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Article

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

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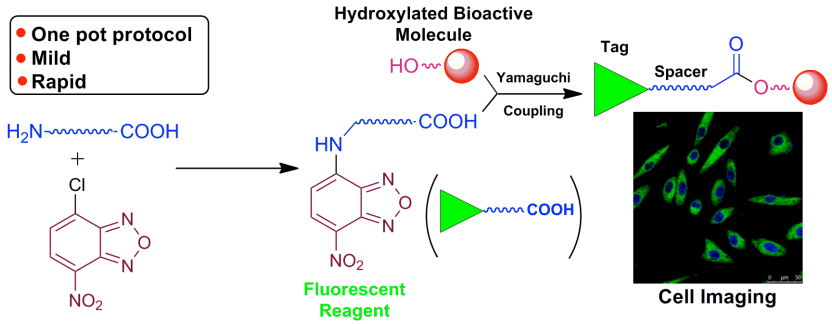
Author Contributions. [#]These authors contributed equally.

Table of Contents (TOC) Graphic.



ABSTRACT

Tagging of small bioactive molecules with 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 the fluorescence detecting instruments.[1-5] Natural products or biochemical probes covalently linked with the 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 rapid, inexpensive and effective fluorescent tagging protocol for the bioactive natural products and successful implementation of the developed procedure will facilitate the integration of fluorescence imaging and investigation of cellular events.

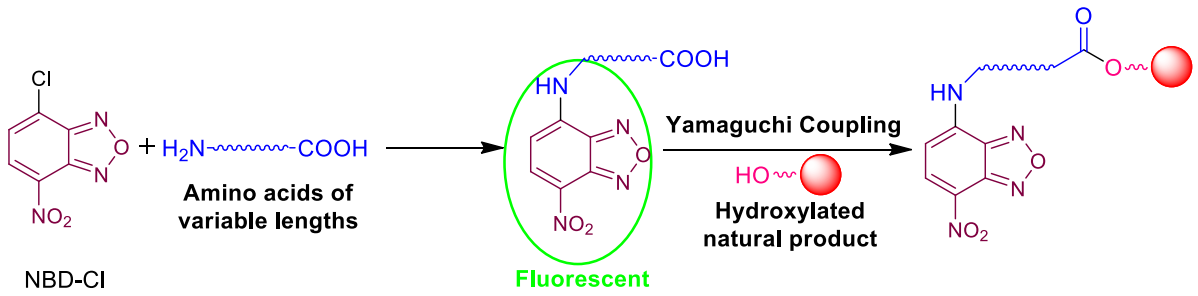
Reported protocols for the fluorescent labeling of natural products involving several reaction steps along with purification and protection-deprotection procedures result in low overall yield and make the process tedious.[12,13] Indeed the availability of natural products is scanty in most of the cases due to their low abundance in the natural sources, making it difficult to carry out all the required steps. Secondly, the 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 ºC at basic pH) which restrict their use for tagging the 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 the labeled derivative. In this article we have demonstrated a one-pot labeling protocol for the 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 semi-synthetic 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

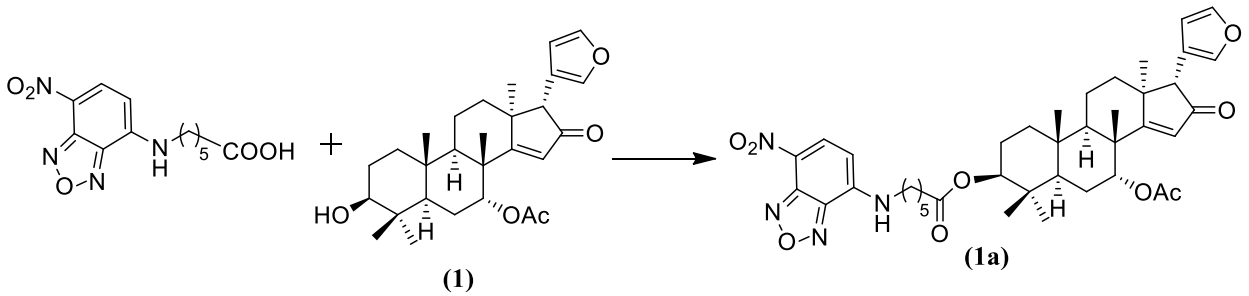
Chemistry. NBD-Cl is a non-fluorescent molecule, which undergoes nucleophilic substitution by amine functionality and becomes a highly sensitive fluorescent entity (depending on the

polarity of the environment) (Supporting information). This technique is being used over the years for the labeling of proteins and amine containing small molecules.[8,22-28] But, the reactivity of NBD-Cl towards hydroxyl group is very poor and this method can't 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 fluorescent reagent followed by coupling between the free carboxylic end and the hydroxylated bioactive molecule in the same vessel (Scheme 1). It is always preferable to synthesize fluorescent reagent freshly in one-pot rather than using it as an individual tagging dye due to several reasons: (i) 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 one-pot protocol utilizing the commercially available amino acids of own choice 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 fluorescent reagent freshly in one-pot from two non-fluorescent starting materials (NBD-Cl and amino acids). (iii) After the preparation of fluorescent reagent it is essentially need to be purified before storage. But, the reported one-pot protocol is devoid of one additional purification step making it less tedious.

Scheme 1. One-pot protocol for the fluorescent labeling of hydroxylated bioactive molecules.



Comparative Kinetics among Coupling Reactions. Among several well-established coupling reactions available between carboxylic acid and alcohol, Yamaguchi was preferred on the basis of outcome of 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 presence of various coupling reagents (DCC, EDC, DIC, Yamaguchi) (Figure 1). 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 profile. 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 till 10 h (Figure 1). The higher stoichiometric ratios of coupling reagents (DCC, EDC and DIC) also didn't alter the progress or rate of the reaction significantly even after the prolonged reaction time (Supporting information). On the other hand, Yamaguchi coupling showed quantitative conversion in 30 min; hence was chosen as the preferred coupling reaction for other molecules.



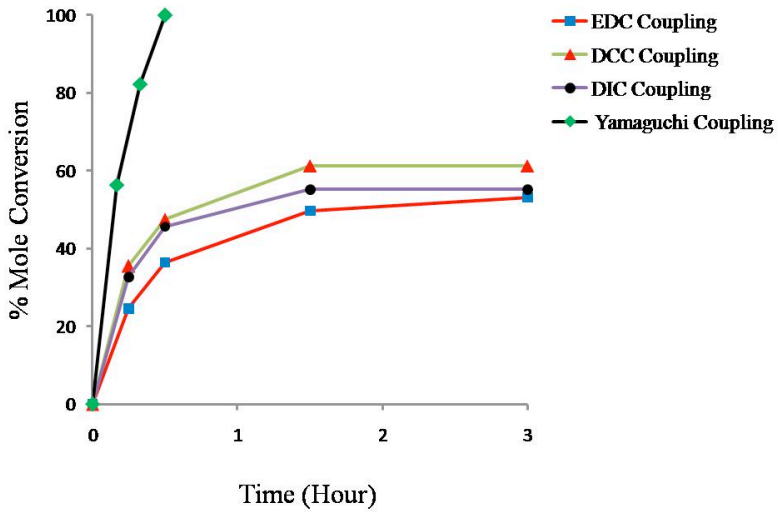
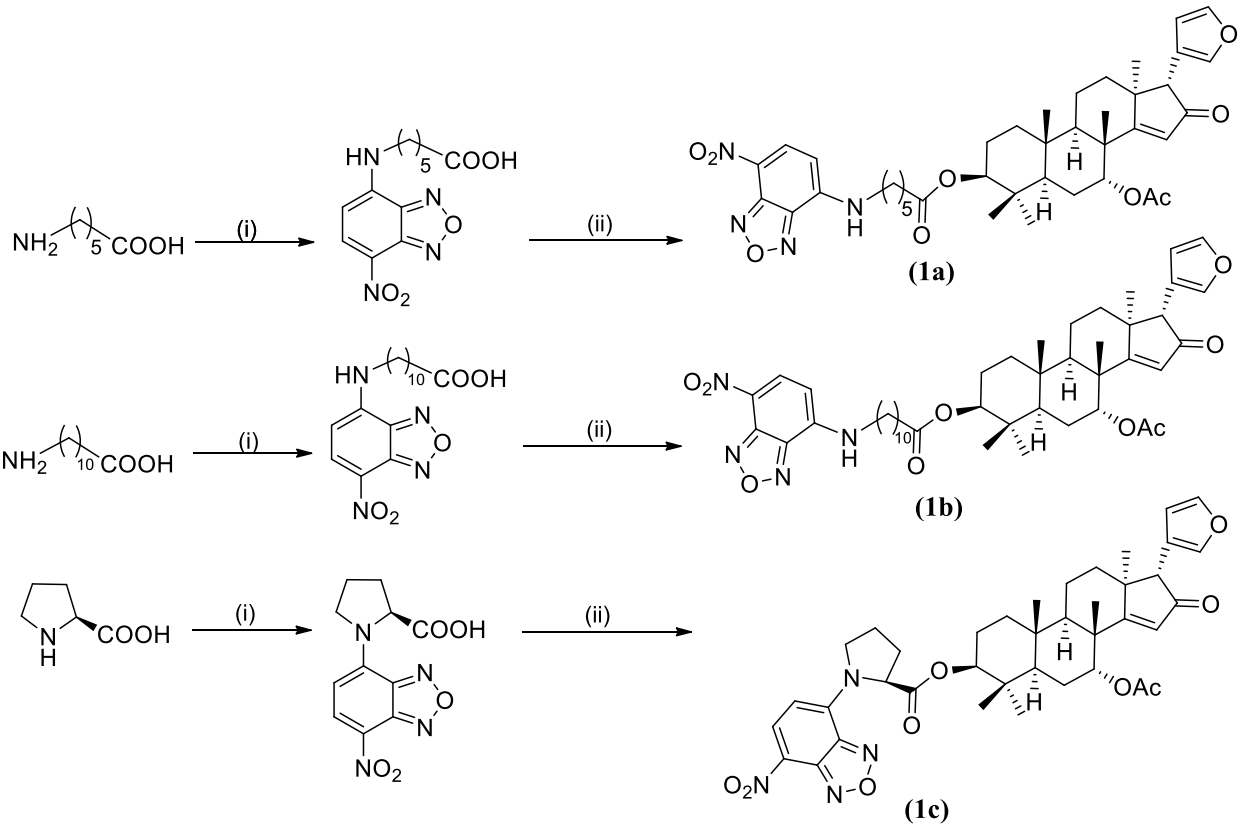


Figure 1. Comparative kinetics among various well-known coupling reactions between **1** and 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid.

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, spacer of optimum length is important for retaining the activity observed with 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 turn in the structure) (Scheme 2).

Scheme 2.[a] Selection of the amino acid in the initial step varies the length of spacer in between the tag and studied molecule.

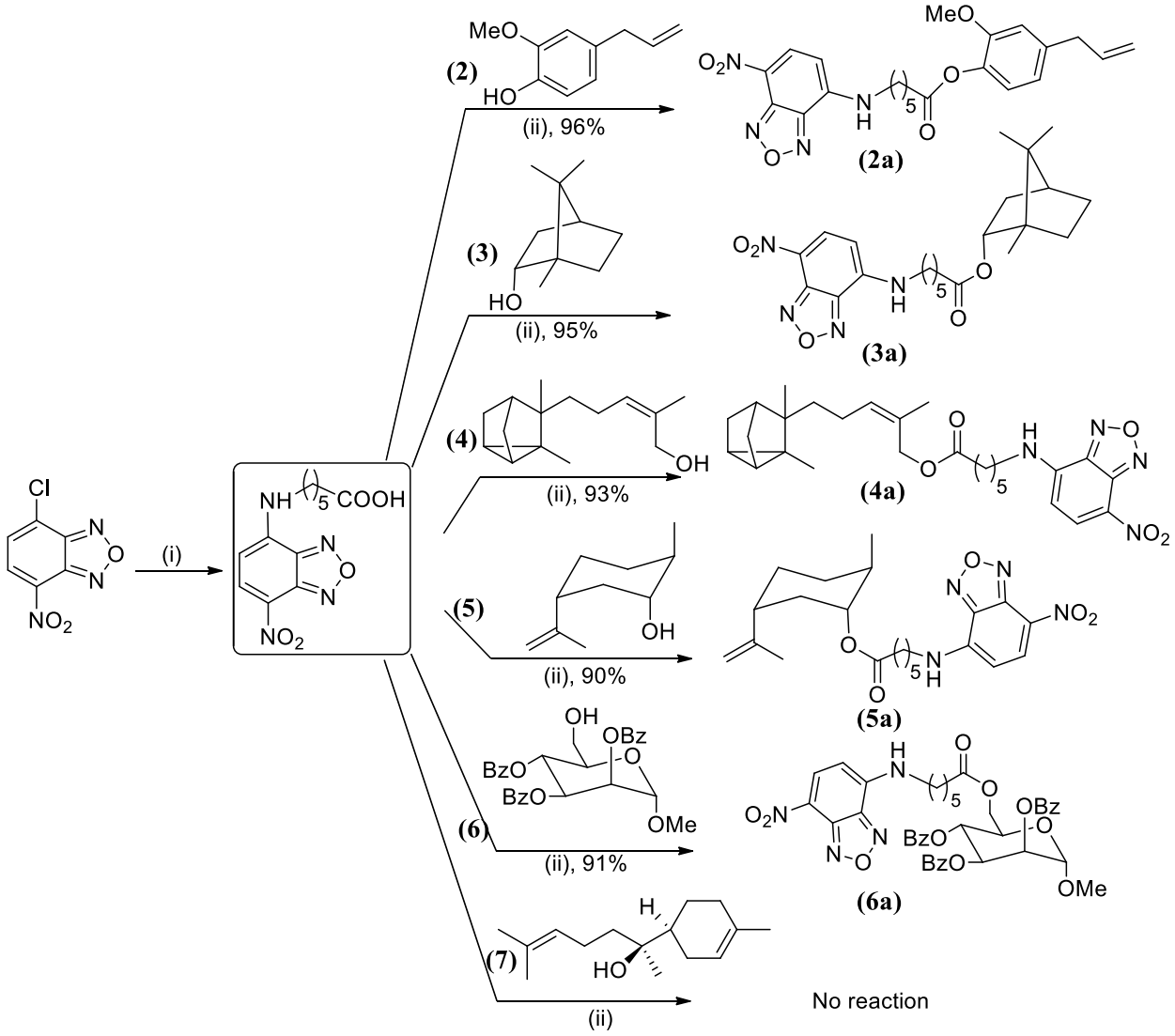


[a]Reagents and conditions: (i) NBD-Cl, $NaHCO_3$, acetonitrile, water, 55 ºC; (ii) **1**, 2,4,6-trichlorobenzoylchloride, TEA, DMAP, anhydrous THF, 85-90% for two steps.

Nature of the Hydroxyl Group. To investigate the efficiency of Yamaguchi coupling with varying nature of hydroxyl groups, several structurally simple compounds bearing different kind of hydroxyl functionalities (such as phenolic, primary, secondary and tertiary) were chosen and subjected to 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 primary hydroxyl furnished the labeled product with excellent yields. However, the coupling did not work out when α-(-)-bisabolol, (**7**) a sesquiterpene carrying 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.

Scheme 3.[a] Efficiency of Yamaguchi coupling varies with the nature of hydroxyl group.

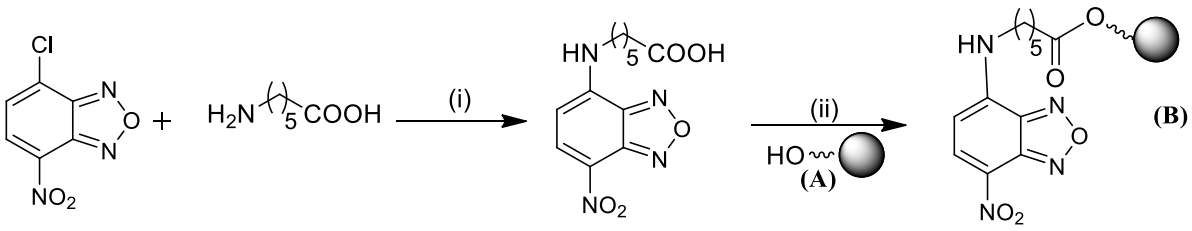


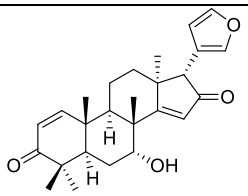
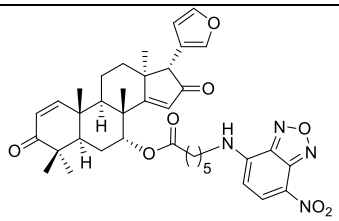
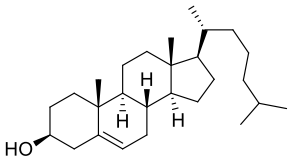
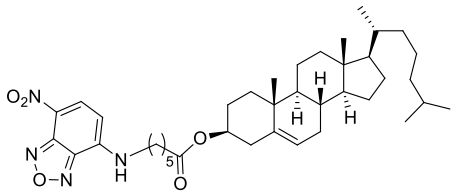
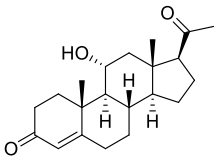
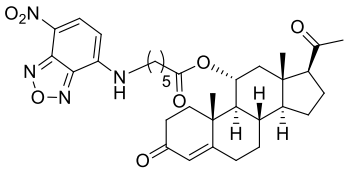
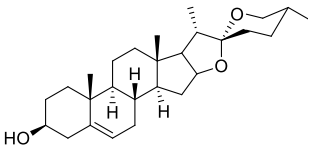
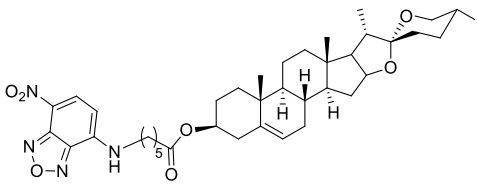
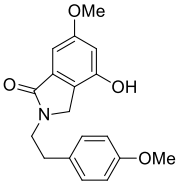
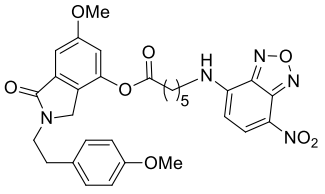
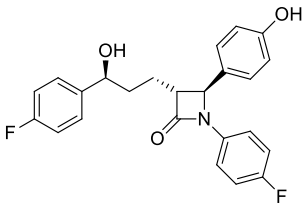
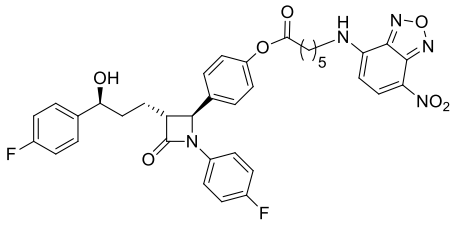
[a] Reagents and conditions: (i) 6-aminocaproic acid, $NaHCO_3$, acetonitrile, water, 55 ºC; (ii) 2,4,6- trichlorobenzoylchloride, TEA, DMAP, anhydrous THF.

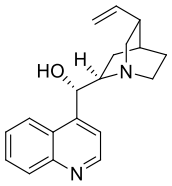
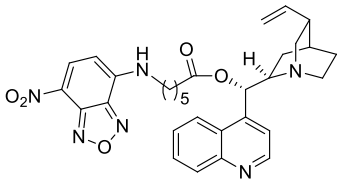
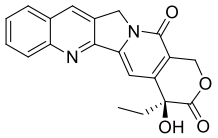
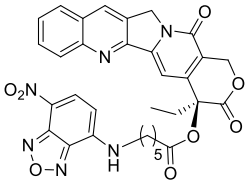
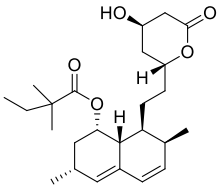
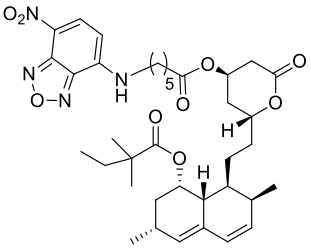
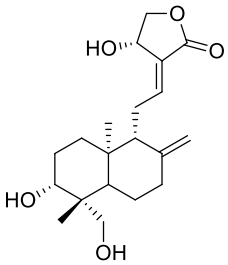
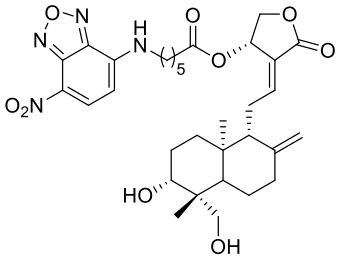
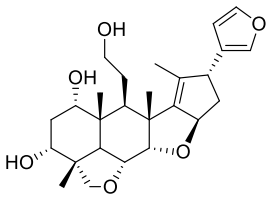
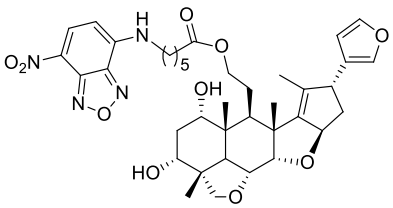
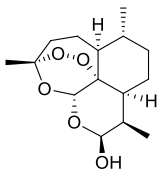
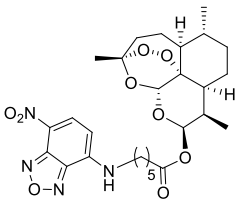
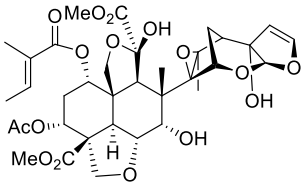
Applicability with Complex Molecules. To showcase broad applicability of the present methodology several structurally diverse and multifunctional molecules were selected for

fluorescent labeling (Table 1) using 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 basic limonoid skeleton was used as the substrate. Similarly, cholesterol (**9**), 11α-hydroxyprogesterone (**10**) and diosgenin (**11**, a steroidal sapogenin containing a spiroketal moiety)[30] furnished corresponding tagged compounds **9a**, **10a** and **11a** in good to excellent yields. Phenolic hydroxyl on the isoindolin skeleton of compound **12** responded to the reaction with an excellent yield. Ezetimibe (**13**, a cholesterol lowering drug)[31] possessing both alkylic and phenolic hydroxyl underwent a competitive reaction. However, at -15 ºC with 5 min of reaction time, tagged phenolic hydroxyl was obtained as a major product (52%) (For the detailed characterization consult supporting information) along with doubly tagged product (27%). An antimalarial alkaloid cinchonine[32] (**14**) carrying a secondary hydroxyl group was labeled with very good yield (82%) whereas another anticancer alkaloid (S)-(+)-camptothecin[33] (**15**) yielded the coupled product with a poor yield (09%) due to the tertiary nature of its hydroxyl functionality. A hypolipidemic drug simvastatin[31] (**16**) carrying a secondary hydroxyl group on a six membered lactone ring produced the tagged derivative with 81% yield. Andrographolide[34] (**17**), an anticancer natural product has three different hydroxyl groups around its skeleton; an allylic secondary hydroxyl on five membered lactone ring, one primary and another hindered secondary hydroxyl group. Although at room temperature the same reaction condition produced a mixture of products, at -15 ºC with 5 min stirring the mono-labeled (on allylic secondary hydroxyl) product was obtained with a moderate yield (33%) and characterized by 1D- and 2D-NMR studies as discussed in the supporting information. Interestingly, a reduced product of salanin (**18**) having

Table 1.[a] Entries showing applicability of the developed one-pot protocol for fluorescent labeling of complex bioactive hydroxylated compounds.



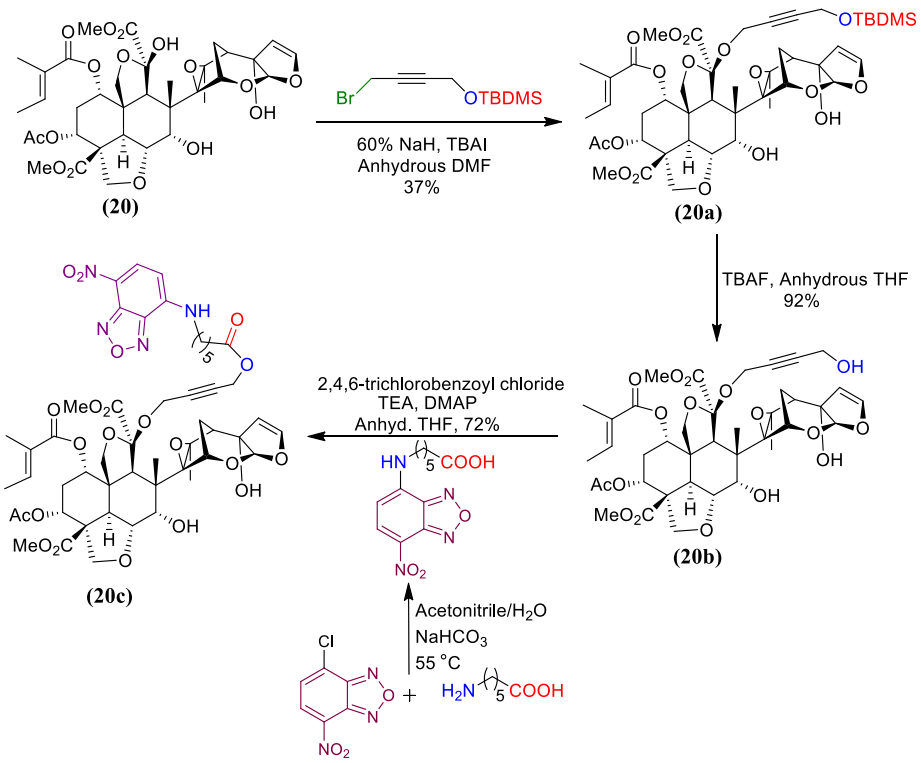
Entry	(A)	(B)	Yield
1.	 **(8)**	 **(8a)**	71%
2.	 **(9)**	 **(9a)**	96%
3.	 **(10)**	 **(10a)**	88%
4.	 **(11)**	 **(11a)**	94%
5.	 **(12)**	 **(12a)**	90%
6.	 **(13)**	 **(13a)**	52%

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-trichlorobenzoylchloride, TEA, DMAP, anhydrous THF. Parentheses are showing numbering of compounds.

two hindered secondary hydroxyl and another primary hydroxyl groups, furnished mono-labeled product on primary hydroxyl with an 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 with 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 towards 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. Inability of azadirachtin A (**20**) to produce any product may be due to the extreme steric hindrance around hydroxyl groups and their involvement in the intramolecular hydrogen bonding network.[37] To avoid these obstacles, an alternative approach was adopted.

Scheme 4. Fluorescent labeling protocol for azadirachtin A.



Initially, a short spacer carrying a protected hydroxyl group at the tail was attached followed by deprotection and Yamaguchi coupling. Azadirachtin A (**20**) was reacted with a propargyl bromide derivative carrying a TBDMS protected hydroxyl group at the other end in presence of sodium hydride. After nucleophilic substitution by 11-OH (as characterized on the basis of earlier reports),[38,39] hydroxyl was deprotected by TBAF and further subjected to one-pot protocol to get 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 their 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 further they were 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 with varying concentrations (0-100 μg/mL)

and MTT assay was performed. The results depicted comparable decrease in cell viability in labeled and unlabeled limonoids in a concentration dependent manner (Figure 2).

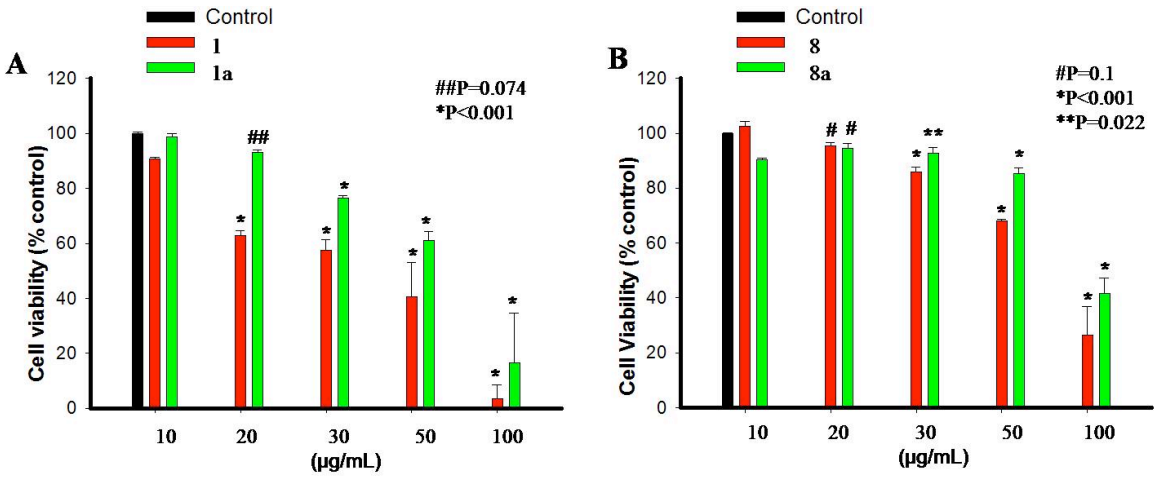


Figure 2. (**A** &**B**) MDA-MB-231 cells were treated with labeled and unlabeled limonoids and cell viability was assessed by MTT assay. The data obtained were analyzed statistically and represented graphically. Column, mean ± SE, *P<0.001, **P=0.022, #P=0.1, ##P=0.074 vs. control. The results are the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

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, treated with labeled (**1a** and **8a**) as well as 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 increasing concentration of limonoids (labeled as well as unlabeled) (Figure 3).

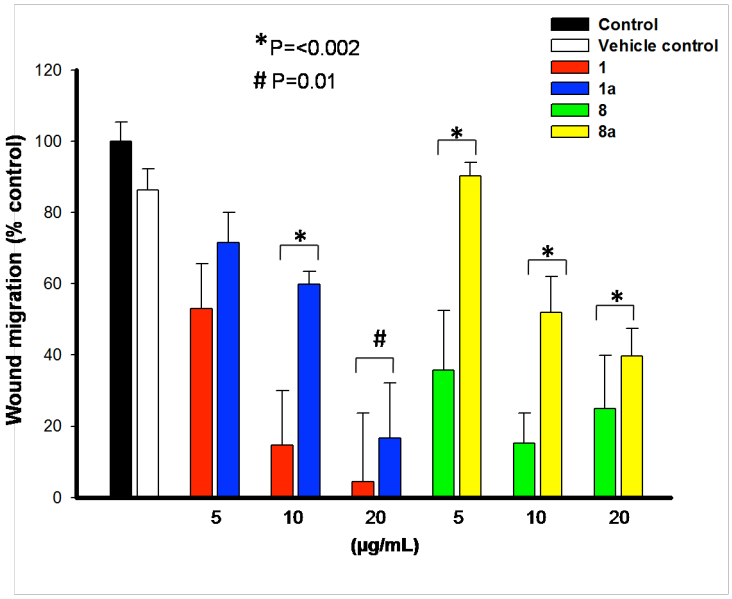


Figure 3. Wound migration is inhibited by limonoids in MDA-MB-231 cells. Cells were treated with **1**, **1a**, **8** and **8a** (0-20 μg/mL) and wounds were photographed after 12 h. Graphical representation for the quantification of wound migration assay; Column, mean $\pm$ SE, *P<0.002, #P=0.01 vs.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

Cell Imaging Studies. The comparative inhibition studies of cancer cell viability and motility have indicated the concentration dependent cytotoxic activity for both the 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 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 NBD tag and esterification of hydroxyl functionality. Therefore, considering the extent of cytotoxic activity preserved, **1a** and **8a** can be considered as the valid cancer cell imaging probe for azadiradione derivatives.

Cancer cells (A375, HeLa and MDA-MB-231) were treated either with **1a** or **8a** (20 µg/mL) for 1 h and analyzed for cellular internalization and localization under 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 sub-cellular organelles. To demonstrate specific examples, HeLa cells treated either with **1a** or **8a** were stained by Mito-tracker Red and analyzed through confocal microscopy, which didn't 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).

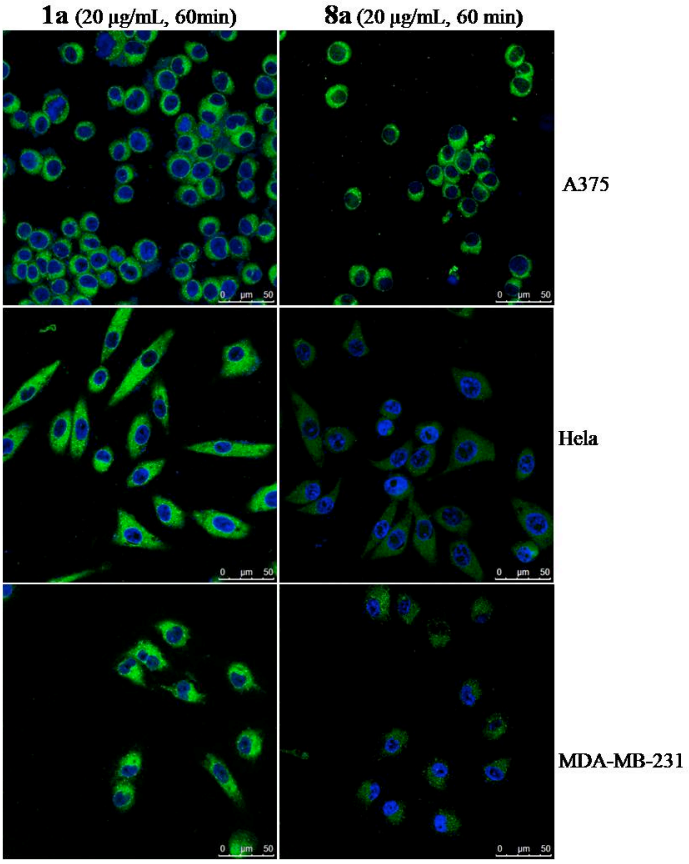


Figure 4. Cancer cells (A375, Hela and MDA-MB-231) were treated (20 μg/mL) with NBD labeled limonoids (**1a** and **8a**) for 1 h and analyzed under confocal microscope. Green: compounds labeled with NBD, Blue: Nucleus staining with DAPI. Micron bar: 50 μm.

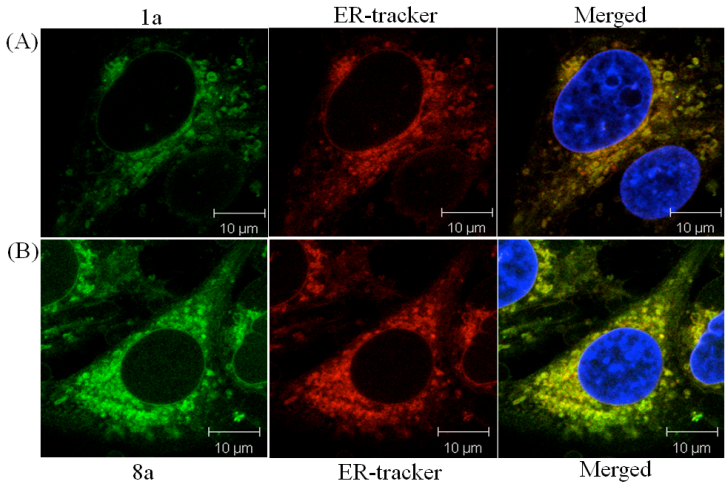


Figure 5. (A) MDA-MB-231 cells were co-stained with **1a** (20 μg/mL) and ER-tracker red (1 µM) and analyzed under confocal microscopy. **(B)** MDA-MB-231 cells were co-stained with **8a** (20 μg/mL) and ER-tracker red (1 µM) and analyzed under confocal microscopy. Nuclei were stained with DAPI. Blue: 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 best on the basis of yield and kinetic studies. Protocol was executed with a mild condition so that it was able to sustain sensitive 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 single purification step. Even the natural products isolated in trace amount (a few milligrams) can be labeled in sufficient yield and more economic way using the present protocol. Importantly, coupling reaction was very selective between acid and alcohol functionalities, which allows to tag the complex molecules with multiple functional groups. Fluorescein, a widely used fluorescent tag, was replaced by NBD as it is less bulkier than former. Again, length of the spacer can easily be modified by a suitable choice of the amino acid. Further, evaluation of the two NBD tagged azadiradione derivatives for cytotoxic activity indicated that the labeling did not alter the activity drastically. 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 ºC and incubated for 1 h. Then acetonitrile was concentrated under reduced pressure and pH of the aqueous reaction mixture was adjusted to ~2.0 using 1N HCl. Further it was concentrated to dryness under 20 mbar pressure and 62 ºC temperature. The deep orange crude solid was again dissolved in minimum amount of acetonitrile and dried in the same condition to make sure that there is no residual moisture remaining. 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 under 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 continued with stirring for another 30 min. The reaction was quenched by adding few drops of water. Then it was concentrated to dryness and directly purified over silica gel column. All the steps were performed under dim light and labeled compounds were stored at -20 ºC 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, anhydrous DCM (30 mL/mmol) was added. The mixture was stirred to dissolve all the components followed by addition of coupling reagent (1.00 equiv) (DCC, EDC or DIC) in stirring condition. After addition, reaction mixture was left for stirring at room temperature.

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

Preparation of 21b. 1M TBAF (62 μL, 0.062 mmol) was added dropwise to **21a** (46.72 mg, 0.052 mmol) taken 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 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)**: 41.9 mg (0.057 mmol) of **1a** was isolated from 30.0 mg (0.066 mmol) of **1** with 87% yield. Orange solid. $[\alpha]^{25}_{D}$ = -15.5 (*c* 3.0, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3327, 1719, 1700. ^{1}H 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)**: 47.3 mg (0.059 mmol) of **1b** was isolated from 30.0 mg (0.066 mmol) of **1** with 89% yield. Orange solid. $[\alpha]^{25}_{D}$ = -12.6 (*c* 1.7, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3327, 1715, 1700. ^{1}H 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)**: 40.0 mg (0.056 mmol) of **1c** was isolated from 30.0 mg (0.066 mmol) of **1** with 85% yield. Orange solid. $[\alpha]^{25}_D$ = +101.2 (*c* 2.25, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 1736, 1701. ^{1}H 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)**: 77.4 mg (0.176 mmol) of **2a** was isolated from 30.0 mg (0.183 mmol) of **2** with 96% yield. Orange solid. $[\alpha]^{25}_D$ = +0.15 (*c* 4.1, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3330, 1754. ^{1}H 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)**: 79.1 mg (0.184 mmol) of **3a** was isolated from 30.0 mg (0.195 mmol) of **3** with 95% yield. Orange solid. $[\alpha]^{25}_D$ = -18.6 (*c* 3.8, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3326, 1719. ^{1}H 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)**: 62.5 mg (0.126 mmol) of **4a** was isolated from 30.0 mg (0.136 mmol) of **4** with 93% yield. Orange solid. $[\alpha]^{25}_D$ = +3.51 (*c* 1.3, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3328, 1719. ^{1}H 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)**: 75.2 mg (0.175 mmol) of **5a** was isolated from 30.0 mg (0.195 mmol) of **5** with 90% yield. Orange solid. $[\alpha]^{25}_{D}$ = -34.7 (*c* 2.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3325, 1727. ^{1}H 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)**: 42.2 mg (0.054 mmol) of **6a** was isolated from 30.0 mg (0.059 mmol) of **6** with 91% yield. Orange solid. $[\alpha]^{25}_{D}$ = -55.3 (*c* 2.5, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3339, 1732. ^{1}H 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)**: 35.7 mg (0.052 mmol) of **8a** was isolated from 30.0 mg (0.073 mmol) of **8** with 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)**: 49.4 mg (0.075 mmol) of **9a** was isolated from 30.0 mg (0.078 mmol) of **9** with 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)**: 48.5 mg (0.080 mmol) of **10a** was isolated from 30.0 mg (0.091 mmol) of **10** with 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. ^{1}H 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)**: 46.9 mg (0.068 mmol) of **11a** was isolated from 30.0 mg (0.072 mmol) of **11** with 94% yield. Orange solid. $[\alpha]^{25}_{D}$ = -64.2 (*c* 3.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3324, 1728. ^{1}H 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)**: 50.8 mg (0.086 mmol) of **12a** was isolated from 30.0 mg (0.096 mmol) of **12** with 90% yield. Orange solid. $[\alpha]^{25}_{D}$ = +0.29 (*c* 1.48, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3333, 1759, 1683. ^{1}H 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)**: 26.1 mg (0.038 mmol) of **13a** was isolated from 30.0 mg (0.073 mmol) of **13** with 52% yield. Orange solid. $[\alpha]^{25}_D$ = -25.7 (*c* 2.5, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3336, 1743. ^{1}H 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)**: 47.7 mg (0.084 mmol) of **14a** was isolated from 30.0 mg (0.102 mmol) of **14** with 82% yield. Orange solid. $[\alpha]^{25}_D$ = +43.7 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3334, 1734. ^{1}H NMR ($CDCl_3$, 400 MHz) 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)**: 8.1 mg (0.013 mmol) of **15a** was isolated from 50.0 mg (0.144 mmol) of **15** with 09% 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)**: 40.3 mg (0.058 mmol) of **16a** was isolated from 30.0 mg (0.072 mmol) of **16** with 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)**: 17.7 mg (0.028 mmol) of **17a** was isolated from 30.0 mg (0.086 mmol) of **17** with 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,10aS,10bR,10cR) **(18a)**: 30.2 mg (0.042 mmol) of **18a** was isolated from 20.0 mg (0.045 mmol) of **18** with 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)**: 42.0 mg (0.075 mmol) of **19a** was isolated from 30.0 mg (0.106 mmol) of **19** with 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)**: 46.7 mg (0.052 mmol) of **20a** was isolated from 100.0 mg (0.139 mmol) of

20 with 37% yield. White solid. $[\alpha]^{25}_{D}$ = -20.1 (*c* 3.2, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3480, 1741. ^1H 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**): 37.7 mg (0.048 mmol) of **20b** was isolated from 46.7 mg (0.052 mmol) of **20a** with 92% yield. White solid. $[\alpha]^{25}_{D}$ = -20.2 (*c* 0.9, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3460, 1740. ^1H 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-dicarboxylate **(20c)**: 36.9 mg (0.035 mmol) of **20c** was isolated from 37.7 mg (0.048 mmol) of **20b** with 72% yield. Orange solid. $[\alpha]^{25}_{D}$ = -12.3 (*c* 1.8, $CHCl_3$). IR ($CHCl_3$) ν_{max} (cm^{-1}): 3413, 1738. ^1H 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 previous report.[34] Briefly, MDA-MB-231 cells ($2x10^4$ 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 separate experiment, cells were treated either with **1**or **1a** (0-100 μg/mL) for 24 h. 200 μL of MTT (0.5 mg/mL) was added into each well and incubated at 37 ºC for 4 h. Formazan crystals were dissolved with isopropanol and optical density of formazan solution were measured using microplate reader at 570 nm. Experiments were performed in triplicates. Mean value of the reading were calculated, analyzed statistically and percent change with respect to control was defined. Graph was plotted as cell viability (% control) versus doses using sigma plot software.

Cell motility assay. The wound migration assay using MDA-MB-231 was performed as described in previous report.[34] Briefly, cells were grown, synchronized for 24 h in serum-free medium and wounded uniformly using sterile tip. The wound photograph 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 phase contrast microscope and analyzed.

Confocal microscopy. Confocal microscopy analysis to study cellular localization of NBD labeled compound was performed as reported earlier.[41] MDA-MB-231, A375 and HeLa cells were grown on cover slips, 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 mito-tracker (50 nM) for 20 min. Treated cells were fixed with 2% paraformaldehyde for 10 min, washed twice with PBS and mounted onto glass slide with mounting media. Nuclei were stained with DAPI and visualized under confocal microscope (excitation 460 nm, emission 530 nm) with 60x magnification. Similarly, colocalization experiment was performed with fluorescently (green) tagged derivatives of azadiradione (**1a** and

8a) and red ER tracker in MDA-MB-231 cells. Cells treated either with **1a** or **8a** (20 μg/mL for 1 h) were stained with ER-tracker (1 μM for 20 min) and analyzed under confocal microscope with 100x magnification after fixation and nuclear staining with DAPI (blue).

ASSOCIATED CONTENT

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

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