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Preliminary Communication

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Fluorescent labeling of oleanolic acid using 'click' chemistry

Abstract: The natural product oleanolic acid (OA) was functionalized and labeled with the non-aggregated fluorescent dye BOPIM (boron 2-(2′-pyridyl)imidazole complex) using 'click' chemistry, yielding a highly fluorescent probe. The pharmacological activity of the probe is comparable to that of unmodified OA, making it suitable for studying the therapeutic mechanisms of OA derivatives *in vitro*.

Keywords: fluorescence; labeling; oleanolic acid; pharmacological activity.

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Introduction

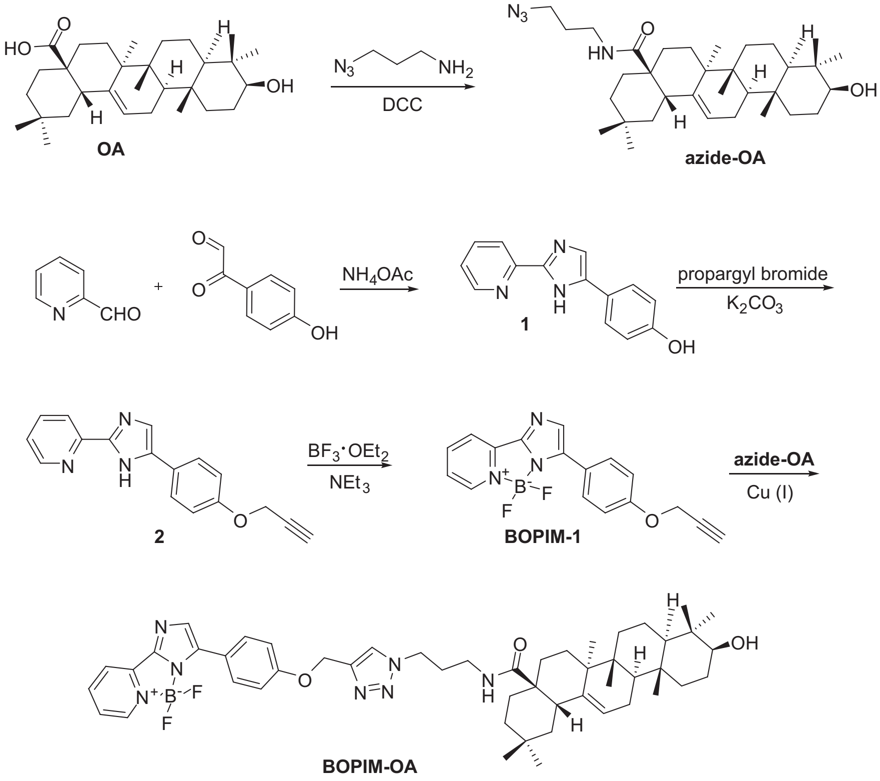
The broad range of biological activities expressed by natural products has led to their widespread use in the treatment of diseases, and a great deal of effort has been directed towards their synthesis and structural modification. The biological activity of natural products is generally evaluated using MTT assays in cell cultures or by administration to animals [1]. Because the tests are highly sensitive and enable quantitative analysis at the molecular level, fluorescent labeling is a major research tool in molecular and cellular biology for elucidating therapeutic mechanisms and metabolic routes [2]. However, there have been relatively few reports describing the fluorescent labeling of natural products [3–7]. The probes typically contain intensely fluorescent organic fluorophores, and although they fulfill analytical requirements, they may be unsuitable in terms of other characteristics such as good photo- and chemical stability, low toxicity, or resistance to aggregation in aqueous media. Many chromophores have been used as probes for bio-imaging, including cyanines, squaraine rotaxenes, and difluorobordiaza-*s*-indacenes (BODIPYs). However, none of these chromophores possess all of the desirable attributes mentioned above. We recently explored the boron-fluorine analog boron 2-(2′-pyridyl)imidazole (BOPIM), which is highly fluorescent, non-aggregated, and non-toxic [8]. We used the BOPIM chromophore to label the natural product oleanolic acid (**OA**), which shows anti-inflammatory [9–11] and anti-HIV [12, 13] activity, induces apoptosis in human leukemia cells [14], and inhibits tumor growth [12, 15–18]. Pharmacological activity evaluations using H9c2 and HeLa cells have indicated that the biological activity of the labeled compound is comparable to natural **OA** and that it may be used as a probe for investigating the pharmacological mechanisms of **OA**.

Results and discussion

Although various synthetic procedures may be used to covalently link the BOPIM chromophore to **OA**, the 'click' chemistry methodology developed by Sharpless and coworkers is preferred. This method facilitates the synthesis of target substances by joining small molecules through a convenient heterocycle formation reaction with good yield and regioselectivity. The reactions conditions are generally mild, and in many cases water may be used as a solvent. Another important feature of 'click' chemistry is that it tolerates a variety of functional groups, reducing the need for protection and de-protection of functional groups and yielding the triazole product with minimal work-up and purification.

OA contains a carboxylic acid unit that can react with an amine to produce an amide with biological activity similar to that of **OA** [12]. Reaction of **OA** with 3-azidopropylamine [19] produced the intermediate **azide-OA** in high yield (80%) following chromatographic purification.

The BOPIM chromophore core was synthesized according to a previously reported procedure [8]. A



Scheme 1 Synthesis of fluorescently labeled oleanolic acid (**OA**).

three-component, one-pot reaction of (4-hydroxylphenyl) glyoxal, pyridine-2-carbaldehyde, and ammonium acetate produced intermediate **1** which was converted to **2** in 55% yield (Scheme 1). The functional alkoxy linker is an electron-donating group and can red-shift the emission band of BOPIM dyes [20]. Treatment of **2** with $BF_3·OEt_2$ under basic conditions yielded **BOPIM-1** in 40% yield following chromatographic purification. **BOPIM-1** contains an aliphatic alkyne group than can readily undergo click chemistry with **azide-OA** to produce the target product **BOPIM-OA** in quantitative yield.

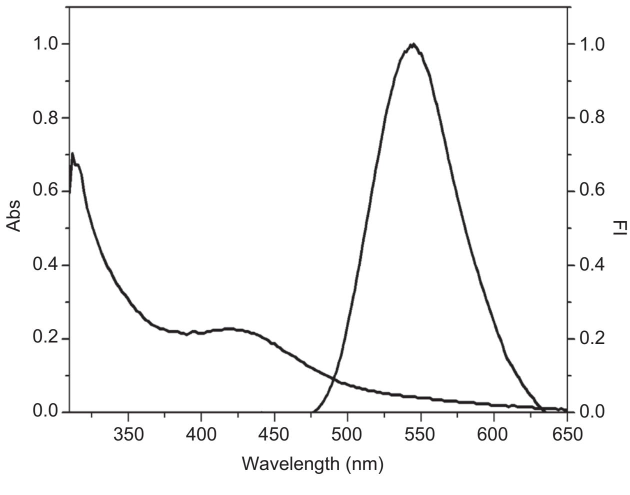


Figure 1 Absorption and fluorescence spectra of **BOPIM-OA** (2×10^{-5} M, DMSO/H_2O 1:1, excitation at 380 nm).

Although *in vitro* and *in vivo* studies are ideally conducted under physiological conditions, the aqueous solubilities of **OA** and **BOPIM-OA** are fairly low. For this reason, the spectroscopic properties of **BOPIM-OA** were investigated in a mixture of DMSO and water, which is also suitable for studies of biological activity. **BOPIM-OA** exhibits a broad absorption band centered at 422 nm with a molar absorptivity of 11 400 L/mol/cm^{-1} (Figure 1). Unlike other chromophores that aggregate in aqueous media, this probe displays a sharp fluorescence peak in polar media. The non-parallel packing mode plays an important role in inhibiting fluorescence quenching under these conditions [8]. The maximum emissive peak is observed at 545 nm with a fluorescence quantum yield of 0.24. The probe's Stokes shift in aqueous solution was 123 nm. The large shift coupled with high fluorescence intensity makes this molecule a suitable probe for *in vitro* imaging.

To evaluate the pharmacological activity of the labeled probe, normal H9c2 and cancerous HeLa cells were treated using **OA**, **BOPIM-1**, and **BOPIM-OA** at concentrations of 25, 50, and 100 μM for 48 h (Table 1).

Table 1 Effect of **OA**, **BOPIM-1**, and **BOPIM-OA** on normal and cancer cell viability.

Compound	H9c2 cell viability (vs. control)			HeLa cell viability (vs. control)		
	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
OA	1.24±0.11	1.46±0.09	1.89±0.13	0.68±0.06	0.61±0.01	0.23±0.08
BOPIM-1	1.13±0.06	1.35±0.05	1.77±0.15	0.37±0.09	0.13±0.07	0.03±0.01
BOPIM-OA	0.90±0.08	1.13±0.12	1.30±0.04	0.88±0.06	085±0.02	0.67±0.02

Data are expressed as mean±SD, n=3 (see Experimental section for details).

OA and **BOPIM-1** promoted H9c2 cell viability in a dose-dependent manner. The viability of H9c2 cells treated with **BOPIM-OA** decreased a small amount, suggesting that the compound has relatively low toxicity for normal cells. The growth of HeLa cells was inhibited by **OA** and more strongly by **BOPIM-1**, suggesting that **BOPIM-1** alone may have potential in anticancer therapy. Covalent attachment to **OA** reduces the effect, but the reduction is small at low concentrations compared to natural **OA**, indicating that the fluorescently labeled derivative may be used to study the therapeutic mechanisms through fluorescence imaging [21].

Conclusions

The highly fluorescent BOPIM dye containing a functionalized alkyne group was synthesized and used to label the natural product **OA**. The labeled probe exhibits intense fluorescence in polar media and pharmacological activity comparable to native **OA**. These properties make it a suitable probe for studying the therapeutic mechanisms of OA derivatives *in vitro*. However, both **OA** and the labeled probe are poorly soluble in water, and further research to develop more soluble derivatives is still in progress.

Experimental

General

All chemicals were obtained commercially as analytical grade and used without further purification. All yields refer to isolated products. Melting points were determined using an optical digital melting point apparatus (WRS-1A). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker 400 NMR spectrometer. Chemical shifts are reported in ppm and referenced to $CDCl_3$ (7.26 ppm for ^{1}H NMR and 77.0 ppm for ^{13}C NMR). MS data were obtained on a Waters Quattro Micro API LC-MS spectrometer (Waters, USA) or an Applied Biosystems Voyager-DE STR mass spectrometer. UV-vis and fluorescence spectra were obtained using Hitachi U-3010 and F-4500 spectrometers. The fluorescence quantum yield was calculated using Rhodamine B as a reference.

Synthesis of 3-azidopropyl oleanolamide (azide-OA)

Oleanolic acid (2.34 g, 5.20 mmol) in dichloromethane (100 mL) was added to 3-azidopropylamine (0.52 g, 5.20 mmol) under nitrogen. HOBt (0.70 g, 5.20 mmol) and DCC (1.07 g, 5.20 mmol) were added to the solution at 0°C. The mixture was stirred overnight at room temperature. Following evaporation of the solvent, the crude product was subjected to column chromatography (silica gel, petroleum ether/ethyl acetate, 8:1) to provide the target product as a white solid (80%); ^{1}H NMR: δ 6.04 (t, *J* = 5.5 Hz, 1H), 5.31 (t, *J* = 3.4 Hz, 1H), 3.42 (m, 2H), 3.29 (t, *J* = 6.6 Hz, 2H), 3.15 (m, 1H), 3.01 (m, 1H), 2.45 (dd, *J* = 13.0, 3.6 Hz, 1H), 1.10, 0.92, 0.85, 0.84, 0.83, 0.72, 0.69 (s, each 3H); HRMS: m/z Calcd for $C_{33}H_{55}N_4O_2$ 539.4325 $[M+H]^+$. Found 539.4313.

Synthesis of boron 2-(5-(4-(prop-2-ynyloxy) phenyl)-imidazol-2-yl)pyridine difluoride (BOPIM-1)

$BF_3 \cdot Et_2O$ (9.0 mL, 70 mmol) was added dropwise at 0°C to a stirred mixture of 2-(5-(4-(prop-2-ynyloxy)phenyl)-1*H*-imidazol-2-yl)pyridine (0.20 g, 0.73 mmol) and triethylamine (8.0 mL) in anhydrous dichloromethane (30 mL). The mixture was warmed to room temperature and stirred overnight. The organic phase was washed with brine and several times with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel (petroleum ether/EtOAc, 2:1) to provide the pale yellow solid of **BOPIM-1** (40%); ^{1}H NMR: δ 8.45 (d, *J* = 5.6 Hz, 1H), 8.18 (t, *J* = 7.8 Hz, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.8 Hz, 2H), 7.48(m, 2H), 7.06 (d, *J* = 8.8 Hz, 2H), 4.74 (d, *J* = 2.4 Hz, 2H), 2.55 (t, *J* = 2.4 Hz, 1H); HRMS: m/z Calcd for $C_{17}H_{13}BN_3OF_2$ $[M+H]^+$ 324.1120. Found 324.1112.

Synthesis of boron 2-(5-(4-((1-(3-propyl oleanolamide)-1,2,3-triazol-4-yl)methoxy) phenyl)-1*H*-imidazol-2-yl)pyridine difluoride (BOPIM-OA)

BOPIM-1 (96 mg, 0.30 mmol) and **azide-OA** (150 mg, 0.28 mmol) were dissolved in a mixture of chloroform and water (10 mL, 1:1 v/v). $CuSO_4 \cdot 5H_2O$ (17 mg, 0.07 mmol) and sodium ascorbate (24 mg, 0.12 mmol) were successively added and the mixture was vigorously stirred overnight at room temperature. The organic layer was separated and washed with an EDTA solution and the solvent was evaporated. The target product **BOPIM-OA** was obtained as a yellow solid in quantitative yield following purification using column chromatography (silica gel, petroleum ether/EtOAc, 2:1); ^{1}H NMR: δ 8.44 (d, *J* = 5.7 Hz, 1H), 8.18 (t, *J* = 8.0 Hz, 1H), 8.00 (d, *J* = 8.1 Hz, 1H), 7.76 (m, 3H), 7.50 (s, 1H), 7.48 (m, 1H), 7.07 (d, *J* = 8.8 Hz, 2H), 6.19 (t, *J* = 5.8 Hz, 1H), 5.43 (t, *J* = 3.4 Hz, 1H), 5.26 (s, 2H), 4.42 (m, 2H), 4.12 (q, *J* = 7.2 Hz, 1H), 3.45 (m, 1H), 3.21 (m, 1H), 3.00 (m, 2H), 2.88 (s, 1H), 1.16, 0.98, 0.92, 0.91, 0.90, 0.77, 0.73 (s, each 3H); ^{13}C NMR: δ 178.9, 158.2, 146.2, 145.8, 144.6, 144.1, 141.2, 138.5, 132.6, 127.3, 123.3, 123.1, 123.1, 122.6, 117.5, 115.3, 78.9, 77.27, 77.0, 76.7, 62.1, 55.1, 47.9, 47.5, 46.7, 46.4, 42.0, 39.3, 38.7, 38.4, 36.9, 36.3, 34.1, 32.9, 32.8, 32.3, 30.7, 30.2, 29.7, 28.1, 27.2, 25.7, 23.8, 23.6, 23.5, 21.1, 18.2, 16.9, 15.5, 15.3, 14.2, 1.0; IR (KBr, cm^{-1}): 3421 (OH), 1635 (C=C, C=N), 1150 (B-F), 995 (B-N); HRMS: m/z Calcd for $C_{50}H_{67}BF_2N_7O_3$ 862.5367 $[M+H]^+$. Found 862.5347.

Cell culture

Cells from the rat embryonic heart-derived myogenic cell line H9c2 and the human cervical carcinoma HeLa cell line (Shanghai Institute of Cell Biology, Chinese Academy of Science, China) were grown in DMEM or RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) in a humidified incubator containing 5% CO_2 at 37°C.

MTT assay for cell viability assay

Cell viabilities were measured *in vitro* in the H9c2 and HeLa cell lines using a modified MTT assay [22] After 48 h of incubation with one of the compounds, the culture medium was carefully removed and 20 µL of MTT was added to each well. The plates were incubated at 37°C for 4 h. The medium was removed and the resulting formazan crystals were dissolved in 100 µL DMSO. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. The plates were mixed on a microshaker for 10 min and the absorbance of the samples was measured at 570 nm using a microplate reader (Tecan). For each compound, the percent viability was plotted against concentration and the IC_{50} (concentration required to reduce viability by 50%) value was calculated relative to the untreated controls.

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