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Design and synthesis of novel photoaffinity probes for study of the target proteins of oleanolic acid

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ABSTRACT

To explore the molecular mechanisms of oleanolic acid, two novel photoaffinity probes were synthesized based on the structure–activity relationship reported previously. Their potency were evaluated in an enzyme inhibition assay against rabbit muscle glycogen phosphorylase a (RMGPa), a known target protein of oleanolic acid. The inhibitory activity of probe **2** was only about two-fold less potent than the mother compound oleanolic acid. The photoaffinity labeling experiments were also performed and two proteins were specifically tagged by probe **2**. The results suggest that the synthesized probes could be used as powerful tools to isolate and identify the target proteins of oleanolic acid.

The identification of target proteins for bioactive molecules is always an essential task in modern drug development that can extend our understanding of the drug's mechanisms and provide information for designing new drug candidates.[1] Furthermore, the discovery of new druggable target proteins may provide the biochemical evidence for their therapeutic effects in different diseases.[2]

Oleanolic acid (**OA**, **1**), a naturally occurring bioactive molecule, has been successfully used as an anti-hepatitis drug in China for more than 20 years.[3] It has been reported to possess various biological activities including antitumor, anti-inflammation, anti-oxidation, anti-HIV, anti-hyperglycemia, hepatoprotective and cardioprotective acitvities.[4] Given the clinical significance and biological importance of **OA**, the identification of its target proteins is highly desirable. To date, partial target proteins of **OA** have been proposed, such as glycogen phosphorylase, protein tyrosine phosphatase 1B (PTP1B) and α-glycosidase[5–8] in the modulation of glucose metabolism, and peroxisome proliferators activated receptors (PPARs), nuclear factor κB (NF-κB) and phospholipase A_2 (PLA_2) for its anti-cancer activity.[9–12] Although the mechanisms mentioned above cover many therapeutic facets of **OA**, the target proteins of **OA** are still not completely realized so that many preventive and therapeutic functions of **OA** can not be explained until now. Therefore, more studies in this area are warranted.

For investigation of the potential target proteins of **OA**, two trifunctional photoaffinity probes were designed based on the previous primary structure–activity relationship which indicated that the modifications of **OA** at C-3 position had little effect on its activities.[6] Consequently, we modified **OA** at C-3 position with a benzophenone photophore for covalent labeling and a biotin reporter group for detecting and purifying the target proteins. Moreover, in order to avoid the sterically hindrance caused by the biotin tag, we designed another tag-free probe which employed an azide handle for downstream conjugation to reporter tag via the click-chemistry reaction after proteome labeling. Herein, we describe the synthesis of those probes and the evaluation by the glycogen phosphorylase inhibition assay and photoaffinity labeling experiments, the reaction of the tag-free probe **3** with the biotin-derived alkyne on a simple model system was also studied.

The synthesis of intermediates **8** and **12** is outlined in Scheme 1. Treatment of toluene **4** with 4-nitrobenzoyl chloride **5** in the presence of $AlCl_3$ at room temperature afforded benzophenone **6** in 78% yield. Acid-catalyzed oxidation of compound **6** with CrO_3 gave the corresponding acid **7**. Acid **7** was treated with $SOCl_2$ to yield acyl chloride **8**. On the other hand, reaction of 2-(2-aminoethoxy)ethanol **9** with benzyl bromide in the presence of K_2CO_3 in CH_3CN at 50 °C gave amino–alcohol **10**. Alkylation of **10** with bromoacetic acid afforded the corresponding acid **11**. Acyl chloride **12** was obtained upon treating **11** with $SOCl_2$.

The synthesis of probe **2** is shown in Scheme 2. According to the procedures reported previously,[6] reaction of **1** (**OA**) with benzyl chloride afforded benzyl ester **13** in high yield. Esterification of

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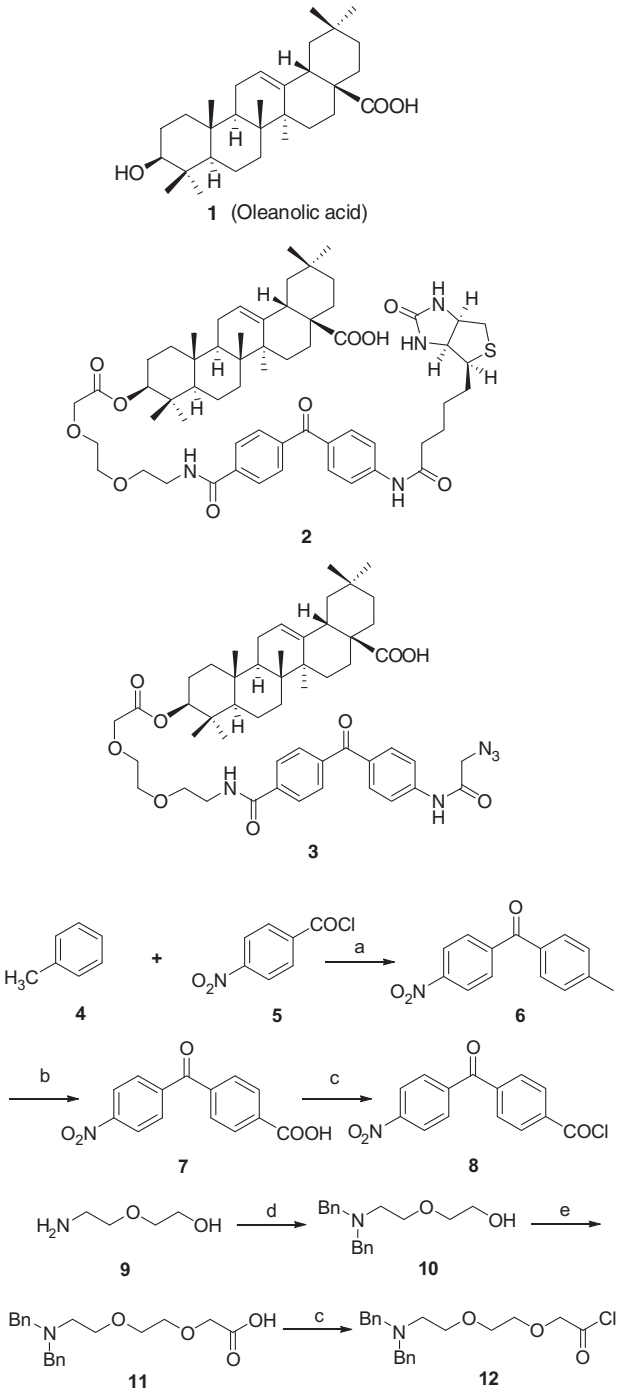


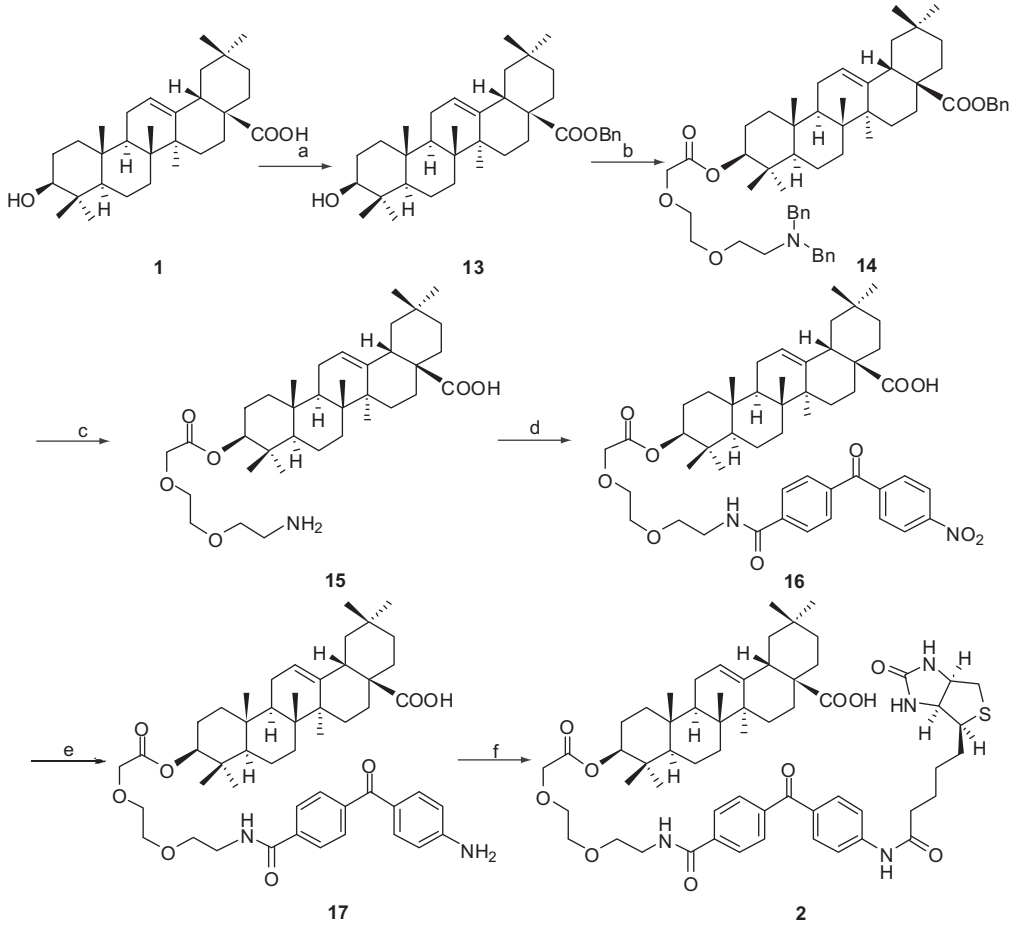
Scheme 1. Reagents and conditions: (a) $AlCl_3$, rt; (b) CrO_3, H_2SO_4, HAc, 0 °C; (c) $SOCl_2$, 80 °C; (d) BnBr, K_2CO_3, CH_3CN, 50 °C; (e) $BrCH_2COOH$, NaH, THF, reflux.

13 with **12** afforded ester **14**. Hydrogenolysis of **14** over Pd-C in THF furnished acid **15** in 47% yield. Amidation of **15** with **8** afforded amide **16**. Reduction **16** with stannous chloride afforded amine **17**. Coupling of amine **17** with D-biotin in DMF in the presence of isobutyl chloroformate and *N*-methylmorphorpholine as the condensing agents gave the probe **2**.

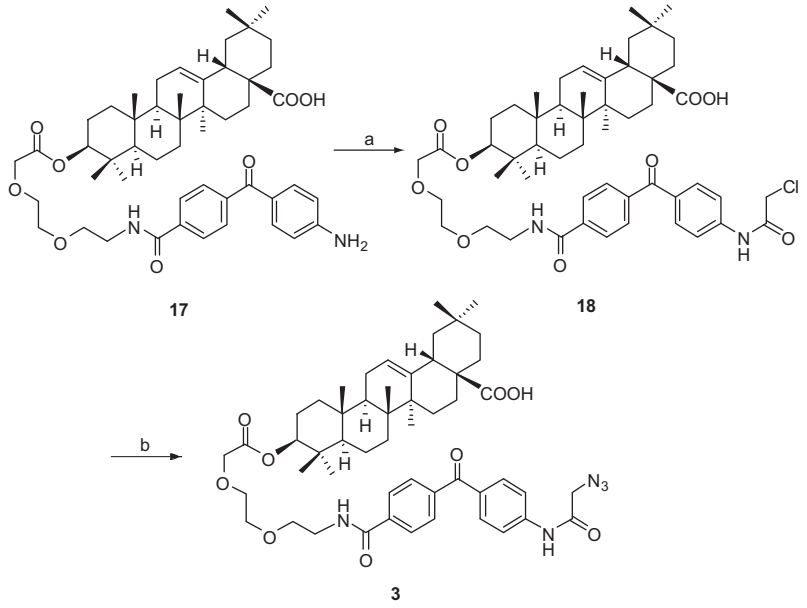
The synthesis of tag-free probe **3** is summarized in Scheme 3. Amidation of **17** with chloroacetyl chloride afforded compound **18**, which was converted to probe **3** via nucleophilic substitution reaction with sodium azide.

Next, we attempted to study the click-chemistry process about the tag-free probe **3** on a simple model system (Scheme 4). Esterification of **19** (biotin) with propargyl bromide afforded alkyne **20**. Treatment of **20** with probe **3** under typical click conditions (copper sulfate, sodium ascorbate as reducing agent) resulted in the ligantion product **21** in high yield.

It is obviously important that the synthesized probes retain potency in bioassays. Therefore, probes **2** and **3** were evaluated in an enzyme inhibition assay against rabbit muscle glycogen phosphorylase a (RMGPa), a known target protein of **OA**. The activity of RMGPa was measured through detecting the release of phosphate from glucose-1-phosphate in the direction of glycogen synthesis based on the published method.[13] The results are summarized in Table 1. The newly synthesized probes exhibited moderate

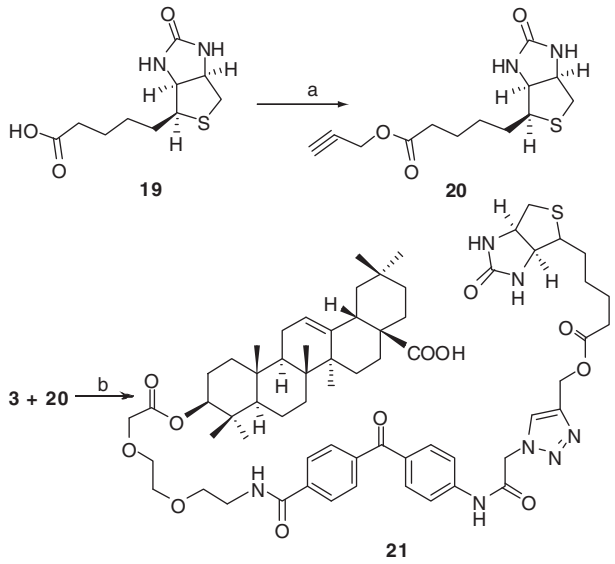


Scheme 2. Reagents and conditions: (a) BnCl, K_2CO_3, DMF, 60 °C; (b) **12**, Et_3N, CH_2Cl_2, rt; (c) H_2, 10% Pd-C, THF, rt; (d) **8**, pyridine, rt; (e) $SnCl_2$, ethanol, reflux; (f) D-biotin, isobutyl chloroformate, HMM, DMF.



Scheme 3. Reagents and conditions: (a) chloroacetyl chloride, Et_3N, CH_2Cl_2, rt; (b) NaN_3, DMF, 55 °C.

inhibitory activity against RMGPa in spite of the incorporation of benzophenone photophore and reporter group. Especially, probe **2** (IC_{50} = 41.4 μM) exhibited only about two-fold less potent than the mother compound **OA**. Therefore, the probe **2** may be very



Scheme 4. Reagents and conditions: (a) propargyl bromide, K_2CO_3, DMF, rt; (b) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2–H_2O, rt.

Table 1
Inhibition of rabbit muscle GPa by compounds **1–3**

Compound	RMGPa[a] IC_{50} (μM)
1	22.9 ± 1.8
2	41.1 ± 3.4
3	114.9 ± 10.5
Caffeine[b]	75.3 ± 6.6

[a] Each value represents the mean ± SD of three experiments.
[b] Caffeine was used as a positive control.

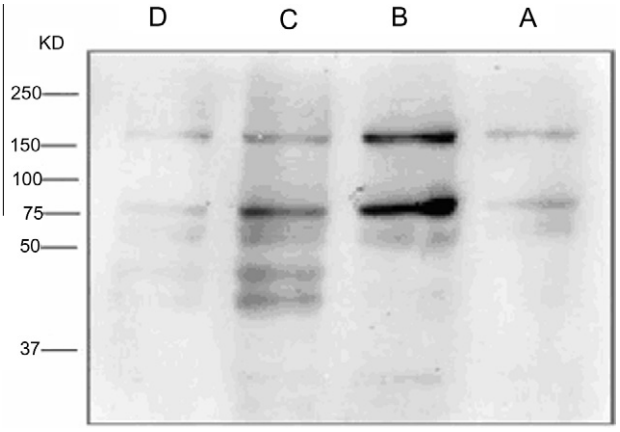


Figure 1. Results of SDS–PAGE analysis of the photoaffinity labeling experiment by synthesized probe **2**. Photoaffinity labeling of the soluble proteomes prepared from HepG2 cells followed SDS–PAGE electrophoresis and then transfer onto PVDF membrane and detection with streptavidin-HRP. Samples were prepared by incubating 2.0 mg/mL proteomes at different conditions: (A) with 0 μM probe **2** and exposed to UV for 30 min; (B) with 10 μM probe and exposed to UV light for 0 min; (C) with 10 μM probe and exposed to UV light for 30 min; (D) with 10 μM probe and 1 mM **OA** and then exposed to UV light for 30 min.

promising tool for isolation of the target proteins of **OA**. To our surprise, the incorporation of azide moiety resulted in greatly diminished activity in the assay.

We next tested the synthesized probe **2** in photoaffinity labeling experiments to detect the target proteins of **OA**. The soluble proteomes prepared from HepG2 cells were incubated with 10 μM probe **2** and then exposed to UV light for 30 min, and separated by SDS–PAGE. The results showed that two proteins, whose molecular weights were about 40–50 kDa, were tagged by probe **2**. This labeling was specific since it was competed by **OA** and no such labeling was seen in the absence of UV irradiation condition (Fig. 1). These results demonstrated that the synthesized probe **2** might be used to label the target proteins of **OA**.

In conclusion, the aim of this study was to develop specific reagents for isolation of **OA** target proteins. We have synthesized two photoaffinity probes and evaluated their potency in an enzyme inhibition assay against RMGPa. The results showed that the probe **2** exhibited inhibitory activity against RMGPa with an IC_{50} value of 41.4 μM. Photoaffinity labeling experiments were also performed and two proteins with 40–50 kDa in MW were specifically tagged by probe **2**. These data suggest that the synthesized probes might be used to label, identify and purify the target proteins of **OA**. We are now in the process of isolating the protein bands and microsequencing.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.123.

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