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Sensitive and Rapid Analysis of Protein Palmitoylation with a Synthetic Cell-Permeable Mimic of Src Oncoproteins

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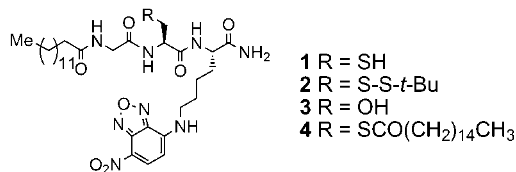
Members of the Src and Ras families of oncoproteins function at the inner leaflet of cellular plasma membranes to control biological signal transduction pathways critical for cancer proliferation.[1] These proteins are anchored to plasma membranes by covalently linked lipids, which are attached posttranslationally by enzymes including protein myristoyltransferase, protein farnesyltransferase (FTase), and the membrane-associated palmitoyl acyltransferase (PAT).[2] These enzymes couple myristoyl (Myr), farnesyl, and palmitoyl lipids to defined amino acid residues to enable insertion of attached hydrophobic lipid chains into the hydrophobic inner leaflet of plasma membranes.[2] For example, most Src family proteins are intracellularly myristoylated and palmitoylated at their N-terminus as shown in Figure 1.

Plasma membrane localization of oncogenic Src and Ras proteins is critical for the proliferation of specific cancers.[3] As a consequence, intense efforts are underway to discover anticancer agents that inhibit specific lipid modification pathways such as prenylation catalyzed by the well-characterized FTases.[4] In contrast, very little is known about the enzymology and biochemistry of protein palmitoylation, studies of which are hindered by the dynamic and reversible nature of cysteine acylation and the lack of convenient assays of PAT activity. These assays are urgently needed because palmitoylation is critical for plasma membrane association of Src and Ras oncoproteins, and the identification of selective inhibitors of protein palmitoylation has the potential to define a new class of antitumor agents.[5]

To enable the rapid identification of compounds that affect protein palmitoylation, we report here the solid-phase synthesis of a cell-permeable lipopeptide (**1**) that mimics the Myr-Gly-Cys N-terminus of Src family proteins. Although elegant solution-phase syntheses of Ras and Src protein fragments have been described in the literature,[6] we report the first rapid solid-phase synthesis of a cell-permeable PAT substrate that enables efficient, high-throughput fluorescence-based assays of protein palmitoylation. This novel approach is much more convenient and sensitive than classical assays of PAT activity, which include multistep treatment of cells with radiolabeled palmitate, precipitation of palmitoylated protein, and scintillation counting to determine the amount of radiolabel incorporated.[5a,6d]



The solid-phase synthesis of fluorescent lipopeptides **1**−**4** employed Fmoc methodology and incorporation of the solvochromic green fluorophore 7-nitrobenz-2-oxa-1,3-diazole (NBD) as outlined in Scheme 1.

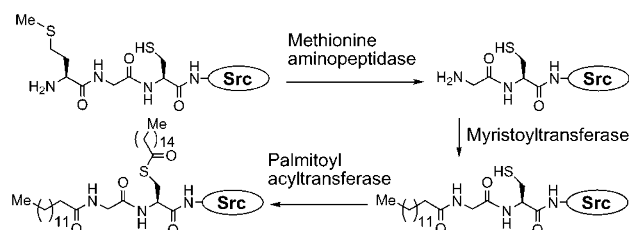
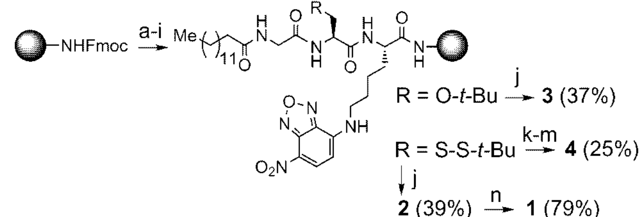


Figure 1. Lipid modification of Src family proteins.

Scheme 1 [a]



[a](a) Piperidine, DMF. (b) Fmoc-Lys(Alloc)-OH, PyBOP, HOBT, DIEA, DMF. (c) Piperidine, DMF (d) Fmoc-Cys(S-*t*-Bu)-OH or Fmoc-Ser(*t*-Bu)-OH, PyBOP, HOBT, DIEA, DMF. (e) Piperidine, DMF. (f) Myr-Gly-OH, PyBOP, HOBT, DIEA, DMF. (g) $Pd(PPh_3)_4$, Morpholine, CH_2Cl_2. (h) $Et_2NCS_2Na \cdot 3H_2O$, DMF. (i) NBD-Cl, Et_3N. (j) TFA. (k) $HOCH_2CH_2SH$, DIEA. (l) Palmitoyl-Cl, DIEA. (m) TFA. (n) $HOCH_2CH_2SH$, Tris, DMSO, 55 °C.

Living Jurkat lymphocytes were employed to investigate whether **1** comprises a functional synthetic mimic of the Src N-terminus. Compound **3** provided a negative control because PAT is known to palmitoylate Cys but not analogous Ser residues.[6d] Compound-treated cells were washed with bovine serum albumin (BSA) to remove excess peptides, and cells were investigated by extraction of radiolabeled metabolites, examination by fluorescence microscopy, and analysis by flow cytometry.

To determine whether **1** is palmitoylated intracellularly, Jurkat lymphocytes were radiolabeled with ^{14}C-palmitic acid and treated with combinations of **1** and the palmitoylation inhibitor 2-bromopalmitic acid.[5c] 2-Bromopalmitic acid has been reported to block palmitoylation of the Myr-Gly-Cys N-terminus of the Src family tyrosine kinases Fyn and Lck at high concentrations (100 μM).[5c] Lipids were extracted from compound-treated cells with dichloromethane and radiolabeled/fluorescent products analyzed by thin-layer chromatography (TLC) and HPLC-MS (Supporting Information). These experiments revealed that **1** is palmitoylated intracellularly to yield radiolabeled **4**, and this conversion is blocked by treatment with 2-bromopalmitic acid (Figure 2).

Fluorescence microscopy of Jurkat lymphocytes treated with **1** revealed living cells bearing intense green fluorescence primarily at the cellular periphery (Figure 3), which was confirmed to

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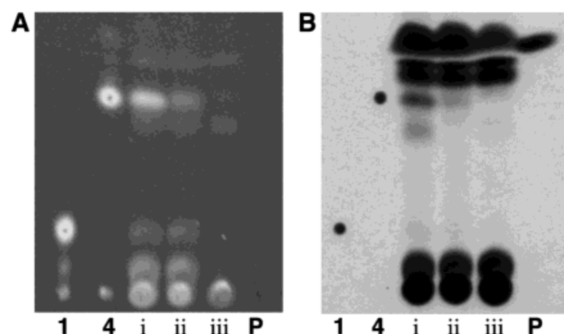

Figure 2. TLC of extracts of Jurkat lymphocytes treated with **1** and ^{14}C-palmitic acid. (A) Fluorescent products imaged by UV-irradiation (365 nm). (B) Autoradiography of the TLC plate. (i) Extract of cells treated with **1** (1 μM). (ii) Extract of cells treated with **1** (1 μM) and 2-bromopalmitic acid (10 μM). (iii) Extract of cells treated with ^{14}C-palmitic acid alone. **P**: ^{14}C-palmitic acid. After elution, **1** and **4** in control lanes were marked with ^{14}C-palmitic acid to enable detection on film.

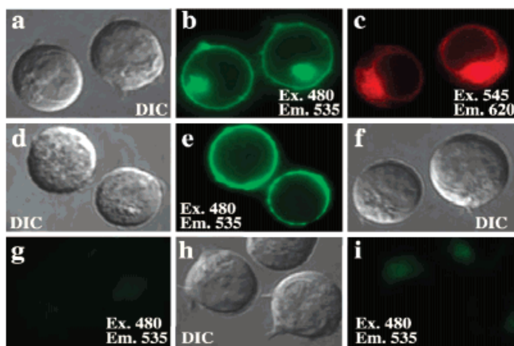

Figure 3. Subcellular localization of compounds **1** and **3** added to living Jurkat lymphocytes for 2 h at 37 °C. Panels a−c: Cells treated with green-fluorescent **1** (1 μM) and red-fluorescent golgi-specific BODIPY TR ceramide stain. Panels d and e: Control experiments identifying the plasma membrane by labeling extracellular amines with sulfosuccinimidyl biotin and FITC-streptavidin. Panels f and g: Cells treated with **3** (1 μM). Panels h and i: Cells treated with **1** (1 μM) and 2-bromopalmitic acid (10 μM). DIC: Differential Interference Contrast. Exposure times are identical for all samples. Fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

correspond to the cellular plasma membrane by comparison with cells bearing plasma membranes labeled with sulfosuccinimidyl biotin and green fluorescent FITC-conjugated cell-impermeable streptavidin (Figure 3e),[7] or cells treated with a previously described synthetic plasma membrane probe.[8] Intracellular fluorescence was observed in the golgi complex as established by fluorescence co-localization with the golgi-specific red-fluorescent BODIPY TR ceramide probe (Figure 3c).

In contrast to the persistent plasma membrane fluorescence resulting from addition of **1** to cells, **3** engendered only weak cellular fluorescence (Figure 3g) consistent with the hypothesis that **1** becomes palmitoylated intracellularly to yield **4**, whereas **3** remains metabolically unchanged. Addition of **1** and 2-bromopalmitic acid (10 μM) to Jurkat cells reduced plasma membrane fluorescence to levels similar to those conferred by **3**. This result suggested that changes in the fluorescence of **1** could provide a novel assay for inhibitors of palmitoylation.

To quantify inhibition of palmitoylation, cellular fluorescence was analyzed by flow cytometry. This method revealed that **1** comprises a highly sensitive molecular probe of PAT activity. Curve fitting the data shown in Figure 4 yielded an IC_{50} value for 2-bromopalmitic acid of 2.4 $\pm$ 1.0 μM, indicating that **1** detects inhibition of palmitoylation with ca. 50-fold higher sensitivity than previously reported assays.[5c]

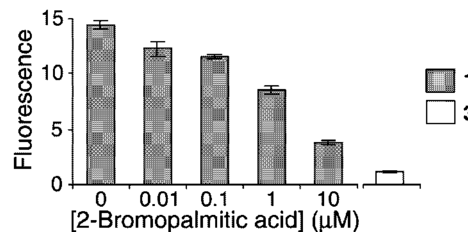

Figure 4. Analysis of inhibition of palmitoylation using flow cytometry. Cells were treated with **1** (1 μM) and 2-bromopalmitic acid at 37 °C for 2 h. Treatment with **3** (1 μM) is shown as a negative control. Each bar represents the mean of three independent replications measuring the median fluorescence of 10 000 living Jurkat lymphocytes.

The use of flow cytometry to screen combinatorial libraries of synthetic compounds has been relatively unexplored.[9] However, considering that a typical sample containing 10 000 cells can be analyzed in less than 10 s, this method has significant potential for high-throughput screening, particularly as described herein for the discovery of compounds that affect lipid modification pathways. This endeavor would be significantly facilitated by compound **1**, which comprises a highly sensitive molecular probe of palmitoyl acyltransferase activity that enables unprecedented flow cytometry-based assays of protein palmitoylation.

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Supporting Information Available: Experimental procedures and characterization data for new compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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