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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3208–3216
Bioorganic & Medicinal Chemistry

Synthesis of carbamate derivatives of iejimalides. Retention of normal antiproliferative activity and localization of binding in cancer cells

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Received 4 January 2007; revised 16 February 2007; accepted 19 February 2007
Available online 23 February 2007

Abstract—The syntheses of six iejimalide carbamate derivatives are described. Their biological activity and those of the unmodified iejimalides A and B against breast and prostate cancer cell lines were determined. These results show that the serine hydroxyl group of iejimalides A and B is a permissive site that can be functionalized to form carbamate derivatives without significant loss of normal biological activity. This method of derivatization will be valuable for cellular target identification, mechanism of action studies, and drug development efforts. A fluorescent derivative does not exhibit binding to the cytoskeletal features of cancer cells.

1. Introduction

Iejimalides A–D (**1a**–**d**) constitute a class of naturally occurring marine macrolides. These compounds occur in minute quantities (0.0003–0.0006% of wet weight) in *Eudistoma* cf. *rigida*, a species of marine tunicate native to coral reefs in the vicinity of Ie Island (Iejima) near Okinawa, Japan. They were originally isolated by J. Kobayashi and co-workers.[1–3] Their structures consist of a 24-membered lactone ring bearing an *N*-formyl serine terminated side chain (Fig. 1). The only difference between iejimalide A (**1a**) and iejimalide B (**1b**) is the simple replacement of the hydrogen of the C(2) carbon by a methyl group.

The Kobayashi laboratory measured high levels of cytotoxicity of the iejimalides in a few cancer cell lines.[1,2] We have also confirmed the significant biological activity of these compounds against several other human cancer cell lines. As we will report in detail elsewhere, we have data for over 70 cell lines to date, including the standard NCI panel and others. As of now, only limited studies of the biological target(s) of the iejimalides have been reported. In a recent study, iejimalides were shown to have an antiosteoporotic effect via the inhibition of a V-ATPase.[4] Total syntheses of iejimalide B have been accomplished in the Fürstner[5] and Helquist laboratories.[6–10] As of now, no iejimalide analogues for use in drug development efforts have been reported.

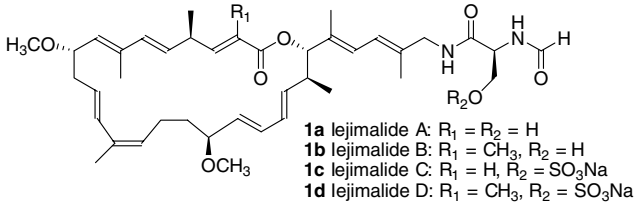

Figure 1. Structures of iejimalides A through D.

In order to perform these further investigations, a permissive site must be found in the iejimalides to allow incorporation of appropriate structural modifications

Keywords: Iejimalide; Coumarin; Cancer; Growth inhibition; Cellular localization; Cell imaging microscopy; Microtubules.
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doi:10.1016/j.bmc.2007.02.046

without adversely affecting the biological activity. For example, it would be useful to be able to attach a fluorescent or an affinity label to track cellular uptake and subcellular sites of accumulation of the iejimalides. Likewise, drug development efforts may require incorporation of groups to improve physiological transport and other therapeutic properties.

In this paper, we describe our initial studies of the preparation of derivatives of the iejimalides and the study of their biological activity. These early investigations have been successful in elucidating appropriate means for obtaining active, stable derivatives of the iejimalides and for providing an initial indication of the intracellular binding sites of the compounds.

2. Results and discussion

2.1. Synthesis of iejimalide carbamates

The iejimalides are nearly devoid of functional groups that can serve as sites for direct derivatization with minimal changes of structure. The most accessible site for derivatization is the hydroxyl group of the serine subunit of iejimalides A and B (**1a** and **1b**). Less useful reactivity is provided by the amide and ether groups. Direct reactions of any of these less reactive groups would disrupt the overall structural integrity of the iejimalide system. In order to probe for the feasibility of using the serine hydroxyl group to provide derivatives that retain normal biological activity, the synthesis of carbamate derivatives was undertaken. Although the formation of esters could also be considered, carbamates are expected to be more robust than esters under physiological conditions. Esters may be more appropriate choices as possible prodrug forms for later drug development studies; for the purpose of other studies, we have in fact synthesized simple iejimalide ester derivatives.

The reactions of iejimalides A (**1a**) and B (**1b**), with phenyl isocyanate and β-naphthyl isocyanate in the presence of 4-dimethylaminopyridine (DMAP) and *N*,*N*-diisopropylethylamine, resulted in the formation of the corresponding carbamate derivatives **2a,b** and **3a,b** in reasonable yields (Scheme 1). Due to the limited availability of the natural iejimalides, these reactions were necessarily performed on small scales. The products were purified effectively by HPLC. These results show that the hydroxyl functionality of iejimalides is an accessible point for derivatization. The resulting carbamates are the first reported derivatives of the iejimalides.[9]

In order to probe the influence of a linker between the naphthyl unit and the iejimalide core on biological activity, a β-naphthol iejimalide B carbamate derivative (**6b**) containing a 1,5-pentylene linker was also prepared (Scheme 2). β-Naphthol was first alkylated with 1,5-diiodopentane. The resulting alkyl iodide (**4**) was treated with AgOCN to form an alkyl isocyanate (**5**),[11] which was allowed to react with iejimalide B under the standard conditions.

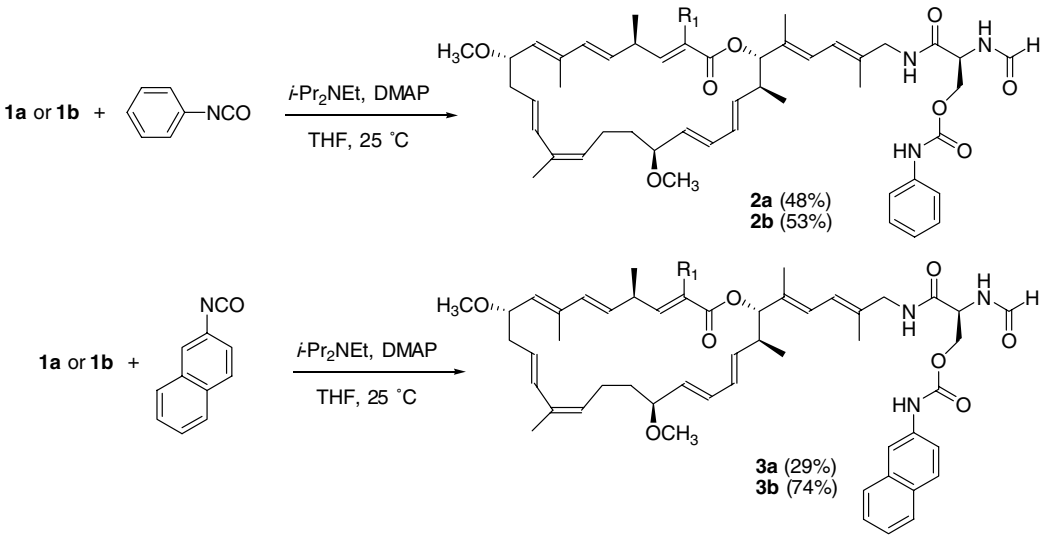
2.2. Biological activity of unmodified iejimalides and iejimalide carbamates

As mentioned above, we have assay data for the ie-jimalides in over 70 cell lines. In the present study, we have employed the cell lines MCF7, MDA231, and SKBR3 derived from breast tumors, and the PC3 cell line derived from a prostate tumor. In these cell lines and others which we have studied, iejimalide B (**1b**) was observed to be an order of magnitude more potent than iejimalide A (**1a**) despite the minimal structural difference between them. The cell growth inhibition activities of **2a**, **2b**, **3a**, **3b**, and **6b** against the same human cancer cell lines as the unmodified iejimalides were determined. The GI_{50} values are summarized in Table 1.

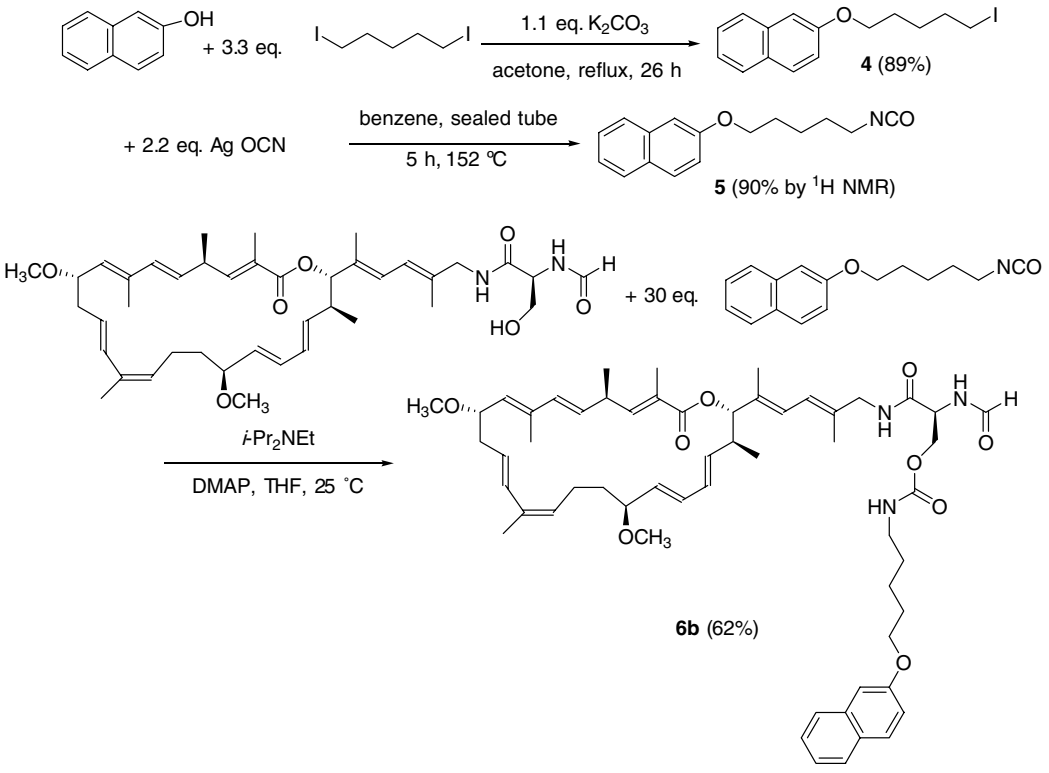
Consistent with previous results, the data indicate that the parent compound iejimalide B (**1b**) is a potent agent causing complete growth arrest at very low nM concentrations. Most importantly, the new data show that the phenyl carbamates **2a** and **2b** have the same activity profiles and very nearly the same levels of activity as the unmodified iejimalides. However, there is an order of magnitude difference in activity between the phenyl carbamates **2a** and **2b** and the β-naphthyl carbamates **3a** and **3b**, with the latter being less potent. Even with this reduction of potency, the activity of most of the derivatives is sufficient for the purposes of this work.

2.3. Synthesis of iejimalide B coumarin conjugate

Since a carbamate substitution of the serine side chain produced iejimalide derivatives which retained significant levels of activity, we reasoned that the attachment of a fluorophore of approximately the same size as a naphthyl group might produce an iejimalide fluorophore conjugate that also retains significant biological activity. Thus, such a derivative may be suitable to study the cellular localization of the iejimalides. We therefore decided to pursue the attachment of a 7-diethylaminocoumarin-3-carbonyl substituent to an iejimalide through carbamate formation (Scheme 3). Recently, the attachment of 7-diethylaminocoumarin-3-carbonyl to another natural product through a carbamate linkage was reported in the literature.[12] We chose to synthesize the coumarin derivative of iejimalide B (**1b**), since ie-jimalide B is on average about one order of magnitude more active than iejimalide A towards several cell lines (Table 1). Heating a benzene solution of 7-diethylaminocoumarin-3-carbonyl azide for 5 h at 85 °C resulted in a Curtius rearrangement to form 7-diethylaminocoumarin-3-isocyanate, which then reacted with iejimalide B (**1b**) in the same manner as employed for the other carbamate derivatives to form the 7-diethylaminocoumarin-3-carbamate of iejimalide B (**7b**). In order to access a control compound for the biological studies, the 7-diethylaminocoumarin-3-carbamate derivative (**8**) of benzyl alcohol was also prepared following the same procedure.



Scheme 1. Syntheses of phenyl carbamate (**2a** and **2b**) and β-naphthyl carbamate derivatives (**3a** and **3b**) of iejimalides A and B (**a**, $R_1 = H$; **b**, $R_1 = CH_3$).

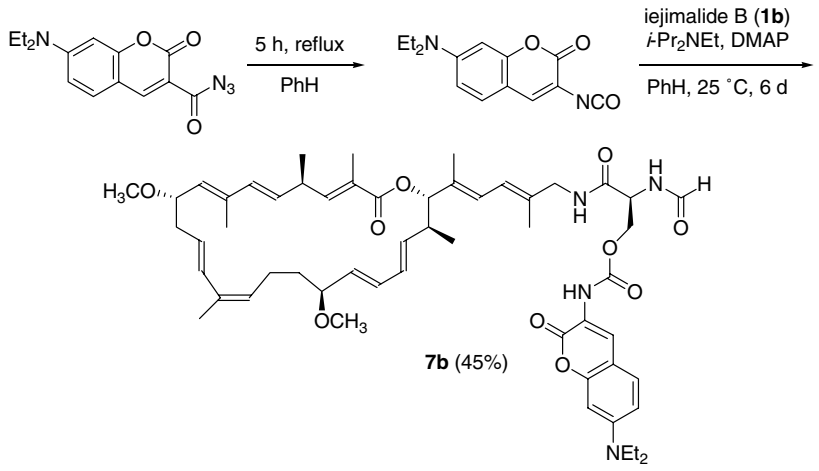


Scheme 2. Synthesis of β-naphthol iejimalide B carbamate derivative (**6b**).

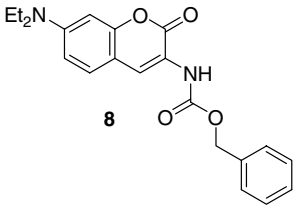
Table 1. GI_{50} (nM) of iejimalides A and B, and their derivatives

Cell line	1a	2a	3a	1b	2b	3b	6b	7b
MCF7	6.9	7.5	126	1.2	8.3	44	12.7	13.6
MDA231	9.4	12.0	221	1.2	8.8	44	12.7	13.6
PC3	6.1	15.3	99	1.4	9.9	60	18.6	16.4
SKBr3	<5.0	20.0	77	1.0	10.5	82	41	10.9

1a, iejimalide A; **2a**, iejimalide A phenyl carbamate; **3a**, iejimalide A β-naphthyl carbamate; **1b**, iejimalide B; **2b**, iejimalide B phenyl carbamate; **3b**, iejimalide B β-naphthyl carbamate; **6b**, β-naphthol iejimalide B carbamate derivative; **7b**, 7-diethylaminocoumarin-3-carbamate of iejimalide B.



Scheme 3. Synthesis of 7-diethylaminocoumarin-3-carbamate of iejimalide B (**7b**).



The biological activity of this coumarin standard was tested against the four cell lines employed in this study. It did not show growth inhibition activity against any of these cell lines (data not shown).

2.4. Biological activity and cellular localization of iejimalide B coumarin conjugate

We tested the biological activity of iejimalide B 7-diethylaminocoumarin-3-carbamate (**7b**) against human cancer cell lines (Table 1). Interestingly, we found that iejimalide B coumarin conjugate **7b** has an activity profile similar to that observed with iejimalide B phenyl carbamate (**2b**).

To investigate the cellular localization of this fluorescent iejimalide B derivative, PC3 live cell imaging was conducted with confocal microscopy (Fig. 2). Iejimalide B coumarin conjugate **7b** showed a significantly different cell staining pattern than that of the control benzyl coumarin **8**. It can be concluded that the difference in cellular localization of iejimalide coumarin conjugate **7b** as compared to coumarin standard **8** is due to its parent iejimalide portion.

Figure 2A shows that iejimalide B coumarin **7b** does not accumulate in the nucleus, but it instead accumulates at a number of specific locations within the cytoplasm. Although these data may suggest a higher concentration of **7b** in the vicinity of mitochondria, none of our other studies have suggested inhibition of mitochondrial function. In contrast to **7b**, its coumarin standard **8** is contained in the endosomes. While an endosomal localization of the iejimalides cannot be ruled out by these studies, Figure 2A indicates that the uptake and apparent cellular distribution of the iejimalide-coumarin conjugate is elevated in these cells when compared to dye alone.

NCI COMPARE analyses,[13,14] which allow tentative predictions of the biochemical mechanisms of action of novel drugs on the basis of their in vitro activity profiles, were conducted on both iejimalide A and iejimalide B.[15] A modest correlation was found between P3616, S236580 (destruxin B), P3618, and iejimalide A, and between S24818 (podophyllotoxin), S648784, and iejimalide B. Among the possible mechanisms of action of these compounds is the disruption of microtubules.[16]

However, co-staining of PC3 cells with both a monoclonal anti-microtubule antibody and iejimalide B coumarin **8b** revealed that a significant portion of iejimalide B coumarin **8b** does not co-localize with the microtubules (Fig. 3). Cells treated with iejimalide B at 10 nM for 12 h did not show microtubule binding or disruption as indicated by comparison with paclitaxel[17,18] (Fig. 4). Furthermore, **8b** appears mainly perinuclear localized. The results suggest that iejimalides may have a different, non-microtubule-based mechanism of action, which is also in agreement with other recent studies.[4,9] However, the significant intracellular distribution of iejimalide indicates that additional sites of binding and initiation of a drug action may exist and contribute to the tumor cell growth inhibition.

3. Significance of the work

The most important finding from the present study is that the serine subunit of the iejimalide core provides a conveniently manipulated, permissive site for derivatization without adversely affecting the tumor cell growth inhibitory activity. Using a fluorophore-labeled iejimalide B, we have shown that the iejimalide core structure is responsible for cellular uptake and that the subcellular distribution does not appear to involve nuclear localization. A combination of results reported here and other

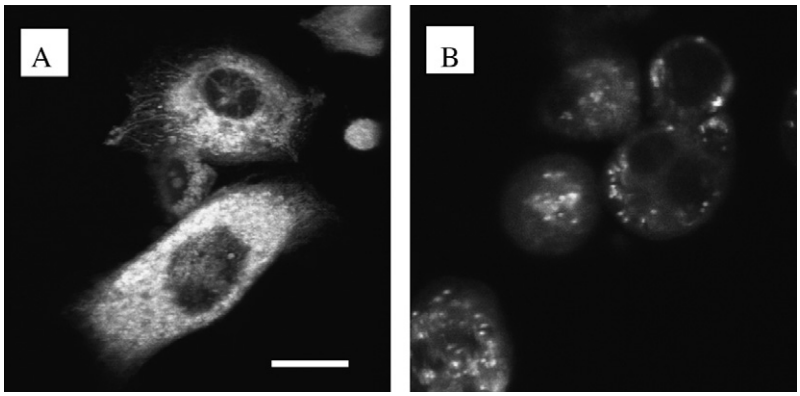


Figure 2. Confocal images of iejimalide B coumarin **7b** (A) and its coumarin standard **8** (B). PC3 cells were treated with either 200 nM iejimalide coumarin or 200 nM coumarin standard for 3 h and then washed with PBS three times. Live cell images were obtained by confocal microscopy. The length of the scale bar is 10 μm.

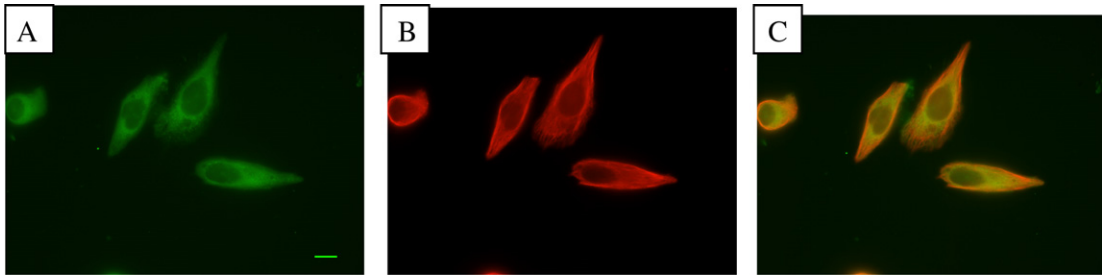


Figure 3. Differential staining of iejimalide B coumarin **7b** and microtubules on fixed PC3 cells. Fixed cells were co-stained with iejimalide B coumarin **7b** (A) and a mouse monoclonal anti-microtubule antibody (B). The merged image (C) does not show co-localization of iejimalide B coumarin with the microtubules. The length of the scale bar is 10 μm.

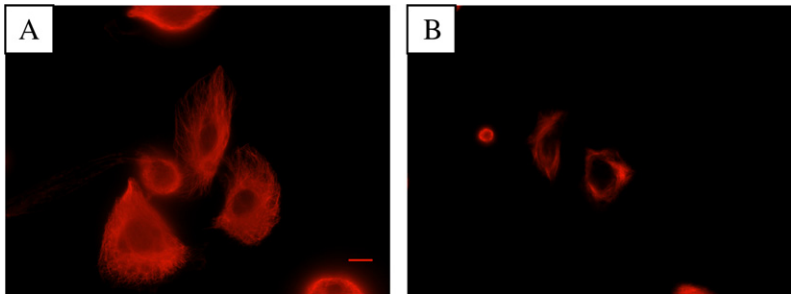


Figure 4. Staining of the microtubules in fixed PC3 cells treated with 10 nM iejimalide B (**1b**) (A) and 100 nM paclitaxel (B). The cells were treated in both cases for 12 h with 10 nM iejimalide B (**1b**) and 100 nM paclitaxel, respectively, and were then fixed and stained with a mouse monoclonal anti-microtubule antibody. The microtubule structure is not disrupted in the case of iejimalide B treated PC3 cells (A), whereas it is in the case of paclitaxel treated cells (B).

studies from our laboratories has further substantiated Kobayashi's earlier reports of potent inhibitory activity of the iejimalides against several cancer cell lines. In work to be reported elsewhere, cell line selectivity is also indicated. Ongoing studies are being directed at identification of the specific binding sites and mechanism(s) of action.

4. Experimental

4.1. General methods

Unless stated otherwise, all reagents and solvents were used as received from commercial suppliers. 7-Diethylaminocoumarin-3-carbonyl azide (D-1446) was obtained from Molecular Probes. All moisture sensitive reactions were performed using dried solvents in flame-dried glassware under an atmosphere of Ar. ^{1}H NMR spectra were measured at 300 MHz on a Varian VXR-300 spectrometer. All chemical shifts (δ) are relative to residual solvent. Mass spectral data were measured on a JEOL JMS-AX505 HA spectrometer. All HPLC separations were performed using a C18 reverse phase column (RCM 8×10, Radial-Pak Cartridge, Type: 8NV C18 4μ or Novo-Pak C18, 3.9×150 mm) on a Waters® HPLC system with absorption detection at 254 nm.

4.2. Isolation of iejimalides from tunicates

Specimens of *Eudistoma* cf. *rigida* (3.7 kg wet) were collected near Ie Island, Japan, cut up, and repeatedly

extracted with acetone. A portion (1.038 g) of the crude acetone extract (7.24 g) was filtered through a pad of silica gel (60 mL, ICN 60 Å silica gel, 32–63 μ) using methanol (400 mL). After removal of the solvent, the residue was purified on a silica gel column (2.2×16 cm, gradient: 100% $CH_2Cl_2 \rightarrow 16\%$ MeOH/CH_2Cl_2). Fractions containing iejimalides were identified by the presence of the *N*-formyl signal at 8.3 ppm in the ^1H NMR spectrum. All iejimalide-containing fractions were combined, and the solvent was removed on a rotary evaporator. The residue was purified on a second silica gel column (2.2×16 cm, gradient: 100% $CH_2Cl_2 \rightarrow 5\%$ MeOH/CH_2Cl_2) to yield 35 mg of iejimalide-containing material. This material was purified on a Sephadex LH-20 column (2.2×14 cm, gradient: 100% $CH_2Cl_2 \rightarrow 50\%$ MeOH/CH_2Cl_2), followed by a third silica gel column (2.2×12 cm, gradient: 100% $CH_2Cl_2 \rightarrow 5\%$ MeOH/CH_2Cl_2) to yield 25 mg of iejimalide-containing material. On this material, HPLC (C18, isocratic MeCN/H_2O: 70:30, 1 mL/min, t_R (iejimalide A) = 13.0 min, t_R (iejimalide B) = 15.0 min) was performed to yield 6.5 mg of iejimalide A and 7.7 mg of iejimalide B as white, amorphous solids.

4.3. Phenyl carbamate (2a) of iejimalide A

DMAP (0.9 mg, 0.0074 mmol) was added to a flask containing iejimalide A (**1a**) (3.8 mg, 0.0056 mmol), followed by dry THF (1.5 mL). Phenyl isocyanate (5 μL, 0.0459 mmol) and *N*,*N*-diisopropylethylamine (2 μL, 0.0115 mmol) were added, followed by dry THF (0.5 mL). The mixture was stirred at 25 °C for 45 h. The reaction was quenched by adding five drops of H_2O, and the mixture was evaporated to dryness. The residue was taken up in CH_2Cl_2/H_2O. After extraction and phase separation, the aqueous phase was extracted with additional CH_2Cl_2. The combined organic phase was evaporated to dryness, redissolved in $CDCl_3$, and filtered through cotton in a pipette. The product was purified by HPLC (C18, isocratic MeCN/H_2O: 70:30, 1 mL/min, t_R = 28.0 min) to provide **2a** as an off-white solid (2.1 mg, 0.0027 mmol, 48% yield): ^1H NMR (300 MHz, $CDCl_3$): δ 8.30 (s, 1H, H_{34}), 8.26 (m, 1H, aromatic), 7.35 (m, 3H, aromatic), 7.11 (m, 1H, aromatic), 6.91 (m, 2H, NH_{30}, NH_{33}), 6.80 (dd, 1H, J = 9.6, 15.4 Hz, H_3), 6.70 (m, 1H, NH_{47}), 6.45 (d, 1H, J = 15.8 Hz, H_{12}), 6.26 (d, 1H, J = 12.0 Hz, H_{26}), 6.13 (d, 1H, J = 12.0 Hz, H_{27}), 6.04 (m, 2H, H_{19}, H_{20}), 5.90 (d, 1H, J = 15.4 Hz, H_6), 5.71 (d, 1H, J = 15.6 Hz, H_2), 5.58–5.36 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.12 (m, 3H, H_8, H_{14}, H_{23}), 4.81 (m, 1H, H_{32}), 4.52 (m, 2H, H_{35}), 4.15 (m, 1H, H_9), 3.91 (m, 2H, H_{29}), 3.34 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.03 (s, 3H, H_{39}), 2.96 (m, 1H, H_4), 2.62 (m, 1H, H_{10}), 2.51 (m, 2H, H_{15}, H_{22}), 2.35 (m, 1H, H_{10}), 2.02 (m, 1H, H_{15}), 1.81–1.74 (4 overlapping s, 12H, H_{36}, H_{37}, H_{40}, H_{42}), 1.61 (m, 1H, H_{16}), 1.41 (m, 1H, H_{16}), 1.10 (d, 3H, J = 6.8 Hz, H_{43}), 0.91 (d, 3H, J = 6.8 Hz, H_{38}).

4.4. β-Naphthyl carbamate (3a) of iejimalide A

The reaction was performed as for **2a** using DMAP (2.0 mg, 0.0164 mmol), β-naphthyl isocyanate (6.0 mg, 0.0355 mmol), *N*,*N*-diisopropylethylamine (2 μL, 0.0115 mmol), and iejimalide A (**1a**) (2.8 mg, 0.0041 mmol). The product was purified by HPLC (C18, MeCN/H_2O: 80:20, 1 mL/min, t_R = 16.5 min) to provide **3a** as an off-white solid (1.0 mg, 0.0012 mmol, 29% yield): ^1H NMR (300 MHz, $CDCl_3$): δ 8.33 (s, 1H, H_{34}), 8.15 (m, 2H, aromatic), 7.97 (s, 1H, aromatic), 7.79 (m, 2H, aromatic), 7.44 (m, 2H, aromatic), 7.14 (m, 1H, NH_{30} or NH_{33}), 6.98 (m, 1H, NH_{33} or NH_{30}), 6.79 (dd, 1H, J = 9.5, 15.3 Hz, H_3), 6.70 (m, 1H, NH_{47}), 6.51–6.38 (m, 2H, H_{12}, H_{26}), 6.30–5.85 (m, 4H, H_6, H_{19}, H_{20}, H_{27}), 5.70 (d, 1H, J = 15.4 Hz, H_2), 5.64–5.32 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.20 (m, 1H, H_{14}), 5.11 (m, 1H, H_{23}), 5.02 (d, 1H, J = 10.0 Hz, H_8), 4.84 (m, 1H, H_{32}), 4.57 (m, 2H, H_{35}), 4.14 (m, 1H, H_9), 3.92 (m, 2H, H_{29}), 3.34 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.03 (s, 3H, H_{39}), 2.96 (m, 1H, H_4), 2.62 (m, 1H, H_{10}), 2.51 (m, 2H, H_{15}, H_{22}), 2.35 (m, 1H, H_{10}), 2.02 (m, 1H, H_{15}), 1.81–1.74 (4 overlapping s, 12H, H_{36}, H_{37}, H_{40}, H_{42}), 1.61 (m, 1H, H_{16}), 1.41 (m, 1H, H_{16}), 1.10 (d, 3H, J = 6.8 Hz, H_{43}), 0.91 (d, 3H, J = 6.8 Hz, H_{38}).

4.5. Phenyl carbamate (2b) of iejimalide B

The reaction was performed as above using DMAP (3.0 mg, 0.0245 mmol), iejimalide B (**1b**) (1.3 mg, 0.0018 mmol), phenyl isocyanate (6 μL, 0.0550 mmol), and *N*,*N*-diisopropylethylamine (5 μL, 0.0286 mmol). The product was purified by HPLC (C18, isocratic MeCN/H_2O: 80:20, 1 mL/min, t_R = 14.5 min) to provide **2b** as an off-white solid (0.8 mg, 0.0010 mmol, 53% yield): ^1H NMR (300 MHz, $CDCl_3$): δ 8.31 (s, 1H, H_{34}), 8.15 (m, 1H, aromatic), 7.35 (m, 3H, aromatic), 7.11 (m, 1H, aromatic), 6.88 (m, 2H, NH_{30}, NH_{33}), 6.71 (m, 1H, NH_{47}), 6.62 (d, 1H, J = 9.6 Hz, H_3), 6.50 (d, 1H, J = 16.3 Hz, H_{12}), 6.41 (m, 1H, H_{19} or H_{20}), 6.28 (d, 1H, J = 12.0 Hz, H_{26}), 6.14 (d, 1H, J = 12.0 Hz, H_{27}), 6.02 (m, 1H, H_{20} or H_{19}), 5.86 (d, 1H, J = 15.6 Hz, H_6), 5.58–5.34 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.15 (m, 3H, H_{14}, H_{23}, H_8), 4.83 (m, 1H, H_{32}), 4.54 (m, 2H, H_{35}), 4.14 (m, 1H, H_9), 3.92 (m, 2H, H_{29}), 3.30 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.14 (m, 1H, H_4), 2.96 (s, 3H, H_{39}), 2.67 (m, 1H, H_{10}), 2.56 (m, 2H, H_{15}, H_{22}), 2.32 (m, 1H, H_{10}), 1.90 (m, 1H, H_{15}), 1.81–1.75 (5 overlapping s, 15 H, H_{36}, H_{37}, H_{40}, H_{42}, H_{44}), 1.67 (m, 1H, H_{16}), 1.32 (m, 1H, H_{16}), 1.05 (d, 3H, J = 6.4 Hz, H_{43}), 0.92 (d, 3H, J = 6.4 Hz, H_{38}). HRMS (FAB+) m/z ($[(M-CH_3O)H]^+$): Calcd for $C_{47}H_{62}N_3O_7$: 780.4588. Found: 780.4594.

4.6. β-Naphthyl carbamate (3b) of iejimalide B

The reaction was performed as above using DMAP (3.0 mg, 0.0245 mmol), β-naphthyl isocyanate (7.1 mg, 0.0419 mmol), iejimalide B (**1b**) (1.3 mg, 0.0018 mmol), and *N*,*N*-diisopropylethylamine (5 μL, 0.0286 mmol). The product was purified by HPLC (C18, MeCN/H_2O: 80:20, 1 mL/min, t_R = 19.5 min) to provide **3b** as an off-white solid (1.2 mg, 0.0014 mmol, 74% yield): ^1H NMR (300 MHz, $CDCl_3$): δ 8.33 (s, 1H, H_{34}), 8.20 (m, 2H, aromatic), 7.97 (s, 1H, aromatic), 7.78 (m, 2H, aromatic), 7.43 (m, 2H, aromatic), 6.84 (d, 1H, J = 9.0 Hz,

NH_{33}), 6.61 (m, 3H, H_3, NH_{30}, NH_{47}), 6.49 (d, 1H, $J = 15.4$ Hz, H_{12}), 6.27 (d, 1H, $J = 11.6$ Hz, H_{26}), 6.14 (d, 1H, $J = 11.1$ Hz, H_{27}), 6.00 (m, 2H, H_{19}, H_{20}), 5.85 (d, 1H, $J = 15.2$ Hz, H_6), 5.58–5.26 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.11 (m, 3H, H_8, H_{14}, H_{23}), 4.87 (m, 1H, H_{32}), 4.62 (dd, 1H, $J = 5.7$, 11.8 Hz, H_{35}), 4.49 (dd, 1H, $J = 5.7$, 11.8 Hz, H_{35}), 4.13 (m, 1H, H_9), 3.92 (m, 2H, H_{29}), 3.30 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.14 (m, 1H, H_4), 2.96 (s, 3H, H_{39}), 2.67 (m, 1H, H_{10}), 2.56 (m, 2H, H_{15}, H_{22}), 2.32 (m, 1H, H_{10}), 1.90 (m, 1H, H_{15}), 1.81–1.75 (5 overlapping s, 15 H, H_{36}, H_{37}, H_{40}, H_{42}, H_{44}), 1.67 (m, 1H, H_{16}), 1.32 (m, 1H, H_{16}), 1.04 (d, 3H, $J = 6.6$ Hz, H_{43}), 0.93 (d, 3H, $J = 6.6$ Hz, H_{38}). HRMS (FAB+) *m/z* ($[M-CH_3O]^+$): Calcd for $C_{51}H_{64}N_3O_7$: 830.4744. Found: 830.4708.

4.7. 5-(1-Iodopentyl)-β-naphthol (4)

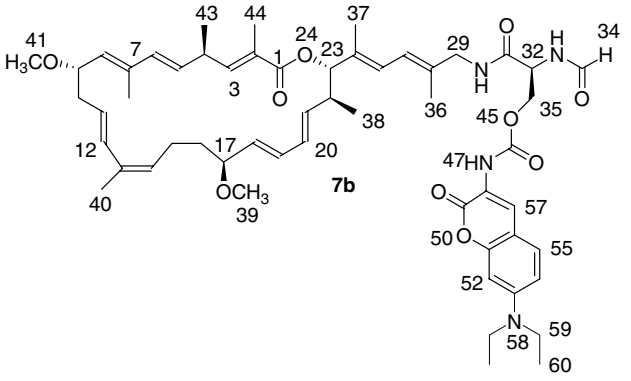
A stirred mixture containing β-naphthol (1.00 g, 6.93 mmol), K_2CO_3 (1.03 g, 7.45 mmol), freshly distilled 1,5-diiodopentane (7.46 g, 23.0 mmol), and acetone (50 mL) was heated under reflux for 27 h. After filtration and concentration, the residue was purified twice by chromatography (SiO_2, 2.8 × 19 cm, gradient: 100% hexanes → 20% ethyl acetate/hexanes) to provide **4** as a nearly colorless oil (2.09 g, 6.13 mmol, 89% yield) ($R_f = 0.65$ using 40% ethyl acetate/hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (m, 3H, aromatic), 7.48 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.37 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.21 (dd, 1H, $J = 9.6$, 2.6 Hz, aromatic), 7.11 (d, 1H, $J = 2.4$ Hz, aromatic), 3.94 (t, 2H, $J = 6.3$ Hz, H_5), 3.14 (t, 2H, $J = 7.0$ Hz, H_1), 1.76 (m, 4H, H_2, H_4), 1.52 (m, 2H, H_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 157.0, 134.7, 129.3, 129.0, 127.7, 126.7, 126.3, 123.5, 118.9, 106.9, 67.6, 33.2, 28.2, 27.2, 6.5; IR (thin film): 3056, 2939, 2868, 1621, 1600, 1511 cm^{-1}; HRMS (FAB+) *m/z* (M^+): Calcd for $C_{15}H_{17}OI$: 340.0324. Found: 340.0303.

4.8. 1-(5-Isocyanatopentyl)-β-naphthol (5)

Under stirring, 5-(1-iodopentyl)-β-naphthol (**4**) (151 mg, 0.44 mmol), AgNCO (145 mg, 0.97 mmol), and benzene (1.0 mL) were heated in a closed Ace pressure tube ($V = 60$ mL) to 152 °C for 5 h. After cooling, the content was filtered and concentrated to yield an oil. ^{1}H NMR indicated ca. 90% conversion to the desired product. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (m, 3H, aromatic), 7.48 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.37 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.18 (m, 2H, aromatic), 4.10 (t, 2H, $J = 6.3$ Hz, H_1), 3.36 (t, 2H, $J = 6.5$ Hz, H_5), 1.78 (m, 2H, H_2), 1.72 (m, 2H, H_3 or H_4), 1.63 (m, 2H, H_4 or H_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 157.2, 157.1, 134.8, 129.6, 129.1, 127.8, 126.9, 126.5, 123.7, 119.1, 106.7, 67.7, 43.0, 31.2, 28.8, 23.4; IR (thin film): 2274 cm^{-1}; HRMS (FAB+) *m/z* (M^+): Calcd for $C_{16}H_{17}NO_2$: 255.1259. Found: 255.1254.

4.9. β-Naphthol iejimalide B carbamate derivative (6b)

1-(5-Isocyanatopentyl)-β-naphthol (**6**) (114 mg, 0.446 mmol) was dissolved in THF (12 mL). A portion (1.5 mL, 0.056 mmol) of this solution was added to a flask containing iejimalide B (1.3 mg, 0.0019 mmol) and DMAP (4 mg, 0.033 mmol). *N*,*N*-Diisopropylethylamine (10 μL, 0.057 mmol) was added, and the mixture was stirred at 25 °C for 120 h. It was taken up in CH_2Cl_2/H_2O (20:15 mL). After extraction and phase separation, the aqueous phase was twice extracted with additional CH_2Cl_2 (20 mL). The combined organic phase was evaporated to dryness. The residue was dissolved in CH_2Cl_2 and filtered through cotton in a pipette. The residue was purified by HPLC (Novo-Pak C18, 3.9 × 150 mm, MeCN/H_2O: 85:15, 1 mL/min, $t_R = 8.2$ min) to provide **6b** as a white solid (1.1 mg, 0.0012 mmol, 62% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.27 (s, 1H, H_{34}), 7.75 (m, 3H, aromatic), 7.44 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.34 (dt, 1H, $J = 7.6$, 1.2 Hz, aromatic), 7.25 (s, 1H, NH_{47}), 7.14 (m, 2H, aromatic), 6.93 (d, 1H, $J = 6.9$ Hz, NH_{33}), 6.62 (d, 1H, $J = 10.3$ Hz, H_3), 6.49 (d, 1H, $J = 15.4$ Hz, H_{12}), 6.36 (t, 1H, $J = 5.6$ Hz, NH_{30}), 6.28 (d, 1H, $J = 11.1$ Hz, H_{26}), 6.12 (d, 1H, $J = 10.5$ Hz, H_{27}), 6.02 (m, 2H, H_{19}, H_{20}), 5.85 (d, 1H, $J = 15.4$ Hz, H_6), 5.57–5.32 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.13 (m, 3H, H_8, H_{14}, H_{23}), 4.89 (m, 1H, H_{32}), 4.71 (m, 1H, H_{35}), 4.42 (m, 1H, H_{35}), 4.13 (m, 1H, H_9), 4.09 (t, 2H, $J = 6.2$ Hz, H_{59}), 3.90 (m, 2H, H_{29}), 3.29 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.23 (m, 2H, H_{48}), 3.14 (m, 1H, H_4), 2.95 (s, 3H, H_{39}), 2.69 (m, 1H, H_{10}), 2.56 (m, 2H, H_{15}, H_{22}), 2.33 (m, 1H, H_{10}), 1.88 (m, 1H, H_{15}), 1.81–1.73 (5 overlapping s, 15 H, H_{36}, H_{37}, H_{40}, H_{42}, H_{44}), 1.59 (m, 7 H, H_{16}, H_{49}, H_{50}, H_{51}), 1.31 (m, 1H, H_{16}), 1.04 (d, 3H, $J = 6.6$ Hz, H_{43}), 0.92 (d, 3H, $J = 6.8$ Hz, H_{38}). HRMS (FAB+) *m/z* (M^+): Calcd for $C_{57}H_{77}N_3O_9$: 947.5660. Found: 947.5634.



4.10. 7-Diethylaminocoumarin-3-carbamate (7b) of iejimalide B

7-Diethylaminocoumarin-3-carbonyl azide (11.1 mg, 0.0387 mmol) was dried in a flask on a high vacuum line for 1 h. It was dissolved in dry benzene (3.0 mL) under Ar, and the solution was heated for 5 h at 85 °C. During this time, iejimalide B (**1b**) (2.6 mg, 0.0037 mmol) and DMAP (2.7 mg, 0.0221 mmol) were dried in a flask on a high vacuum line for 3 h. Once the benzene solution was cooled to 25 °C, it was added to the second flask. *N*,*N*-Diisopropylethylamine (10 μL, 0.0572 mmol) was added. The mixture was stirred at 25 °C for 136 h. The

reaction was quenched by adding five drops of H_2O, and the resulting mixture was evaporated to dryness. The residue was taken up in CH_2Cl_2/H_2O. After extraction and phase separation, the aqueous phase was extracted with additional CH_2Cl_2. The combined organic phase was evaporated to dryness and filtered through cotton in a pipette using CH_2Cl_2. The product was purified by HPLC (C18, MeCN/H_2O: 80:20, 1 mL/min, t_R = 28.5 min) to provide **7b** as a light yellow solid (1.6 mg, 0.0017 mmol, 45% yield): ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.32 (s, 1H, H_{34}), 8.20 (s, 1H, NH_{47} or H_{57}), 8.17 (s, 1H, H_{57} or NH_{47}), 7.43 (s, 1H, aromatic), 6.72 (m, 2H, NH_{33}, aromatic), 6.62 (m, 2H, H_3, NH_{30}), 6.49 (d, 1H, J = 14.1 Hz, H_{12}), 6.28 (m, 2H, H_{26}, aromatic), 6.14 (d, 1H, J = 11.3 Hz, H_{27}), 6.02 (m, 2H, H_{19}, H_{20}), 5.85 (d, 1H, J = 15.6 Hz, H_6), 5.57–5.32 (m, 4H, H_5, H_{11}, H_{18}, H_{21}), 5.13 (m, 3H, H_8, H_{14}, H_{23}), 4.82 (m, 1H, H_{32}), 4.62 (dd, 1H, J = 5.8, 11.3 Hz, H_{35}), 4.46 (dd, 1H, J = 5.8, 11.3 Hz, H_{35}), 4.13 (m, 1H, H_9), 3.93 (m, 2H, H_{29}), 3.42 (q, 4H, J = 7.1 Hz, H_{59}), 3.30 (m, 1H, H_{17}), 3.26 (s, 3H, H_{41}), 3.14 (m, 1H, H_4), 2.95 (s, 3H, H_{39}), 2.67 (m, 1H, H_{10}), 2.56 (m, 2H, H_{15}, H_{22}), 2.32 (m, 1H, H_{10}), 1.90 (m, 1H, H_{15}), 1.81–1.75 (5 overlapping s, 15 H, H_{36}, H_{37}, H_{40}, H_{42}, H_{44}), 1.67 (m, 1H, H_{16}), 1.32 (m, 1H, H_{16}), 1.21 (t, 6 H, J = 7.1 Hz, H_{60}), 1.04 (d, 3H, J = 6.6 Hz, H_{43}), 0.92 (d, 3H, J = 6.8 Hz, H_{38}). HRMS (FAB+) m/z ($[M-H]^+$): Calcd for $C_{55}H_{73}N_4O_{10}$: 949.5327. Found: 949.5295.

4.11. Benzyl-7-diethylaminocoumarin-3-carbamate (8)

7-Diethylaminocoumarin-3-carbonyl azide (6.6 mg, 0.023 mmol) was dried in a flask on a high vacuum line for 1 h. It was dissolved in benzene (3.0 mL) under Ar, and the solution was heated for 5 h at 85 °C. Once the benzene solution was cooled to 25 °C, benzyl alcohol (50 μL, 0.482 mmol), DMAP (10.9 mg, 0.089 mmol), and *N*,*N*-diisopropylethylamine (45 μL, 0.260 mmol) were added. The mixture was stirred at 25 °C for 208 h. After concentration, the residue was purified twice by chromatography (SiO_2, 2.2 × 19 cm, gradient: 100% hexanes → 20% ethyl acetate/hexanes) to provide **8** as a white solid (5.7 mg, 0.0156 mmol, 68% yield) (R_f = 0.50 using 40% ethyl acetate/hexanes). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (s, 1H, H_4), 7.41–7.24 (m, 7 H, Ph, NH, H_5 or H_6), 6.61 (d, 1H, J = 8.8 Hz, H_6 or H_5), 6.50 (s, 1H, H_8), 5.21 (s, 2H, H_{14}), 3.40 (q, 4H, J = 7.1 Hz, H_{15}, H_{16}), 1.20 (t, 6 H, J = 7.1 Hz, H_{17}, H_{18}); HRMS (FAB+) m/z (M^+): Calcd for $C_{21}H_{22}N_2O_4$: 366.1580. Found: 366.1595.

4.12. Cell culture

Human breast cancer cell lines SKBr3, MCF7, MDA231, and human prostate cancer cell line PC3 were obtained from ATCC and grown in T-75 tissue culture flasks (Falcon Labware, Oxnard, CA) at 37 °C in a humidified atmosphere containing 5% CO_2. The compositions of the growth media used for the different cell lines were: (i) SKBR3 cells: McCoy's 5a medium containing 1.5 mM L-glutamine, 1.5 g/L $NaHCO_3$, and 10% fetal bovine serum; (ii) MDA231 cells: RPMI 1640 medium containing 1.5 mM L-glutamine, 1.5 g/L $NaHCO_3$, and 10% fetal bovine serum; (iii) MCF7 cells: Basal Medium Eagle containing Earle's balanced salts (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, 0.1 mM sodium pyruvate, 0.01 mg/mL bovine insulin, and 10% fetal bovine serum; and (iv) PC3 cells: Ham's F-12 medium containing 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, and 10% fetal bovine serum. To every liter of medium, 10 mL of 10,000 U/mL penicillin Sodium, 10 mg/mL streptomycin sulfate solution, and 1 mL of Fungizone (Invitrogen, Carlsbad, CA) were added and then adjusted to pH 7.2 before filter sterilization.

4.13. Screening for biological activity

Cells were harvested from T-75 flasks by removing the medium and rinsing with 0.25% trypsin containing 0.03% EDTA. The flasks were stored at 37 °C until the cells detached. Cells were plated at 3000 cells/100 μL in 96-well plates and allowed to incubate at 37 °C overnight. The medium in each well was replaced with fresh medium containing appropriate concentrations of the compound of interest. Six wells were used for each compound concentration. The number of viable cells was determined using the MTT assay.[19] A stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5 mg/mL was made in PBS. After 72 h compound treatment, the compound containing media were replaced with 98 μL of fresh media and 2 μL of MTT stock solution in each well. The cell plates were incubated at 37 °C for 4 h to allow MTT to be metabolized. The plates were then dried, and MTT's metabolic product, formazan, was dissolved in 200 μL DMSO. The plates were shaken for 2 min, and the optical density in each well was determined at 560 nm. The cell numbers were plotted as percentage of day zero, with 100% indicating that the cells did not grow after treatment with the compound of interest for 3 days. Each dose response experiment was conducted twice. GraphPad Prism 4 software (San Diego, CA) was used to calculate the GI_{50} value of each compound for each cell line. Two-way ANOVA statistical analysis was performed using GraphPad Prism 4.

4.14. Fluorescence microscopy on fixed cells

Cells grown in 12-well plates covered with microscope cover glasses were fixed with cold methanol for 8 min, washed three times with PBS, permeabilized (0.3% Triton-100, 1% BSA, 10% FBS in PBS) for 1 h, and stained with either mouse monoclonal antibody (1:100 dilution) for 30 min or 1 μM iejimalide coumarin **7b** for 2 h. Cells were then washed with PBS five times, 5 min per wash, and the cells stained with mouse monoclonal antibody were then incubated with Alexa Flour 594 goat anti-mouse secondary antibody (Molecular Probes) for 1 h. Fluorescence images were acquired using an E1000 microscope (Nikon, Tokyo, Japan) equipped with a SPOT RT (Diagnostic Instruments, Sterling Heights, MI) monochromatic camera.

4.15. Confocal microscopy on live cells

For drug uptake studies, PC3 cells were grown on coverglass plates to 60–70% confluency, treated with 200 nM iejimalide-coumarin **7b** or coumarin standard **8** solutions in media for 3 h at 37 °C, and then washed three times with PBS to remove excess drug–dye conjugate or control dye before collecting images. An incubation period of 3 h was necessary to obtain a sufficiently strong fluorescence signal of the coumarin derivative within the cell. The confocal system used was a MRC-1024UV (Bio-Rad, Hemel Hempstead, England) on a Diaphot 300 (Nikon, Tokyo, Japan) inverted microscope at the Purdue University Cytometry Laboratory.

Acknowledgments

We wish to acknowledge funding provided by the Department of the Army, US. Army Research Office, and collaboration with the Walther Cancer Institute. We thank Dr. John Kane for initial ester and carbamate functionalization studies. We wish to thank Prof. Holly Goodson for the mouse monoclonal antibody and Professor Olaf Wiest, Professor Martin Tenniswood, and Dr. Rudolph Navari for very helpful discussions.

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