for $C_{10}H_{19}O_2$ $[M]^+$: 171.1380, found 171.1383.

(1R,2R)-1-((1S,2R)-2-((2S,3S)-3-Methoxypentan-2-yl)cyclo-propyl)-2-methylbut-3-yn-1-ol (13a)—A solution was prepared containing aldehyde **10** (50.0 mg, 0.29 mmol) and *m*-allenylstannane **12** (152.0 mg, 0.44 mmol) in CH_2Cl_2 (6 mL). After cooling to −78 °C, BF_3•Et_2O (0.11 mL, 0.88 mmol) was added drop wise over 5 min. After stirring for 1 h, a mixture of MeOH (1.6 mL) and satd. $NaHCO_3$ (0.3 mL) was added and the solution was warmed. After reaching rt, the organic layer was collected and the aqueous layer was further extracted with CH_2Cl_2 (5 mL). The combined organic layers were washed with brine (3 mL), dried with Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash column chromatography eluting with 5:1 hexanes/EtOAc provided a mixture of alkynes **13a** and **13b** (51 mg, 80%). Repeated column chromatography eluting with 10:1 hexanes/EtOAc could separate the two isomers. Alkyne **13a** ^1H NMR (500 MHz, C_6D_6) δ 3.23 (s, 3H), 2.88 (dt, *J* = 4.4, 6.3 Hz, 1H), 2.62 (m, 1H), 2.57 (m, 1H), 1.83 (d, *J* = 2.4 Hz, 1H), 1.59 (pd, *J* = 7.2, 14.5 Hz, 1H), 1.42 (m, 1H), 1.39 (m, 1H), 1.22 (d, *J* = 7.0 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3H), 0.86 (t, *J* = 7.4 Hz, 3H), 0.77 (m, *2*H), 0.61 (dddd, *J* = 8.5, 9.3, 9.4, 9.8 Hz, 1H), 0.47 (m, 1H), 0.30 (td, *J* = 4.8, 8.2 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 86.6, 85.3, 78.5, 71.0, 58.3, 40.8, 33.1, 24.0, 23.3, 21.3, 18.0, 14.8, 10.8, 10.4. HR–ESI–MS *m/z* calcd. for $C_{14}H_{24}O_2Na$ $[M]^+$: 247.1669, found 247.1673. Alkyne **13b** ^1H NMR (500 MHz, C_6D_6) δ 3.22 (s, 3H), 2.88 (dt, *J* = 4.4, 6.3 Hz, 1H), 2.80 (ddd, *J* = 4.1, 5.7, 7.5 Hz, 1H), 2.60 (ddq, *J* = 2.5, 5.8, 7.0 Hz, 1H), 1.87 (d, *J* = 2.5 Hz, 1H), 1.61 (dqd, *J* = 6.5, 7.4, 13.8 Hz, 1H), 1.47 (d, *J* = 4.7 Hz, 1H), 1.41 (dqd, *J* = 6.0, 7.5, 13.6 Hz, 1H), 1.21 (d, *J* = 7.0 Hz, 3H), 1.03 (d, *J* = 6.9 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 3H), 0.83 (m, 2H), 0.67 (ddd, *J* = 4.9, 8.7, 10.2 Hz, 1H), 0.37 (td, *J* = 4.8, 8.3 Hz, 1H), 0.33 (ddd, *J* = 4.7, 5.5, 8.5 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 86.8, 86.3, 78.3, 70.4, 58.1, 39.7, 33.1, 23.9, 21.1, 20.5, 16.7, 14.4, 10.9, 10.4. HR–ESI–MS *m/z* calcd. for $C_{14}H_{24}O_2Na$ $[M]^+$: 247.1669, found 247.1674.

(2S,3S,6S,7R,10R,E)-7,10-Dihydroxy-2-((2E,4E,6R,7S)-7-hydroxy-7-((1S,2R)-2-((2S,3S)-3-methoxypentan-2-yl)cyclopropyl)-6-methylhepta-2,4-dien-2-yl)-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl acetate (7a)—Compound **7a** was prepared via two-step procedure that began with the hydrostannlation of **13a** to vinystannane **14a** followed by Stille coupling of **14a** to vinyl iodide **15**. This procedure was best conducted in a rapid fashion without storing **14a**. $PdCl_2(PPh_3)_2$ (2.1 mg, 3.0 μmol) was added to solution of alkyne **13a** (14.0 mg, 62.0 μmol) in THF (5 mL) cooled to 0 °C. This

was followed by the addition of n-Bu_3SnH (35 uL, 0.127 mmol) in a drop wise fashion over 5 min. After stirring for 30 min, the reaction mixture was concentrated under reduced pressure and quickly run through a short silica gel column eluting with 15:1 hexanes/EtOAc containing 2% Et_3N to give the crude vinyl stannane **14a** (16.0 mg). Immediately after preparation, $Pd_2(dba)_3$ (0.4 mg, 0.4 μmol), Ph_3As (3 mg, 8.8 μmol), and LiCl (.5 mg, 13 μmol) were added sequentially to a solution of vinyl iodide **15** (2 mg, 4.2 μmol) and stannane **14a** (2.2 mg, 4.2 μmol) in dried and degassed *N*-methylpyrrolidone (NMP) (0.3 mL). The reaction was stirred for 24 h at which point an additional $Pd_2(dba)_3$ (0.4 mg, 0.4 μmol) and Ph_3As (3.0 mg, 8.8 μmol) were added. After an additional 24 h at rt, H_2O (1 mL) was added and the mixture was extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with H_2O (5 mL) and brine (5 mL), dried with Na_2SO_4 and concentrated. The remaining NMP was removed under a stream of N_2. Flash chromatography with a gradient from 1:1 hexanes/EtOAc to 2:1 EtOAc/hexanes followed by pTLC in a chamber containing 2:1 EtOAc/hexanes afforded isomer **7a** (1.7 mg, 73 %), a white wax. ^{1}H NMR (500 MHz, C_6D_6) δ 6.40 (ddd, *J* = 1.0, 10.9, 15.2 Hz, 1H), 6.24 (dd, *J* = 1.6, 10.9, Hz, 1H), 5.98 (dd, *J* = 8.7, 15.2 Hz, 1H), 5.82 (dd, *J* = 9.7, 15.3 Hz, 1H), 5.62 (dd, *J* = 9.9, 15.3 Hz, 1H), 5.29 (d, *J* = 10.6 Hz, 1H), 5.25 (d, *J* = 9.7 Hz, 1H), 3.63 (d, *J* = 11.0 Hz, 1H), 3.48 (m, 1H), 3.24 (s, 3H), 2.88 (dt, *J* = 3.6, 6.3 Hz, 1H), 2.67 (dd, *J* = 3.6, 8.0 Hz, 1H), 2.55 (ddd, *J* = 4.0, 7.3, 7.4 Hz, 1H), 2.42 (m, 3H), 2.22 (dd, *J* = 4.0, 15.0 Hz, 1H), 2.17 (dd, *J* = 3.1, 14.9 Hz, 1H), 1.67 (d, *J* = 1.2 Hz, 3H), 1.62 (s, 3H), 1.57 (m, 1H), 1.41 (s, 1H), 1.35 (m, 3H), 1.18 (d, *J* = 6.9 Hz, 3H), 1.06 (d, *J* = 6.3 Hz, 3H), 1.01 (s, 3H), 0.92 (m, 2H), 0.84 (t, *J* = 7.4 Hz, 3H), 0.72 (d, *J* = 6.8 Hz, 3H), 0.65 (m, 2H), 0.34 (td, *J* = 4.5, 7.3 Hz, 1H), 0.20 (td, *J* = 4.8, 7.7 Hz, 1H).^{13}C NMR (125 MHz, $CDCl_3$) δ 172.1, 169.0, 140.7, 138.8, 131.9, 130.9, 126.5, 126.4, 86.5, 82.7, 79.4, 79.2, 73.3, 69.3, 57.8, 43.9, 41.2, 41.1, 38.5, 35.9, 24.8, 24.2, 23.4, 21.8, 20.8, 18.0, 16.5, 15.2, 11.8, 10.4, 10.3, 1.4. HR–ESI–MS *m/z* calcd. for $C_{32}H_{52}O_8Na$ [M]+: 587.3554, found 587.3553.

(2S,3S,6S,7R,10R,E)-7,10-Dihydroxy-2-((2E,4E,6R,7S)-7-hydroxy-7-((1S,2R)-2-((2S,3S)-3-methoxypentan-2-yl)cyclopropyl)-6-methylhepta-2,4-dien-2-yl)-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl acetate (7b)—$PdCl_2(PPh_3)_2$ (1.7 mg, 2.4 μmol) was added to a solution of alkyne **13b** (11.0 mg, 49.0 μmol) in THF (5 mL) cooled to 0 °C. This was followed by the addition of n-Bu_3SnH (27 μL, 101.0 μmol) in a drop wise fashion over 5 min. After stirring for 30 min, the reaction was concentrated under reduced pressure and quickly run through a short silica gel column eluting with 15:1 hexanes/EtOAc with 2% Et_3N to afford the crude **14b** (18 mg, 73%). Immediately after preparation, $Pd_2(dba)_3$ (0.4 mg, 0.4 μmol), Ph_3As (3 mg, 8.8 μmol), and LiCl (0.5 mg, 13 μmol) were added sequentially to a solution of vinyl iodide **15** (2 mg, 4.2 μmol) and the crude stannane **14b** (2.2 mg, 4.2 μmol) in dried and degassed NMP (0.3 mL). The reaction was stirred for 24 h at which point an additional $Pd_2(dba)_3$ (0.4 mg, 0.4 μmol) and Ph_3As (3 mg, 8.8 μmol) were added. After an additional 24 h at rt, H_2O (1 mL) was added and the mixture was extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with H_2O (5 mL) and brine (5 mL), dried with Na_2SO_4 and concentrated. The remaining NMP was removed under a stream of N_2. Flash chromatography with a gradient from 1:1 hexanes/EtOAc to 2:1 EtOAc/hexanes followed by pTLC in a chamber containing 2:1 EtOAc/hexanes afforded isomer **7b** (1.9 mg, 80 %) a white wax. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.37 (ddd, *J* = 1.1, 10.8, 15.2 Hz, 1H), 6.23 (dd, *J* = 1.5, 10.8 Hz, 1H), 5.85 (dd, *J* = 7.9, 14.8 Hz, 1H), 5.81 (dd, *J* = 9.6, 15.3 Hz, 1H), 5.62 (dd, *J* = 10.0, 15.2 Hz, 1H), 5.29 (d, *J* = 10.7 Hz, 1H), 5.24 (d, *J* = 9.8 Hz, 1H), 3.62 (d, *J* = 11.1 Hz, 1H), 3.48 (m, 1H), 3.21 (s, 3H), 2.86 (dt, *J* = 4.2, 6.3 Hz, 1H), 2.67 (dd, *J* = 5.6, 8.1 Hz, 1H), 2.46 (dq, *J* = 1.2, 6.9 Hz, 1H), 2.39 (m, 1H), 2.25 (dd, *J* = 3.9, 14.9 Hz, 1H), 2.17 (dd, *J* = 3.0, 14.9 Hz, 1H), 2.10 (m, 1H), 1.68 (d, *J* = 1.3 Hz, 3H), 1.61 (s, 3H), 1.56 (m, 1H), 1.36 (s, 1H), 1.29 (m, 4H), 1.18 (d, *J* = 6.85 Hz, 3H), 1.00 (s, 3H), 0.96 (d, *J* = 6.8 Hz, 3H), 0.91 (m, 2H), 0.83 (t, *J* = 7.4 Hz, 3H), 0.72

(d, J = 6.8 Hz, 3H), 0.60 (m, 2H), 0.27 (d, J = 6.8 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.1, 169.0, 140.7, 139.8, 131.9, 131.0, 126.4, 125.5, 86.6, 82.7, 79.4, 79.2, 73.3, 69.3, 57.6, 44.2, 41.2, 40.1, 38.5, 35.8, 32.4, 24.8, 24.0, 22.6, 21.1, 20.7, 16.5, 16.2, 14.5, 11.9, 10.7, 10.4, 1.4. HR–ESI–MS *m/z* calcd. for $C_{32}H_{52}O_8Na$ $[M]^+$: 587.3554, found 587.3552.

Chronic lymphocytic leukemia-samples and cell culture conditions

Chronic lymphocytic leukemia (CLL) samples were obtained from the CLL Research Consortium (CRC) tissue bank. The blood samples were collected after obtaining a written informed consent from the patients under a protocol approved by the institutional review board of The University of California, San Diego. All patients met criteria for CLL. The peripheral blood mononuclear cells were separated from heparinized venous blood of the CLL patients by density gradient centrifugation using Ficoll-Hypaque separation (GE Healthcare, USA). The samples that were used had more than 95% positivity for B cells confirmed by staining using CD19 and CD5+, followed by flow cytometry. CLL B cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C in an atmosphere of 5% CO_2.

Apoptosis Induction Assay

Apoptotic and viable cells were discriminated via flow cytometry of cells stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, OR, USA) and propidium iodine (PI) (Sigma, St Louis, MO, USA). Using this method, viable cells exclude PI and stain brightly positive for $DiOC_6$.

RT-PCR Splicing Assay

B-CLL cells were treated with 100 nM **1a**, 100 nM **1b**, 10 μM fludarabine (F-Ara-A), and a varied concentration of **7b** (100–1000 nM) for 4 h. Total RNA was extracted using mirVana™ miRNA isolation kit (Life Technologies, Grand Island, NY USA). After DNase treatment, 200 ng total RNA was reverse-transcribed using a SuperScript™ III first-strand synthesis system (Life Technologies, Grand Island, NY USA). PCR was performed on 10 ng of the obtained cDNA or human genomic DNA as a control in 20 μl of reaction mixture containing Plantinum PCR SuperMix® High-fidelity (Life Technologies, Grand Island, NY USA), and 0.1 μM of each primer of the appropriate pair. PCR conditions were 94 °C for 3 min; 35 cycles of 94 °C for 30 s, annealing temperature (58°C and 55 °C for *DNAJB1* and *GAPDH*, respectively) for 30 s, and 72 °C for 1 min; followed by 72 °C for 5 min. PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

In Vitro Stability Assays

Compounds were dissolved in NMR grade solvents and stored at 37 °C for the ascribed period. NMR analyses were collected at 500 MHz on a Joel ECA500 NMR machine or VX500 NMR machine equipped with an X-Sens probe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was support by financial support from the American Cancer Society (RSG–06–011–01–CDD), NIH (R01GM086225), and Lymphoma Research Foundation grant (#285871). We thank Dr. Yongxuan Su (UC San Diego) for mass spectral analyses, and Drs. Anthony Mrse and Xuemei Huang (UC San Diego) for assistance with acquiring NMR spectral data.

ABBREVIATIONS USED

CLL	chronic lymphocytic leukemia
$DiOC_6$	3,3′-dihexyloxacarbocyanine iodide
DNAJB1	DnaJ (Hsp40) homolog subfamily B member 1
F-Ara-A	fludarabine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HR–ESI–MS	high–resolution electrospray ionization mass spectrometry analysis
MOA	mode of action
PBMCs	peripheral blood mononuclear cells
PI	propidium iodine

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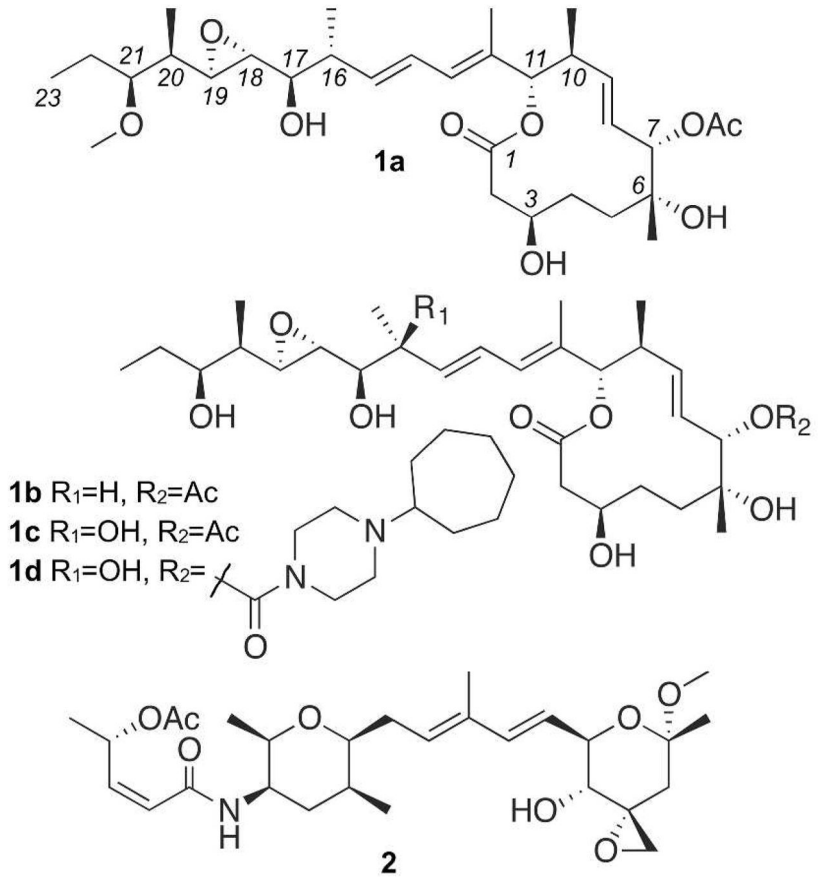


Figure 1.
Structures of **1a** (FD-895), **1b** (pladienolide B), **1c** (pladienolide C), the clinical entry **1d** (E7107), and the related spliceosome targeting natural product derivative spliceostatin (**2**).

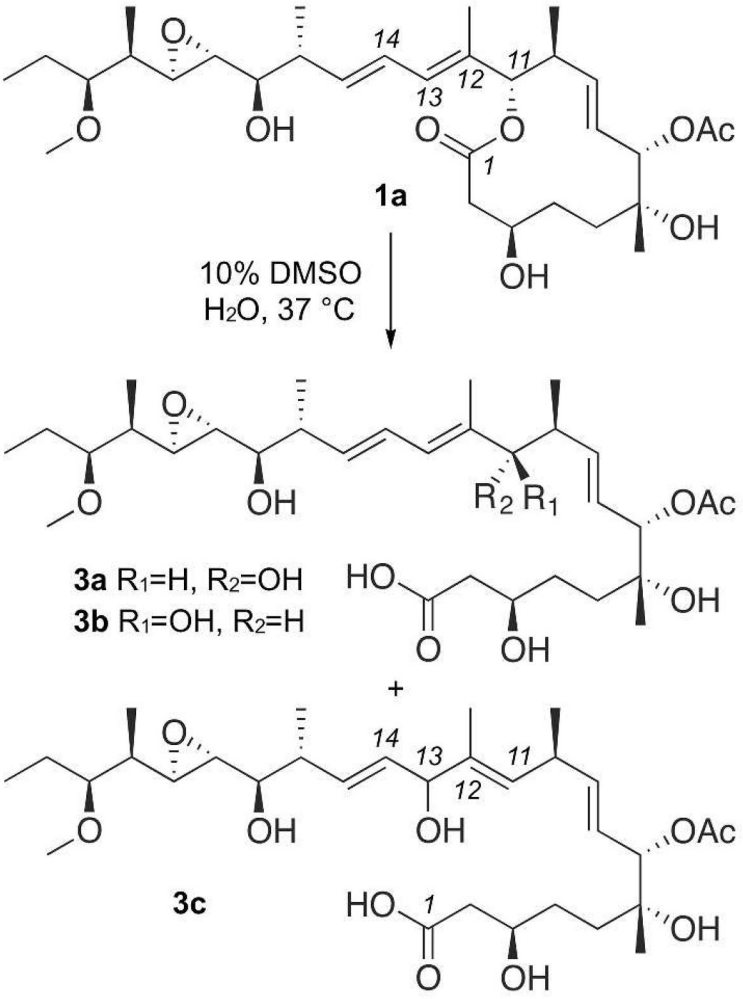


Figure 2.
In aqueous media, both natural and synthetic FD–895 (**1a**) undergo hydrolysis resulting in acids **3a**–**3c**.

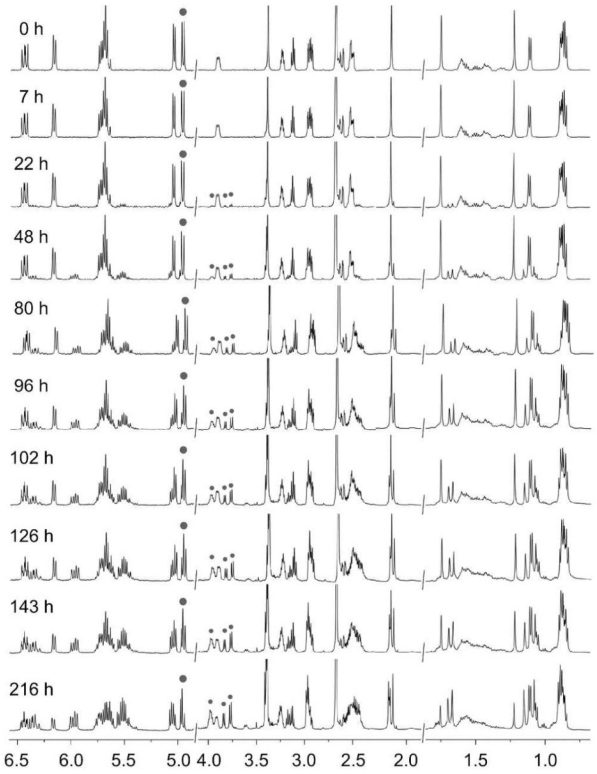


Figure 3.

NMR monitor of the hydrolytic decomposition of 10 mM **1a** in D_2O/DMSO-d_6 (10:1) at 37 °C. Hydrolysis occurs as marked by a shift of the C11 proton (large to small circles). Assignments of **3a**, **3b** and **3c** were made based on coupling constant and gCOSY analyses.

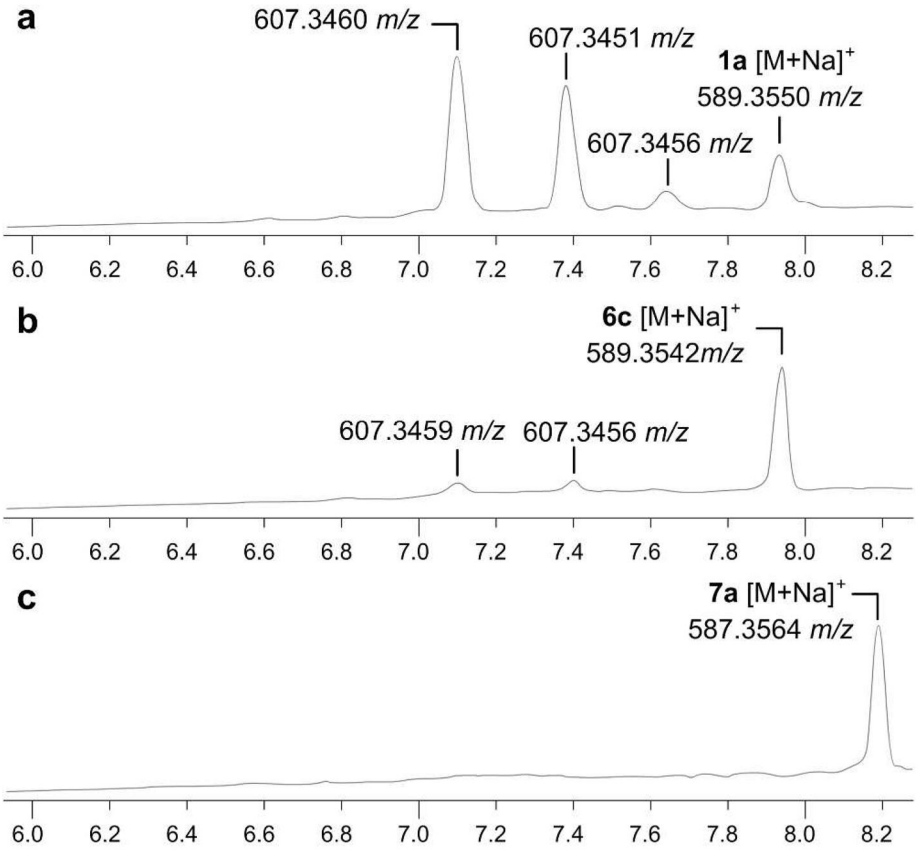


Figure 4.

LC-HRMS traces depicting the products from incubating samples of a) 10 mM **1a** in D_2O/DMSO-d_6 (10:1); b) 10 mM **6c** in D_2O/DMSO-d_6 (10:1); c) 10 mM **7a** in D_2O/DMSO-d_6 (10:1) at 37 °C for 143 h. The plot in a) corresponds to the NMR point at 143 h in Fig. 3. Hydrolysis of **1a** including acids **3a**–**3c**, while derivatives **1e** and cyclopropane **7a** remained stable. Exact masses $[M+Na]^+$ are provided for each peak.

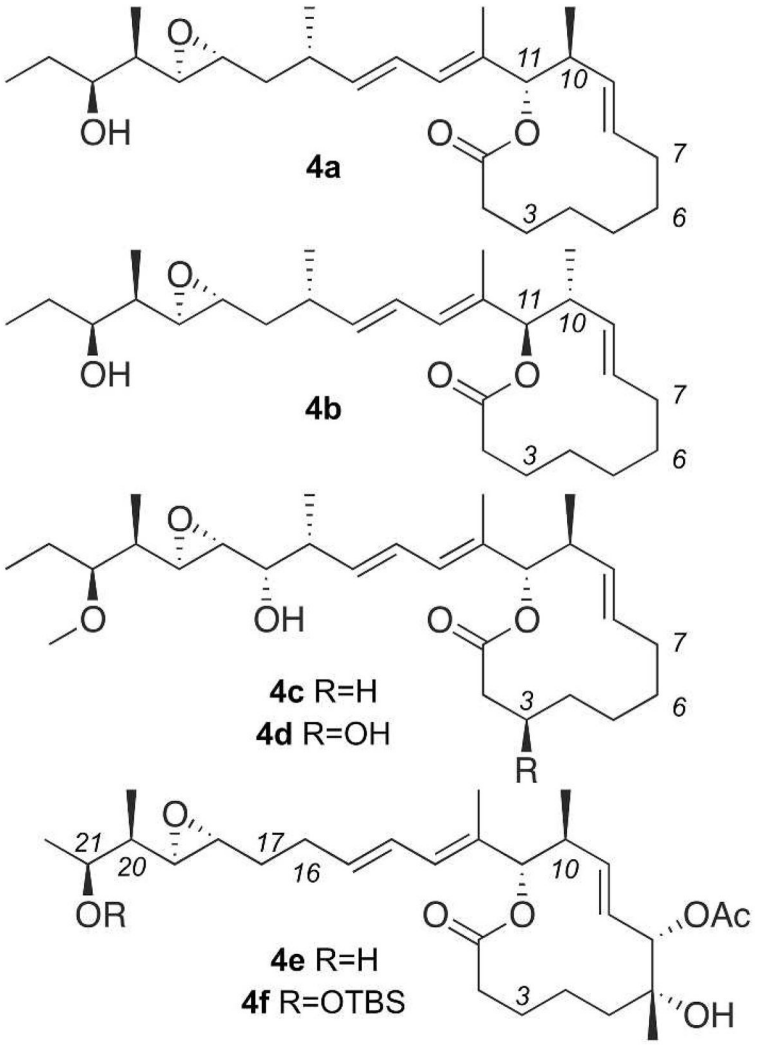


Figure 5.

Synthetic analogs **4a**–**4d** proved to be devoid of activity (see Table 1), indicating that gross modifications to the core would not be tolerated. This was confirmed by comparable analogs such as **4e**. Other analogs such as TBS-ether **4f** displayed modest activity.[10]

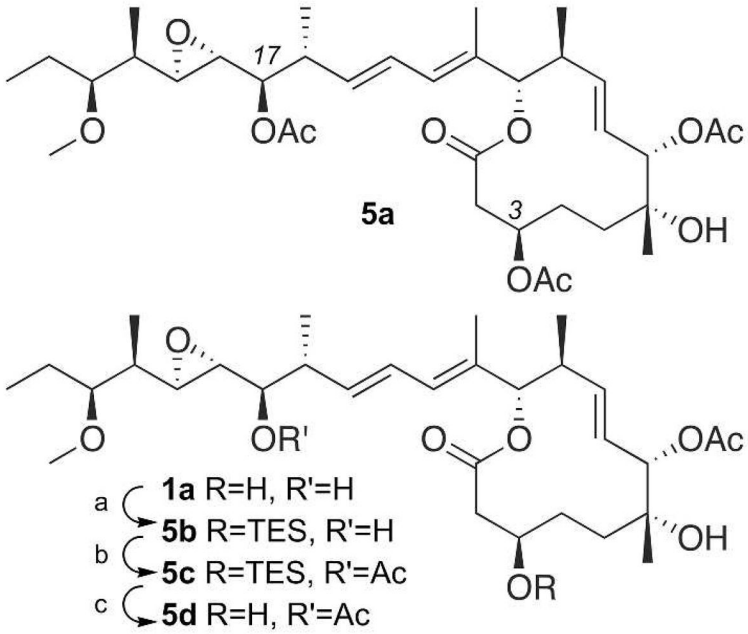


Figure 6.

Semisynthetic analogs **5a**–**5d** also failed to enhance the activity of **1a**. Reagents and conditions: (a) Ac_2O, DMAP, pyridine, 82%; (b) TESCl, imidazole, DMAP, THF, 8 h, rt, 87%; (c) Ac_2O, DMAP, pyridine, 75%; (d) TBAF, wet THF, 16 h, 52%.

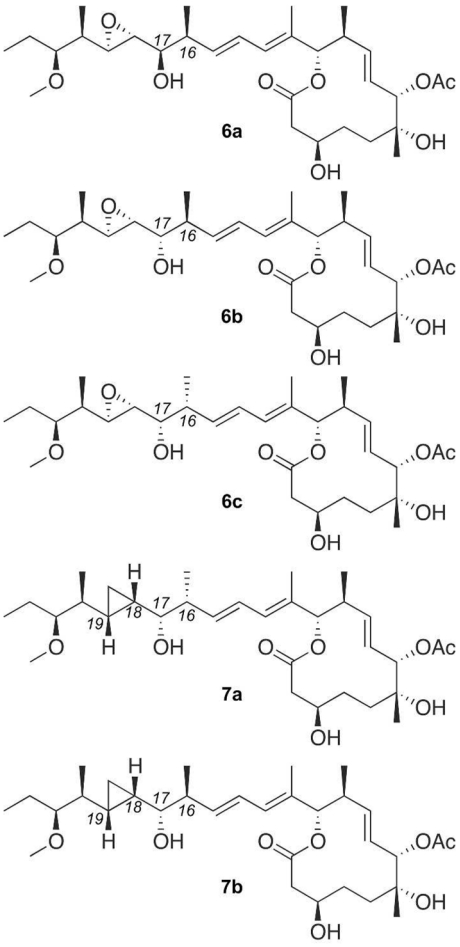


Figure 7.
Structures of the C16-C17-isomers **6a**–**6c** and C18-C19 cyclopropanes **7a** and **7b**.

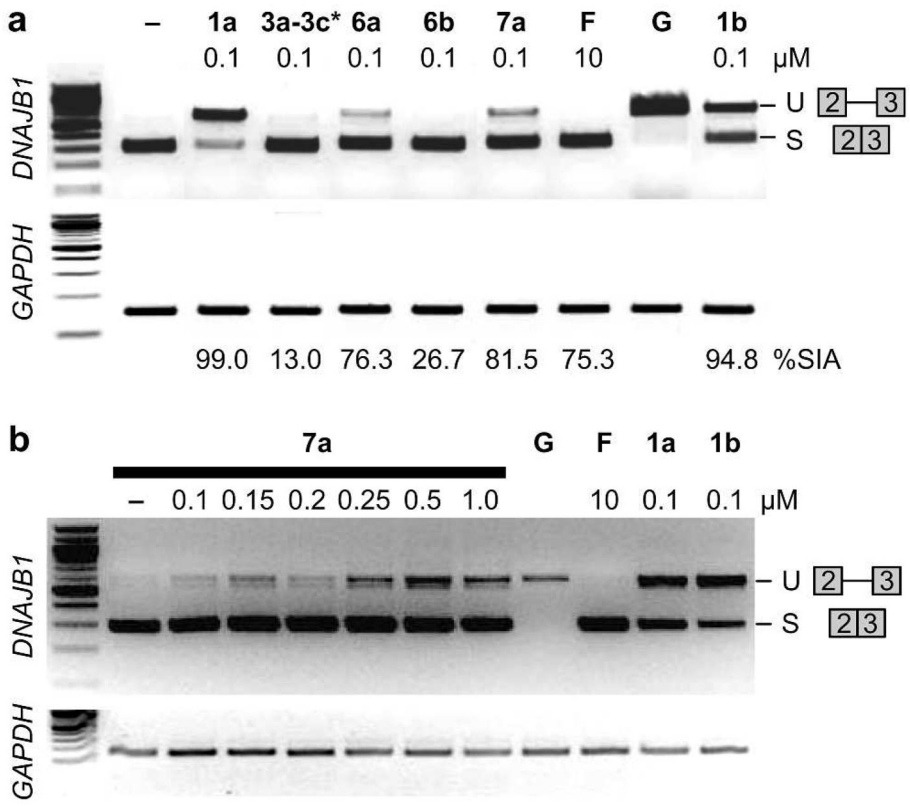
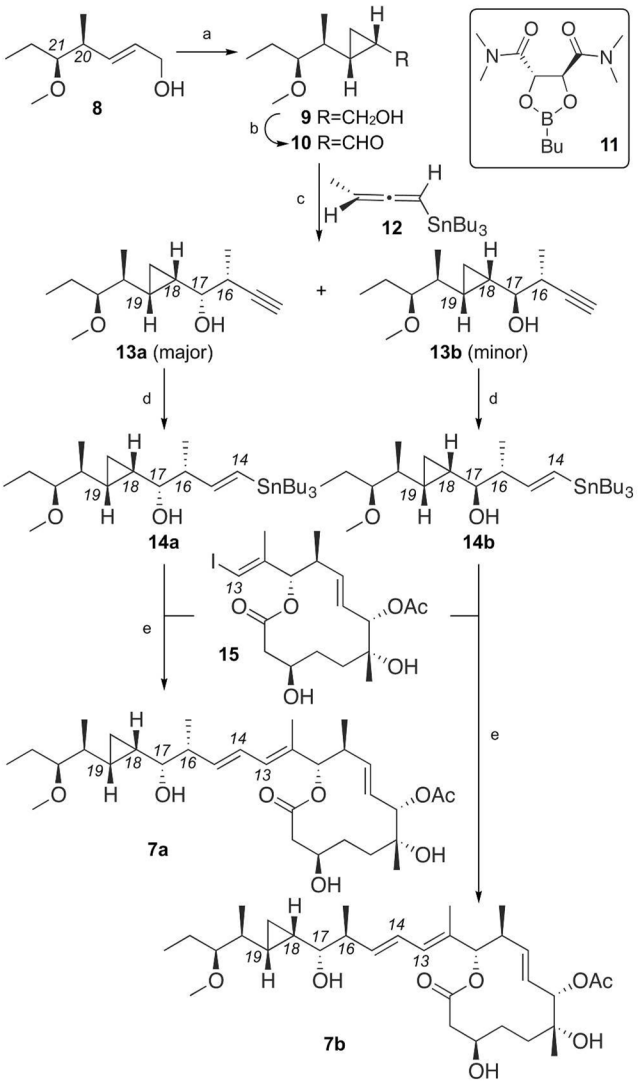


Figure 8.

RT-PCR analysis of splicing inhibitory activity. Splicing activity was determined by monitoring the increase of unspliced the *DNAJB1* gene as compared to *GAPDH* (a housekeeping gene). a) Compound screening identified **1a** as the most potent inhibitor with derivatives **6a** and **7a** also imparting splicing inhibition. %SIA denotes the percentage of apoptotic induction by this material. b) Splicing ability correlates with the concentration of **7a**. Controls are indicated by: (−) negative control or no compound added; (G) genomic DNA; (F) fludarabine. Spliced (S) and unspliced (U) forms of *DNAJB1* are shown. *A mixture of **3a**–**3c** was obtained from the hydrolysis of **1a** after 143 h at 37 °C in H_2O/DMSO (10:1). Acids **3a**–**3c** were tested as a mixture.



Scheme 1.

Synthesis of cyclopropane analogs **7a** and **7b**.[a]

[a]Reagents and conditions: (a) **11**, Zn, CH_2I_2, DME, −10 °C to rt, 83%; (b) Dess-Martin periodinane, CH_2Cl_2, 0 °C to rt, 80%; (c) **12**, BF_3•Et_2O, CH_2Cl_2, −78 °C to rt, 80% of a 3:2 mixture of chromatographically separable **13a:13b**; (d) $PdCl_2(PPh_3)_2$, (n-Bu)$_3$SnH, THF, rt; (e) $Pd_2(dba)_3$, $AsPh_3$, LiCl, NMP, 73% for **7a** over two steps from **13a** and 80% for **7b** from **13b**.

Table 1

Growth Inhibition Data

	HCT-116[a] IC_{50} (nM)	CLL IC_{50} (nM)
1a	23.5 ± 0.2	2.1 ± 0.1
1b	24.8 ± 0.4	30.5 ± 0.2
3a–3c (mixture)	11,200 ± 1,400	142.0 ± 0.3
4a	>50,000	
4b	>50,000	
4c	>50,000	
4d	>50,000	
5a	542.0 ± 12.1	
5d	142.2 ± 6.3	
6a	3.7 ± 0.2	108.9 ± 0.2
6b	23.0 ± 1.2	>50,000
6c	0.8 ± 0.1	
7a	42.9 ± 1.3	102.9 ± 0.1
7b	82.3 ± 3.2	

[a] IC_{50} values were collected using the MTT assay and data was presented as an average of 3–5 repetitions.

[b] IC_{50} values were calculated by GraphPad Prism v5.0 to determine the concentration to induce 50% SIA using at least three different concentrations as noted in the experimental procedures.