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A Central Strategy for Converting Natural Products into Fluorescent Probes

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Nearly forty years after Wieland's studies on the labeling of phalloidin with fluorescein isothiocyanate,[1] fluorescent modifications have become an important tool in the study of natural product biology.[2–5] Since Wieland's efforts, there has been a profound increase in the number of labels. This increase has led to the creation of a complex compendium of natural product probes, as illustrated by the generation of **Aa**–**Dd** from the appendage of labels **A**–**D** to natural products **a**–**d** (Figure 1 A). The resulting compendium **Aa**–**Dd** now contains modifications within three distinct variables: the label, linkage, and natural product. While variations in the label and linker might be of use in activity optimization, the extended diversity in **Aa**–**Dd** profoundly inhibits the ability to conduct comparative analyses. By using a single label and a common set of linkers, the number of variables displayed within a collection of natural product probes is reduced. As a result, data collected from the refined set of probes **Da**–**Dd** are homogenized (Figure 1 B).

Our studies began by identifying a route for labeling a diverse set of natural products from a single dye. In choosing the optimal dye, we considered a combination of criteria such as good photophysical properties, sufficient cellular uptake, lack of subcellular localization and lack of intrinsic biological activity. For small-molecule conjugation, carbocyanine dyes and rhodamine dyes, including rhodamine 123 and rhodamine B, are taken up into the mitochondria, while other common labels, such as cyanine dyes or BODIPY dyes localize in the nucleus and endoplasmic reticulum (ER), respectively. Ethyl 7-dimethylaminocoumarin-4-acetate (**1**)[6–7] was chosen as the central dye for these studies based on its lack of biological activity. Coumarin **1** is readily taken up into cells, however, it does not localize and can be removed by washing the cells. Additionally, its water-solubility,[8] small size,[9] availability,[10] photostability,[11] and photophysical properties ($\lambda_{ex}=370$ nm, $\varepsilon=22\,000\,\text{M}^{-1}\text{cm}^{-1}$, $\lambda_{em}=459$ nm, $\Phi=0.1$–0.4) were favorable to the development of synthetic applications, while providing viable fluorescence for a diverse set of photophysical experiments.[12]

Coumarin **1** was converted into a set of functionalized labels **2**–**9** by using procedures developed by members of this team.[7–8] By using this route, 10–100 g batches of **1** were routinely prepared from *m*-(*N*,*N*)-dimethylaminophenol and diethyl 1,3-acetonedicarboxylate and converted to acid **2**. A set of labels was prepared from either ester **1** or acid **2**. As depicted in Scheme 1, the coupling of **2** with glycine *tert*-butyl ester, followed by deprotection with TFA and esterification with *N*-hydroxysuccinimide provided ester **3** in 25% overall yield. Comparable amide couplings were also effective in synthesizing labels **4**–**6** and **8**–**9** (Scheme 1). Alternatively, labels **3**–**9** could be prepared by thiol-assisted addition of a primary amine to ester **1**, as illustrated by the reaction of cysteamine with **1** to afford **7**.[13–14] The addition of 5–20 mol% of sodium thiophenoxide, sodium ethanethiolate, or group IV metal alkoxides[15] can be used to facilitate this process.

With labels **2**–**9** in hand, our efforts turned to screening reaction conditions so as to optimize their ability to tag a collection of natural products, natural product precursors, and intermediates from total synthetic efforts (Scheme 1). Amide-bond formation was perhaps the most intuitive means of conjugation,[16] as metabolic processes in prokaryotic and eukaryotic cells have been shown to conduct comparable amidation in vivo.[17–20] The carboxylic acid-containing natural products, okadaic acid[21–22] and (−)-borrelidin[23–25] were effectively condensed with **4** to provide the respective fluorescent amides **14** and **15**. In a complementary process, fluorescent amides were also obtained from amine-containing natural products by condensation with succinimidyl ester **3**, as illustrated by the conversion of amphotericin B to **13**.[26–27]

Olefins also served as a viable function for the appendage of a label, as the mild conditions of olefin metathesis were ideally suited for functionally complex molecules.[28] Application of

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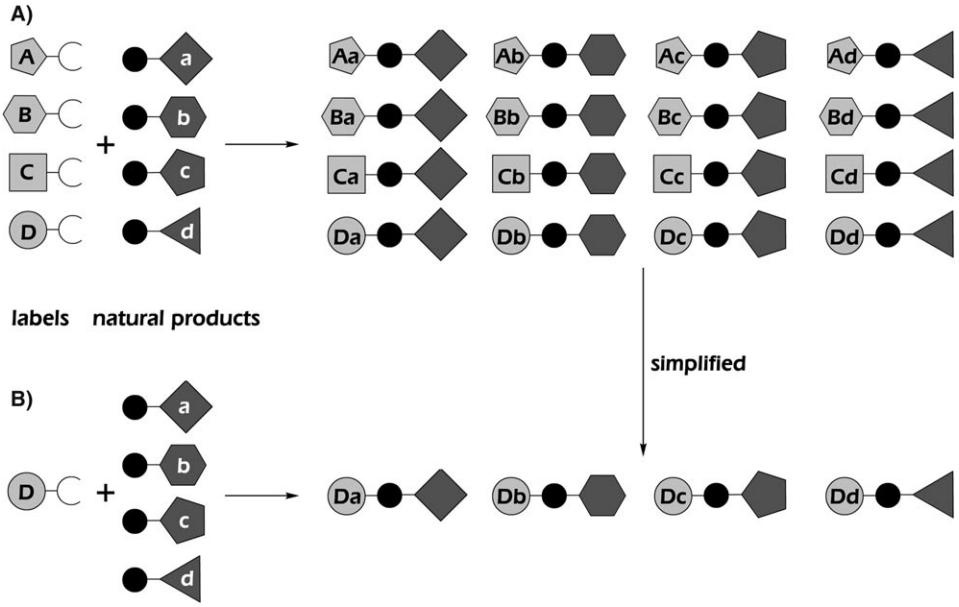


Figure 1. Logistical analysis in the design of natural product probes. A) The conventional system for preparing natural product probes appends a label, **A**–**D** (light gray), through a linker (black) to a natural product, **a**–**d** (dark gray) to form conjugates **Aa**–**Dd**. B) A streamlined approach eliminates complications arising from the use of multiple labels by replacing a set of affinity labels with a single reporter. The biological activity of probe **Da** does not necessarily reflect the sum of the biological activities of its constituents **D** and **a**. Therefore, conclusions derived from the relation of **A** to **B** cannot be used to relate probes **Aa** to **Bb**. In contrast, the adoption of a single label eliminates this concern and limits materials to an abridged set of natural product probes, **Da**–**Dd**. Each of these probes **Da**–**Dd** now bears the same label and therefore can be analyzed and compared by using a centralized system.

this method was demonstrated by the synthesis of probe **16** from microsclerodermin F and **5**. Alternatively, conjugate addition reactions offer a means to label olefins with nucleophilic labels, such as thiol **7**.[29] Examples such as the Michael addition of **7** to lissoclinolide, affording **18**–**20**, illustrate the potential of this strategy.

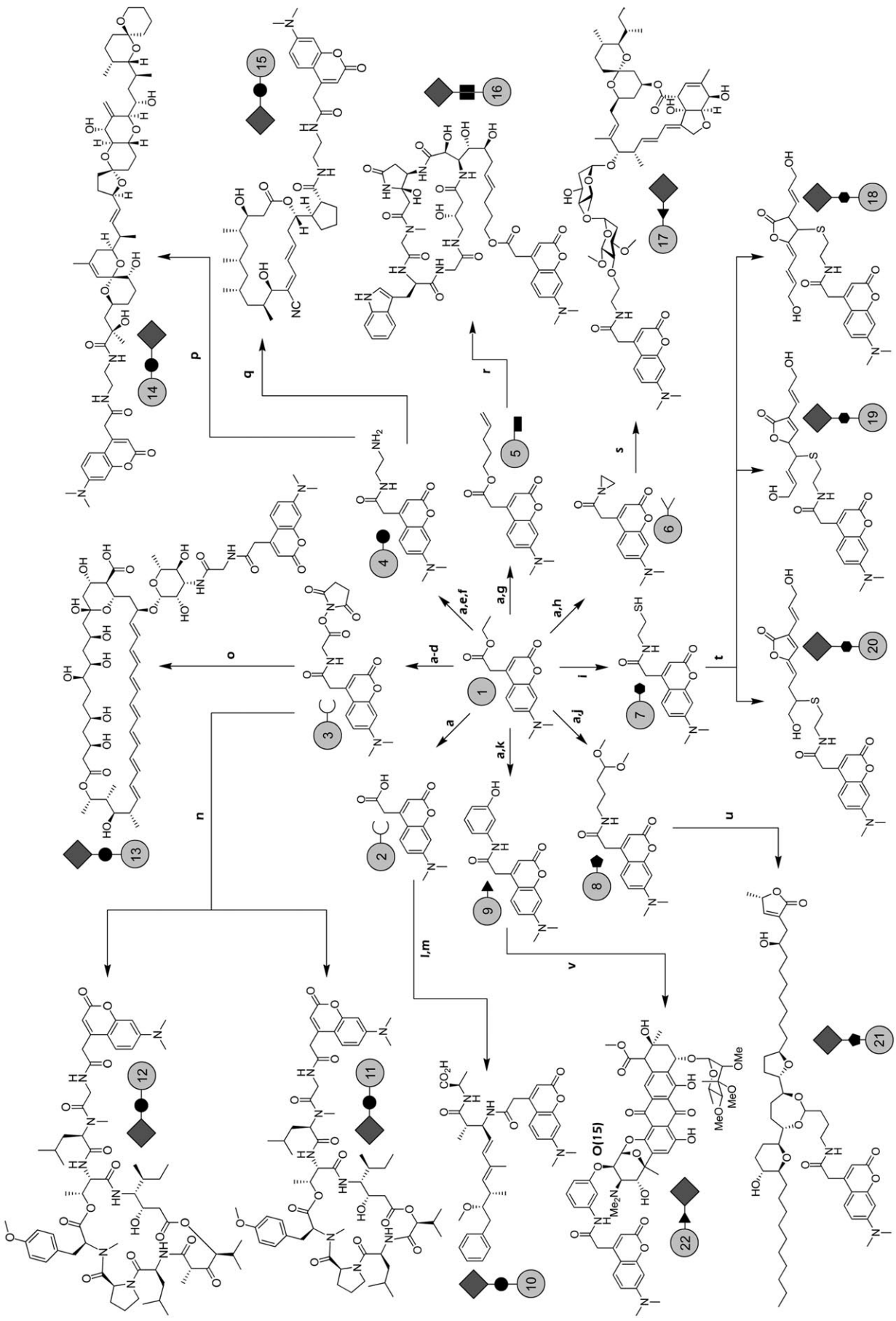
Hydroxyl groups were another effective target, as their conversion to ethers provided a stable conjugate for in vivo analysis. Aziridine ring-opening reactions were advantageous for this process, as illustrated by the preparation of **17** from 22,23-dihydroavermectin B1a and **6**.[30–31] This strategy circumvents the problems associated with the preparation of labile functionality, as the reaction occurred without additional reagents. Alternatively, ethers such as nogalamycin[32–33] analogue **22** can be prepared by a Mitsunobu reaction with phenol **9**. Finally, diols could be labeled through formation of an acetal, as illustrated by the conversion of (−)-mucocin[34] to fluorescent acetal **21**.

To ensure that the probes provided reliable bioactivity, each analogue was assayed by using the methods developed to evaluate the activity of their corresponding natural products (Table 1). All but two of the analogues **10**–**22** retained an activity that was within an order of magnitude of that of their parent natural product. Considerable activity was lost when lissoclinolide[34–35] was converted to **18** and **19**. This loss in activity suggests that Michael additions to lissoclinolide in a 1,4- and 1,6-fashion might play a role in the mode of action of lissoclinolide.

We investigated the utility of probes **10**–**22** by examining their uptake and localization in mammalian (HeLa) and plant (onion epidermal) cells. Our studies began by calibrating the uptake of each analogue by quantitative fluorescence microscopy (see Experimental Section). Each of the analogues was taken up into HeLa cells (Figure 2A) and onion epidermal cells (Figure 2B). After exposure and extensive washing with media, **10**–**22** remained in live cells at micromolar levels. Under the same conditions, less than 10 nM of controls **1**, **23**, **24**, **25**, and **26** were observed in cells after washing cells extensively with media. The inactive lissoclinolide analogues **18**–**19** displayed the lowest uptake in HeLa cells, while the borrelidin analogue **15** and 22,23-dihydroavermectin B1a analogue **17** displayed a reduced uptake in onion epidermal cells. To determine that this response was associated with the bioactivity of the natural product, the uptake of **10**–**22** was determined after incubating cells with their corresponding natural product. As indicated in Figure 2, the uptake of **10**–**17** and **21**–**22** was reduced in cells pretreated with 10 μM aliquots of their corresponding natural product, thereby suggesting a correlation between uptake and bioactivity. The lack of blocking the uptake of analogues **18**–**19** suggests that these probes do not provide an accurate mimic. Comparable blocking of **10**–**22** was also observed in onion cells. In these cells, pretreatment with lissoclinolide was only modestly effective in blocking the uptake of **18**–**19**.

Satisfied by our activity correlations, our attention turned to examining the subcellular localization of probes **10**–**22** by LED microscopy. Our studies began by examining materials whose intracellular trafficking had been described. In HeLa cells, the two protein phosphatase inhibitors **10** and **14** were indistinguishable (Figure 3, left), appearing exclusively in the ER. This observation was in accordance with the established localization of associated protein phosphatases in the ER.[21] Comparable localization of **10** and **14** in the ER was also observed in plant cells (Figure 3, right).

Similar affinity events can be used to target the coumarin label to oligonucleotides. Nogalamycin analogue **22**, an antibiotic that intercalates DNA with its nogalose sugar (lower right of **22**, Scheme 1) in the minor groove and its aminoglucose sugar (upper left of **22**) in the major groove,[33] localized as expected within the nucleus of HeLa cells (Figure 3, left). This



Scheme 1. Fluorescent probe synthesis networked from single fluorescent core **1**. Reagents and conditions: a) LiOH, THF, H_2O, 99%; b) glycine *tert*-butyl ester, EDAC, EtN*i*Pr$_2$, DMF, 69%; c) TFA, CH_2Cl_2, 99%; d) *N*-hydroxysuccinimide, EDAC, EtN*i*Pr$_2$, DMF, 36%; e) *N*-Boc-ethylenediamine, EDAC, EtN*i*Pr$_2$, DMF, 76%; f) TFA, CH_2Cl_2, 99%; g) pent-4-en-1-ol, EDAC, DMF, 89%; h) aziridine, DCC, THF, DMF, 65%; i) cysteamine, EtN*i*Pr$_2$, DMAP, THF, 56%; j) 4,4-dimethoxybutylamine, EDAC, EtN*i*Pr$_2$, DMF, 55%; k) 3-aminopropan-1-ol, EDAC, EtN*i*Pr$_2$, DMF, 94%; l) see ref. [7]. Me-ADDA-D-Ala, HATU, collidine; m) LiOH, H_2O, THF, 29% from **2**; n) see ref. [58]; o) amphotericin B; DMSO, RT, 67%; p) okadaic acid, EDAC, EtN*i*Pr$_2$, DMF, 61%; q) (−)-borrelidin, EDAC, Et_3N, CH_2Cl_2, 86%; r) microsclerodermin F, bis(tricyclohexylphosphine)benzylidine ruthenium(IV) chloride (10 mol%), DMF, methanol, 45%; s) 22,23-dihydroavermectin B1a, pyridine, CH_2Cl_2, 33%; t) lissoclinolide, EtN*i*Pr$_2$, THF, **18** (12%), **19** (25%), **20** (39%); u) (−)-mucocin, (1*S*)-(+)-10-camphorsulfonic acid, CH_2Cl_2, 75%; v) nogalamycin, DEAD, PPh_3, CH_2Cl_2, 35%. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DEAD = diethyl azodicarboxylate, DMAP = 4-dimethylaminopyridine, EDAC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

Table 1. Bioactivity data for **10**–**22** and their corresponding natural products.[a]

Assay	pNPP			NCI-DTP screen			GI	AA	GI	GI	MTT	BST	affinity
target	PP1	PP2A1	PP2A2										polydA50dC50
organism							*S. cerevisiae*		*C. albicans*	*C. elegans*	*H. sapiens*	*A. salina*	
cell line											HeLa		
material	IC_{50}	IC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	MIC	IC_{50}	IC_{50}	MIC	IC_{50}	IC_{50}	K_d
10	0.42	0.152	0.21										
microcysin LR	0.012	0.021	0.092										
11				0.049	0.589	2.24							
tamandarin A				0.0015									
12				0.011	0.295	7.2							
didemnin B				0.013	0.066	3.8							
13							4.2						
amphotericin B							1.0						
14	0.0011	0.064	0.079										
okadaic acid	0.00091	0.014	0.056										
15								0.0052					
(−)-borrelidin								0.0008					
16									3.2				
microsclerodermin F									1.2				
17										0.12			
22,23-dehydroavermectin B1										2.00			
18											29		
19											1.3		
20											0.26		
lissoclinolide											0.194		
21												0.3/0.5	
(−)-mucocin												1.3	
22													0.021
nogalamycin													0.0012

[a]Abbreviations: pNPP (*p*-nitrophenyl phosphate assay), PP1 (protein phosphatase 1), PP2A1 (protein phosphatase 2A1), PP2A2 (protein phosphatase 2A2), GI (growth inhibition assay), AA (angiogensis assay), MTT (mitochondrial dehydrogenase enzyme assay), and BST (brine shrimp lethality test).

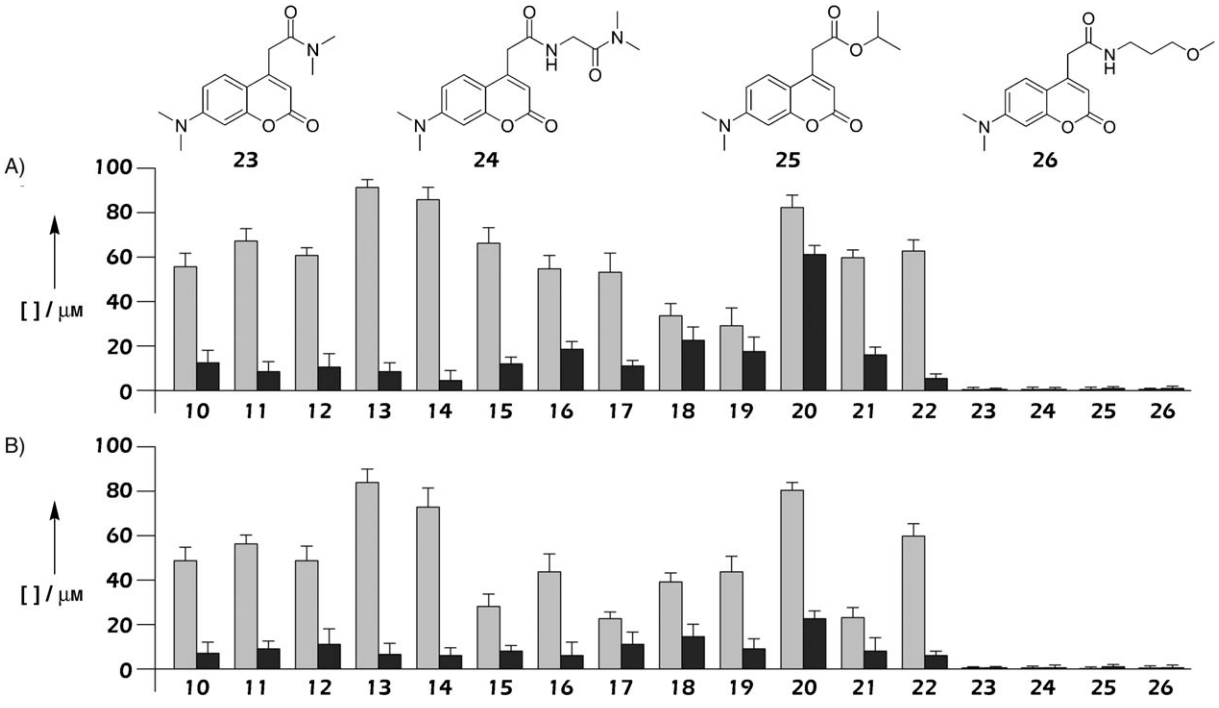


Figure 2. Probe uptake in A) HeLa and B) onion epidermal cells. A) The average cellular concentration of probes **10**–**26** in HeLa cells cultured in DMEM containing 0.1% DMSO after exposure of 10^6 cells to probe stock (100 nM, 100 μL) in DMEM for 5 min at 32 °C followed by washing three times with DMEM (1 mL). B) The average cellular concentration of probes **10**–**26** in onion epidermal cells after exposure of a 25 mm^2 square section of skin (~10^4 cells per mm^2) to probe stock (100 nM, 100 μL) in PBS (10 mM; pH 7.0) containing 5% DMSO for 5 min at 23 °C followed by washing 3× with 1 mL aliquots of DMEM. Light gray bars denote cells that were treated with the probe. Dark gray bars denote cells that were incubated with 10 μM solution of the natural product (100 μL) before being exposed to its corresponding probe.

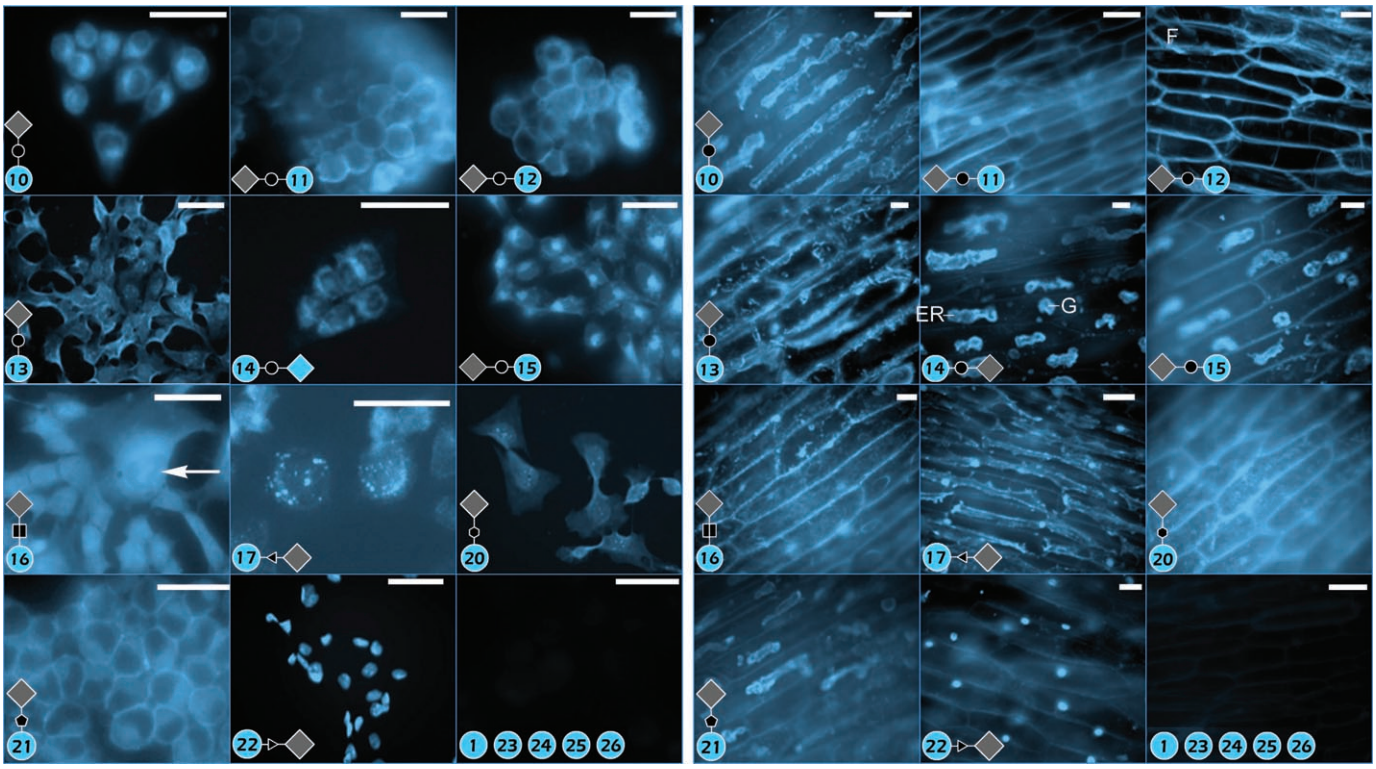


Figure 3. Comparative subcellular localization analysis by using LED fluorescence microscopy. Left: Localization of probes **10**–**26** in HeLa cells. Cells were cultured to a density of ~10^6 cells per mm in phenol red-free DMEM containing 10% fetal bovine serum (FBS) and synchronized by using a double thymidine block. Cells were treated at the S-stage with 100 nM probe in phenol red-free DMEM (100 μL) for 5 min at 32 °C followed by washing twice with phenol red-free DMEM (100 μL). Right: Localization of probes **10**–**26** in onion epidermal cells. A 4 mm^2 skin of cells was treated with probe (100 nM, 100 μL) in PBS (10 mM; pH 7.2) containing 5% DMSO. Cells were washed twice with PBS (100 μL, 10 mM; pH 7.2) containing 5% DMSO. Negative control was again collected by using **1**. The bars denote 50 μm.

activity was again observed in the plant-cell model (Figure 3, right).

Coumarin-labeled natural products can also be used to study membrane association. Analogue **13**, prepared from amphotericin B, localized exclusively within the cell membrane of HeLa cells (Figure 3, left) and the cell wall in onion epidermal cells (Figure 3, right). This observation correlated directly with evidence collected by Carreira,[26] whose efforts illustrated comparable localization of a related fluorescent amphotericin B analogue in the plasma membrane of yeast (*Saccharomyces cerevisiae* FY250) and Jurkat cells.

With probes developed for proteins, oligonucleotides, and membranes, we examined the scope of this method. We were particularly interested in determining what facets of natural product biology could be explored with the developed probes. Natural products are often associated with more than one pathway.[36] Frequently the elucidation of secondary systems is neglected, as the first elucidated is often viewed as predominant. Coumarin labeling can be used to screen for such interactions. To illustrate this point, analogue **17** was prepared from 22,23-dihydroavermectin B1a, an agonist for the neurotransmitter γ-aminobutyric acid (GABA).[31,37] In mammals, the primary target of 22,23-dihydroavermectin B1a is the neuronal membrane. Based on this evidence, one would not expect 22,23-dihydroavermectin B1a to localize within non-neuronal mammalian cells or in plant cells. However, analogue **17** was taken up into HeLa cells and localized within the vacuoles and lysosomes (Figure 3, left).[38] This uptake remained after washing of the cells with media and suggested the entry of these materials into metabolic pathways.[39–41] In onion epidermal cells, localization of **17** was not as refined, as indicated by accumulation in the nuclear membrane, cytosol, ER, and vacuole (Figure 3, right).

Coumarin labeling can also be used to provide clues as to the mode of action of natural products. Didemnin B and tamandarin A provided an ideal model to illustrate this potential, as preliminary evidence has suggested that they bind to palmitoyl protein thioesterase 1 (PPT1)[42] and elongation factor 1α (EF-1α).[43] Probes **11** and **12** localized in the membrane of HeLa cells and cell wall of onion epidermal cells. This localization was not identical to that of amphotericin B analogue **13**, which located within the membrane (Figure 3, left). Instead, probes **11** and **12** localized on the external matrix of the membrane (Figure 3, left) or cell wall (Figure 3, right). Staining of the extracellular matrix was used to verify this conclusion, as the addition of 0.1 μM negrosin[44] to cell culture led to a complete quenching of the fluorescence from **11** and **12** and not from amphotericin B analogue **13**. This specificity suggests that **11** and **12** bind to a component of the extracellular matrix, such as a membrane protein or channel. Unfortunately,

neither EF-1α nor PPT1 satisfy these requirements, thereby suggesting that the target of **11** and **12** in HeLa cells has yet to be discovered.

Studies then turned to examining (−)-borrelidin, an antiangiogenic polyketide that has been shown to inhibit threonyl-tRNA synthetase in *E. coli*,[25] reduce cyclin-dependent kinase activity of Cdc28p/Cdk2p,[24] and partake in the induction of amino acid biosynthesis through a GCN4 transcription-factor-regulated pathway.[45] When presented at the S-stage, **15** localized predominantly in the Golgi apparatus of HeLa cells, as less than 10% of the fluorescence from **15** was found in the cytosol, mitochondria, and ER when dosed at 100 nM (Figure 3, left). When presented at 1 μM, the amount of **15** in the cytosol was comparable to that observed in the Golgi. In onion epidermal cells, **15** was observed in both the Golgi and ER (Figure 3, right). While the target for this response has not yet been identified, information gathered from these observations can be used to guide further studies towards the identification of the function of **15** with regards to regulation of processes within the Golgi.

Not all the probes prepared in Scheme 1 demonstrated a clear cellular target. Analogue **21**, from the annonaceous acetogenin (−)-mucocin,[46–47] and analogue **16**, from the nonribosomal peptide microsclerodermin F,[48–49] were found ubiquitously throughout HeLa cells (Figure 3, left). While these analogues lacked a sub-cellular specification, HeLa cells exposed to **16** during mitosis were particularly affected, and underwent a dramatic slowing in G2, which resulted in cells (arrow in Figure 3, left) whose growth was ten times greater than that of cells that were exposed to **16** outside of mitosis or cells that were not treated with **16**. A comparable response was not obtained with **21**. While the mechanism guiding this response remains undetermined, this example illustrates that coumarin-labeled natural products can also be used to simultaneously induce fluorescently coded morphogenic responses.

Finally, this labeling procedure can be used to establish structure–activity correlations. To demonstrate, we conducted a comparative analysis of the three conjugates **18**, **19**, and **20** obtained from lissoclinolide. These materials provided an ideal internal control as the activity of **18** and **19** was significantly diminished as compared to lissoclinolide and **20**. While the differentiation of **18**, **19**, and **20** was not evident in the plant-cell model (Figure 3, right), analogue **20** was observed in the nuclear and plasma membranes of HeLa cells (Figure 3, left). Comparable localization was not observed with **18** and **19** as these probes were contained in the cytosol (Figure 3, left). These observations suggest that the activity of lissoclinolide as expressed in **20**, and diminished in **18**–**19**, is derived from nuclear and plasma-membrane association.

The facile conversion of a single core reporter to eight labels (**2**–**9**) and thirteen natural products probes (**10**–**22**) was completed in a parallel manner through a total of 29 steps. The longest linear route was five steps. Fortuitously, the fluorescence of the 7-dimethylaminocoumarin label not only simplifies chromatographic purification but also extends the detection limit; this permits synthetic operations with micrograms of material. For instance, 10 μg (12.4 nmols) of okadaic acid was routinely coupled with **4** to provide **14** in 80–90% yield after chromatographic purification. Large panels of reaction conditions can be screened rapidly by reduction to micrograms of material. This allows the optimization of the labeling conditions and the preparation of fluorescent probe sets from milligram samples of a natural product.

The synthesis of a set of functionally diverse labels from a single dye offers probes whose activity can be used for live-cell imaging. Probes developed through this route are orthogonal to fluorescently tagged proteins,[50–51] fluorescent particles,[52] and genetically engineered fluorescent amino acids.[53–54] While the engineering of fluorescent proteins may be advantageous to identify expression and localization events within the cell,[55–56] the tuning of natural products through their labeling with a single fluorescent dye provides an activity that can be generally applied as a multifaceted biological probe.[57]

Experimental Section

Probe synthesis: The natural products used in this report were obtained from various sources. Amphotericin B, 22,23-dihydroavermectin B1a, okadaic acid, and nogalamycin were purchased from Sigma–Aldrich Inc., Tocris–Cookson Inc. (Ellisville, MO), or Calbiochem Inc. (San Diego, CA). Microsclerodermin F[48–49] was extracted by Faulkner from a lithistid sponge *Microscleroderma* sp. (−)-Borrelidin,[24] lissoclinolide,[34–35] and (−)-mucocin[46] were obtained through total synthesis. Fragments of microcystin-LR,[58–59] didemnin B,[7] and tamandarin A[8] were also obtained synthetically. A detailed description of the synthesis of **1**, labels **2**–**9**, and probes **10**–**22** is provided in the Supporting Information.

Bioactivity calibration: The activity of each analogue developed in Scheme 1 was compared to its parent natural product by using established assays. The activity of each analogue was analyzed by using published procedures (Table 1). The *p*-nitrophenylphosphate assay (*p*NPP)[60] was used for microcystin analogue **10** and okadaic acid analogue **14** by following the protocol of Chamberlin.[58–59] The activities of the tamandarin A analogue **11** and didemnin B analogue **12** were determined by screening at the Developmental Therapeutics Program.[61–62] Procedures and data sets for these materials can be found online at http://dtp.nci.nih.gov/. The activity of the amphotericin analogue **13** was determined by using a growth-inhibition assay in yeast (*S. cerevisiae*).[63] The activity of the (−)-borrelidin analogue **15** was characterized by an angiogenesis assay.[24–25] The activity of microsclerodermin F analogue **16** was determined by using a yeast growth-inhibition assay with *Candida albicans*, as described by Faulkner.[48] The activity of 22,23-dehydroavermectin B1a analogue **17** was identified by using a growth-inhibition assay in *Caenorhabditis elegans*.[31] The activities of the three lissclinolide analogues **18**–**20** were tested by using an MTT assay[64] in HeLa cells, as described by Ireland.[34–35] (−)-Mucocin analogue **21** was screened for its activity by the brine shrimp lethality test (BST), as described by McLaughlin.[65] The activity of the nogalamycin analogue was determined by measuring its affinity to polydA50dC50, as described by Waring[32] and Wang.[66]

Cell materials: HeLa cells (ATCC CCL-2) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g L^{-1}), L-glutamine (4.5 g L^{-1}), and 5% heat-inactivated fetal calf serum (FCS) in glass-bottom dishes. As noted, cells were synchronized by using a double thymidine block. Bulbs of the common

onion, *Allium cepa*, were obtained from vendors, and the epidermal tissue was removed under sterile water and soaked for 1 h in phosphate-buffered saline (PBS; 10 mM, pH 7.2) with 5% DMSO.

Cell uptake: Uptake studies were conducted in 96-well plates (BD Biosciences, Franklin Lakes, NJ). HeLa cells were cultured in phenol red-free DMEM with glucose (4.5 g L^{-1}), L-glutamine (4.5 g L^{-1}), and 5% heat-inactivated FCS and washed with fresh DMEM prior to use. It was important that phenol red, a common additive in DMEM, was not used during the culturing or experimentation due to its interference with uptake and cellular microscopy. Uptake in onion epidermal cells was examined by exposing a 4 mm^2 square to probes dissolved in PBS (10 mM, pH 7.2) containing 5% DMSO. The skins were washed twice with PBS (10 mM, pH 7.2) containing 5% DMSO and imaged after mounting the skin on a Superfrost microscope slide (VWR, West Chester, PA). Concentrations of fluorophore were determined by calibration with 100 μm polystyrene microspheres (Polysciences Inc., Warrington, PA) that were impregnated with **2** and standardized at concentrations of 10.0 ± 0.5 nM, 100.0 ± 0.2 nM, 1.00 ± 0.1 μM, 5.00 ± 0.1 μM, 10.0 ± 0.1 μM, 25.0 ± 0.1 μM, and 50.0 ± 0.1 μM. The amount of fluorescence per cell was collected by photon-counting microscopy using an Electron Bombardment CCD Camera, EBCCD (Hamamatsu Photonics K.K., Shizuoka, Japan). The camera was coupled via fiber-optic relay to an Oil Immersion Epiplan Neofluar objective (Zeiss). The light was filtered by using a dichroic filter set FF409 (Semrock Inc., Rochester, NY) with an excitation at 377 ± 50 nm, dichroic with >98% reflection at 344–404 nm, >98% transmission at 415–570 nm, and an emission filter at 447 ± 60 nm. Samples were illuminated by using a mercury vapor lamp (Nikon). Experiments were run in triplicate. A total of 10 images were collected per plate or slide, and the same 200 cells were sampled per image. The average (bar) and deviation (line) are reported. Alternatively, the cell uptake data in Figure 2 could be collected by using quantitative fluorescence microscopy.[67–69] Such data were collected on an LED microscope by using a self-built time-correlated single-photon-counting PCI card. Software written in C/C++ and Labview was used to collect and process the signal at pixel resolution. While this system is not commercially available, comparable instrumentation, such as the TimeHarp 200 PC card (PicoQuant GmbH) or Digital Photon Counting System Model DPC-1 (C&L Instruments Inc.), could be used for these measurements.

LED fluorescence microscopy: All images were collected on a self-built inverted microscope (Xenobe Research Institute) engineered for one-switch conversion between wide-field, confocal, and two-photon imaging. LED fluorescence images were collected by using the excitation from a 100 mW 370 nM GaN UV-LED (Roithner Lasertechnik, Vienna, Austria). The emitted light was filtered through a dichroic filter set (FF409, Semrock) with excitation at 377 ± 50 nm, dichroic with >98% reflection at 344–404 nm, and >98% transmission at 415–570 nm, and an emission filter at 447 ± 60 nm. Focusing was conducted with an Oil Immersion Epiplan Neofluar objective (Zeiss), and image collection was conducted by using a Electron Bombardment CCD Camera, EBCCD (Hamamatsu Corp.).

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