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An inexpensive fluorescent labeling protocol for bioactive natural products utilizing Cu(I)-catalyzed Huisgen reaction

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Abstract—Labeling of bioactive small molecules with organic dyes for various applications in cell biology has been emerging as an attractive research field. Using an easily prepared and inexpensive fluorescein derivative **1** and a Cu(I)-catalyzed Huisgen reaction, an efficient fluorescent labeling strategy is developed generally for bioactive natural products. Essentials of a successful labeling include the personalized introduction of an azido functionality to specific targets by a selective and efficient manner, and the strategic adjustment of reaction sequence to avoid possible side reactions under the 'click' reaction conditions. Such a protocol has been successfully applied to the fluorescent labeling of four bioactive small molecules in different chemical categories in this study. Advantages of this labeling protocol include the use of inexpensive reagents, ease of operation, free-of-protections at the 'click' step, and suiting a wide range of bioactive molecules bearing the reactive functionalities.

1. Introduction

Fluorescence allows qualitative and quantitative determinations of cellular events at molecular level.[1] Such visualizations are usually performed easily and reliably by rapid and economic methods with improved sensitivity and selectivity. In various chemical biology studies, proper labeling of the bioactive compounds (including natural products and biochemical probes) with organic dyes has been recognized as a very useful technique in recent years.[2,3] Therefore, development of convenient labeling protocols and acquirement of suitably labeled bioactive molecules are of great importance and are becoming an attractive area in organic chemistry. In recent years, Sharpless and co-workers have identified a number of reactions called 'click chemistry'.[4] The foundation of 'click chemistry' is termed by generation of chemical substances by joining small units together through a convenient heterocycle-formation reaction in high chemical yield and selectivity in a wide range of substrates. Among these, the most powerful example is to utilize the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition[4,5] between an azide and a terminal alkyne (to afford the 1,2,3-triazole regioselectively, Fig. 1). Cu(I)-based catalysts dramatically improve the regioselectivity and reaction rate of Huisgen 1,3-dipolar cycloaddition and thus eliminate the need for elevated temperatures. This high-yielding reaction also tolerates a variety of functional groups and affords the 1,2,3-triazole product with minimal work-up and purification. These significances make Huisgen reaction to be an ideal research tool in many applications, including the fluorescent labeling of biomolecules.[3a,6,7] Another focus involved in the fluorescent labeling is fluorescent reagents. Though many choices are commercially available today and ready to use in most experiments, most fluorescent reagents are relatively expensive and required for feasible examinations in individual cases. Up to date, fluorescent

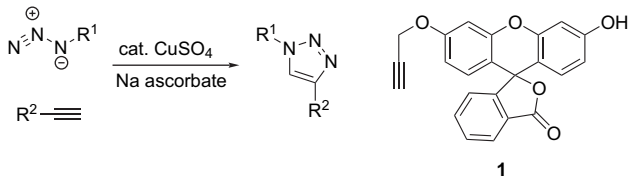

Figure 1. Cu(I)-catalyzed Huisgen reaction and the inexpensive fluorescein derivative **1**.

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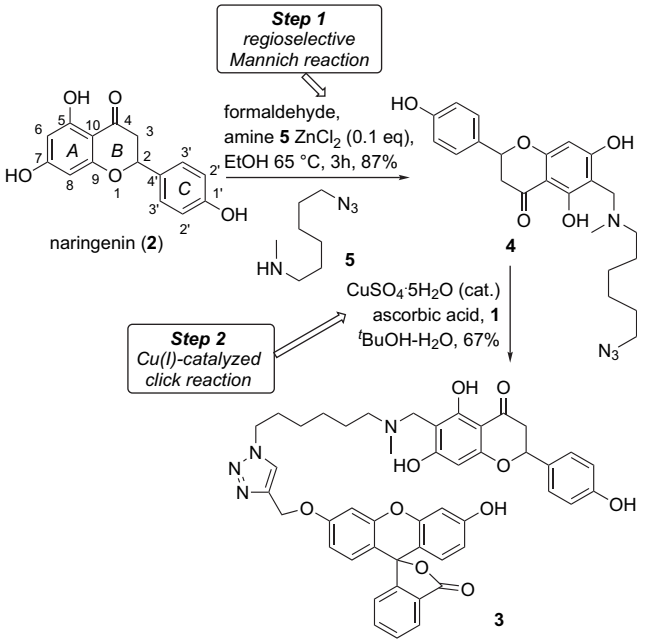
labeling of the bioactive small molecules is still a high-cost and time-consuming work, especially for those bioactive compounds naturally bearing multiple reactive functionalities. Herein, we report our explorations and results in developing a versatile and economic 'click' fluorescent labeling protocol, which is suitable for a wide range of bioactive compounds and natural products in different chemical categories. An easily prepared and inexpensive fluorescein derivative **1** bearing a terminal alkyne functionality[8,9] is successfully used as the fluorescent reagent.

2. Results and discussions

For genetic reasons, all original natural products have no azido group, which is essentially required for the 'click' reaction. Therefore, selective and efficient introduction of an azido group to a proper position of the bioactive targets becomes a critical work for this 'click' labeling strategy. Usually, it is a difficult and time-consuming work because most bioactive targets contain a variety of reactive functional groups. Also, they present similar reactivity in many cases. Such contradictory prevents people from convenient acquirement of corresponding azido derivatives starting from their parents. However, existence of complex but slightly different functionalities in the bioactive targets also provides us the chance to find a selective azido-group introduction directly or indirectly. Undoubtedly, such operations (to introduce an azido group selectively into a specific target) have to be done in a 'personalized' fashion because of the different chemical environment of individual targets.

The first natural product we examined is naringenin (**2** in Scheme 1), a representative flavonone involved in plant root nodulation and nitrogen fixation.[10] Of course, a fluorescent probe based on naringenin will be much more helpful to those biochemical visualization studies, such as the interactions between naringenin and nod-D protein. The above-mentioned 'click-labeling' concept was successfully employed in our recent study on naringenin, in which a quick synthesis of a fluorescent labeled naringenin derivative **3** was accomplished in a site-selective and protection-free fashion.[8] In naringenin, three hydroxyl groups and one ketone carbonyl group exist in the different rings of one molecule, bringing great complexity to the site-selective azido-group introduction by a direct manner (without protection manipulations). However, fine structural analysis indicates that the A ring of naringenin, especially C-6 and C-8 positions, might be more reactive toward the electrophiles than the C ring. Relatively mild Mannich reaction[11] was thus considered as a direct derivation method for naringenin. After careful optimizations of reaction conditions, a direct and regioselective Mannich reaction of naringenin with a variety of secondary amines and formaldehyde was successfully developed.[8]

From the above successful two-step labeling of naringenin, several conclusions were drawn in general. First, convenient preparation of an ideal derivative bearing an azido group is the most important issue for this 'click' labeling protocol with fluorescein derivative **1**. Unambiguously, direct and selective introduction of an azido functionality to a specific target (a natural product or a bioactive small molecule) can be achieved, of course, using the 'personalized' innovative chemistry. Second, the 'click' labeling in a 'free-of-protection' fashion proves to be a versatile and efficient method, especially for those targets bearing multiple hydrophilic and reactive functionalities. Encouraged by the above results, further applications of this 'click' labeling protocol was examined to the representative bioactive natural products, including muramyl dipeptide (MDP, **6**, a bioactive unit of peptidoglycan),[12] sinomenine (**7**, an immunosuppressive alkaloid from traditional Chinese medicine),[13] and artemisinin (qinghaosu, **8**, an anti-malarial terpenoid from traditional Chinese medicine)[14] (Fig. 2). These three bioactive natural



Scheme 1. 'Click' fluorescent labeling of naringenin (**2**) using a direct and regioselective Mannich reaction.

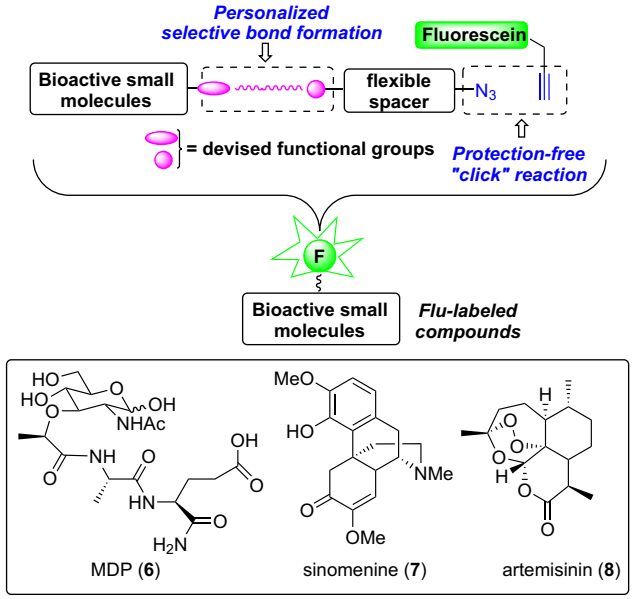


Figure 2. General concepts for 'click' fluorescent labeling, and three selected bioactive targets **6**–**8** for further studies.

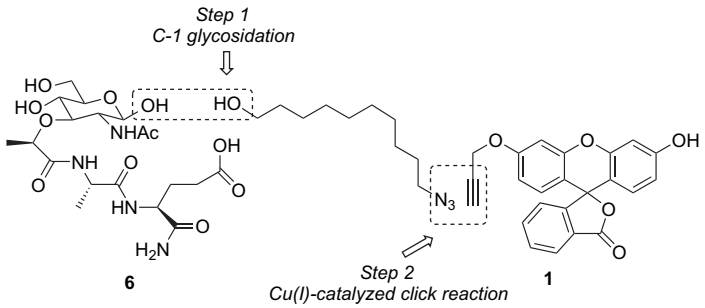
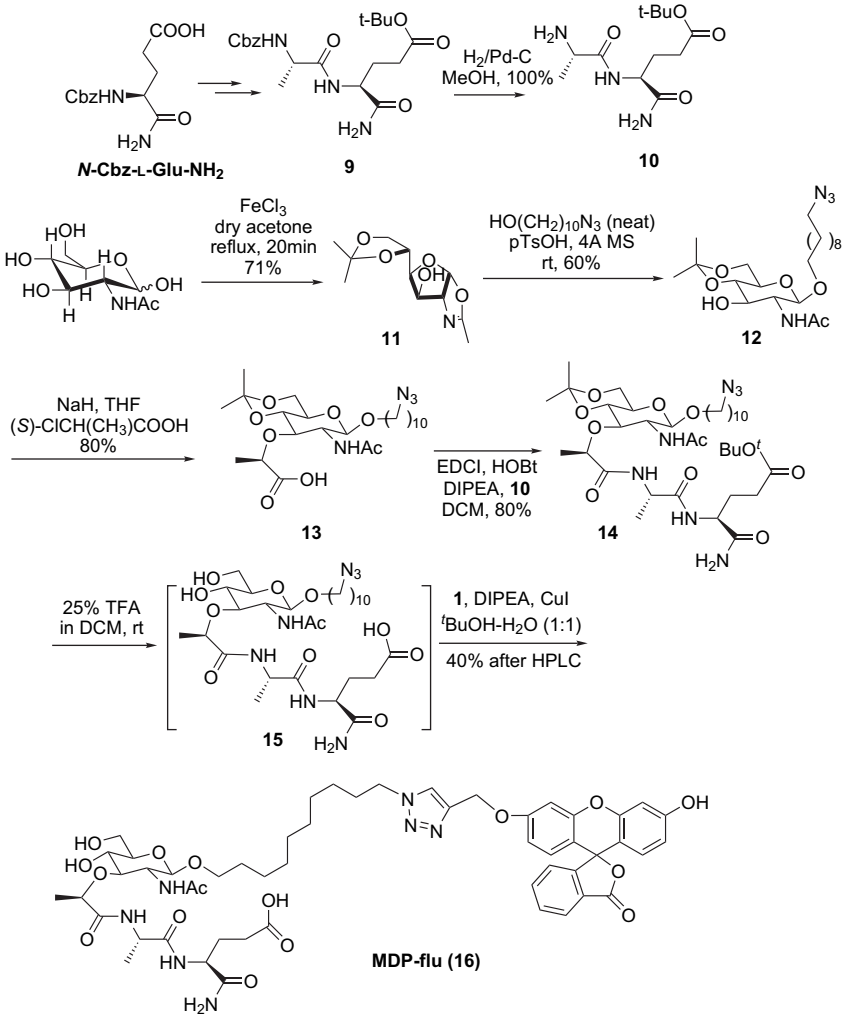


Figure 3. A personalized protocol for 'click' fluorescent labeling of MDP (**6**).

products belong to different chemical categories, presenting different chemical and biological properties. Of course, they represent the substrate generality to some extent.

Muramyl dipeptide (MDP, **6**), the minimal bioactive unit of peptidoglycan of Gram-positive bacterial cell walls, is known to have pleiotropic stimulatory effects on host immune systems and to elicit defensive responses. MDP is also known to have many effects on both the humoral- and cell-mediated immune systems, and has been implicated in the stimulation of macrophage.[14] A fluorescent-labeled MDP would be useful to visualize the binding of MDP to various cells in the study of immuno-adjuvant cellular interactions in experimental model system. According to Chirva's previous work,[15] the alkyl β-glycosides of muramyl dipeptide (MDP) having different hydrocarbon-chain lengths exerted no substantial effect on the corresponding immunostimulating activity. Thus, its labeling attachment point to an azido functionality was decided to fix at C-1 position via glycosylation of muramyl dipeptide (Fig. 3).

The synthesis of MDP derivative **15** bearing an internal azido-spacer is shown in Scheme 2. Both the dipeptide amine **10** and sugar precursor **11** were conveniently prepared



Scheme 2. Synthesis of MDP-flu **16** by click chemistry.

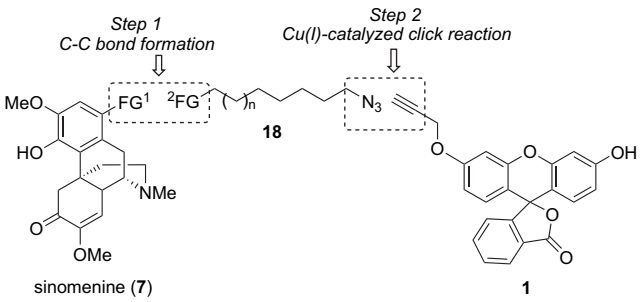


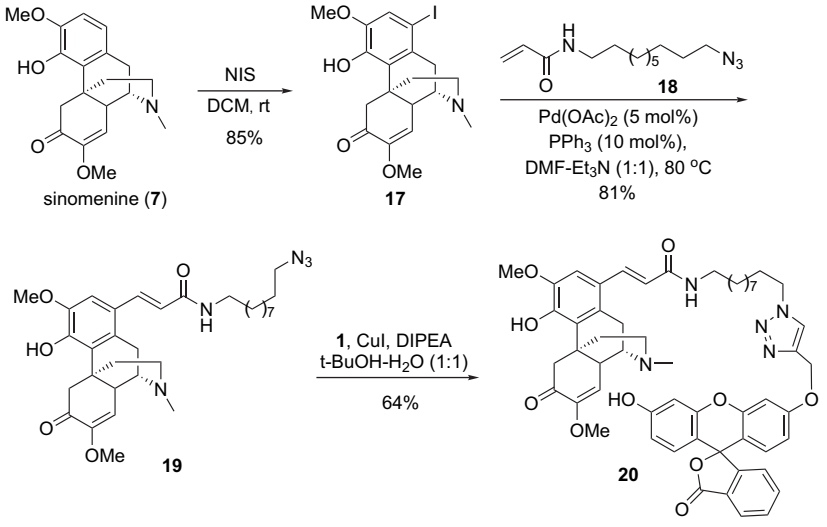
Figure 4. A personalized 'click' fluorescent-labeling protocol for sinomenine (**8**).

in good yield.[16] Treatment of the protected sugar derivative **11** with 10-azidodecanol[8] afforded exclusively unprotected β-glucopyranosides **12** in the presence of *p*-TsOH and 4A MS. *O*-Alkylation of free alcohol **12** with (*S*)-2-chloropropionic acid gave the carboxylic acid **13**. Coupling of **13** with dipeptide **10** using EDCI/HOBt gave the protected MDP derivative **14**. Final global deprotection of **14** was carried out in a DCM solution containing 25% of TFA at room temperature. This highly polar and water-soluble crude product **15** was successfully used for the last 'click' reaction without any further purification. The 1,3-dipolar Huisgen cycloaddition between MDP derivative **15** and fluorescein derivative **1** was finally accomplished in ^{t}BuOH/H_2O (v/v= 1:1) in the presence of catalytic amount of CuI and excess of DIPEA (partially to neutralize the remaining TFA in crude **15**). The reaction smoothly proceeded at room temperature, and the crude product was isolated in high yield by simple filtration of solid CuI. A pure sample of the labeled MDP derivative **16** was collected in 40% yield after a reverse-phase HPLC purification.

Sinomenine (**7**) is a naturally occurring alkaloid from Chinese medicinal plant *Sinomenium acutum*, exhibiting immunomodulating and anti-inflammatory activities with unambiguous clinical effects.[17] Guided by our previous results on modification of sinomenine,[18] maintenance of originally existing functional groups would be more favorable for keeping its bioactivities. Considering this, a personalized 'click' labeling plan for sinomenine was designed (Fig. 4). By this strategy, the C–C bond formation was fixed at the *para*-position of its phenol ring through a selective halogen introduction. Such a strategy might lead to the accomplishment of a very short and highly efficient route.

Treatment of natural sinomenine (**7**) with *N*-iodosuccinimide at room temperature afforded the iodide **17** in 85% yield, regioselectively (Scheme 3). Heck reaction of aryl iodide **17** with pre-prepared acrylamide **18** was smoothly performed under $Pd(OAc)_2$-catalyzed conditions to give the azido-containing derivative **19** in 81% yield. Final 'click' labeling was successfully carried out again in a mixture of *tert*-butanol and water (1:1), affording sinomenine-flu (**20**) in 65% yield after purification by a silica gel column chromatography. Using this personalized protocol, the fluorescent labeling of sinomenine was efficiently achieved in three steps.

Artemisinin (Qinghaosu, **8**) is a unique sesquiterpene 1,2,4-trioxane isolated from the Chinese medicinal herb Qinghao (*Artemisia annua* L.).[14] Artemisinin and many of its derivatives are highly effective against multidrug-resistant malaria caused by *Plasmodium falciparum*. The detailed mechanisms of these drugs remain unclear yet. However, many studies have shown that the activity of artemisinin is a consequence of the cleavage of the endoperoxide group by intraparasitic heme of associated heme alkylation, and of inhibition of hemin polymerization to hemozoin.[19] In order to demonstrate the mechanism of artemisinin compounds through real-time imaging study, acquirement of suitable artemisinin derivatives containing a fluorescent group is of great value. However, this class of peroxides are rather reactive when they are exposed to the Cu(I) species, leading to cleavage of their endoperoxide bridge and loss of bioactivity. Therefore, a strategic consideration has to be required in order to successfully employ this 'click' labeling protocol to the artemisinin derivatives. After various comparisons, a protocol by simply reversing the order of labeling sequence was finally adopted. According to this protocol, coupling of



Scheme 3. Click chemistry-based short synthesis of sinomenine-flu (**20**).

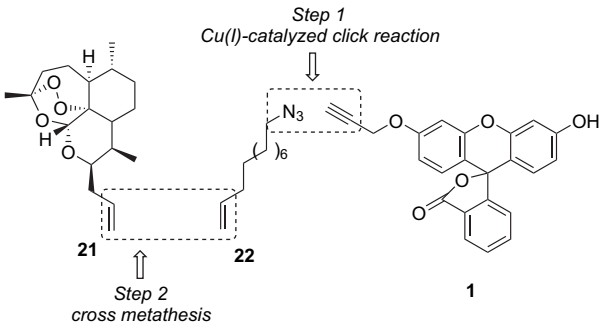


Figure 5. A reverse sequence for 'click' labeling of artesiminin derivatives using cross metathesis and Huisgen reaction.

fluorescein derivative **1** with an azido-containing spacer was executed at first by a click reaction in the presence of Cu(I) catalyst, so that the final attachment to the peroxide target could be done under Cu(I)-free conditions. Such a strategic modification can avoid the exposure of artemisinin endoperoxides to the Cu(I) species in the same reaction media, and does not require additional steps. Under these considerations, an easily prepared and known artemisinin derivative, 10β-allyldeoxoartemisinin **21** was chosen as the labeling precursor, in which its terminal olefin functionality was devised as the final C–C bond formation position by a Ru-catalyzed cross metathesis.[20] In addition, a hydrocarbon spacer **22** bearing a terminal olefin and azido group was designed to join both the artemisinin derivative **21** and fluorescein derivative **1** in this personalized protocol (Fig. 5).

Starting from the commercially available 10-undecen-1-ol, a linear spacer **22** was prepared (Scheme 4). 'Click' linkage of fluorescein derivative **1** was efficiently accomplished with azido-containing spacer **22** under the Cu(I)-catalyzed conditions. Final cross metathesis of newly produced fluorescein derivative **24** with 5 equiv of artemisinin derivative **21** in the presence of 20 mol % of the second generation Grubbs catalyst (added in three portions) afforded the labeled compound **25** (*E*-isomer only) in 33% yield after purification by a preparative TLC.

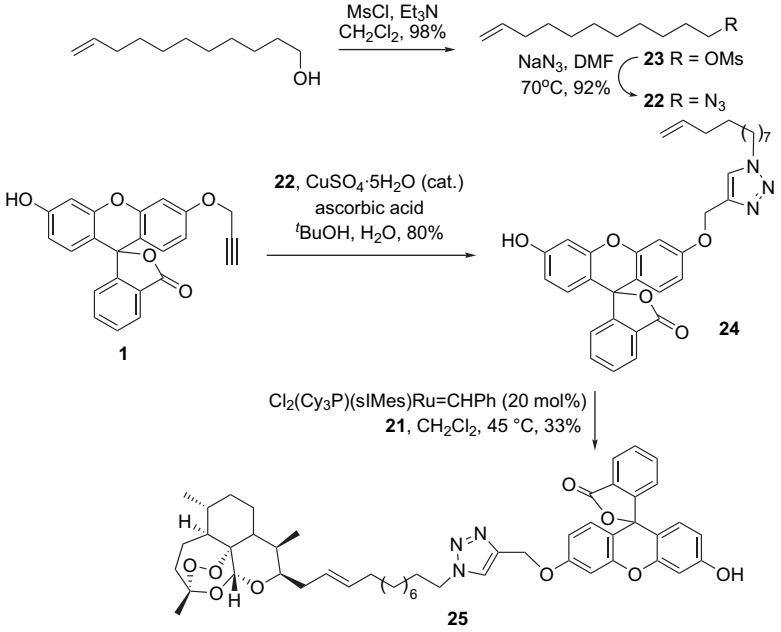
3. Conclusion

In summary, a flexible and efficient 'click' fluorescent labeling strategy is described. Personalized introduction of an azido functionality to specific targets by selective reactions and strategic order adjustment of labeling sequence are two essentials for a successful labeling. Applications to naringenin, MDP, sinomenine, and artemisinin were successfully achieved in short routes. Advantages of this labeling protocol include ease of operation, use of an inexpensive fluorescein reagent, and a catalyzed Huisgen cycloaddition in a free-of-protection fashion, as well as high efficiency achieved. It thus provides a practical and useful tool for developing bioactive small molecule-based probes and the corresponding studies on biological mechanisms. Further applications of these labeled compounds to related biological studies are underway in this laboratory and will be reported in due course.

4. Experimental

4.1. General

Optical rotations were measured at room temperature. Infrared spectra were recorded on a Perkin–Elmer 983 FTIR spectrophotometer, with all samples examined as films on a KBr disk. NMR spectra were recorded at Bruker Avance instruments (300 MHz or 500 MHz for ^{1}H NMR, and 75 MHz or 125 MHz for ^{13}C NMR) and are reported in parts per million (δ). HRMS spectra were recorded on Kratos Concept, Q-Tof Micro or APEXIII 7.0 TESLA FTMS. Elemental analyses were preformed on VARIO EL Elemental



Scheme 4. A two-step synthesis of artemisinin-flu (**25**) using cross metathesis and click reaction.

Apparatus. All melting points were uncorrected. Flash column chromatography was performed on silica gel (10~40 μm) using a mixture of petroleum ether and ethyl acetate as the eluant.

4.1.1. Compound 4. To a solution of naringenin (272 mg, 1 mmol), paraformaldehyde (60 mg, 2 mmol), and $ZnCl_2$ (14 mg, 0.1 mmol) in ethanol (4 mL) was added the secondary amine **5** (234 mg, 1.5 mmol) at room temperature. The mixture was then heated to 65 °C, and the reaction process was judged by TLC monitoring. After the reactants were consumed, EtOAc (30 mL) and acidic water (30 mL, pH 3) were added to the mixture. The pH of aqueous phase was then adjusted to 7. The aqueous phase was extracted with EtOAc (15 mL×3). The combined extracts were dried over anhydrous Na_2SO_4. The crude product was purified by silica gel chromatography (CH_2Cl_2/MeOH=50:1) to afford pure **4** (383 mg, 87%). IR (KBr): ν_{max} 3025, 2938, 2862, 2096, 1646, 1614, 1540, 1519, 1459, 1367, 1184, 1168, 1082, 835 cm^{-1}. ^1H NMR (acetone-d_6, 300 MHz): δ 7.34 (2H, d, J=8.4 Hz), 6.88 (2H, d, J=8.4 Hz), 5.78 (1H, s), 5.35 (1H, dd, J=12.6, 3.0 Hz), 3.83 (2H, s), 3.30 (2H, t, J=6.9 Hz), 3.09 (1H, dd, J=17.1, 12.6 Hz), 2.70 (2H, t, J=7.4 Hz), 2.66 (1H, dd, J=17.1, 3.0 Hz), 2.44 (3H, s), 1.69–1.56 (4H, m), 1.39–1.37 (4H, m). ^{13}C NMR (acetone-d_6, 75 MHz): δ 195.49, 171.13, 162.70, 161.25, 157.94, 129.90, 128.03, 115.32, 100.67, 100.01, 95.85, 78.84, 56.05, 52.65, 51.02, 42.57, 40.05, 28.49, 26.33, 26.23, 25.87. HRMS (ESI, m/z) calcd for $C_{23}H_{29}N_4O_5$ $(M+H)^+$: 441.2133, found: 441.2139. Anal. Calcd for $C_{23}H_{28}N_4O_5$: C, 62.71; H, 6.41; N, 12.72. Found: C, 62.49; H, 6.40; N, 12.58.

4.1.2. Naringenin-flu (3). To a mixture of compounds **4** (400 mg, 0.91 mmol) and **1** (504 mg, 1.36 mmol) in t-BuOH/H_2O (20 mL, v/v=1:1) were added $CuSO_4 \cdot 5H_2O$ (2 mg, 0.008 mmol) and ascorbic acid (5 mg, 0.028 mmol). The mixture was stirred at room temperature for 24 h. EtOAc (200 mL) was added to dilute the reaction. The organic phase was washed by saturated brine, and dried over anhydrous Na_2SO_4. The crude product was purified by silica gel chromatography (CH_2Cl_2/MeOH=20:1), affording pure naringenin-flu **3** (493 mg, 67%). IR (KBr): ν_{max} 2937, 1759, 1637, 1616, 1505, 1459, 1364, 1250, 1174, 1110, 1085, 834 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ 8.24 (1H, s), 8.00 (1H, d, J=7.5 Hz), 7.79–7.69 (2H, m), 7.32 (1H, s), 7.28 (2H, d, J=8.4 Hz), 7.09 (1H, d, J=2.7 Hz), 6.81–6.74 (4H, m), 6.65 (1H, d, J=9.3 Hz), 6.59 (2H, d, J=1.2 Hz), 5.66 (1H, s), 5.35 (1H, dd, J=12.6, 2.4 Hz), 5.22 (2H, s), 4.36 (2H, t, J=6.6 Hz), 3.83 (2H, s), 3.15 (1H, dd, J=17.1, 12.6 Hz), 2.69 (2H, t, J=7.2 Hz), 2.61 (1H, dd, J=17.1, 2.4 Hz), 2.40 (3H, s), 1.82 (2H, t, J=6.6 Hz), 1.56 (2H, br m), 1.27 (4H, br m). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 194.8, 173.4, 169.1, 162.6, 161.7, 160.2, 158.1, 152.8, 152.3, 142.6, 136.1, 130.6, 129.7, 129.5, 129.4, 128.7, 126.6, 125.1, 125.0, 124.5, 115.6, 113.4, 112.9, 111.9, 109.8, 102.7, 102.2, 100.0, 99.8, 96.6, 83.4, 78.5, 62.1, 55.7, 52.1, 49.8, 42.3, 40.4, 29.9, 26.1, 25.9, 25.3. HRMS (ESI, m/z) calcd for $C_{46}H_{43}N_4O_{10}$ $(M+H)^+$: 811.2974, found: 811.2993.

4.1.3. Compound 12. To a mixture of oxazoline **11**[16b] (240 mg, 1 mmol) and 10-azidodecan-1-ol (813 mg, 4 mmol) were added molecular sieves (4 Å, 300 mg) and anhydrous p-TsOH (172 mg, 1 mmol). The mixture was stirred at room temperature overnight. The reaction was quenched by adding K_2CO_3 slowly until the pH reached 7, when TLC indicated that reactant disappeared. After removal of the solid by filtration, the filtrate was concentrated under reduced pressure. The residue was washed several times with CH_2Cl_2 to afford a brown syrup. This syrup was then suspended in a mixture of acetone (8 mL) and 2,2-dimethoxypropane (5 mL). p-Toluenesulfonic acid (17 mg, 0.1 mmol) was added, and the mixture was stirred for 3 h at room temperature. The solvent was removed under reduced pressure, and the residue was then taken up into dichloromethane. This solution was washed with dilute sodium bicarbonate, water, and brine successively, dried over magnesium sulfate, filtered, and concentrated. The resulting light brown oil was purified by chromatography on silica gel (2% methanol/DCM) to yield **12** as a yellow syrup (265 mg, 60%); $[\alpha]_D^{20}$ −39.8 (c 1.83, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz): δ 5.77 (br s, 1H), 4.61 (d, 1H, J=8.1 Hz), 4.31 (br s, 1H), 3.95–3.80 (m, 5H), 3.59–3.46 (m, 3H), 3.27 (t, 3H, J=6.6 Hz), 2.05 (s, 3H), 1.59–1.25 (m, 25H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.1, 101.2, 99.8, 74.3, 71.6, 70.0, 67.1, 62.0, 57.9, 51.4, 32.7, 29.5, 29.5, 29.4, 29.4, 29.3, 29.1, 29.0, 28.8, 26.7, 25.9, 23.3, 19.1. HRMS (ESI, m/z) calcd for $[C_{21}H_{38}N_4O_6+Na]$ $(M+Na)^+$: 465.2683, found: 465.2678.

4.1.4. Compound 13. Sodium hydride (100 mg, 2.52 mmol, 60% dispersion in oil) was placed in a 25-mL round-bottom flask, washed with hexane three times, and finally suspended in dry THF (3 mL). A solution of compound **12** (0.35 g, 0.80 mmol) in dry THF (2 mL) was added dropwise over 10 min under nitrogen. To this mixture was added dropwise over 10 min a solution of (*S*)-(−)-2-chloropropionic acid (77 mg, 0.72 mmol) in dry THF (1 mL). The mixture was heated at reflux for 18 h, cooled, quenched with ethanol, diluted with water, and washed with ether. The aqueous layer was acidified with 1 M phosphoric acid and extracted with dichloromethane three times. The organic solution was again extracted with dilute sodium bicarbonate solution. The aqueous layer was then re-acidified with 1 M phosphoric acid and extracted with ethyl acetate. The ethyl acetate solution was washed with brine, dried over magnesium sulfate, filtered, and concentrated to yield **13** as an amorphous solid (298 mg, 80%); $[\alpha]_D^{20}$ 18.1 (c 0.75, $CHCl_3$). IR (KBr): ν_{max} 3338, 2993, 2933, 2858, 2098, 1722, 1624, 1561, 1459, 1376, 1110 cm^{-1}. ^1H NMR (MeOH-d_4, 500 MHz): δ 4.45 (d, 1H, J=6.7 Hz), 4.39 (d, 1H, J=8.3 Hz), 3.84 (m, 2H), 3.67–3.40 (m, 5H), 3.30–3.25 (m, 3H), 1.94 (s, 3H), 1.59–1.51 (m, 5H), 1.38–1.30 (m, 17H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 187.9, 177.9, 174.0, 103.0, 83.5, 78.1, 77.1, 72.5, 70.9, 63.0, 56.5, 52.8, 33.3, 31.0, 30. 9, 30.8, 30.6, 30.2, 28.1, 27.4, 23.5, 19.9. HRMS (ESI, m/z) calcd for $[C_{24}H_{42}N_4O_8+Na]$ $(M+Na)^+$: 537.2894, found: 537.2914.

4.1.5. Compound 14. To a solution of **13** (925 mg, 1.8 mmol) and **10** (410 mg, 1.5 mmol) in dry dichloromethane (20 mL) were added DIPEA (232 mg, 1.8 mmol), *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide (EDCI) (350 mg, 1.8 mmol), and *N*-hydroxybenzotriazole hydrate (HOBt) (275 mg, 1.8 mmol). The reaction was stirred for 24 h at room temperature. The mixture was diluted with

dichloromethane, washed successively with saturated $NaHCO_3$, water and brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography on silica gel (2% methanol in DCM) to yield **14** as an amorphous solid (922 mg, 80%); $[\alpha]_D^{20}$ 5.59 (*c* 1.69, $CHCl_3$). IR (KBr): ν_{max} 3367, 2934, 2859, 2098, 1736, 1660, 1561, 1458, 1370, 1267, 1203, 1158 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.58–7.45 (m, 2H), 6.93 (s, 1H), 6.64 (d, 1H, *J*=7.6 Hz), 6.00 (s, 1H), 4.82 (d, 1H, *J*=8.2 Hz), 4.45–4.40 (m, 1H), 4.32–4.27 (m, 1H), 4.17–4.14 (m, 1H), 3.97–3.90 (m, 2H), 3.82–3.75 (m, 2H), 3.59 (t, 1H, *J*=9.2 Hz), 3.47–3.33 (m, 2H), 3.32–3.28 (m, 1H), 3.25 (t, 2H, *J*=6.9 Hz), 2.44–2.34 (m, 2H), 2.17–2.11 (m, 1H), 2.01–1.93 (m, 4H), 1.61–1.27 (m, 37H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 174.4, 174.2, 172.9, 172.7, 171.3, 100.7, 99.3, 80.9, 79.9, 78.0, 74.6, 69.9, 66.6, 62.1, 57.0, 52.6, 51.4, 49.4, 31.9, 29.5, 29.4, 29.4, 29.3, 29.1, 28.8, 28.1 (3C), 28.0, 27.0, 26.7, 25.8, 23.6, 19.5, 19.0, 18.0. HRMS (ESI, *m/z*) calcd for $[C_{36}H_{63}N_7O_{11}+Na]$ $(M+Na)^+$: 792.4478, found: 792.4506.

4.1.6. MDP-flu (16). Treatment of **14** (40 mg, 0.05 mmol) with TFA (0.25 mL) in methylene chloride (0.75 mL) at room temperature for 4 h afforded crude **15** (37 mg, 100%). To a mixture of crude **15** (37 mg, 0.05 mmol) and fluorescein derivative **1** (22 mg, 0.06 mmol) in *t*-BuOH/H_2O (1 mL/1 mL) were added CuI (1 mg, 0.005 mmol) and DIPEA (7 mg, 0.05 mmol). The mixture was stirred at room temperature for 24 h. The whole mixture was evaporated to dryness under reduced pressure. The residue was re-dissolved into MeOH and purified by reverse-phase HPLC (conditions: Vydac C18 column (monomeric 120A, 250×10 mm); 40% acetonitrile in water to 60% acetonitrile in water in 20 min, and then to 100% acetonitrile in 15 min; flow rate 1 mL/min; UV 224 nm; retention time of product is 16.8 min), affording pure **16** (21 mg, 40%) as a light yellow syrup. ^1H NMR (MeOH-d_4, 300 MHz): δ 8.11 (s, 1H), 8.05 (d, 1H, *J*=8.1 Hz), 7.82–7.70 (m, 2H), 7.22 (d, 1H, *J*=7.5 Hz), 7.05 (s, 1H), 6.82–6.74 (m, 3H), 6.68–6.60 (m, 2H), 5.27 (s, 2H), 4.93 (d, 1H, *J*=8.2 Hz), 4.44–4.35 (m, 4H), 4.29–4.20 (m, 2H), 4.08–3.95 (m, 1H), 3.90–3.68 (m, 3H), 3.46–3.42 (m, 2H), 2.40 (t, 2H, *J*=7.5 Hz), 2.24–2.22 (m, 1H), 1.96–1.94 (m, 2H), 1.93 (s, 3H), 1.52–1.26 (m, 22H). HRMS (MALDI, *m/z*) calcd for $[C_{52}H_{65}N_7O_{16}+Na]$ $(M+Na)^+$: 1066.4380, found: 1066.4390.

4.1.7. Compound 17. To a solution of sinomenine (**7**, 3.29 g, 10.0 mmol) in CH_2Cl_2 (80 mL) was added NIS (2.36 g, 10.5 mmol) at room temperature. After 2 h, the reaction mixture was quenched by adding aqueous $Na_2S_2O_3$ solution. The organic layer was separated and washed with H_2O and then brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH=50:1 to 20:1) to give **17** (3.87 g, 85%) as a pale yellow solid; $[\alpha]_D^{20}$ 5.8 (*c* 0.46, $CHCl_3$); mp: 108–110 °C. IR (KBr): ν_{max} 2934, 2837, 1689, 1628, 1471, 1435, 1278, 1202, 1148, 1052, 885, 749 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.12 (1H, s), 6.67 (1H, br s), 5.42 (1H, s), 4.32 (1H, d, *J*=15.6 Hz), 3.75 (3H, s), 3.46 (1H, s), 3.21 (1H, d, *J*=3.9 Hz), 2.97 (1H, s), 2.86 (1H, d, *J*=18.9 Hz), 2.53–2.43 (3H, m), 2.39 (3H, s), 2.00–1.85 (3H, m). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 193.7, 152.2, 145.8, 145.1, 131.9, 124.3, 119.2, 114.5, 87.8, 57.0, 56.0, 54.8, 48.7, 46.9, 45.5, 42.7, 41.1, 35.4, 31.8. HRMS (ESI, *m/z*) calcd for $C_{19}H_{23}INO_4$ $(M+H)^+$: 456.0666, found: 456.0661.

4.1.8. Compound 19. To a mixture of iodo-sinomenine derivative **17** (282 mg, 0.62 mmol), *N*-(10-azidodecyl)acrylamide **18** (156 mg, 0.62 mmol), PPh_3 (16 mg, 0.062 mmol), and $Pd(OAc)_2$ (7 mg, 0.031 mmol) were added degassed DMF (15 mL) and Et_3N (3 mL) under nitrogen atmosphere. The resulting mixture was heated to 80 °C for 2 h, cooled down to room temperature and filtered over a pad of Celite. The pad was washed with ethyl acetate (20 mL) and the filtrate was washed by H_2O and brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography (CH_2Cl_2/CH_3OH=50:1 to 20:1) to give **19** (290 mg, 81%) as a pale yellow solid; $[\alpha]_D^{20}$ 28.9 (*c* 0.52, $CHCl_3$); mp: 125–127 °C (decomp.). IR (KBr): ν_{max} 2930, 2854, 2096, 1692, 1654, 1617, 1465, 1422, 1290, 1203, 1148, 1109, 1073 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.92 (1H, d, *J*=15.3 Hz), 6.86 (1H, s), 6.39 (1H, br s), 6.15 (1H, d, *J*=14.7 Hz), 5.69 (1H, t, *J*=5.1 Hz), 5.45 (1H, s), 4.33 (1H, d, *J*=15.6 Hz), 3.82 (3H, s), 3.47 (3H, s), 3.38 (2H, q, *J*=6.6 Hz), 3.28–3.24 (3H, m), 3.12 (1H, d, *J*=18.0 Hz), 3.02 (1H, s), 2.68 (1H, dd, *J*=5.1, 18.6 Hz), 2.41 (3H, s), 2.02–1.88 (3H, m), 1.62–1.55 (4H, m), 1.30–1.26 (12H, m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 194.0, 166.2, 152.3, 146.4, 145.2, 137.8, 130.7, 123.6, 123.2, 119.9, 115.0, 106.5, 56.1, 55.8, 54.7, 51.4, 49.1, 47.0, 45.4, 42.8, 40.6, 39.8, 35.5, 29.7, 29.4 (2C), 29.3, 29.1, 28.8, 26.9, 26.7, 22.0. HRMS (ESI, *m/z*) calcd for $C_{32}H_{46}N_5O_5$ $(M+H)^+$: 580.3494, found: 580.3489.

4.1.9. Sinomenine-flu (20). To a solution of azido-sinomenine derivative **19** (92 mg, 0.16 mmol), acetylene-fluorescein derivative **1** (59 mg, 0.16 mmol), and CuI (3 mg, 0.016 mmol) in *t*-BuOH/H_2O (5 mL, v/v=1:1) was added DIPEA (83 μL, 0.48 mmol) dropwise under nitrogen atmosphere. The resulting mixture was stirred at room temperature for 4 h. The mixture was extracted with 5% MeOH/CH_2Cl_2 for three times. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH=30:1 to 10:1) to afford **20** (96 mg, 64%) as a yellow solid; $[\alpha]_D^{20}$ 22.8 (*c* 0.52, $CHCl_3$); mp: 138–140 °C (decomp.). IR (KBr): ν_{max} 2928, 2854, 1764, 1687, 1611, 1465, 1427, 1288, 1249, 1180, 757 cm^{-1}. ^1H NMR (DMSO, 500 MHz): δ 10.15 (1H, br s), 8.93 (1H, br s), 8.24 (1H, s), 8.00 (1H, d, *J*=7.7 Hz), 7.97 (1H, t, *J*=5.5 Hz), 7.79 (1H, t, *J*=7.3 Hz), 7.71 (1H, t, *J*=7.3 Hz), 7.66 (1H, d, *J*=15.4 Hz), 7.27 (1H, d, *J*=7.7 Hz), 7.09 (1H, d, *J*=2.3 Hz), 6.96 (1H, s), 6.77 (1H, dd, *J*=2.4, 8.8 Hz), 6.73 (1H, s), 6.66 (1H, d, *J*=8.8 Hz), 6.59 (2H, s), 6.41 (1H, d, *J*=15.4 Hz), 5.69 (1H, s), 5.22 (2H, s), 4.34 (2H, t, *J*=7.0 Hz), 4.16 (1H, d, *J*=15.4 Hz), 3.78 (3H, s), 3.34 (3H, s), 3.23 (1H, s), 3.15 (2H, q, *J*=6.4 Hz), 2.92–2.88 (2H, m), 2.73–2.69 (1H, m), 2.44 (2H, d, *J*=15.3 Hz), 2.30 (3H, s), 1.91–1.74 (5H, m), 1.44–1.42 (2H, m), 1.24–1.22 (12H, m). ^{13}C NMR (DMSO, 125 MHz): δ 192.5, 168.6, 165.2, 159.7, 159.6, 152.4, 151.8, 151.7, 151.4, 146.5, 145.5, 142.1, 135.58, 135.55, 130.1, 130.1, 129.0, 128.9, 126.0, 124.6, 124.5,

123.9, 122.8, 120.9, 115.9, 112.8, 112.4, 111.3, 109.4, 106.9, 102.2, 101.6, 82.6, 62.8, 61.5, 55.5, 55.2, 54.2, 49.3, 46.6, 44.4, 42.3, 40.1, 38.6, 34.6, 29.6, 29.1, 28.82, 28.75, 28.65, 28.3, 26.4, 25.8, 21.6. HRMS (ESI, *m/z*) calcd for $C_{55}H_{59}N_5O_{10}Na$ $(M+Na)^+$: 927.4154, found: 927.4115.

4.1.10. Compound 24. To a suspension of 11-azido-undec-1-ene **22** (918 mg, 4.7 mmol) and acetylene-fluorescein derivative **1** (1.446 g, 3.9 mmol) in a 1:1 mixture of water and *tert*-butyl alcohol (60 mL) were added cupric sulfate (17 mg, 0.04 mmol) and ascorbic acid (43 mg, 0.14 mmol) successively. The mixture was stirred vigorously at room temperature for 24 h. The reaction mixture was diluted with ethyl acetate (400 mL), washed with water (10 mL×3), saturated brine solution (10 mL), dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was chromatographed on silica gel to afford **24** as a yellow solid (1.03 g, 80%); mp 78–80 °C. IR (KBr): ν_{max} 2927, 1765, 1634, 1615, 1504, 1465, 1430, 1249, 1183, 1108, 760, 692 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 8.00 (d, 1H, J=7.2 Hz), 7.70–7.58 (m, 3H), 7.15 (d, 1H, J=7.2 Hz), 6.86 (d, 1H, J=1.5 Hz), 6.77 (s, 1H), 6.69–6.56 (m, 4H), 5.84–5.73 (m, 1H), 5.22 (s, 2H), 5.01 (dd, 1H, J=3.9, 1.5 Hz), 4.96–4.90 (m, 1H), 4.36 (t, 1H, J=6.9 Hz), 2.06–1.99 (m, 2H), 1.92 (t, 2H, J=6.6 Hz), 1.32–1.20 (m, 12H). MS (ESI, *m/z*): 566 $(M+H)^+$, 588 $(M+Na)^+$. Anal. Calcd for $C_{34}H_{35}N_3O_5$: C, 72.19; H, 6.24; N, 7.43. Found: C, 71.96; H, 6.50; N, 7.43.

4.1.11. Fluorescein-labeled artemisinin derivative 25. A suspension of **24** (48 mg, 0.085 mmol) and 12β-allyldeoxoartemisinin **21** (131 mg, 0.425 mmol) in anhydrous CH_2Cl_2 (0.2 mL) was added the second generation Grubbs catalyst (7 mg, 0.0085 mmol) over 2 h via a syringe pump under N_2 at 45 °C. After 12 h, the second portion of Grubbs catalyst (3.5 mg, 0.0042 mmol) in dichloromethane (0.1 mL) was added. This mixture was stirred for 24 h, and then the third portion of Grubbs catalyst (3.5 mg, 0.0042 mmol) in dichloromethane (0.1 mL) was added. After additional 12 h, the reaction was allowed to cool down to room temperature and filtered through a pad of Celite. The filtrate was concentrated in vacuo. The residue was purified by a preparative thin layer chromatography to afford **25** as a yellow solid (24 mg, 33%); mp 68–69 °C; $[\alpha]_D^{23}$ −59.5 (*c* 0.073, $CHCl_3$). IR (KBr): ν_{max} 2926, 1769, 1637, 1563, 1504, 1374, 1248, 1179, 1108, 1038, 840, 764, 668, 420 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 8.01 (d, 1H, J=7.6 Hz), 7.66 (t, 1H, J=7.5 Hz), 7.62–7.59 (m, 2H), 7.15 (d, 1H, J=7.6 Hz), 6.86 (s, 1H), 6.75 (d, 1H, J=2.2 Hz), 6.69–6.55 (m, 4H), 5.50 (dt, 1H, J=15.5, 6.8 Hz), 5.44 (dt, 1H, J=14.7, 6.0 Hz), 5.24 (s, 2H), 5.23 (s, 1H), 4.35 (t, 2H, J=7.2 Hz), 4.15–4.11 (m, 1H), 3.56 (s, 1H), 2.25–2.14 (m, 3H), 2.00–1.91 (m, 6H), 1.81–1.70 (m, 3H), 1.56 (s, 3H), 1.30–1.19 (m, 18H), 0.91–0.87 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.8, 159.6, 158.8, 152.5, 152.3, 143.2, 135.1, 132.1, 129.6, 129.5, 129.1, 127.1, 126.7, 124.9, 124.0, 122.9, 115.3, 112.7, 111.8, 110.2, 107.0, 103.1, 102.0, 96.8, 83.0, 69.8, 69.0, 61.9, 53.7, 50.6, 41.2, 39.9, 35.2, 34.4, 34.3, 32.6, 31.7, 30.4, 30.1, 29.7, 29.4, 29.3, 29.2, 28.9, 26.4, 25.1, 20.3, 18.5, 14.1, 12.0. MS (MALDI, *m/z*): 846 $(M+H)^+$, 868 $(M+Na)^+$. HRMS (MALDI, *m/z*) calcd for $C_{50}H_{59}N_3O_9$ $(M)^+$: 845.4251, found: 845.4246.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.04.069.

References and notes

1. Recent reviews, see: (a) Lucitti, J. L.; Dickinson, M. E. *Pediatr. Res.* **2006**, *60*, 1–5; (b) Demchenko, A. P. *FEBS Lett.* **2006**, *580*, 1951–2957; (c) Dirks, R. W.; Tanke, H. J. *Biotechniques* **2006**, *40*, 489–496; (d) Lang, P.; Yeow, K.; Nichols, A.; Scheer, A. *Nat. Rev. Drug Discov.* **2006**, *5*, 343–356; (e) Hoffman, R. M. *IDrugs* **2006**, *9*, 632–635; (f) Machleidt, T.; Robers, M.; Hanson, G. T. *Methods Mol. Biol.* **2007**, *356*, 209–220.
2. Reviews, see: (a) Finney, N. S. *Curr. Opin. Chem. Biol.* **2006**, *10*, 238–245; (b) Mertz, J. *Curr. Opin. Neurobiol.* **2004**, *14*, 610–616; (c) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell. Biol.* **2002**, *3*, 906–918.
3. Recent examples of fluorescent probes: (a) Evans, M. J.; Saghatelian, A.; Sorensen, E. J.; Cravatt, B. F. *Nat. Biotechnol.* **2005**, *23*, 1303–1307; (b) Zhao, X.; Hilliard, L. R.; Mechery, S. J.; Wang, Y.; Bagwe, R. P.; Jin, S.; Tan, W. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15027–15032; (c) Qu, L. W.; Luo, P. G.; Taylor, S.; Lin, Y.; Huang, W. J.; Anyadike, N.; Tzeng, T. R. J.; Stutzenberger, F.; Latour, R. A.; Sun, Y. P. *J. Nanosci. Nanotechnol.* **2005**, *5*, 319–322; (d) Dhayal, B.; Henne, W. A.; Doorneweerd, D. D.; Reifenberger, R. G.; Low, P. S. *J. Am. Chem. Soc.* **2006**, *128*, 3716–3721; (e) Bettegowda, C.; Foss, C. A.; Cheong, I.; Wang, Y.; Diaz, L.; Agrawal, N.; Fox, J.; Dick, J.; Dang, L. H.; Zhou, S.; Kinzler, K. W.; Vogelstein, B.; Pomper, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1145–1150.
4. (a) Huisgen, R. *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; Wiley: New York, NY, 1984; pp 1–176; (b) Gothelf, K. V.; Jorgensen, K. A. *Chem. Rev.* **1998**, *98*, 863–909.
5. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.
6. Review, see: Kolb, H. C.; Sharpless, K. B. *Drug Discov. Today* **2003**, *8*, 1128–1137.
7. Selective applications of 'click' chemistry: (a) Slater, M.; Snauko, M.; Svec, F.; Frechet, J. M. J. *Anal. Chem.* **2006**, *78*, 4969–4975; (b) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687; (c) Speers, A. E.; Cravatt, B. F. *J. Am. Chem. Soc.* **2005**, *127*, 10018–10019; (d) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047; (e) Kiick, K. L.; Saxon, E.; Tirrel, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19–24.
8. Chen, L.; Hu, T.-S.; Zhu, J.; Wu, H.; Yao, Z.-J. *Synlett* **2006**, 1225–1229.

9. Derbre, S.; Roue, G.; Poupon, E.; Susin, S. A.; Hocquemiller, R. *ChemBioChem* **2005**, *6*, 979–982.
10. (a) McIver, J.; Djordjevic, M. A.; Weinman, J. J.; Bender, G. L.; Rolfe, B. G. *Mol. Plant-Microbe Interact.* **1989**, *2*, 97–106; (b) Spaink, H. P.; Okker, R. J. H.; Wijffelman, C. A.; Tak, T.; Goosen-deRoo, L.; Dees, E.; van Brussel, A. A. N.; Lugtenberg, B. J. J. *J. Bacteriol.* **1989**, *171*, 4045–4053.
11. A review on Mannich reaction: Arend, M.; Westermann, B.; Risch, N. *Angew. Chem., Int. Ed.* **1998**, *37*, 1044–1070.
12. Cummings, N. P.; Pabst, M. J.; Johnson, R. B., Jr. *J. Exp. Med.* **1980**, *152*, 1659–1669.
13. Vieregge, B.; Resch, K.; Kaever, V. *Planta Med.* **1999**, *65*, 80–82.
14. Butler, A. R.; Wu, Y.-L. *Chem. Soc. Rev.* **1992**, *21*, 85–90.
15. Zemlyakov, A. E.; Tsikalov, V. V.; Kalyuzhin, O. V.; Kur'yanov, V. O.; Chirva, V. Y. *Russ. J. Bioorg. Chem.* **2003**, *29*, 286–292.
16. (a) Cai, Y.; Ling, C.-C.; Bundle, D. R. *Org. Lett.* **2005**, *7*, 4021–4024; (b) Furneaux, R. H.; Gainsford, G. J.; Lynch, G. P.; Yorke, S. C. *Tetrahedron* **1993**, *42*, 9605–9612.
17. (a) Liu, L.; Riese, J.; Resch, K.; Kaever, V. *Arzneimittelforschung* **1994**, *44*, 1223–1226; (b) Liu, L.; Resch, K.; Kaever, V. *Int. J. Immunopharmacol.* **1994**, *16*, 685–691; (c) Candinas, D.; Mark, W.; Kaever, V.; Miyatake, T.; Koyamada, N.; Hechenleitner, P.; Hancock, W. W. *Transplantation* **1996**, *62*, 1855–1860; (d) Liu, L.; Buchner, E.; Beitze, D.; Schmidt-Weber, C. B.; Emmrich, F.; Kinne, R. W. *Int. J. Immunopharmacol.* **1996**, *18*, 529–543.
18. (a) Yao, Z.-J.; Zhou, H.-B. Chinese Patent ZL 2005 1 0024479.3; *Chem. Abstr.* **2006**, *145*, 189061; (b) Yao, Z.-J.; Zhou, H.-B. Chinese Patent ZL 2005 1 0024478.9; *Chem. Abstr.* **2006**, *145*, 230784.
19. O'Neill, P. M.; Posner, G. H. *J. Med. Chem.* **2004**, *47*, 2945–2964.
20. Trnka, T. M.; Grubbs, R. H. *Acc. Chem. Res.* **2001**, *34*, 18–29.