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THE CHEMICAL RECORD

Development of Chemical Probes for Functional Analysis of Anticancer Saponin OSW-1

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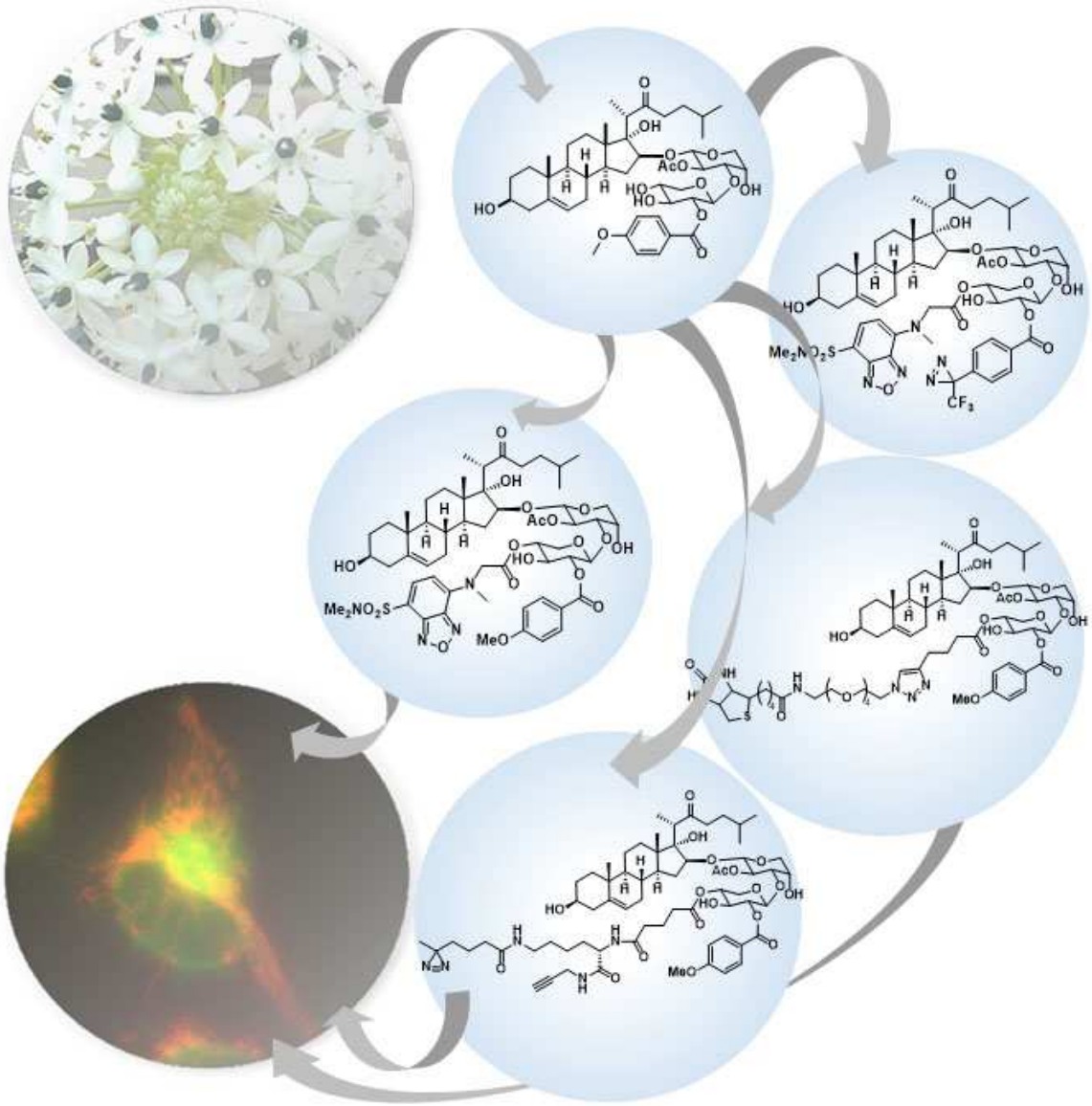

Abstract: Chemical probe-based approaches have proven powerful in recent years in the target identification studies of natural products. OSW-1 is a saponin class of natural products with highly potent and selective cytotoxicity against various cancer cell lines. Understanding its mechanism of action is important for the development of anticancer drugs with potentially novel target pathways. This account reviews recent progress in the development of OSW-1 derived probes for exploring the mechanism of its action. The key to the probe development is a judicious choice of functionalization sites and a selective functionalization strategy. The types of probes include fluorescent probes for cellular imaging analysis and affinity probes for target identification analysis.

Keywords: OSW-1, anticancer natural products, saponin, chemical probes, mechanism of action

1. Introduction

OSW-1 is a steroidal saponin isolated from the bulbs of *Ornithogalum saundersiae* by Sashida *et al.* more than 20 years ago.[1] It is structurally unique among the saponins having glycosylated at the D-ring of the sterol core and the acylation at each ring of the carbohydrate moiety.[2] It was initially identified as an active agent in a screening for cyclic AMP phosphodiesterase inhibitors. Subsequently in the National Cancer Institute 60-cell in vitro screen, it was revealed that OSW-1 displays not only a highly potent anticancer activity with an average GI_{50} of 0.78 nM but also a 10–100 fold selective cytotoxicity against cancer cell lines over non-malignant cells.[3] The bioinformatics analysis on the panel assay data predicted that it exerts cytotoxicity by a potentially novel mechanism, which is distinct from other known anticancer agents. In 2010, Shair *et al.* reported that OSBP and ORP4 L, two members of the oxysterol binding protein (ORP) superfamily implicated in lipid/sterol transport and sensing are the major protein targets of OSW-1.[4,5] Using competitive binding assays based on recombinant OSBP and ORP4 L and [^{3}H]-25-HC, their endogenous ligand, OSW-1 exhibits high affinity toward these proteins ($K_i = 26$ nM for OSBP and 54 nM for ORP4 L). However, the link between the inhibition of OSBP and ORP4 L and apoptotic induction has remained unclear. As a consequence, the molecular basis of the cancer selective activity of OSW-1 is not well understood.

Elucidation of the mechanism of action of natural products entails a complex challenge, having to identify the cellular site of action, the target proteins and the target cellular pathways.[6] Recently, chemical probe-based approaches have emerged as powerful methods for mechanistic studies of natural products in the cellular proteome.[7] Here we referred to chemical probes as functional analogues of compounds of interest, which are derivatized with functionalities required for mechanistic analysis. For example, fluorescently derivatized probes can reveal cell internalization and intracellular localization properties of the compound whereas affinity probes with an affinity tag such as biotin moiety enables the binding analysis between the compound and the target proteins and the affinity purification of target proteins for their subsequent sequence identification by mass spectrometry. The major advantage of the chemical probe-based approaches to target identification of natural products lies in their ability to directly determine the target proteins. The discovery of target proteins should provide the first critical step toward deciphering the target cellular pathway leading to the phenotypes of interest.

Because of the biomedical interest, several groups have achieved total synthesis of OSW-1 and a number of its analogues have been synthesized to decipher the structure-activity relationship (SAR) and to explore more potent analogues.[8] In contrast, a relatively few studies have been reported on the development of chemical probes of OSW-1 for investigation of its biological role.[4,9,10] Here, we summarize our recent efforts in the synthesis, biological properties of probes derived from naturally occurring OSW-1 and their application to the analysis of intracellular localization and the interaction with known target proteins.

2. Synthesis of Fluorescent Probe by Site-Selective Acylation of OSW-1

As a first step toward understanding the cellular effects of OSW-1, we aimed to elucidate its site of action and its cellular targets. It was then unknown whether OSW-1 translocates plasma membrane and has a specific distribution in cells. To investigate the cellular localization of OSW-1, we thus planned to develop a fluorescent probe based on naturally occurring OSW-1. We chose to employ a chemical modification approach to develop chemical probes based on naturally

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occurring OSW-1 for two reasons. First, OSW-1 is not commercially available nor is readily available through synthesis but could be prepared from natural sources. Secondly, several congeners with varying degrees of activity have been identified from the same plant source,[1] which could be used as negative control compounds in the biological studies of OSW-1. For example, congener **1** displays a cinnamoyl group instead of *p*-methoxybenzoyl (MBz) group at C2″ position of the xylose residue, which has been found with a 4-fold higher activity than OSW-1. Of particular interest are two deacylated congeners: **2** lacks MBz group at C2″ of the xylose residue with 10-fold decreased anticancer activity whereas **3** lacks both MBz group and acetyl (Ac) group at C2' of the arabinose residue with 100-fold decreased activity.[1,3]

Successful design of chemical probes for natural products needs to meet the following criteria: 1) the derivatized analogue retains the activity of the parent natural product, 2) functional groups should be appropriately positioned to serve their purposes. In the case with fluorescent probes, a fluorophore should be introduced distal to the pharmacophore of the parent molecule to avoid imposing steric hindrance. To fulfill these requirements, prior knowledge on the SAR of natural products is critical. The early studies on SAR of OSW-1 indicated that both the sterol aglycone and the sugar moiety are required and their relative orientation to each other are important for its extremely potent cytotoxicity.[1,3,8] In particular, the sterol side chain at C21 and the acyl groups of the disaccharide residue were found essential. Our structural analysis on OSW-1 and the cinnamoyl analogue by NMR and the X-ray crystallography showed that the C21-sterol side chain, C2'-Ac group and C2″-MBz group form a hydrophobic cluster, leading us to propose it as a protein binding site (Figure 1).[11] Based on these findings, C3″ or C4″-hydroxyl groups at the terminal xylose residue was selected as the suitable site of functionalization.[12,13]

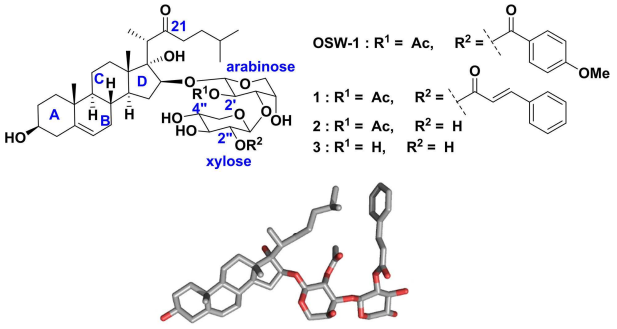

Figure 1. Structures of OSW-1, its naturally occurring congeners (**1**–**3**), crystal structure of **1**.[11]

The synthesis of the fluorescent probe of OSW-1 (**4**) is shown in Scheme 1a. We employed 4-(*N*,*N*-dimethyl aminosulfonyl)-2,1,3-benzoxadiazole (DBD) group as a fluorescent tag with an expectation that its small size should have minimal structural perturbation. Our initial effort to synthesize DBD-probe by standard acylation method proved low yielding.[12] Because naturally occurring OSW-1 is limited in supply, we subsequently developed an efficient site-selective functionalization strategy to modify OSW-1.[13] Based on the report by Onomura *et al.*,[14] we developed a method to site-selectively acylate C4'-OH of the monomeric xyloside derivative with a variety of substituents in the presence of Me_2SnCl_2. Me_2SnCl_2 serves to activate 1,2-diols and with the use of a hindered base such as *N*,*N*-diisopropylethylamine (DIPEA), and allows acylation to occur at the less sterically hindered hydroxyl group. It was found that catalytic amounts of Me_2SnCl_2 are inefficient in acylating the xyloside with alkyl acid chlorides such as DBD-COCl and hexynoyl chloride. Screening of molar equivalents of the catalyst, acylating reagents and substrate concentration showed that 2 to 4-fold excess amounts

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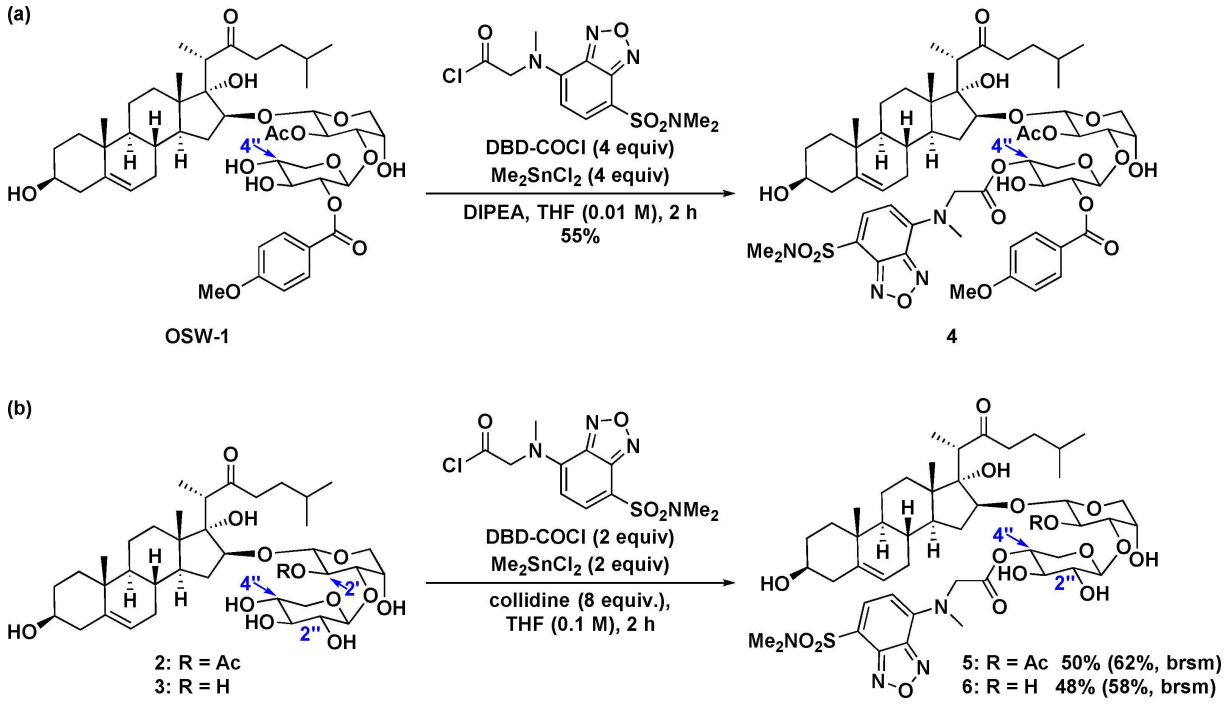
Kaori Sakurai received her Ph. D. in chemistry from Princeton University in 2002. From 2003 to 2006, she studied as a postdoctoral fellow at the Department of Chemistry and Chemical Biology, Harvard University. She started her independent research group at the Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology in 2006, where she is currently an associate professor. Her research interests are focused on the development and application of new chemical probes for identification and analysis of protein targets of natural products and bioactive small-molecules.



Scheme 1. Synthesis of fluorescent probes (**4**–**6**) derived from (a) OSW-1 and (b) its congeners **2**–**3** by Me_2SnCl_2-mediated site-selective monoacylation strategy.

of the catalyst are required to achieve improve acylation yields. Since OSW-1 was available in minute quantity, we further optimized the reaction conditions to efficiently functionalize it with acyl groups under a dilute substrate concentration. Upon treatment of OSW-1 at 0.01 M in CH_2Cl_2 with 4 equivalents of the Me_2SnCl_2 and DBD-COCl, the desired 4″-*O*-acylated product **4** was obtained in 55% yield (Scheme 1a) with the 3″-*O*-acylated product in 6%, while no bis-acylated product was observed.[13] It was thus demonstrated Me_2SnCl_2 can efficiently promote site-selective acylation of a polyol natural products.

To study the structure-cellular localization property relationship, the fluorescent probes of the deacylated congeners of OSW-1 were also synthesized (**5**-**6**, Scheme 1b). The Me_2SnCl_2-mediated site-selective acylation method was applied to synthesize fluorescent probes **5** using the deacylated OSW-1 **2** with six hydroxyl group and the acetylated OSW-1 **6** using the dideacylated OSW-1 **3** with seven hydroxyl groups. Deacetylated congeners were successfully acylated at C4″-OH to give **5** in a 50% yield (62%, based on the recovered starting material, brsm)[15] and **6** in 48% (58%, brsm).[16]

The fluorescent probes **4**–**6** were tested using XTT assay for their antiproliferative activity in HeLa cells.[16] The IC_{50} values of the probes **6** (**4**: 1.4 nM, **5**: 38 nM, **6**: 3300 nM) were similar to these of the parent compounds (**1**: 0.46 nM, **2**: 83 nM, **3**: 5200 nM). These results strongly suggested that the fluorescently-tagged analogues serve as valid functional mimics of the parent natural products in live cell studies. We thus successfully prepared a series of related fluorescent probes from OSW-1 and its congeners in one step in good yields and in a site-selective fashion by using Me_2SnCl_2.

With the appropriate fluorescent probes in hand, we then applied them to the cell-imaging studies to investigate cell internalization and intracellular distribution of OSW-1 and its congeners.[16] All three fluorescent probes **4**–**6** were rapidly untaken up by cells and co-localized with specific markers of endoplasmic reticulum (ER) and Golgi apparatus (Figure 2). We thus showed for the first time that OSW-1 is internalized into cells and that it potentially targets these subcellular organelles. Comparison of the data for **4**–**6** suggested that differences in cytotoxicity of these compounds are not due to differences in their cell permeability nor subcellular localization but more likely due to differences in the direct interaction with target proteins. They also suggested that the sterol moiety is responsible for transporting OSW-1 and the congeners to ER and Golgi apparatus. In contrast, Ac and MBz groups on the sugar residues do not affect their cell localization property but are likely involved in target protein binding. More recently, we have demonstrated by using high-resolution microscopy that fluorescent OSW-1 **4** selectively targets Golgi apparatus. The study furthermore led us to reveal that OSW-1 induces the Golgi stress response pathways leading to cell death.[17]

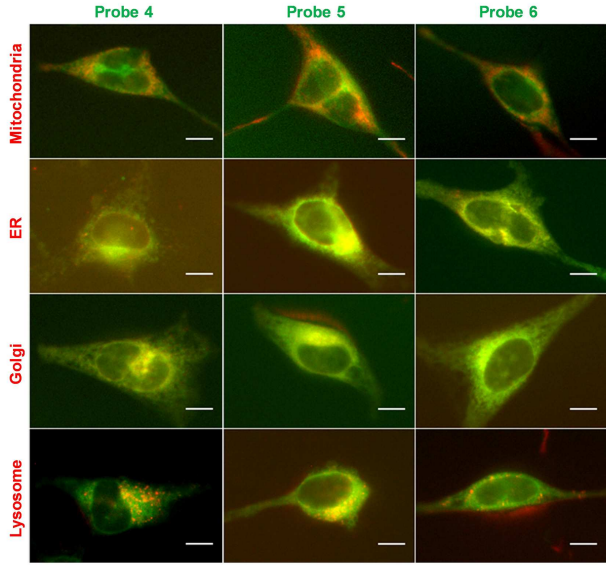


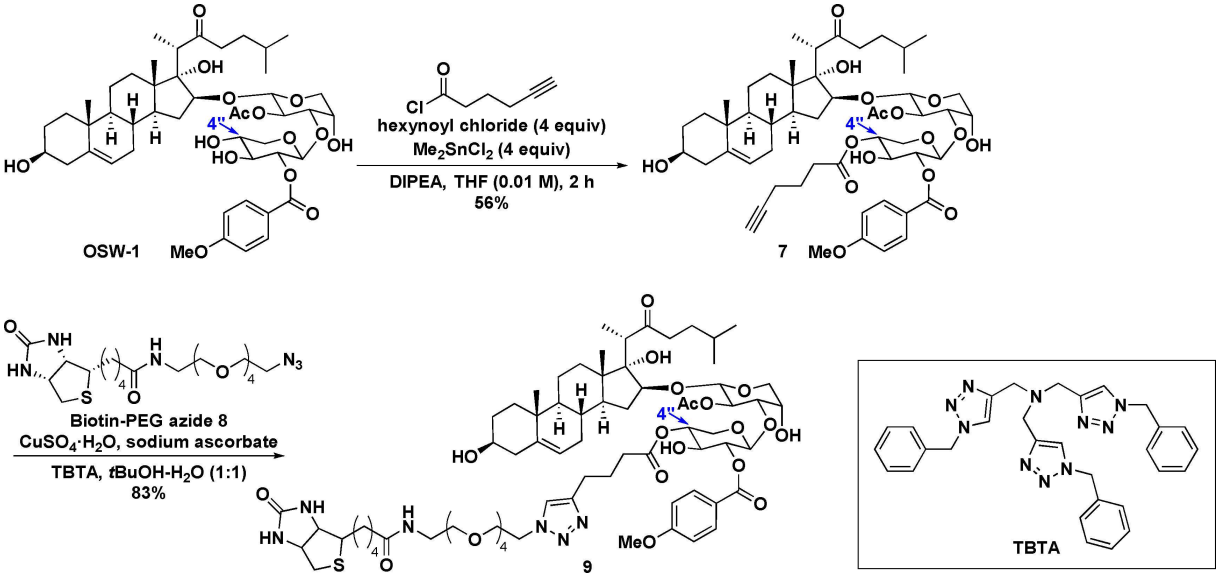
Figure 2. Intracellular localization of fluorescent probes **4**–**6** shown in green. HeLa cells were incubated with probes (1 μM) for 1 h and co-stained for mitochondria by tetramethylrhodamine etheyl ester, the Golgi apparatus by BODIPY-TRX ceramide, endoplasmic reticulum by ER Tracker Red and lysosome by Lysosome Tracker Red (shown in red). Scale bars: 10 μM.

3. Development of Affinity Probes for Target Protein Analysis

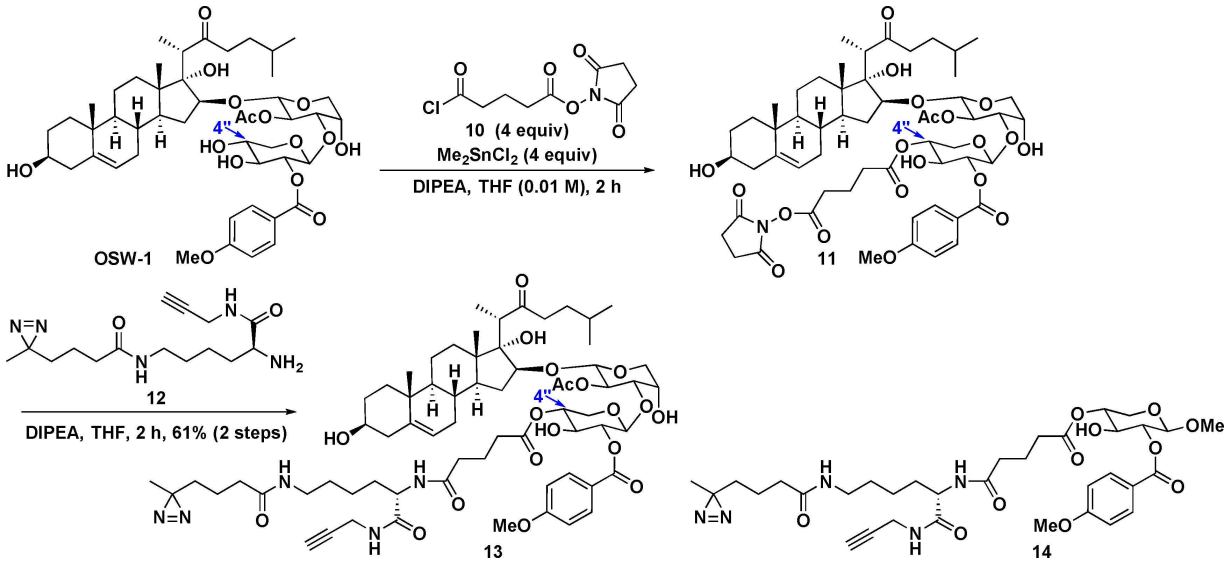
We next developed a set of affinity probes toward investigation of cellular targets of OSW-1.[13,18] For simple target enrichment experiments, we designed a biotinylated derivative of OSW-1 (**9**). A biotin tag has been widely utilized in affinity purification of target proteins using avidin-immolized resins by virtue of the strong biotin-avidin interaction. It has been shown that the linker length for appending the biotin tag has a strong impact on the efficiency of affinity purification.[19] We included tetraethylene glycol as a hydrophilic linker between a biotin tag and OSW-1 so that the steric hindrance would be minimal.[20] As in the case with a fluorescent tag, a biotin tag was to be attached at C4″-OH to avoid the putative pharmacophore of OSW-1 and to facilitate ready synthesis.

To synthesize the biotin probe **9**, alkyne group was first introduced to C4″-OH of OSW-1 by Me_2SnCl_2 promoted site-selective acylation using hexynoyl chloride to give **7** in 55% yield (Scheme 2). Subsequently, biotin-PEG-azide **8** was conjugated to alkyne-tagged OSW-1 (**7**) by click chemistry under copper-promoted alkyne – azide cycloaddition condition in 83% yield.[13]

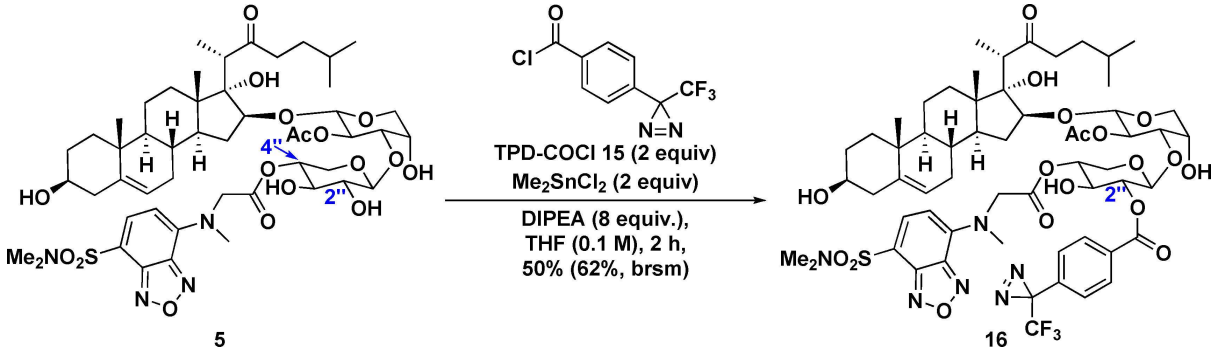
We next designed photoaffinity probes **13** (Scheme 3) and **16** (Scheme 4) involving a photoreactive group to crosslink target proteins and a reporter tag for photoaffinity labeling (PAL), which have proven powerful in recent years in the chemical probe-based approaches to target identification studies.[7b,21] They are particularly useful in detecting and analyzing target proteins in live cells, which is not possible with the affinity chromatography based approaches. Alkyl diazirine group[22] was included in probe **13** as a photoreactive group to crosslink the target proteins based on its small size and selective reactivity. Our previous studies comparing the major photoreactive groups (benzophenone, phenylazide and



Scheme 2. Synthesis of biotinylated OSW-1 **9** as an affinity probe.



Scheme 3. Two-step synthesis of OSW-1-based clickable photoaffinity probe **13**.



Scheme 4. Synthesis of OSW-1-based fluorescent photoaffinity probe **16** by sequential site-selective acylation reaction using Me_2SnCl_2.

alkyl diazirine) found that alkyl diazirine provides the most selective photoreactive group in capturing low affinity carbohydrate-protein interactions.[23] Moreover, the terminal alkyne group was employed for **13** as a small and clickable chemical handle, which facilitates conjugation of a reporter group of choice such as a fluorophore or biotin.[24] We planned a two-step approach to capturing target proteins, where PAL and click chemistry are sequentially conducted before the affinity purification step (Figure 3a). We employed a two-step synthesis where we first site-selectively derivatized C4″-OH with a glutaryl linker **10** to generate an *N*-hydroxysuccinimide ester **11**, which was then coupled with a photoaffinity label **12** to give the desired alkyne-tagged photoaffinity probe **13** in 61% yield (Scheme 3).[18]

For the design of photoaffinity probes, a photoreactive group should ideally be placed at the protein binding site while a detection group at any site distant from the protein binding residues to achieve maximal crosslinking efficiency without compromising the bioactivity and effective post-labeling analysis. We designed another type of a photoaffinity probe, in which a photoreactive group is positioned at C2′-OH, a putative protein binding site of OSW-1. It has been known that various aromatic carboxylic acid ester groups can substitute C4″-OH of OSW-1 without loss of activity.[1,3,25] We thus employed trifluorophenyl diazirine (TPD) group[26] as an aromatic photoreactive group to replace MBz group of OSW-1. To simplify the synthetic scheme, a fluorescent tag was introduced to C4″-OH of OSW-1. We envisioned that probe **16** could be synthesized by sequential acylation of a naturally occurring deacylated OSW-1 (**2**, Scheme 4) at C4″-OH and then at C2″-OH by using Me_2SnCl_2.[15] Treatment of **5** under the Me_2SnCl_2-mediated acylation conditions using acid chloride **15** gave the desired C2″-OH acylated product in 50% (62% brsm). While extensive structure–activity relation-

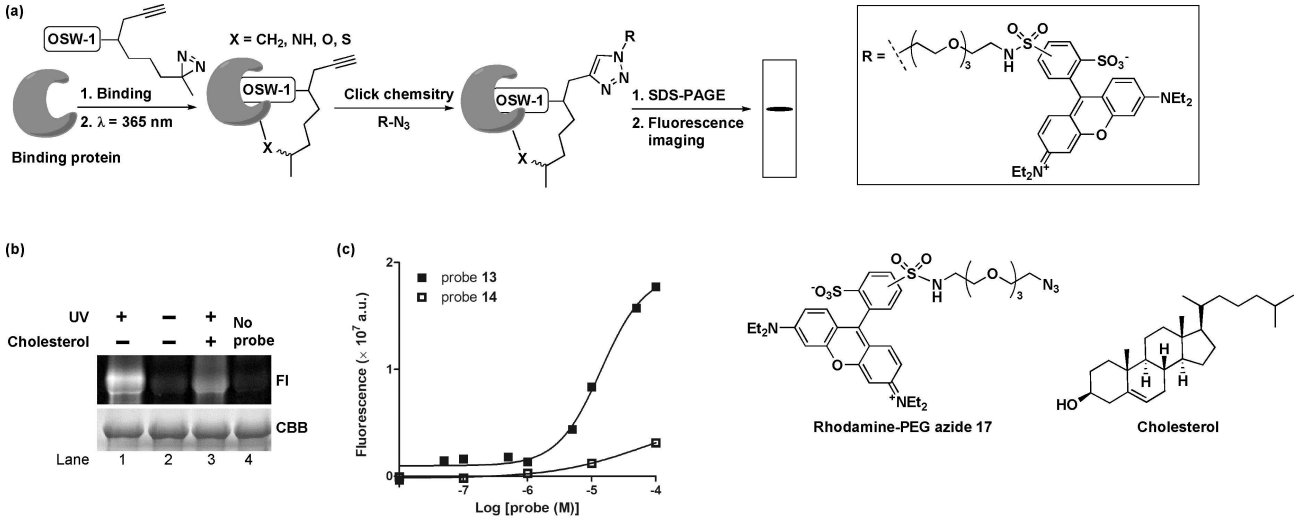


Figure 3. (a) Photoaffinity labeling-click chemistry approach to capture and detection of binding proteins by using a clickable photoaffinity probe. (b) Photoaffinity labeling of BSA by using clickable photoaffinity probe **13**. Rhodamine-PEG azide **17** was used to detect the probe-crosslinked BSA. Fl: fluorescently scanned image; CBB: Coomassie Brilliant Blue stained image. Cholesterol was used as a competitive ligand. (c) Dose-dependence of photoaffinity labelling by probe **13** in comparison with a control probe **14**.

ship studies have been conducted for OSW-1, probe **16** represented the first example in the synthetic modification of the biologically essential C2″-moiety. It thus demonstrated the versatility and straightforward utility of our Me_2SnCl_2-mediated site-selective modification method, which can be used to access not only the C4″-substituted analogs of OSW-1 but also the C2″-analogues.

The antiproliferative activity of probe **13** and **16** were evaluated by XTT assay.[13,18] The IC_{50} of the probes were found to be comparable (**13**: 1.1 nM; **16**: 3.2 nM) to that of OSW-1 (0.46 nM). It thus suggested that they serve as suitable cell permeable probes to explore OSW-1 binding proteins in live cell studies.

To first assess the reactivity of the photoaffinity probe **13**, PAL and click chemistry were sequentially performed using bovine serum albumin (BSA) as a model sterol binding protein (Figure 3a).[18] PAL experiments were typically conducted by incubating a photoaffinity probe with the protein solution at 4 °C and then were irradiated at 365 nm at 0 °C for 10 min. The reaction mixture was precipitated by cold acetone to remove the unreacted probe. The probe-crosslinked protein was conjugated to rhodamine-PEG azide **17** (Figure 3) by click chemistry. The resultant rhodamine-labeled protein was resolved by SDS-PAGE, which was then quantitated by in-gel fluorescence imaging. We confirmed that probe **13** reacted with BSA only under the reaction condition involving UV irradiation (Figure 3b, lane 1 and 2). To validate the specific reactivity of probe **13**, we also conducted a PAL reaction of BSA and **13** in the presence of cholesterol as a competitive ligand in 400-fold excess of **13** (Figure 3b, lane 1 and 3). These results demonstrated that **13** can photocrosslink a protein in a specific ligand-dependent fashion and that the protein crosslinked with probe **13** can be detected either by a fluorophore or biotin label through click chemistry. To demonstrate the affinity dependent reactivity, we reacted BSA with varied concentrations of probe **13** and subsequently with rhodamine-azide **17** by click chemistry (Figure 3c).[18] Probe **14** presents a negative control displaying a substructure of OSW-1 lacking the sterol moiety, which should not specifically bind BSA. A titration curve for probe **13** in Figure 3c shows that BSA was crosslinked by **13** in a dose-dependent fashion with the EC_{50} value of 14 μM. On the other hand, probe **14** showed marginal reactivity toward BSA even at 100 μM. Taken together, we have shown that probe **13** retains potent anticancer activity of the parent natural product and is capable of effectively photocrosslink specific binding proteins.

4. Summary and Outlook

In this review, we have described the design and synthesis of our chemical probes derived from naturally occurring OSW-1. Based on the previous structural analysis, C4″-OH was chosen as a functionalization site and the site-selective monoacylation strategy was successfully developed using Me_2SnCl_2 to access various OSW-1 analogues bearing a fluorescent tag, an alkyne tag, a biotin tag or a bifunctional photoaffinity label. In addition to the fluorescent-tagged OSW-1, less active congeners of OSW-1 lacking acyl groups were modified with a fluorophore at C4″ position using the Me_2SnCl_2-mediated

acylation method, to investigate the role of biologically essential acyl groups. Furthermore, a sequential site-selective acylation method was developed to install two different functional groups (a fluorophore and a photoreactive group) at different positions (C4″-OH and C2″-OH) of OSW-1. This method enabled the introduction of a photoreactive group at the putative protein binding site of OSW-1, which is expected to maximize the efficiency of crosslinking the target proteins. Cell imaging studies using the fluorescent probe **4** have shown that OSW-1 selectively localizes to Golgi apparatus, which raised the possibility that they are the target subcellular organelles. Comparative analysis of the fluorescent analogues of OSW-1 congeners indicated that the sterol moiety confers the intracellular transporting function while the acyl groups of the disaccharide moiety likely play a separate important role in the direct interaction with the target proteins. Reactivity analysis of alkyne photoaffinity probe **13** demonstrated that it can effectively crosslink a model binding protein in an affinity dependent fashion. Target identification studies using biotin probe **9** and biotinylated derivative of **13** are currently in progress.

Acknowledgements

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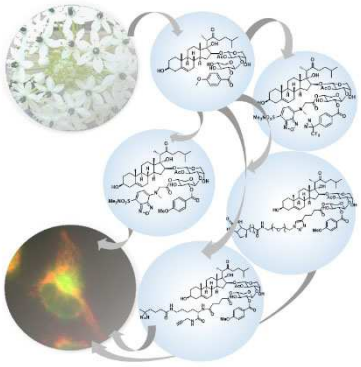
PERSONAL ACCOUNT

OSW-1 is an extremely potent and selective anticancer saponin with a potentially novel mechanism. This account reviews recent progress in the development of OSW-1 derived probes for exploring its mechanism of action.



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Development of Chemical Probes for Functional Analysis of Anticancer Saponin OSW-1