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2-Bromopalmitate Analogues as Activity-Based Probes To Explore Palmitoyl Acyltransferases

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Supporting Information

ABSTRACT: Reversible S-palmitoylation is an important post-translational modification that regulates the trafficking, localization, and activity of proteins. Cysteine-rich Asp-His-His-Cys (DHHC) domain-containing enzymes are evolutionarily conserved protein palmitoyl acyltransferases (PATs). The human genome encodes 23 DHHC-PATs that regulate diverse cellular functions. Although chemical probes and proteomic methods to detect palmitoylated protein substrates have been reported, no probes for direct detection of the activity of PATs are available. Here we report the synthesis and characterization of 2-bromohexadec-15-ynoic acid and 2-bromooctadec-17-ynoic acid, which are analogues of 2-bromopalmitate (2-BP), as activity-based probes for PATs as well as other palmitoylating and 2-BP-binding enzymes. These probes will serve as new chemical tools for activity-based protein profiling to explore PATs, to dissect the functions of PATs in cell signaling and diseases, and to facilitate the identification of their inhibitors.

Protein S-palmitoylation is a dynamic post-translational modification wherein the 16-carbon fatty acid (palmitate) is linked to cysteine residues through a labile thioester bond.[1,2] Palmitoylation increases the hydrophobicity of proteins, facilitates their attachment to intracellular and plasma membranes, and regulates their subcellular localization and functions.[3] Signaling molecules such as G-protein-coupled receptors, Ras GTPases, the Src family of kinases, neurotransmitter receptors, and endothelial nitric oxide synthase are palmitoylated, and dynamic palmitoylation/depalmitoylation is essential for their functions.[3,4]

Genetic studies in yeast have revealed that the Asp-His-His-Cys (DHHC) family of proteins are palmitoyl acyltransferases (PATs) that are evolutionarily conserved in many species.[1,5−7] Emerging evidence has shown that the DHHC family of PATs play important roles in normal physiology, and their deregulation has been linked to various pathological conditions. For example, increased risk of schizophrenia is associated with a single-nucleotide polymorphism in the *DHHC8* gene, which putatively encodes a truncated inactive enzyme.[8] DHHC17 (also known as huntingtin-interacting protein 14, HIP14) palmitoylates huntingtin protein and prevents the formation of toxic aggregates in Huntington's disease.[9] Mutations in *dhhc13* led to alopecia, osteoporosis, and systemic amyloidosis in a mouse model, highlighting its important function in regulating tissue homeostasis and aging.[10] In addition, amplification of *DHHC11* is linked to highly malignant bladder cancer and non-small-cell lung cancer[11,12] and *DHHC9* is overexpressed in the majority of microsatellite-stable colorectal cancers,[13] suggesting that they could be potential therapeutic targets for cancers. In addition to the DHHC family of PATs, some palmitoyl-Coenzyme A (CoA)-binding proteins might possess autoacylation activity.[14] Therefore, it is important to identify additional palmitoylating enzymes or autoacylating proteins that are involved in dynamic S-palmitoylation in signaling and diseases.

Despite their importance in signaling and disease, the detailed cellular functions of PATs have remained elusive. Activity-based protein profiling (ABPP) utilizes chemical probes to label the active sites and assess the functional states of large numbers of enzymes.[15] It has been successfully used to discover disease-relevant proteins from multiple enzyme classes, such as serine hydrolases, cysteine proteases, protein kinases, oxygenases, deiminases, and deubiquitinases. We sought to design activity-based probes that would enable imaging and proteomic studies of PATs via an irreversible inhibitor "warhead" and a "clickable" alkyne tag.[16] 2-Bromopalmitate (2-BP, **1**) is an irreversible pan-inhibitor of PATs with a half-maximal inhibitory concentration (IC_{50}) of 10−15 μM. 2-BP has been widely used as a tool inhibitor to study palmitoylation in signal transduction.[17−20] However, 2-BP also inhibits several other enzymes with IC_{50} values of ∼100 μM in vitro and might also alkylate multiple membrane proteins at higher doses (5−10 mM) in labeling experiments.[21] We synthesized the corresponding terminal alkyne analogues of **1**, 2-bromohexadec-15-ynoic acid (16C-BYA, **2**) and 2-bromooctadec-17-ynoic acid (18C-BYA, **3**) as activity-based probes to explore palmitoylating and other 2-BP-binding enzymes. On the basis of the putative mechanisms of the PAT enzymatic reaction[22] and the reactivity of the α-bromo carbonyl group, we proposed that 2-BP and probes **2** and **3** or their CoA derivatives[19,23] might covalently modify the active-site cysteine or other

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nucleophiles in PATs through the formation of an irreversible and stable thioether adduct (Figure 1).

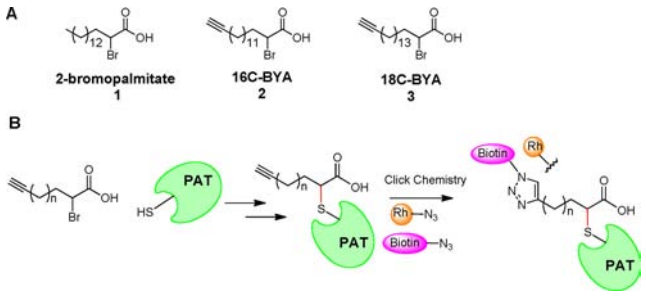

Figure 1. (A) Structures of 2-bromopalmitate (**1**) and activity-based probes **2** and **3**. (B) Labeling and detection of palmitoyl acyltransferase (PAT) by the activity-based probes (n = 11 or 13). Rh = rhodamine.

To test whether these probes can label PATs, HEK293A cells expressing hemagglutinin (HA)-tagged mouse DHHC4 (HA-DHHC4) were treated with the probes (100 μM) for 6 h with subsequent cell lysis and Cu-mediated 1,3-dipolar cycloaddition (click reaction) with biotin azide or rhodamine azide. Both **2** and **3** can effectively label DHHC4 (the lower protein band at ~37 kDa), as detected by streptavidin blot or in-gel fluorescence for the biotin and rhodamine conjugates, respectively (Figure 2A). Labeling of DHHC4 with probe **2**

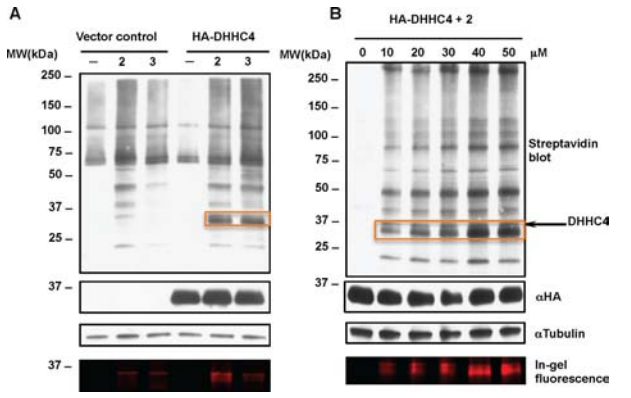

Figure 2. Activity-based probes **2** and **3** labeled HA-tagged DHHC4 protein in HEK293A cells. (A) HEK293A cells transfected with (left) control vector or (right) HA-DHHC4 expression vector were treated with dimethyl sulfoxide (DMSO) control (−) or probe **2** or **3** (100 μM) for 6 h. Click chemistry using biotin azide or rhodamine azide was carried out in cell lysates. The orange box indicates the HA-DHHC4 protein. The expression of HA-DHHC4 was confirmed by anti-HA Western blot. (B) HEK293A cells expressing HA-DHHC4 were treated with various concentrations of **2**, and DHHC4 protein was effectively labeled at **2** concentrations of 20−50 μM (orange box).

was assessed at various concentrations (Figure 2B) and at different time points [Figure S1 in the Supporting Information (SI)]. We observed that DHHC4 in cells could be labeled using **2** at concentrations as low as 10−20 μM, which are comparable to the IC_{50} values of 2-BP against PATs. We further tested whether **2** could directly label DHHC4 in cell lysate or in heat-inactivated lysate. Interestingly, we could not detect labeling of HA-DHHC4 when 100 μM **2** was added directly to the untreated or heat-inactivated lysates (Figure S2), suggesting that the labeling of PATs might require live cells. Next, we transfected HEK293A cells with HA-tagged wild-type DHHC2, -3, and -4 or their catalytically dead mutants (DHHC2 C156S, DHHC3 C157S, and DHHC4 C213S, respectively),[24] in which the Cys in the DHHC domain was mutated to Ser. Consistently, 100 μM **2** could effectively label the wild-type proteins but not the catalytically inactive mutants (Figure 3A), suggesting that **2** is indeed an activity-based probe for DHHC proteins.

Previously, ω-alkynyl fatty acids such as 15-hexadecynoic acid (C16, **4**) and 17-octadecynoic acid (C18, **5**) (Figure S3) have been reported as probes to detect palmitoylated proteins (i.e.,

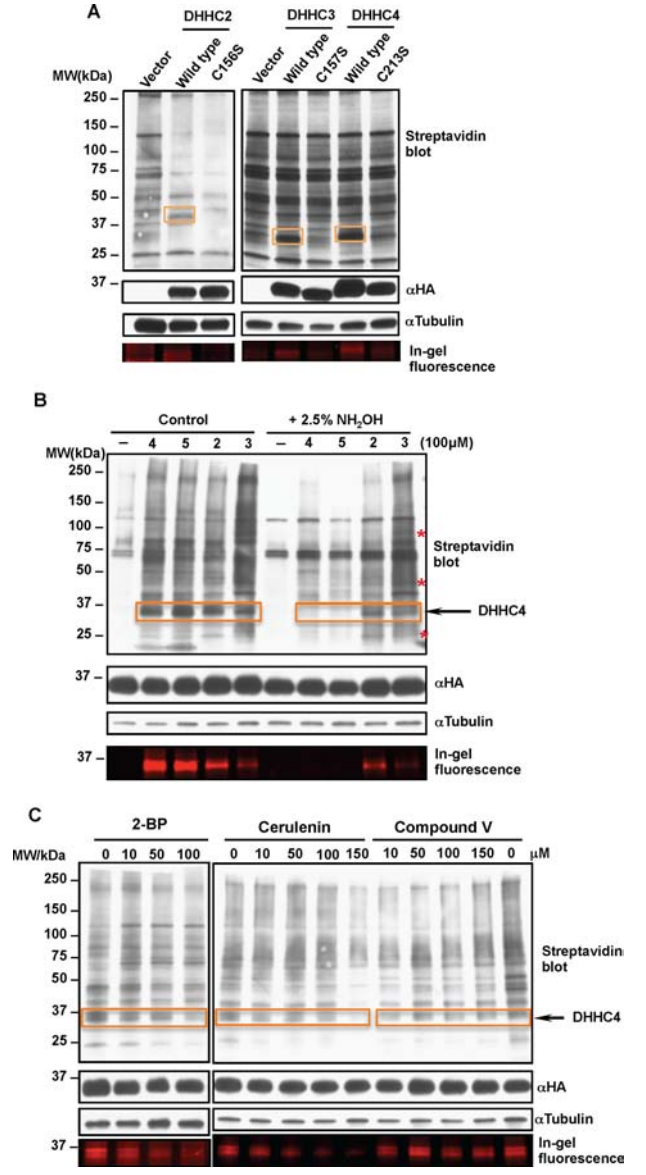

Figure 3. (A) **2** (100 μM) labels wild-type DHHC2, DHHC3 and DHHC4, but not their catalytically inactive mutants. (B) HEK293A cells expressing HA-DHHC4 were treated with DMSO (−) or probe **4**, **5**, **2**, or **3** (100 μM). Cell lysates were treated with or without 2.5% NH_2OH. The orange box indicates the labeled HA-DHHC4 protein. Red asterisks indicate other proteins labeled by **2** and **3**. (C) HEK293A cells expressing HA-DHHC4 were treated with various concentrations of PAT inhibitor (2-BP, cerulenin, or Compound V) and then with **2** (50 μM). Streptavidin blot and in-gel fluorescent scanning showed the competition of the PAT inhibitors with **2**.

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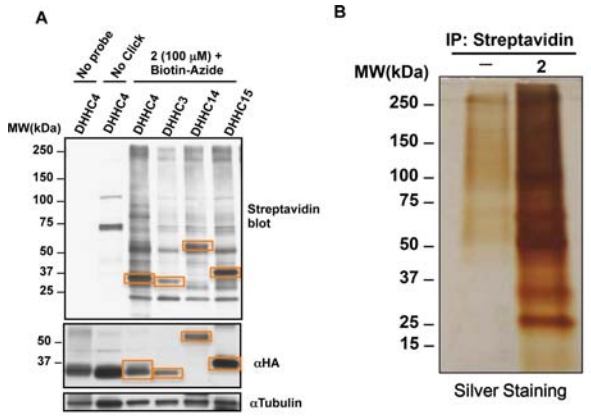
substrates of PATs) for proteomic and imaging studies.[25−27] These probes label the palmitoylated protein by forming a labile thioester linkage that can readily be removed by treatment with 2.5% hydroxylamine (NH_2OH).[28] We compared our PAT activity-based probes (**2** and **3**) with the substrate-labeling probes (**4** and **5**). Palmitoylation probes **4** and **5** (100 μM) could also label the exogenous DHHC4 (Figure 3B), possibly by forming an acyl-enzyme thioester adduct[22] or labeling the S-acylation sites of DHHC4.[29] Treatment with NH_2OH efficiently removed the labile thioester adducts formed with **4** and **5**. In contrast, DHHC4 labeling by **2** and **3** (100 μM) was resistant to NH_2OH treatment (Figure 3B), suggesting that **2** and **3** form a stable, irreversible adduct with the enzyme. Quantitative analyses of the intensities of the protein bands revealed that more than 70% of the DHHC4 remained labeled with **2** or **3** after NH_2OH treatment. In contrast, almost all of the adducts formed with **4** and **5** could be removed by NH_2OH (Figure S4). Although DHHC4 itself could be palmitoylated and the probes might label both the active site and the palmitoylation sites, our results indeed show that the labeling by **2** and **3** is resistant to NH_2OH treatment, in contrast to the labeling by the previously reported probes **4** and **5**. We also observed that in contrast to **4** and **5**, **2** and **3** labeled several unique and NH_2OH-resistant protein bands in HEK293A cells (marked with red asterisks in Figure 3B), which might be endogenous PATs or other palmitoyl-CoA-binding proteins.

2-BP, cerulenin (**6**), and 2-(2-hydroxy-5-nitrobenzylidene)-benzo[*b*]thiophen-3-one (Compound V, **7**) have been reported as inhibitors of DHHC-PATs with IC_{50} values ranging from low to high micromolar (Figure S5).[18,19,30] As activity-based probes could be good chemical tools for inhibitor identification, we set out to test whether these known PAT inhibitors could compete with activity-based probe **2**. We treated HEK293A cells expressing HA-DHHC4 with various concentrations of **1**, **6**, and **7** for 1 h and then added 50 μM **2** for 6 h, after which we carried out the click reaction with biotin azide or rhodamine azide in the cell lysates. We observed that **1** and **6** dose-dependently competed with **2**, while **7** did not compete (Figures 3C and S6). These results suggest that **1**, **6**, and **2** might bind to the same site on DHHC4, presumably the palmitoyl-CoA binding site. It is possible that **7** does not inhibit DHHC4, as it has been reported as a selective inhibitor of type-2 (myristoyl peptide) palmitoylation with an IC_{50} of 0.5 μM for an N-myristoylated peptide[30] and IC_{50}s of 10−50 μM using purified DHHC-PATs and their substrates proteins in enzymatic assays.[18] It is also possible that as a reversible inhibitor, **7** is not able to displace the probes under the current test conditions. As most of the DHHC-PATs are membrane-bound enzymes, the purification and evaluation of their enzymatic activities is not straightforward. Currently, radioactive palmitate or surrogate substrate peptides are often used to screen for small-molecule inhibitors of PATs.[30] By analogy to activity-based probes for other enzyme classes,[31] **2** could be used to develop a cell-based assay to identify palmitoyl-CoA competitive inhibitors of PATs, facilitating drug discovery efforts aimed at targeting PATs.

To evaluate the generality of our labeling method, we sought to use activity-based probe **2** to label other DHHC-family PATs. We expressed HA-tagged DHHC3, DHHC14, and DHHC15 in HEK293A cells and carried out labeling experiments with **2**. Figure 4A shows that HA-tagged PATs were successfully labeled. By Western blotting with anti-HA



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	Probe 2		–
	Exp. 1	Exp. 2	
Palmitoyltransferase ZDHHC18	2	4	0
Palmitoyltransferase ZDHHC5	9	7	0
Palmitoyltransferase ZDHHC6	8	1	0
Membrane bound O-acyltransferase 7 (MBOAT7)	5	2	0
Carnitine O-palmitoyltransferase 1	37	8	0
Acetyl-CoA acyltransferase	20	8	0
Lysophosphatidylcholine acyltransferase 1	20	13	0
Threonylcarbamoyladenosine tRNA methylthiotransferase	16	9	0
Chitobiosyldiphosphodolichol beta-mannosyltransferase	18	8	0
Acyl-CoA oxidase 3	16	3	0
Aldehyde Dehydrogenase 2	48	7	0
Asparagine synthetase	18	8	0
Hydroxyacyl-CoA dehydrogenase	35	9	0

Figure 4. (A) HEK293A cells expressing HA-tagged DHHC4, DHHC3, DHHC14, or DHHC15 were labeled by DMSO ("No probe", lane 1) or **2** (100 μM). Lane 2 ("No Click") shows the protein samples without the click reaction with biotin azide. Lanes 3−6 are samples after the click reaction with biotin azide. The orange boxes indicate the exogenous HA-tagged DHHC-PATs on the basis of their positions in the anti-HA Western blot. (B) A cleavable azide−azobenzene−biotin linker was used to purify probe-labeled endogenous proteins by control (−) or **2** (100 μM) in HEK293A cells. The eluted proteins were silver-stained. (C) Endogenous PATs, acyl transferases, and other proteins labeled by **2** (100 μM) and identified from mass spectrometry studies in two independent experiments. The number of total matched peptide spectra for each protein is listed.

antibody, we observed that the expression levels of DHHC3, DHHC14, and DHHC15 were correlated with the intensity of labeling by probe **2**. Thus, **2** may also be useful in quantifying the relative abundance of these enzymes in native systems. We next tested whether **2** could metabolically label endogenous proteins in HEK293A cells and the pancreatic cancer cell line (PANC1). We observed that **2** labeled proteins with molecular weights ranging from 25 to 100 kDa (Figure S7). To enrich and identify the labeled proteins, we utilized a cleavable azide−azobenzene−biotin linker for click chemistry to pull down the labeled proteins using streptavidin beads and then eluted the bound proteins by cleaving the azo linker using sodium dithionite[32] (Figure S8). We successfully enriched and eluted endogenous proteins labeled by **2** in HEK293A cells (Figure 4B). The eluted proteins were subjected to mass spectrometry studies, and we identified three endogenous DHHC-PATs (ZDHHC18, -5, and -6) from HEK293A cells (Figure 4C). In addition, other acyltransferases and acyl-CoA enzymes are also labeled by **2**. We also detected many known palmitoylated substrates (Table S1 in the SI), suggesting that 2-BP could be

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incorporated into a cellular lipid pool and utilized as an acyl donor in palmitoylation.

In conclusion, we have synthesized and characterized activity-based probes **2** and **3** based on **1**, a known irreversible pan-inhibitor of PATs. As DHHC-PATs are among the proteins labeled by the probes, **2** and **3** could be used to facilitate the identification of additional palmitoylating enzymes as well as in proteomic analyses of PATs in signaling, normal physiology, and diseases. In addition, they will also provide a format for identifying inhibitors of PATs for therapeutic applications. It is noted that DHHC-PATs are not the only cellular targets of **1** and its analogues. Indeed, probe **2** labeled many non-palmitoylating enzymes in our mass spectrometry studies. Because of the high reactivity, off-target effects, and non-selectivity, many classes of activity-based probes often label proteins outside of their intended target class. For example, a probe designed to target protein kinases also labels many nonkinases in mass spectrometry studies.[33] Although the specificity of ABPP probes is not absolute, they are valuable for enriching and characterizing disease-relevant enzymes.[15] The future development of additional activity-based probes with improved specificity and efficiency for targeting PATs might address the limitations of the probes described here.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, experimental protocols, supporting figures, full list of identified proteins from mass spectrometry studies (XLS) and full fluorescent gel images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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