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One-Pot Fluorescent Labeling Protocol for Complex Hydroxylated Bioactive Natural Products

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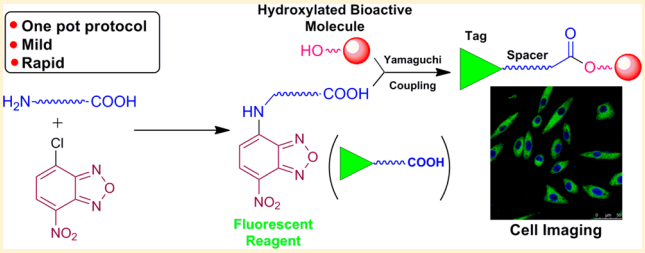
Supporting Information

ABSTRACT: Tagging of small bioactive molecules with a fluorophore is a highly sensitive method to trace their cellular activities through real-time visual information. Here we disclose a 7-nitrobenzo-2-oxa-1,3-diazole (NBD)-based, high-yielding, one-pot labeling protocol for hydroxylated molecules using Yamaguchi coupling as the key reaction. This methodology was successfully applied on several sensitive and complex hydroxylated bioactive compounds including 7-deacetylazadiradione, simvastatin, camptothecin, andrographolide, cinchonine, β-dihydroartemisinin, and azadirachtin A. Further, utility of this protocol was illustrated on the cytotoxic activity of azadiradione derivatives against several cancer cell lines through cell imaging of two qualified fluorescent probes.



INTRODUCTION

Fluorescence is a highly sensitive technique for the real time monitoring of cellular phenomena through qualitative and quantitative optical information obtained from fluorescence-detecting instruments.[1−5] Natural products or biochemical probes covalently linked with fluorescent organic dyes are used to unveil the cellular processes (including localization or specific interactions) involved with their activity. Fluorescence imaging of the living cells to study the cellular events or visualize the associated phenotypes has become an attractive technique in recent years.[6−11] Therefore, exploration of a rapid, inexpensive, and effective fluorescent tagging protocol for bioactive natural products and successful implementation of the developed procedure will facilitate the integration of fluorescence imaging and investigation of cellular events.

Reported protocols for the fluorescent labeling of natural products involving several reaction steps along with purification and protection−deprotection procedures are tedious and result in low overall yield.[12,13] Indeed, the availability of natural products is scanty in most of the cases due to their low abundance in natural sources, making it difficult to carry out all the required steps. Second, widely used tags (such as fluorescein, rhodamine) are bulkier in nature, which may lead to loss of biological activity.[7,12] Although ready to use fluorescent tags are available commercially, they are quite expensive and require extreme reaction conditions for labeling (e.g., NBD-F or DBD-COCl needs 60 °C at basic pH), which restrict their use for tagging sensitive multifunctional natural products.[14−16] Consequently, there is a need for the development of an inexpensive, mild, and high-yielding protocol for the fluorescent labeling of biologically active complex natural products capable of maintaining the activity in a labeled derivative. In this article we have demonstrated a one-pot labeling protocol for hydroxylated bioactive compounds by a 7-nitrobenzo-2-oxa-1,3-diazole (NBD)-based tag which was successfully applied to label several complex multifunctional molecules. Further, two semisynthetic derivatives of azadiradione (as azadiradione and its derivatives are known for their cytotoxic activity against several human cancer cell lines)[17−21] and their fluorescently labeled analogues were subjected to comparative cytotoxicity studies. Also, the cellular uptake and intracellular localization of these labeled compounds in various cancer cell lines were monitored by fluorescence imaging.

RESULTS AND DISCUSSION

Chemistry. NBD-Cl is a nonfluorescent molecule, which undergoes nucleophilic substitution by the amine functionality and becomes a highly sensitive fluorescent entity (depending on the polarity of the environment) (Supporting Information). This technique has been used over the years for the labeling of proteins and amine-containing small molecules.[8,22−28] However, the reactivity of NBD-Cl toward the hydroxyl group is very poor, and this method cannot be used directly for the tagging of hydroxylated molecules.[15] Here, our protocol involves the aromatic nucleophilic substitution on NBD-Cl by the amine functionality of amino acids in the initial step to generate a fluorescent reagent followed by coupling between the

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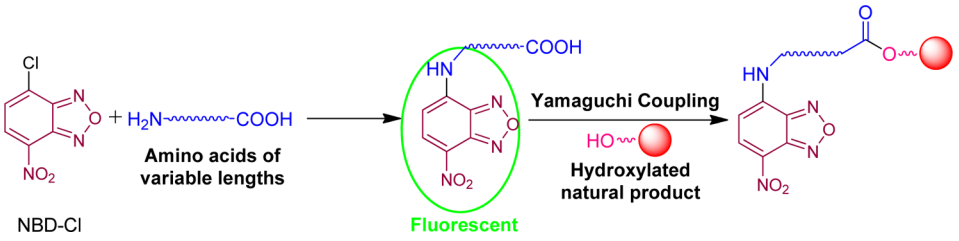
Scheme 1. One-Pot Protocol for the Fluorescent Labeling of Hydroxylated Bioactive Molecules



free carboxylic end and the hydroxylated bioactive molecule in the same vessel (Scheme 1). It is always preferable to synthesize the fluorescent reagent freshly in one pot rather than using it as an individual tagging dye because of several reasons: (i) The length of the amino acid used in the initial step determines the length of spacer between the tag and natural product. Therefore, for achieving different spacer lengths in the labeled analogues, individual fluorescent reagents of various spacer lengths have to be prepared and purified separately. Instead, it is more convenient to use a one-pot protocol utilizing commercially available amino acids corresponding to the desired spacer length. (ii) Fluorescent reagents are photosensitive, and they require special care during preparation and storage such as dim light and low temperature. Preferably they are used immediately after preparation and purification.[29] Therefore, it is more convenient to generate the fluorescent reagent freshly in one pot from two nonfluorescent starting materials (NBD-Cl and amino acids). (iii) After the preparation of a fluorescent reagent, it is essential to purify it before storage. However, our reported one-pot protocol has one less purification step, making it less tedious.

Comparative Kinetics among Coupling Reactions. Among several well-established coupling reactions available between carboxylic acids and alcohols, the Yamaguchi procedure was preferred on the basis of the outcome of a comparative kinetic experiment. **1** was used as the model substrate and allowed to couple with 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid in the presence of various coupling reagents (DCC, EDC, DIC, Yamaguchi) (Figure 1).

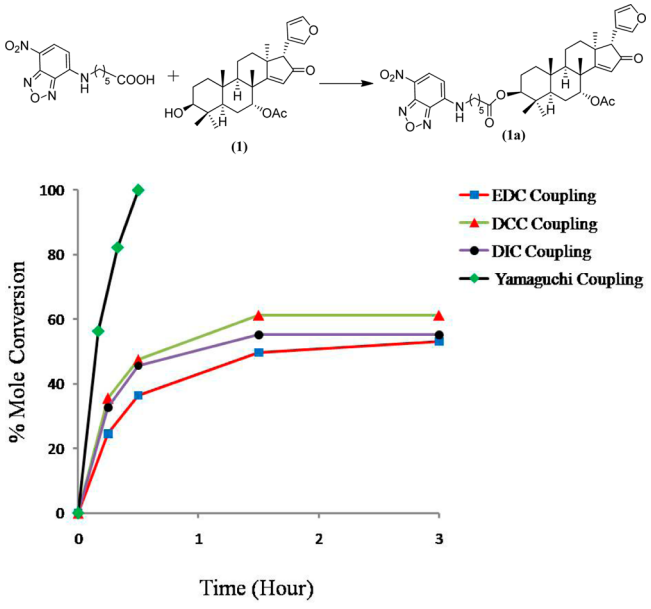


Figure 1. Comparative kinetics among various well-known coupling reactions between **1** and 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)hexanoic acid.

Progress of the reactions was monitored by HPLC using the standard graphs prepared for **1** and **1a**. The coupling reagents DCC, EDC, and DIC showed similar reaction kinetic profiles. About 50−60% of **1** (by mole) was converted to **1a** in 90 min of reaction time by all three coupling agents. However, no significant change in the product level was observed during 10 h (Figure 1). Higher stoichiometric ratios of coupling reagents (DCC, EDC, and DIC) also did not alter the progress or rate of the reaction significantly even after prolonged reaction time (Supporting Information). On the other hand, Yamaguchi coupling showed quantitative conversion in 30 min; hence, it was chosen as the preferred coupling reaction for the other molecules.

Flexibility in Spacer Length. The choice of amino acid to be used in the initial step will determine the length of spacer joining the NBD skeleton and the bioactive molecule of interest. Indeed, the length of the spacer can vary the hydrophobicity of the labeled molecule, which might be a guiding factor for the cellular uptake. Also, a spacer of optimum length is important for retaining the activity observed with the parental molecule.[6] This flexibility in our methodology makes it unique and will be helpful for getting a successful labeling on a complex molecule with preserved activity. To demonstrate, **1** was labeled with spacers of three different lengths using three unlike amino acids: 6-aminocaproic acid (six-carbon spacer), 11-aminoundecanoic acid (eleven-carbon spacer), and L-proline (creating a turn in the structure) (Scheme 2).

Nature of the Hydroxyl Group. To investigate the efficiency of Yamaguchi coupling with varying the nature of the hydroxyl groups, several structurally simple compounds bearing different kinds of hydroxyl functionalities (such as phenolic, primary, secondary, and tertiary) were chosen and subjected to a one-pot protocol using 6-aminocaproic acid (Scheme 3). Eugenol (**2**, phenolic hydroxyl), (−)-borneol (**3**, secondary hydroxyl), α-santalol (**4**, allylic primary hydroxyl), (−)-dihydrocarveol (**5**, secondary hydroxyl), and one partially protected mannose (**6**) carrying a primary hydroxyl furnished the labeled product in excellent yields. However, the coupling did not work out when α-(−)-bisabolol (**7**), a sesquiterpene carrying a tertiary hydroxyl group, was used as the substrate. These results indicated that the developed protocol for NBD-based fluorescent tagging of bioactive molecules possessing phenolic, primary, and secondary hydroxyl groups using Yamaguchi coupling was highly efficient.

Applicability with Complex Molecules. To showcase broad applicability of the present methodology, several structurally diverse and multifunctional molecules were selected for fluorescent labeling (Table 1) using the above-discussed one-pot protocol. The fluorescent tagged compound (**8a**) was obtained in good yield (71%), when nimbocinol[19] (**8**, deacetylated azadiradione) carrying a sterically hindered axial hydroxyl group at C-7 of the basic limonoid skeleton was used as the substrate. Similarly, cholesterol (**9**), 11α-hydroxyprogesterone (**10**), and

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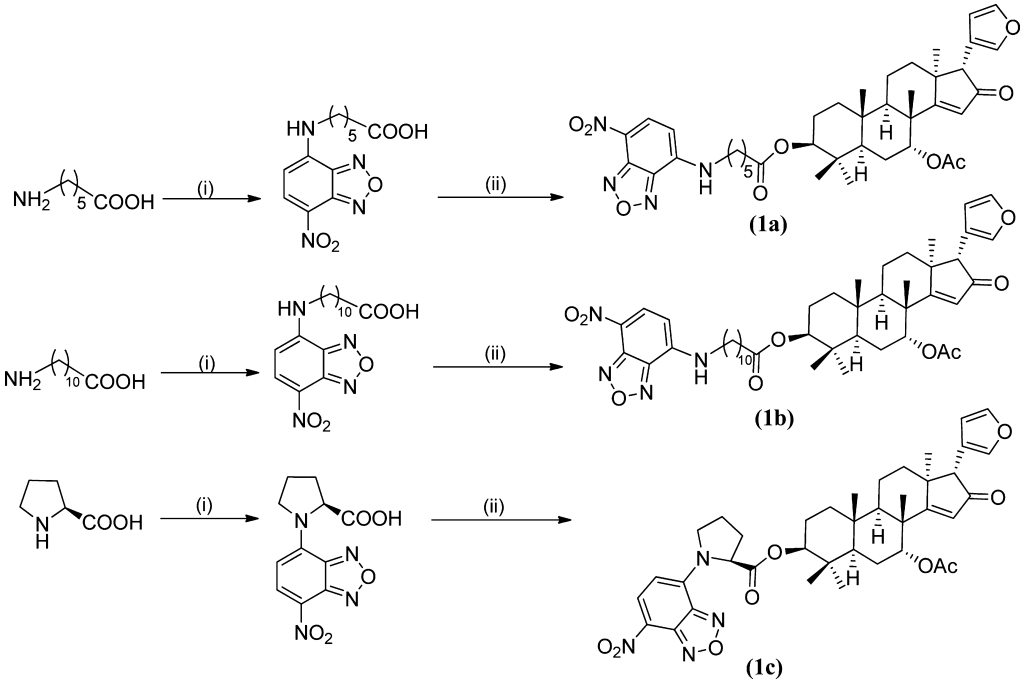
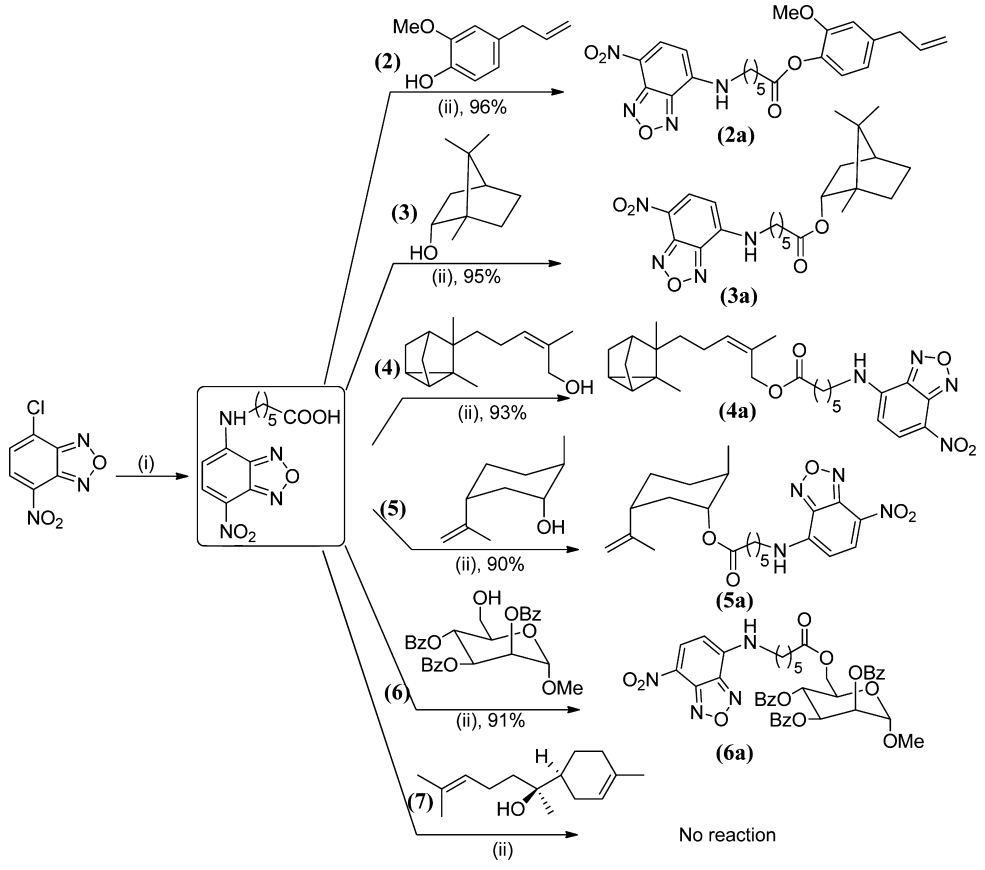
Scheme 2. Selection of the Amino Acid in the Initial Step Varies the Length of Spacer in between the Tag and Studied Molecule[a]



[a]Reagents and conditions: (i) NBD-Cl, $NaHCO_3$, acetonitrile, water, 55 °C; (ii) **1**, 2,4,6- trichlorobenzoyl chloride, TEA, DMAP, anhydrous THF, 85−90% for two steps.

Scheme 3. Efficiency of Yamaguchi Coupling Varies with the Nature of the Hydroxyl Group[a]



[a]Reagents and conditions: (i) 6-aminocaproic acid, $NaHCO_3$, acetonitrile, water, 55 °C; (ii) 2,4,6- trichlorobenzoyl chloride, TEA, DMAP, anhydrous THF.

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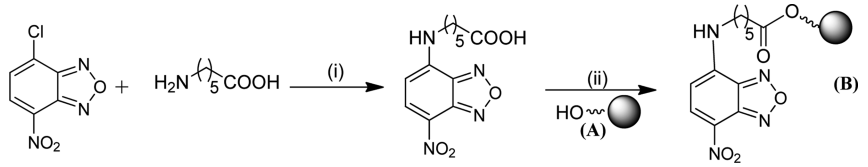
Table 1. [a]Entries Showing Applicability of the Developed One-Pot Protocol for Fluorescent Labeling of Complex Bioactive Hydroxylated Compounds



Entry	(A)	(B)	Yield
1.	(8)	(8a)	71%
2.	(9)	(9a)	96%
3.	(10)	(10a)	88%
4.	(11)	(11a)	94%
5.	(12)	(12a)	90%
6.	(13)	(13a)	52%
7.	(14)	(14a)	82%
8.	(15)	(15a)	09%
9.	(16)	(16a)	81%
10.	(17)	(17a)	33%
11.	(18)	(18a)	93%
12.	(19)	(19a)	71%
13.	(20)	No reaction	–

[a]Reagents and conditions: (i) $NaHCO_3$, acetonitrile, water, 55 °C; (ii) 2,4,6- trichlorobenzoyl chloride, TEA, DMAP, anhydrous THF. Numbered compounds are enclosed within parentheses.

diosgenin (**11**, a steroidal sapogenin containing a spiroketal moiety)[30] furnished corresponding tagged compounds **9a**, **10a**, and **11a** in good to excellent yields. A phenolic hydroxyl on the isoindoline skeleton of compound **12** resulted in an excellent yield. Ezetimibe (**13**, a cholesterol lowering drug),[31] possessing both an alkylic and a phenolic hydroxyl, underwent a competitive reaction. However, at −15 °C with 5 min of reaction time, the tagged phenolic hydroxyl was obtained as the major product (52%) (for a detailed characterization, consult Supporting Information) along with the doubly tagged product (27%). The antimalarial alkaloid cinchonine,[32] (**14**) carrying a secondary hydroxyl group, was labeled in very good yield (82%) whereas another anticancer alkaloid, (*S*)-(+)-camptothecin[33] (**15**), yielded the coupled product in poor yield (9%) due to the tertiary nature of its hydroxyl functionality. The hypolipidemic drug simvastatin,[31] (**16**) carrying a secondary hydroxyl group on a six-membered lactone ring, produced the tagged derivative in 81% yield. Andrographolide[34] (**17**), an anticancer natural product, has three different hydroxyl groups around its skeleton: an allylic secondary hydroxyl on the five-membered lactone ring, one primary hydroxyl group, and one hindered secondary hydroxyl group. Although at room temperature the same reaction condition produced a mixture of products, at −15 °C with 5 min stirring the monolabeled (on allylic secondary hydroxyl) product was obtained in moderate yield (33%) and was characterized by 1D- and 2D-NMR studies as discussed in the Supporting Information. Interestingly, a reduced product of salanin (**18**) having two hindered secondary hydroxyl and another primary hydroxyl group furnished monolabeled product on the primary hydroxyl in excellent yield (93%) (for the detailed characterization, consult Supporting Information). *β*-Dihydroartemisinin (**19**) a well-known antimalarial[35] agent, having a sensitive peroxo bridge along with an adjacent ketal moiety in its skeleton, was also able to produce the coupled product in 71% yield. A well-studied highly potent antifeedant neem limonoid azadirachtin A (**20**) possesses a unique molecular architecture, carrying ester, epoxide, ether, and ketal functionalities, which makes it highly sensitive toward pH and light.[36] Surprisingly, it was not able to furnish any product and remained unreacted under the same reaction condition despite having three hydroxyl groups around its skeleton. The inability of azadirachtin A (**20**) to produce any product may be due to the extreme steric hindrance around the hydroxyl groups and their involvement in the intramolecular hydrogen bonding network.[37] To avoid these obstacles, an alternative approach was adopted.

Initially, a short spacer carrying a protected hydroxyl group at the tail was attached followed by deprotection and Yamaguchi

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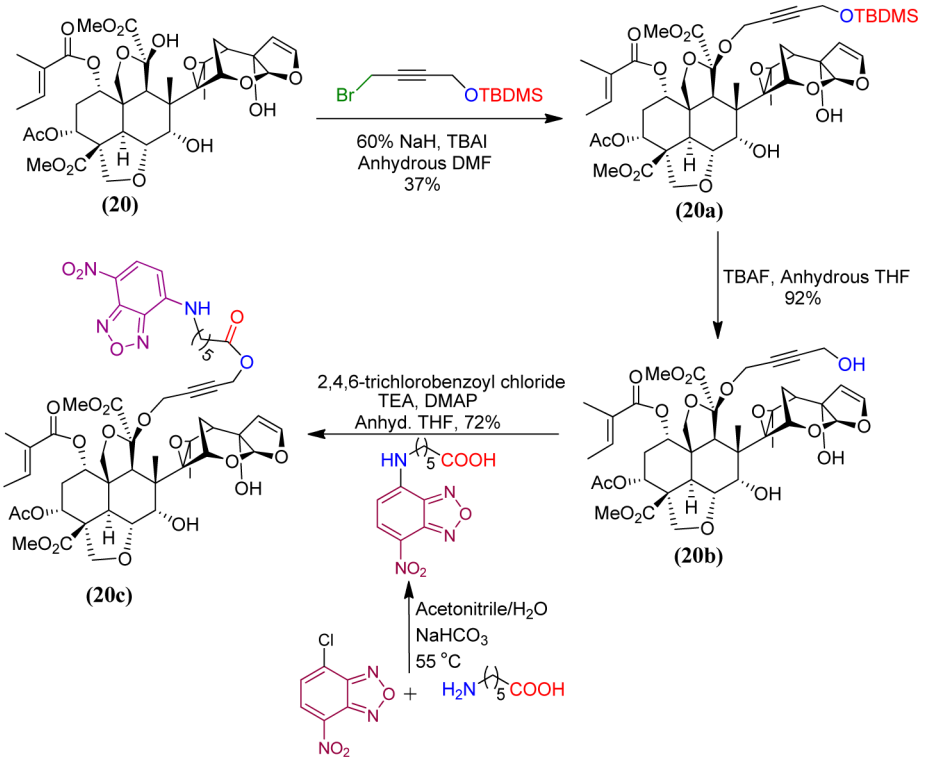
Scheme 4. Fluorescent Labeling Protocol for Azadirachtin A



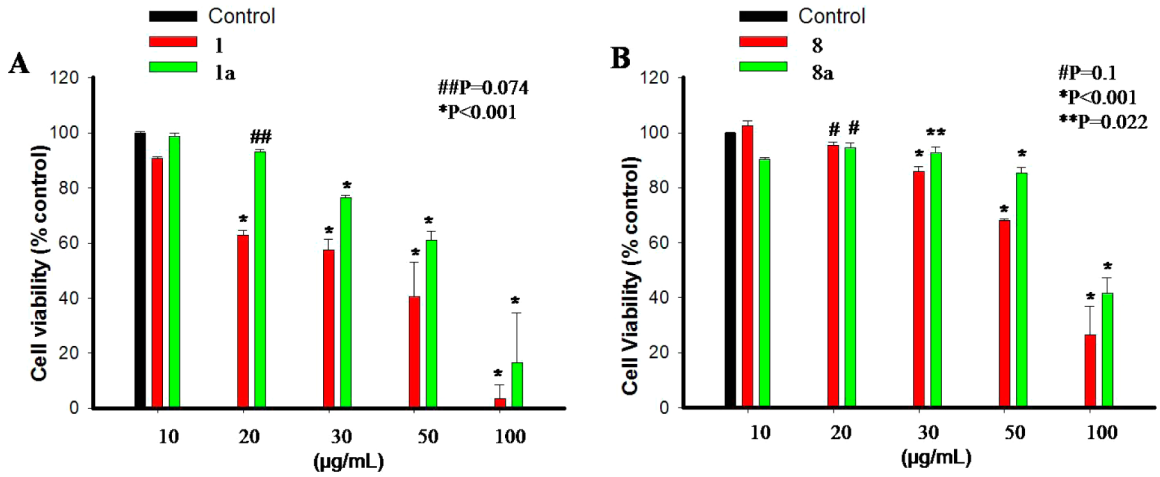


Figure 2. (A and B) MDA-MB-231 cells were treated with labeled and unlabeled limonoids, and cell viability was assessed by MTT assay. The data obtained were analyzed statistically and represented graphically. Column, mean ± SE, $^{*}P < 0.001$, $^{**}P = 0.022$, $^{\#}P = 0.1$, $^{\#\#}P = 0.074$ vs control. The results are the representative of three independent experiments.

coupling. Azadirachtin A (**20**) was reacted with a propargyl bromide derivative carrying a TBDMS-protected hydroxyl group at the other end in the presence of sodium hydride. After nucleophilic substitution by 11-OH (as characterized on the basis of previous reports),[38,39] the hydroxyl was deprotected by TBAF and further subjected to a one-pot protocol to afford labeled azadirachtin (**20c**) in 24% overall yield (Scheme 4).

Biology. To assess the applicability of fluorescent tagged bioactive molecules synthesized in this study (Scheme 2, Table 1), two limonoids were chosen for biological evaluation and cellular imaging. Two azadiradione derivatives (**1** and **8**) and their corresponding labeled analogues (**1a** and **8a**) were evaluated for cytotoxic activity against several cancer cell lines, and they were further used for cell imaging studies.

Cancer Cell Viability Studies. MDA-MB-231 cells were treated with either of labeled (**1a** and **8a**) and parent (**1** and **8**) azadiradione derivatives at varying concentrations (0−100 μg/mL), and MTT assay was performed. The results depicted a comparable decrease in cell viability between labeled and unlabeled limonoids in a concentration-dependent manner (Figure 2).

Cancer Cell Motility Assay. Cancer metastasis is basically the migration of tumor cells from the primary tumors to the distant body parts to develop secondary tumors. Any potent drug should target cancer cell viability as well as motility. Therefore, to check the effect of labeled and unlabeled limonoids on breast cancer cell motility, wound migration assay was performed. Confluent monolayer of MDA-MB-231 cells were wounded uniformly and treated with labeled (**1a** and **8a**) as well as

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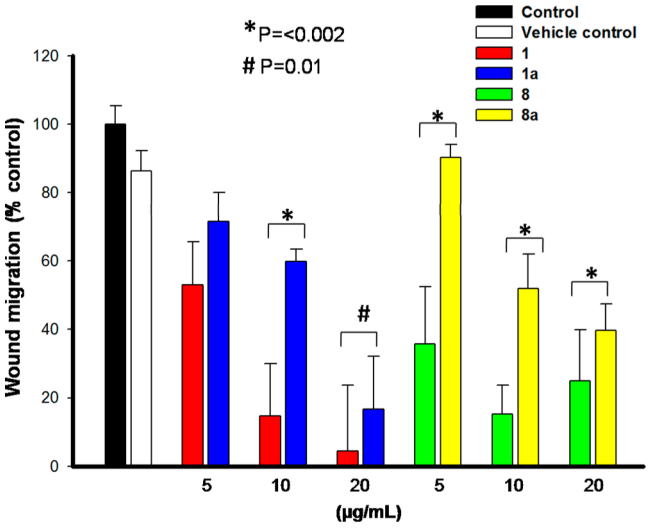

Figure 3. Wound migration is inhibited by limonoids in MDA-MB-231 cells. Cells were treated with **1**, **1a**, **8**, and **8a** (0–20 μg/mL), and wounds were photographed after 12 h. Graphical representation for the quantification of wound migration assay. Column, mean ± SE, $^{*}P < 0.002$, $^{\#}P = 0.01$ vs control.

unlabeled (**1** and **8**) limonoids (0–20 μg/mL) for 12 h, and motility was assessed. The results demonstrated the inhibition in MDA-MB-231 cell motility with an increasing concentration of limonoids (labeled as well as unlabeled) (Figure 3).

Cell Imaging Studies. Comparative inhibition studies of cancer cell viability and motility have indicated concentration-dependent cytotoxic activity for both labeled analogues (**1a** or **8a**) and comparable inhibitory potencies with respect to the parent azadiradione derivatives. The retention of activity in labeled analogues may be attributed to the smaller size of NBD and presence of a highly flexible six-carbon spacer joining the tag and bioactive molecule. However, the slight alternation in potency of tagged derivatives with respect to the parent molecules may be due to the structural modification caused by the attachment of the NBD tag and esterification of the hydroxyl functionality. Therefore, considering the extent of cytotoxic activity preserved, **1a** and **8a** can be considered as valid cancer cell imaging probes for azadiradione derivatives.

Cancer cells (A375, HeLa, and MDA-MB-231) were treated with either **1a** or **8a** (20 μg/mL) for 1 h and analyzed for cellular internalization and localization under a confocal microscope. Results depicted the cellular uptake of NBD-labeled compounds irrespective of the cancer cell lines and no specific localization in the nucleus (Figure 4). Further, these imaging probes can be used for studying the localization in subcellular organelles. To demonstrate specific examples, HeLa cells treated with either **1a** or **8a** were stained by Mito-tracker Red and analyzed through confocal microscopy, which did not show any colocalization with mitochondria (Supporting Information). Similarly, the colocalization experiment was performed with ER-tracker red, and labeled analogues (**1a** and **8a**) were found to be localized in the endoplasmic reticulum (ER) on the basis of yellow fluorescence observed in the overlaid image (Figure 5). Incubation of **1a** with A375 cells and analyses of the cell lysate after the incubation period indicated that the ester bond between tag and compound **1** is stable under intracellular conditions (Supporting Information).

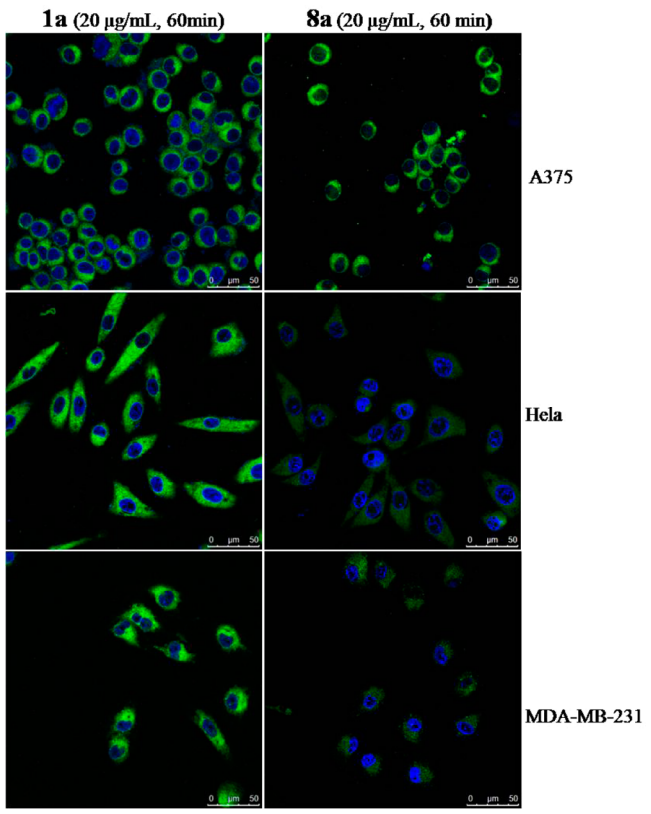

Figure 4. Cancer cells (A375, Hela, and MDA-MB-231) were treated (20 μg/mL) with NBD-labeled limonoids (**1a** and **8a**) for 1 h and analyzed under a confocal microscope. Green: compounds labeled with NBD, blue: nucleus staining with DAPI. Micron bar: 50 μm.

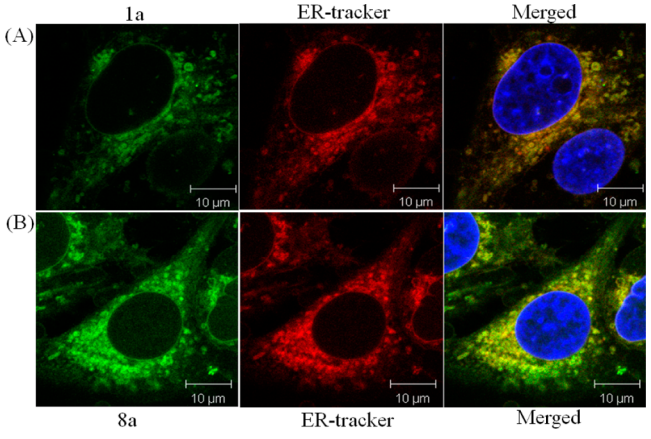

Figure 5. (A) MDA-MB-231 cells were costained with **1a** (20 μg/mL) and ER-tracker red (1 μM) and analyzed under a confocal microscope. (B) MDA-MB-231 cells were costained with **8a** (20 μg/mL) and ER-tracker red (1 μM) and analyzed under a confocal microscope. Nuclei were stained with DAPI. Blue: Nucleus; red: ER; green: NBD-labeled limonoids. Micron bar: 10 μm.

CONCLUSIONS

Simple and highly efficient one-pot methodology for the NBD-based fluorescent labeling of hydroxylated small molecules was developed, which can be applied to track their mode of action through fluorescence cell imaging. Among several common coupling reactions, Yamaguchi was found to be the best on the basis of yield and kinetic studies. The protocol was executed under mild conditions so that it was able to sustain sensitive

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functionalities such as lactone, ketal, ether, peroxo, or epoxide in the reaction environment. Both nucleophilic substitution and coupling steps were carried out in the same vessel and required a single purification step. Even the natural products isolated in trace amounts (a few milligrams) can be labeled in sufficient yield and in a more economic way using the present protocol. Importantly, the coupling reaction was very selective between acid and alcohol functionalities, which allows the tagging of complex molecules with multiple functional groups. Fluorescein, a widely used fluorescent tag, was replaced by NBD, as it is less bulkier than the former. Again, the length of the spacer can easily be modified by a suitable choice of amino acid. Further, evaluation of the two NBD-tagged azadiradione derivatives for cytotoxic activity indicated that the labeling did not drastically alter the activity. The internalization and localization of those probes were imaged successfully inside the cancer cells and found to be localized in the endoplasmic reticulum. Therefore, this methodology can be used to synthesize the fluorescent imaging probes of hydroxylated bioactive compounds to study the cellular processes, especially cellular uptake, localization, or specific interactions.

EXPERIMENTAL SECTION

General Procedure. *A. One-Pot Labeling Protocol.* A solution of NBD-Cl (1.00 equiv) in acetonitrile (18 mL/mmol) was added dropwise to a solution of amino acid (1.00 equiv) and sodium bicarbonate (3.00 equiv) in water (6 mL/mmol) at 55 °C and incubated for 1 h. Then acetonitrile was concentrated under reduced pressure, and the pH of the aqueous reaction mixture was adjusted to ∼2.0 using 1 N HCl. Further, it was concentrated to dryness under 20 mbar pressure and 62 °C temperature. The deep orange crude solid was again dissolved in a minimum amount of acetonitrile and dried under the same conditions to ensure that no residual moisture remained. Further, Yamaguchi coupling was carried out in the same vessel following the reported procedure[40] with slight modifications. The natural product/bioactive molecule (0.75 equiv) with hydroxyl functionality in anhydrous THF (20 mL/mmol) was added to the dried crude product under an inert atmosphere with stirring. 2,4,6-Trichlorobenzoyl chloride (1.00 equiv) and anhydrous TEA (1.00 equiv) were successively added dropwise to the reaction mixture. After 5 min, DMAP (1.00 equiv) was added to the reaction vessel, and stirring was continued for another 30 min. The reaction was quenched by adding a few drops of water. Then it was concentrated to dryness and directly purified over a silica gel column. All the steps were performed under dim light, and labeled compounds were stored at −20 °C in dark.

B. Coupling Reaction (DCC, EDC, and DIC). To a mixture of acid (1.00 equiv), alcohol (0.75 equiv), and DMAP (1.00 equiv) in an inert atmosphere was added anhydrous DCM (30 mL/mmol). The mixture was stirred to dissolve all the components followed by addition of coupling reagent (1.00 equiv) (DCC, EDC, or DIC) while being stirred. After addition, the reaction mixture was stirred at room temperature.

*Preparation of **21a**.* Azadirachtin A (100 mg, 0.14 mmol) in an argon atmosphere was dissolved in 1.0 mL of anhydrous DMF, and the mixture was cooled to 0 °C. A 60% sodium hydride (10.2 mg, 0.42 mmol) solution was added to the mixture and stirred at 0 °C for 8 min. The colorless reaction mixture became light brown by the end of this time period. Then the propargyl bromide derivative was added (52.6 mg, 0.20 mmol) followed by a catalytic amount (pinch) of TBAI. The reaction mixture was stirred at 0 °C for another 4 min and then at room temperature for 20 min. After the reaction was quenched with a few drops of chilled water, ethyl acetate (20 mL) was added and then washed thrice with brine solution (50 mL). The ethyl acetate layer was concentrated and purified by silica gel column chromatography using a gradient mixture of DCM and methanol as eluent to afford the desired product (**21a**) (46.72 mg, 0.052 mmol, yield 37%).

*Preparation of **21b**.* A 1 M TBAF (62 μL, 0.062 mmol) solution was added dropwise to **21a** (46.72 mg, 0.052 mmol) in 2 mL of anhydrous THF, and the reaction mixture was stirred for 10 min in an inert atmosphere. Further, it was concentrated and purified over a silica gel column using 2.0% methanol in DCM as eluent to produce the deprotected product **21b** in 92% yield (37.7 mg, 0.048 mmol).

Analytical Data. *(3S,7R,8R,10S,13S,17R)-7-Acetoxy-17-(furan-3-yl)-4,4,8,10,13-pentamethyl-16-oxo-2,3,4,5,6,7,8,9,10,11,12,13,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**1a**).* A 41.9 mg (0.057 mmol) amount of **1a** was isolated from 30.0 mg (0.066 mmol) of **1** in 87% yield. Orange solid. $[\alpha]^{25}_{D}$ = −15.5 (*c* 3.0, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3327, 1719, 1700. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.47 (d, *J* = 8.55 Hz, 1H), 7.45 (m, 1H), 7.41 (m, 1H), 6.52 (m, 1H), 6.25 (m, 1H), 6.17 (d, *J* = 8.54 Hz, 1H), 5.83 (s, 1H), 5.25 (m, 1H), 4.53 (m, 1H), 3.53 (dd, *J* = 12.51, 6.41 Hz, 2H), 3.37 (s, 1H), 2.37 (m, 2H), 1.95 (s, 3H), 1.25 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.86 (s, 3H), 0.77 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.5, 193.5, 173.1, 169.7, 144.2, 143.9, 142.6, 141.5, 136.5, 123.8, 123.1, 118.6, 111.2, 98.5, 80.2, 74.7, 60.5, 48.1, 48.0, 44.0, 43.6, 43.1, 37.3, 37.2, 34.2, 30.3, 28.0, 27.6, 26.3, 26.2, 26.1, 24.3, 23.3, 22.8, 21.0, 16.4, 15.7, 15.5. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{40}H_{51}N_4O_9$, 731.3659; found, 731.3656. Purity (HPLC): 97.8%.

*(3S,7R,8R,10S,13S,17R)-7-Acetoxy-17-(furan-3-yl)-4,4,8,10,13-pentamethyl-16-oxo-2,3,4,5,6,7,8,9,10,11,12,13,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 11-((7-Nitrobenzo[c][1,2,5]-oxadiazol-4-yl)amino)undecanoate (**1b**).* A 47.3 mg (0.059 mmol) amount of **1b** was isolated from 30.0 mg (0.066 mmol) of **1** in 89% yield. Orange solid. $[\alpha]^{25}_{D}$ = −12.6 (*c* 1.7, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3327, 1715, 1700. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, *J* = 8.72 Hz, 1H), 7.46 (m, 1H), 7.42 (m, 1H), 6.35 (m, 1H), 6.27 (m, 1H), 6.17 (d, *J* = 8.59 Hz, 1H), 5.85 (s, 1H), 5.26 (m, 1H), 4.54 (m, 1H), 3.50 (m, 2H), 3.38 (s, 1H), 2.31 (m, 2H), 1.95 (s, 3H), 1.25 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H), 0.78 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.4, 193.4, 173.6, 169.7, 144.3, 143.9, 143.8, 142.7, 141.6, 136.4, 124.0, 123.2, 118.6, 111.2, 98.5, 79.8, 74.8, 60.6, 48.1, 48.1, 44.0, 44.0, 43.1, 37.5, 37.4, 37.2, 34.7, 30.4, 29.3, 29.2, 29.1, 29.1, 28.5, 27.6, 26.9, 26.2, 26.1, 25.1, 23.4, 22.8, 21.0, 16.4, 15.8, 15.6. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{45}H_{61}N_4O_9$, 801.4438; found, 801.4405.

*(3S,7R,8R,10S,13S,17R)-7-Acetoxy-17-(furan-3-yl)-4,4,8,10,13-pentamethyl-16-oxo-2,3,4,5,6,7,8,9,10,11,12,13,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 1-(7-Nitrobenzo[c][1,2,5]-oxadiazol-4-yl)pyrrolidine-2-carboxylate (**1c**).* A 40.0 mg (0.056 mmol) amount of **1c** was isolated from 30.0 mg (0.066 mmol) of **1** in 85% yield. Orange solid. $[\alpha]^{25}_{D}$ = +101.2 (*c* 2.25, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 1736, 1701. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.46 (d, *J* = 8.84 Hz, 1H), 7.45 (m, 1H), 7.41 (m, 1H), 6.26 (m, 1H), 6.14 (d, *J* = 8.72 Hz, 1H), 5.83 (s, 1H), 5.52 (br m, 1H), 5.26 (m, 1H), 4.52 (m, 1H), 3.78 (br m, 2H), 3.37 (s, 1H), 1.93 (s, 3H), 1.23 (s, 3H), 1.00 (s, 3H), 0.91 (s, 3H), 0.86 (s, 3H), 0.78 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.4, 193.3, 170.5, 169.7, 144.7, 144.3, 143.3, 142.7, 141.6, 135.5, 123.2, 123.1, 118.5, 111.1, 101.7, 82.1, 74.5, 64.7, 60.5, 50.7, 48.1, 48.0, 44.0, 43.0, 37.4, 37.3, 37.2, 31.2, 30.2, 27.6, 26.2, 26.1, 22.9, 22.7, 21.0, 16.3, 15.7, 15.5. HRMS (ESI) *m*/*z*: $[M + Na]^+$ Calcd for $C_{39}H_{46}N_4O_9Na$, 737.3162; found, 737.3157.

*4-Allyl-2-methoxyphenyl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**2a**).* A 77.4 mg (0.176 mmol) amount of **2a** was isolated from 30.0 mg (0.183 mmol) of **2** in 96% yield. Orange solid. $[\alpha]^{25}_{D}$ = +0.15 (*c* 4.1, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3330, 1754. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.47 (d, *J* = 8.85 Hz, 1H), 6.92 (d, *J* = 7.93 Hz, 1H), 6.77 (m, 2H), 6.34 (m, 1H), 6.18 (d, *J* = 8.54 Hz, 1H), 5.96 (m, 1H), 5.11 (m, 2H), 3.79 (s, 3H), 3.54 (dd, *J* = 13.12, 6.17 Hz, 2H), 3.38 (m, 2H), 2.64 (t, *J* = 7.32 Hz, 2H), 1.88 (m, 4H), 1.64 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 171.5, 150.7, 144.2, 143.9, 143.8, 139.1, 137.8, 136.9, 136.4, 124.1, 122.4, 120.7, 116.2, 112.8, 98.5, 55.8, 43.7, 40.0, 33.6, 28.1, 26.2, 24.3. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{22}H_{25}N_4O_6$, 441.1774; found, 441.1753.

*(1R,2R,4S)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**3a**).* A 79.1 mg (0.184 mmol) amount of **3a** was isolated from 30.0 mg (0.195 mmol)

of **3** in 95% yield. Orange solid. $[\alpha]^{25}_{D}$ = −18.6 (*c* 3.8, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3326, 1719. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, *J* = 8.53 Hz, 1H), 6.40 (m, 1H), 6.18 (d, *J* = 8.78 Hz, 1H), 4.90 (m, 1H), 3.52 (m, 2H), 2.39 (m, 3H), 0.91 (s, 3H), 0.87 (s, 3H), 0.82 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.7, 144.2, 143.9, 143.8, 136.5, 123.9, 98.5, 79.9, 48.7, 47.8, 44.8, 43.7, 36.8, 34.2, 28.1, 28.0, 27.1, 26.3, 24.4, 19.6, 18.8, 13.5. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{22}H_{31}N_4O_5$, 431.2294; found, 431.2272.

*(Z)-5-(2,3-Dimethyltricyclo[2.2.1.02,6]heptan-3-yl)-2-methylpent-2-en-1-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)-hexanoate (**4a**).* A 62.5 mg (0.126 mmol) amount of **4a** was isolated from 30.0 mg (0.136 mmol) of **4** in 93% yield. Orange solid. $[\alpha]^{25}_{D}$ = +3.51 (*c* 1.3, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3328, 1719. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, *J* = 8.54 Hz, 1H), 6.34 (m, 1H), 6.18 (d, *J* = 8.54 Hz, 1H), 5.41 (t, *J* = 7.32 Hz, 1H), 4.61 (s, 2H), 3.52 (m, 2H), 2.40 (t, *J* = 7.32 Hz, 2H), 1.73 (s, 3H), 0.99 (s, 3H), 0.82 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.4, 144.2, 143.9, 143.8, 136.4, 132.0, 129.0, 124.1, 98.5, 63.2, 45.8, 43.7, 38.1, 34.6, 33.9, 31.5, 31.0, 28.1, 27.3, 26.3, 24.3, 23.1, 21.4, 19.5, 19.4, 17.5, 10.6. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{27}H_{37}N_4O_5$, 497.2764; found, 497.2740.

*(1R,2R,5R)-2-Methyl-5-(prop-1-en-2-yl)cyclohexyl 6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**5a**).* A 75.2 mg (0.175 mmol) amount of **5a** was isolated from 30.0 mg (0.195 mmol) amount of **5** in 90% yield. Orange solid. $[\alpha]^{25}_{D}$ = −34.7 (*c* 2.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3325, 1727. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.49 (d, *J* = 8.72 Hz, 1H), 6.32 (m, 1H), 6.18 (d, *J* = 8.59 Hz, 1H), 4.68 (m, 2H), 4.50 (m, 1H), 3.50 (m, 2H), 2.37 (m, 2H), 1.70 (s, 3H), 0.89 (d, *J* = 6.32 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.1, 148.8, 144.2, 143.9, 136.5, 123.8, 108.8, 98.5, 78.3, 43.7, 43.6, 37.1, 36.9, 34.2, 33.0, 30.8, 28.1, 26.3, 24.4, 20.8, 18.2. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{22}H_{31}N_4O_5$, 431.2294; found, 431.2285.

*(2S,3R,4S,5R,6R)-2-Methoxy-6-((((6-((7-nitrobenzo[c][1,2,5]-oxadiazol-4-yl)amino)hexanoyl)oxy)methyl)tetrahydro-2H-pyran-3,4,5-triyl Tribenzoate (**6a**).* A 42.2 mg (0.054 mmol) amount of **6a** was isolated from 30.0 mg (0.059 mmol) of **6** in 91% yield. Orange solid. $[\alpha]^{25}_{D}$ = −55.3 (*c* 2.5, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3339, 1732. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.43 (d, *J* = 8.70 Hz, 1H), 8.10−7.22 (m, 15H), 6.53 (m, 1H), 6.13 (d, *J* = 8.24 Hz, 1H), 5.97 (t, *J* = 10.08 Hz, 1H), 5.89 (dd, *J* = 10.07, 3.21 Hz, 1H), 5.68 (m, 1H), 5.01 (d, *J* = 1.83 Hz, 1H), 4.42 (dd, *J* = 12.36, 4.58 Hz, 1H), 4.33 (m, 2H), 3.54 (s, 3H), 3.47 (m, 2H), 2.37−2.52 (m, 2H), 1.71−1.83 (m, 4H), 1.50 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.0, 165.6, 165.4, 165.3, 144.2, 143.9, 143.8, 136.4, 133.5, 133.2, 129.8, 129.7, 129.6, 129.3, 129.0, 128.8, 128.5, 128.5, 128.2, 123.8, 98.6, 98.4, 70.4, 69.9, 68.5, 66.8, 62.6, 55.6, 43.6, 33.6, 28.0, 26.2, 24.0. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{40}H_{39}N_4O_{13}$, 783.2514; found, 783.2508.

*(7R,8R,10R,13S,17R)-17-(Furan-3-yl)-4,4,8,10,13-pentamethyl-3,16-dioxo-4,5,6,7,8,9,10,11,12,13,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-7-yl 6-((7-Nitrobenzo[c][1,2,5]-oxadiazol-4-yl)amino)hexanoate (**8a**).* A 35.7 mg (0.052 mmol) amount of **8a** was isolated from 30.0 mg (0.073 mmol) of **8** in 71% yield. Orange solid. $[\alpha]^{25}_{D}$ = −66.3 (*c* 1.6, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3307, 1729, 1694, 1667. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.48 (d, *J* = 8.85 Hz, 1H), 7.70 (m, 1H), 7.48 (m, 1H), 7.43 (m, 1H), 7.13 (d, *J* = 10.38 Hz, 1H), 6.25 (m, 1H), 6.19 (d, *J* = 8.85 Hz, 1H), 5.88 (m, 2H), 5.35 (m, 1H), 3.53 (s, 1H), 3.50 (m, 2H), 2.50 (m, 1H), 2.32 (m, 1H), 1.37 (s, 3H), 1.27 (s, 3H), 1.09 (s, 3H), 1.06 (s, 3H), 1.06 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 206.2, 203.9, 193.1, 172.1, 156.6, 144.4, 144.1, 142.9, 141.5, 136.7, 125.8, 123.2, 123.0, 118.1, 111.0, 98.3, 73.8, 60.9, 48.3, 46.1, 44.6, 44.0, 43.7, 40.0, 38.2, 34.1, 29.9, 28.1, 26.8, 26.8, 26.4, 26.2, 24.4, 23.4, 21.2, 18.9, 15.7. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{38}H_{45}N_4O_8$, 685.3237; found, 685.3245. Purity (HPLC): 98.5%.

*(3S,10R,13R,17R)-10,13-Dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta-[a]phenanthren-3-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)-amino)hexanoate (**9a**).* A 49.4 mg (0.075 mmol) amount of **9a** was isolated from 30.0 mg (0.078 mmol) of **9** in 96% yield. Orange solid. $[\alpha]^{25}_{D}$ = −20.1 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3331, 1721. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, *J* = 8.54 Hz, 1H), 6.28 (m, 1H), 6.18 (d, *J* = 8.54 Hz, 1H), 5.36 (m, 1H), 4.63 (m, 1H), 3.52 (dd, *J* = 13.12, 6.17 Hz, 1H), 2.35 (m, 2H), 2.30 (m, 2H), 1.02 (s, 3H), 0.92 (d, *J* = 6.41 Hz, 3H), 0.88 (d, *J* = 2.41 Hz, 3H), 0.86 (d, *J* = 2.41 Hz, 3H), 0.68 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.8, 144.3, 143.9, 143.8, 139.5, 136.4, 124.2, 122.8, 98.5, 74.1, 56.7, 56.1, 50.0, 43.6, 42.3, 39.7, 39.5, 38.2, 36.9, 36.6, 36.2, 35.8, 34.2, 31.9, 31.8, 28.2, 28.2, 28.0, 27.8, 26.3, 24.3, 24.3, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 11.8. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{39}H_{59}N_4O_5$, 663.4485; found, 663.4460.

*17-Acetyl-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-11-yl 6-((7-Nitrobenzo[c]-[1,2,5]oxadiazol-4-yl)amino)hexanoate (**10a**).* A 48.5 mg (0.080 mmol) of **10a** was isolated from 30.0 mg (0.091 mmol) amount of **10** in 88% yield. Orange solid. $[\alpha]^{25}_{D}$ = +93.1 (*c* 1.21, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3309, 1727, 1701, 1665. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.49 (d, *J* = 8.54 Hz, 1H), 6.66 (m, 1H), 6.19 (d, *J* = 8.54 Hz, 1H), 5.76 (s, 1H), 5.28 (m, 1H), 3.54 (m, 2H), 2.52 (m, 1H), 2.11 (s, 3H), 1.26 (s, 3H), 0.76 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 208.7, 199.2, 172.3, 169.6, 144.2, 143.9, 143.9, 136.5, 124.7, 123.8, 98.5, 70.7, 62.8, 55.3, 54.7, 45.2, 43.7, 43.6, 39.7, 36.6, 35.0, 34.6, 34.0, 33.2, 31.7, 31.2, 28.1, 26.4, 24.2, 24.0, 23.1, 18.3, 14.0. HRMS (ESI) *m*/*z*: $[M + Na]^+$ Calcd for $C_{33}H_{42}N_4O_7Na$, 629.2951; found, 629.2945.

*(2′R,4S,5′R,6aR,8aS,8bR,9S,11aS)-5′,6a,8a,9-Tetramethyl-1,3,3′,4,4′,5,5′,6,6a,6b,6′,7,8,8a,8b,9,11a,12,12a,12b-icosahydrospiro-[naphtho[2′,1′:4,5]indeno[2,1-b]furan-10,2′-pyran]-4-yl 6-((7-nitrobenzo-[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**11a**).* A 46.9 mg (0.068 mmol) amount of **11a** was isolated from 30.0 mg (0.072 mmol) of **11** in 94% yield. Orange solid. $[\alpha]^{25}_{D}$ = −64.2 (*c* 3.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3324, 1728. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.49 (d, *J* = 8.72 Hz, 1H), 6.29 (m, 1H), 6.18 (d, *J* = 8.59 Hz, 1H), 5.36 (d, *J* = 4.42 Hz, 1H), 4.62 (m, 1H), 4.42 (m, 1H), 3.32−3.57 (m, 4H), 2.35 (m, 4H), 1.03 (s, 3H), 0.98 (d, *J* = 6.69 Hz, 3H), 0.80 (s, 3H), 0.79 (d, *J* = 5.49 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.8, 144.2, 143.8, 139.5, 136.5, 123.8, 122.4, 109.2, 98.5, 80.7, 73.9, 66.8, 62.0, 56.4, 49.9, 43.6, 41.5, 40.2, 39.6, 38.1, 36.9, 36.7, 34.2, 32.0, 31.8, 31.3, 30.2, 28.7, 28.1, 27.7, 26.2, 24.3, 20.8, 19.3, 17.1, 16.2, 14.5. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{39}H_{55}N_4O_7$, 691.4071; found, 691.4073 .

*5-Methoxy-2-(4-methoxyphenethyl)-1-oxoisoindolin-4-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**12a**).* A 50.8 mg (0.086 mmol) amount of **12a** was isolated from 30.0 mg (0.096 mmol) of **12** in 90% yield. Orange solid. $[\alpha]^{25}_{D}$ = +0.29 (*c* 1.48, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3333, 1759, 1683. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.48 (d, *J* = 8.24 Hz, 1H), 7.24 (d, *J* = 2.29 Hz, 1H), 7.13 (d, *J* = 8.70 Hz, 2H), 6.82 (d, *J* = 8.70 Hz, 2H), 6.79 (d, *J* = 1.83 Hz, 1H), 6.50 (m, 1H), 6.18 (d, *J* = 8.70 Hz, 1H), 4.06 (s, 2H), 3.85 (s, 3H), 3.78 (s, 3H), 3.79 (t, *J* = 7.33 Hz, 2H), 3.55 (m, 2H), 2.91 (t, *J* = 7.33 Hz, 2H), 2.63 (t, *J* = 7.33 Hz, 2H), 1.80−1.94 (m, 4H), 1.61 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 170.6, 167.6, 161.0, 158.2, 145.7, 144.2, 143.8, 143.8, 136.4, 135.7, 130.6, 129.6, 125.0, 124.0, 114.0, 112.4, 104.8, 98.6, 56.0, 55.3, 48.2, 44.5, 43.6, 33.9, 33.7, 28.1, 26.3, 24.3. HRMS (ESI) *m*/*z*: $[M + H]^+$ Calcd for $C_{30}H_{32}N_5O_8$, 590.2251; found, 590.2255.

*4-((2S,3R)-1-(4-Fluorophenyl)-3-((S)-3-(4-fluorophenyl)-3-hydroxypropyl)-4-oxoazetidin-2-yl)phenyl 6-((7-Nitrobenzo[c][1,2,5]-oxadiazol-4-yl)amino)hexanoate (**13a**).* A 26.1 mg (0.038 mmol) amount of **13a** was isolated from 30.0 mg (0.073 mmol) of **13** in 52% yield. Orange solid. $[\alpha]^{25}_{D}$ = −25.7 (*c* 2.5, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3336, 1743. ^1H NMR ($CDCl_3$, 400 MHz) δ: 8.47 (d, *J* = 8.53 Hz, 1H), 6.90−7.35 (m, 12H), 6.46 (m, 1H), 6.17 (d, *J* = 8.78 Hz, 1H), 4.72 (t, *J* = 5.77 Hz, 1H), 4.64 (d, *J* = 1.76 Hz, 1H), 3.53 (m, 2H), 3.09 (m, 1H), 2.63 (t, *J* = 7.03 Hz, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 171.7, 167.3, 162.1 (d, *J* = 245 Hz), 159.0 (d, *J* = 244 Hz), 150.6, 144.2, 143.8, 139.9, 136.5, 135.1, 133.6, 127.4 (d, *J* = 8.4 Hz), 126.9, 123.9, 122.3, 118.3 (d, *J* = 7.8 Hz), 115.9 (d, *J* = 22.3 Hz), 115.3 (d, *J* = 20.8 Hz), 98.5, 73.1, 60.7, 60.3, 43.6, 36.5, 33.9, 28.1, 26.2, 25.0, 24.2. HRMS (ESI) *m*/*z*: $[M + Na]^+$ Calcd for $C_{36}H_{33}F_2N_5O_7Na$, 708.2246; found, 708.2222.

*(S)-Quinolin-4-yl((1S,2R,4S,5R)-5-vinylquinuclidin-2-yl)methyl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**14a**).* A 47.7 mg (0.084 mmol) of **14a** was isolated from 30.0 mg (0.102 mmol) amount of **14** in 82% yield. Orange solid. $[\alpha]^{25}_{D}$ = +43.7 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3334, 1734. ^1H NMR ($CDCl_3$, 400 MHz)

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δ: 8.87 (d, J = 4.58 Hz, 1H), 8.46 (d, J = 8.54 Hz, 1H), 8.23 (d, J = 8.24 Hz, 1H), 8.11 (d, J = 7.93 Hz, 1H), 7.72 (m, 1H), 7.61 (m, 1H), 7.37 (d, J = 4.58 Hz, 1H), 6.62 (d, J = 6.71 Hz, 1H), 6.46 (m, 1H), 6.10 (d, J = 8.85 Hz, 1H), 6.01 (m, 1H), 5.11 (m, 2H), 3.36 (m, 3H), 2.95 (m, 2H), 2.82 (m, 1H), 2.74 (m, 1H), 2.46 (m, 2H), 2.30 (q, J = 8.24 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.3, 149.8, 148.5, 145.2, 144.2, 143.9, 143.8, 139.9, 136.4, 130.4, 129.3, 126.9, 125.9, 123.9, 123.3, 118.5, 115.1, 98.5, 73.7, 59.3, 49.7, 49.0, 43.6, 39.5, 33.9, 28.0, 27.7, 26.2, 26.1, 24.1, 23.6. HRMS (ESI) *m/z*: $[M + H]^+$ Calcd for $C_{31}H_{35}N_6O_5$, 571.2669; found, 571.2651.

*(S)-4-Ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3′,4′:6,7]-indolizino[1,2-b]quinolin-4-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**15a**).* A 8.1 mg (0.013 mmol) amount of **15a** was isolated from 50.0 mg (0.144 mmol) of **15** in 9% yield. Orange solid. $[\alpha]^{25}_D$ = −40.4 (*c* 0.6, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3329, 1744, 1661. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.35 (s, 1H), 8.31 (d, J = 8.85 Hz, 1H), 8.12 (m, 1H), 7.90 (m, 1H), 7.77 (m, 1H), 7.63 (m, 1H), 7.19 (s, 1H), 6.54 (m, 1H), 5.99 (d, J = 8.85 Hz, 1H), 5.71 (d, J = 17.09 Hz, 1H), 5.42 (d, J = 17.09 Hz, 1H), 5.29 (s, 2H), 3.45 (m, 2H), 2.53−2.65 (m, 2H), 2.25 (m, 1H), 2.14 (m, 1H), 0.99 (t, J = 7.63 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.4, 167.7, 157.3, 152.3, 148.7, 146.2, 145.9, 144.1, 143.8, 143.8, 136.3, 131.3, 130.7, 129.2, 128.5, 128.3, 128.1, 128.1, 120.1, 98.4, 95.8, 76.0, 67.1, 49.9, 43.7, 33.4, 31.7, 27.9, 26.0, 24.1, 7.5. HRMS (ESI) *m/z*: $[M + H]^+$ Calcd for $C_{32}H_{29}N_6O_8$, 625.2047; found, 625.2044.

*(4R)-2-(2-((1S,2S,6R,8S)-8-((2,2-Dimethylbutanoyl)oxy)-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl)ethyl)-6-oxotetrahydro-2H-pyran-4-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)-amino)hexanoate (**16a**).* A 40.3 mg (0.058 mmol) amount of **16a** was isolated from 30.0 mg (0.072 mmol) of **16** in 81% yield. Orange solid. $[\alpha]^{25}_D$ = +140.0 (*c* 2.6, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3323, 1733. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, J = 8.54 Hz, 1H), 6.70 (m, 1H), 6.20 (d, J = 8.85 Hz, 1H), 5.99 (d, J = 9.77 Hz, 1H), 5.78 (dd, J = 9.77, 6.10 Hz, 1H), 5.52 (t, J = 3.36 Hz, 1H), 5.36 (q, J = 3.05 Hz, 1H), 5.28 (m, 1H), 4.48 (m, 1H), 3.54 (m, 2H), 2.75 (m, 2H), 2.40 (t, J = 7.32 Hz, 2H), 1.13 (s, 3H), 1.12 (s, 3H), 1.09 (d, J = 7.32, 7.02 Hz, 3H), 0.89 (d, J = 7.32, 7.02 Hz, 3H), 0.81 (t, J = 7.63 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 177.9, 172.3, 168.8, 144.3, 144.0, 143.9, 136.5, 132.7, 131.3, 129.8, 128.4, 123.8, 98.5, 77.1, 68.0, 65.7, 43.7, 43.0, 37.4, 36.7, 35.5, 34.0, 33.3, 33.2, 33.0, 32.8, 30.6, 28.1, 27.2, 26.3, 24.8, 24.7, 24.6, 24.4, 23.0, 13.9, 9.3. HRMS (ESI) *m/z*: $[M + Na]^+$ Calcd for $C_{37}H_{50}N_4O_9Na$, 717.3475; found, 717.3434.

*(3S,E)-4-(2-((1R,5R,6R,8aS)-6-Hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl)ethylidene)-5-oxotetrahydrofuran-3-yl 6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)-amino)hexanoate (**17a**).* A 17.7 mg (0.028 mmol) of **17a** was isolated from 30.0 mg (0.086 mmol) amount of **17** in 33% yield. Orange solid. $[\alpha]^{25}_D$ = −80.7 (*c* 0.95, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3409, 1738. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, J = 8.53 Hz, 1H), 7.01 (m, 1H), 6.51 (m, 1H), 6.18 (d, J = 8.53 Hz, 1H), 5.94 (d, J = 5.52 Hz, 1H), 4.85 (s, 1H), 4.56 (m, 1H), 4.47 (s, 1H), 4.23 (m, 1H), 4.15 (d, J = 11.04 Hz, 1H), 3.50 (m, 3H), 3.32 (d, J = 11.04 Hz, 1H), 2.35−2.45 (m, 5H), 1.25 (s, 3H), 0.62 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.9, 169.1, 150.6, 146.9, 144.2, 143.9, 143.8, 136.5, 123.9, 123.8, 108.7, 98.6, 80.3, 71.6, 67.8, 64.1, 55.7, 55.0, 43.6, 42.8, 38.7, 37.6, 36.9, 33.7, 29.7, 28.1, 26.3, 25.3, 24.2, 23.6, 22.7, 15.1. HRMS (ESI) *m/z*: $[M + H]^+$ Calcd for $C_{32}H_{43}N_4O_9$, 627.3030; found, 627.3024.

*2H,3H-Cyclopenta[b]furo[2′,3′,4′:4,5]naphtho[2,3-d]furan-3,5-diol, 8-(3-Furanyl)-2a,4,5,5a,6,6a,8,9,9a,10a,10b,10c-dodecahydro-6-((6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoyl)oxy)-ethyl-2a,5a,6a,7-tetramethyl-, (2aR,3R,5S,5aR,6R,6aR,8R,9aR,10-aS,10bR,10cR) (**18a**).* A 30.2 mg (0.042 mmol) amount of **18a** was isolated from 20.0 mg (0.045 mmol) of **18** in 93% yield. Orange solid. $[\alpha]^{25}_D$ = +73.0 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3140, 1719. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.49 (d, J = 8.85 Hz, 1H), 7.32 (m, 1H), 7.21 (m, 1H), 6.67 (m, 1H), 6.19 (m, 1H), 6.17 (d, J = 8.85 Hz, 1H), 5.36 (m, 1H), 4.22 (m, 1H), 4.18 (d, J = 3.36 Hz, 1H), 3.98 (m, 4H), 3.67 (m, 3H), 3.57 (d, J = 7.02 Hz, 1H), 3.50 (m, 2H), 3.34 (d, J = 6.71 Hz, 1H), 2.61 (d, J = 12.82 Hz, 1H), 2.33 (t, J = 7.02 Hz, 2H), 1.78 (s, 3H), 1.32 (s, 3H), 1.11 (s, 3H), 0.89 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.3, 148.0, 144.2, 144.0, 143.9, 143.1, 138.4, 136.6, 133.4, 127.1, 123.7, 110.4, 98.5, 87.5, 85.9, 77.5, 72.9, 72.6, 71.8, 64.8, 49.2, 49.0, 44.3, 43.7, 41.5, 41.5, 38.5, 37.6, 33.8, 31.4, 28.0, 26.2, 24.9, 24.2, 19.3, 17.1, 15.0, 13.3. HRMS (ESI) *m/z*: $[M + NH_4]^+$ Calcd for $C_{38}H_{52}N_5O_{10}$, 738.3714; found, 738.3698.

*(3R,5aS,6R,8aS,9R,10R,12S,12aR)-3,6,9-Trimethyldecahydro-3H-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10-yl 6-((7-Nitrobenzo-[c][1,2,5]oxadiazol-4-yl)amino)hexanoate (**19a**).* A 42.0 mg (0.075 mmol) amount of **19a** was isolated from 30.0 mg (0.106 mmol) of **19** in 71% yield. Orange solid. $[\alpha]^{25}_D$ = +1.1 (*c* 0.62, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3337, 1747. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.50 (d, J = 8.53 Hz, 1H), 6.56 (m, 1H), 6.19 (d, J = 8.78 Hz, 1H), 5.81 (d, J = 9.79 Hz, 1H), 5.45 (s, 1H), 3.53 (m, 2H), 1.41 (s, 3H), 0.98 (d, J = 6.02 Hz, 3H), 0.84 (d, J = 7.28 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 172.2, 144.2, 143.9, 136.5, 123.8, 104.5, 98.5, 91.9, 91.5, 80.1, 51.5, 45.1, 43.6, 37.2, 36.1, 34.0, 33.8, 31.7, 27.9, 26.1, 25.9, 24.5, 23.8, 22.0, 20.2, 12.1. HRMS (ESI) *m/z*: $[M + Na]^+$ Calcd for $C_{27}H_{36}N_4O_9Na$, 583.2380; found, 583.2370.

*(2aR,2a1R,3S,4S,4aR,5S,7aS,8S,10R,10aS)-Dimethyl 10-Acetoxy-5-((4-((tert-butyldimethylsilyl)oxy)but-2-yn-1-yl)oxy)-3-hydroxy-4-((1aR,2S,3aS,6aR,7S,7aR)-6a-hydroxy-7a-methyl-1a,2,3a,6a,7,7a-hexahydro-2,7-methanofuro[2,3-b]oxireno[2,3-e]oxepin-1a-yl)-4-methyl-8-(((E)-2-methylbut-2-enoyl)oxy)dodecahydronaphtho[1,8-bc:4,4a-c′]difuran-5,10a-dicarboxylate (**20a**).* A 46.7 mg (0.052 mmol) amount of **20a** was isolated from 100.0 mg (0.139 mmol) of **20** in 37% yield. White solid. $[\alpha]^{25}_D$ = −20.1 (*c* 3.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3480, 1741. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.93 (m, 1H), 6.42 (d, J = 2.75 Hz, 1H), 5.63 (s, 1H), 5.48 (t like, 1H), 5.02 (d, J = 3.05 Hz, 1H), 4.74 (m, 1H), 4.71 (t, J = 2.75 Hz, 1H), 4.59 (dd, J = 2.44, 12.51 Hz, 1H), 4.53 (m, 1H), 4.44 (m, 1H), 4.26 (m, 3H), 4.11 (d, J = 9.46 Hz, 1H), 4.05 (d, J = 8.85 Hz, 1H), 3.79 (s, 3H), 3.74 (d, J = 8.85 Hz, 1H), 3.65 (m, 1H), 3.63 (s, 3H), 3.54 (s, 1H), 3.37 (d, J = 12.51 Hz, 1H), 3.01 (1H, br s), 2.92 (1H, br s), 2.34 (m, 1H), 2.28 (d, J = 5.49 Hz, 1H), 2.22 (m, 1H), 1.94 (s, 3H), 1.89 (s, 3H), 1.85 (m, 3H), 1.77 (m, 3H), 1.64 (s, 3H), 1.24 (m, 2H), 0.87 (s, 9H), 0.08 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.4, 169.7, 169.1, 166.4, 146.9, 137.9, 128.5, 108.7, 107.5, 106.5, 84.6, 83.6, 80.6, 76.8, 75.2, 73.7, 72.9, 70.6, 69.4, 66.9, 53.1, 53.0, 52.7, 52.5, 51.7, 50.1, 47.8, 46.2, 45.4, 37.0, 29.7, 25.8, 25.1, 21.0, 20.8, 18.8, 18.2, 14.3, 11.9, (−) 5.2. HRMS (ESI) *m/z*: $[M + Na]^+$ Calcd for $C_{45}H_{62}O_{17}SiNa$, 925.3654; found, 925.3630.

*(2aR,2a1R,3S,4S,4aR,5S,7aS,8S,10R,10aS)-Dimethyl 10-Acetoxy-3-hydroxy-4-((1aR,2S,3aS,6aR,7S,7aR)-6a-hydroxy-7a-methyl-1a,2,3a,6a,7,7a-hexahydro-2,7-methanofuro[2,3-b]oxireno[2,3-e]-oxepin-1a-yl)-5-((4-hydroxybut-2-yn-1-yl)oxy)-4-methyl-8-(((E)-2-methylbut-2-enoyl)oxy)dodecahydronaphtho[1,8-bc:4,4a-c′]-difuran-5,10a-dicarboxylate (**20b**).* A 37.7 mg (0.048 mmol) of **20b** was isolated from 46.7 mg (0.052 mmol) amount of **20a** in 92% yield. White solid. $[\alpha]^{25}_D$ = −20.2 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3460, 1740. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.94 (m, 1H), 6.43 (d, J = 2.75 Hz, 1H), 5.69 (s, 1H), 5.49 (t like, 1H), 5.04 (d, J = 2.75 Hz, 1H), 4.82 (m, 1H), 4.72 (t, J = 2.75 Hz, 1H), 4.58 (dd, J = 12.51, 2.75 Hz, 1H), 4.47 (m, 1H), 4.43 (m, 1H), 4.33 (m, 1H), 4.23 (m, 2H), 4.13 (d, J = 9.77 Hz, 1H), 4.06 (d, J = 8.85 Hz, 1H), 3.80 (s, 3H), 3.74 (d, J = 8.85 Hz, 1H), 3.66 (m, 5H), 3.39 (d, J = 12.51 Hz, 1H), 2.90 (1H, br s), 2.74 (1H, br s), 2.40 (m, 1H), 2.30 (d, J = 5.49 Hz, 1H), 2.22 (m, 1H), 1.94 (s, 3H), 1.91 (s, 3H), 1.87 (m, 3H), 1.78 (dd like, 3H), 1.58 (s, 3H), 1.26 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.4, 169.7, 169.2, 166.5, 147.0, 137.9, 128.5, 108.3, 107.8, 106.5, 84.6, 83.6, 81.8, 75.3, 73.8, 72.9, 70.8, 69.8, 69.5, 66.9, 53.0, 52.7, 52.6, 51.0, 50.2, 47.8, 46.3, 45.5, 37.1, 29.7, 25.4, 20.8, 20.7, 18.9, 14.3, 11.9. HRMS (ESI) *m/z*: $[M + Na]^+$ Calcd for $C_{39}H_{48}O_{17}Na$, 811.2789; found, 811.2741.

*(2aR,2a1R,3S,4S,4aR,5S,7aS,8S,10R,10aS)-Dimethyl 10-Acetoxy-3-hydroxy-4-((1aR,2S,3aS,6aR,7S,7aR)-6a-hydroxy-7a-methyl-1a,2,3a,6a,7,7a-hexahydro-2,7-methanofuro[2,3-b]oxireno[2,3-e]-oxepin-1a-yl)-4-methyl-8-(((E)-2-methylbut-2-enoyl)oxy)-5-((4-((6-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)hexanoyl)oxy)but-2-yn-1-yl)oxy)dodecahydronaphtho[1,8-bc:4,4a-c′]difuran-5,10a-di-carboxylate (**20c**).* A 36.9 mg (0.035 mmol) amount of **20c** was isolated from 37.7 mg (0.048 mmol) of **20b** in 72% yield. Orange solid. $[\alpha]^{25}_D$ = −12.3 (*c* 1.8, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3413,

1738. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.51 (d, J = 8.54 Hz, 1H), 6.93 (m, H-33), 6.50 (m, 1H), 6.44 (d, J = 2.75 Hz, 1H), 6.19 (d, J = 8.85 Hz, 1H), 5.62 (s, 1H), 5.50 (t, J = 2.75 Hz, 1H), 5.04 (d, J = 2.75 Hz, 1H), 4.71 (m, 2H), 4.67 (t like, 2H), 4.59 (dd, J = 12.51,2.75 Hz, 1H), 4.53 (m, 1H), 4.47 (m, 1H), 4.28 (m, 1H), 4.14 (d, J = 9.77 Hz, 1H), 4.06 (d, J = 8.85 Hz, 1H), 3.80 (s, 3H), 3.76 (d, J = 9.16 Hz, 1H), 3.65 (m, 4H), 3.52 (m, 3H), 3.37 (d, J = 12.51 Hz, 1H), 2.92 (m, 2H), 2.38 (t, J = 7.32 Hz, 2H), 2.34 (m, 1H), 2.29 (d, J = 5.49 Hz, 1H), 2.21 (m, 1H), 1.95 (s, 3H), 1.90 (s, 3H), 1.87 (m, 3H), 1.77 (m, 3H), 1.66 (s, 3H), 1.15 (d, J = 6.41 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.4, 172.4, 169.7, 169.0, 166.4, 146.9, 144.3, 143.9, 143.8, 137.9, 136.4, 128.5, 124.0, 108.9, 107.6, 106.4, 98.5, 83.7, 82.7, 79.9, 76.9, 75.2, 73.7, 73.1, 70.6, 69.7, 69.3, 66.9, 53.0, 52.8, 52.8, 52.6, 52.2, 50.2, 47.9, 46.3, 45.4, 43.7, 37.0, 33.6, 29.8, 28.1, 26.3, 25.1, 24.2, 23.5, 20.9, 18.9, 14.3, 11.9. HRMS (ESI) m/z: $[M + Na]^+$ Calcd for $C_{51}H_{60}N_4O_{21}Na$, 1087.3648; found, 1087.3611.

Cell Viability Assay. The cell viability assay was performed as described in a previous report.[34] Briefly, MDA-MB-231 cells (2×10^4 cells per well) were seeded in a 96-well microplate. Cells were treated with either **8** or **8a** (0−100 μg/mL) for 24 h. In a separate experiment, cells were treated with either **1** or **1a** (0−100 μg/mL) for 24 h. A 200 μL amount of MTT (0.5 mg/mL) was added into each well and incubated at 37 °C for 4 h. Formazan crystals were dissolved in 2-propanol, and the optical density of formazan solution was measured using a microplate reader at 570 nm. Experiments were performed in triplicates. The mean value of the reading was calculated and analyzed statistically, and the percent change with respect to control was defined. A graph was plotted as cell viability (% control) versus dose using SigmaPlot software.

Cell Motility Assay. The wound migration assay using MDA-MB-231 was performed as described in a previous report.[34] Briefly, cells were grown, synchronized for 24 h in serum-free medium, and wounded uniformly using a sterile tip. The wound photographs were captured, and the cells were treated with NBD-labeled or unlabeled azadiradione derivatives (0−20 μg/mL). After 12 h, wound closures were photographed using a phase contrast microscope and analyzed.

Confocal Microscopy. Confocal microscopy analysis to study cellular localization of NBD-labeled compound was performed as reported previously.[41] MDA-MB-231, A375, and HeLa cells were grown on coverslips and treated with **1a** or **8a** (20 μg/mL) for 1 h, and fluorescence imaging analysis was performed. For colocalization study, HeLa cells were treated with either **8a** or **1a** for 1 h, followed by treatment with Mitotracker Red (50 nM) for 20 min. Treated cells were fixed with 2% paraformaldehyde for 10 min, washed twice with PBS, and mounted onto a glass slide with mounting media. Nuclei were stained with DAPI and visualized under a confocal microscope (excitation 460 nm, emission 530 nm) with 60× magnification. Similarly, a colocalization experiment was performed with fluorescently (green) tagged derivatives of azadiradione (**1a** and **8a**) and ER-tracker red in MDA-MB-231 cells. Cells treated with either **1a** or **8a** (20 μg/mL for 1 h) were stained with ER-tracker red (1 μM for 20 min) and analyzed under a confocal microscope with 100× magnification after fixation and nuclear staining with DAPI (blue).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of NMR spectra, fluorescence spectra, wound migration photographs, colocalization study, and HPLC profiles and studies for the stability of the ester bond. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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