# Organic & Biomolecular Chemistry

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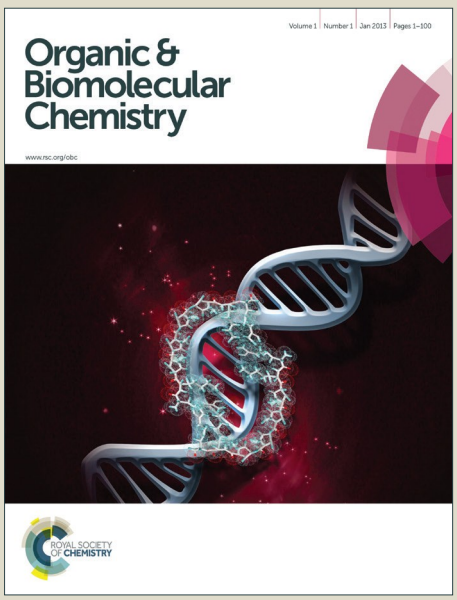





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PAPER

# Binding position analysis of target proteins with the use of amidopyrene probes as LA-LDI enhancing tags †

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Amidopyrene-conjugated compounds can be detected by label-assisted laser desorption/ionization mass spectrometry (LA-LDI MS) without matrixes. When actin, a cytoskeletal protein, was labeled with excess amount of amidopyrene *N*-hydroxysuccinate (apy–OSu), eight apy-labeled actin peptides were predominantly detected by LA-LDI MS. Then actin was labeled with an amidopyrene NHS ester of the antitumor marine macrolide aplyronine A (ApA–apy–OSu) to form a 1:1 conjugate. The sequence of apy-labeled peptide was established as $A^{108}$PLNPKANR$^{116}$ by MS/MS analysis, in which the NHS ester moiety specifically reacted with the ε-amino group of K113. While the fragmentation at the linker part reduced the detection sensitivity of apy-labeled peptides on LA-LDI MS, our chemical probe method is useful for analyzing the binding modes of various ligands and target biomacromolecules that include multiple and weak interactions.

## Introduction

Chemical probes, in which the ligands (small bioactive compounds) are conjugated with reacting and detecting groups, have been used to identify target biomacromolecules and analyze their binding positions.[1] In the case of proteins, enzymatic digestion and subsequent peptide-mass fingerprint (PMF) analysis or Edman degradation of labeled peptides can be used to establish the binding positions of ligands. However, it is sometimes difficult to detect and purify small quantities of labeled peptides from a mixture of unreacted ligands and other degraded products. In 2013, pyrene-conjugated compounds were shown to be selectively detected by label-assisted laser desorption/ionization mass spectrometry (LA-LDI MS) without matrixes.[2] Since then, several compounds have been reported as LA-LDI MS enhancing tags, such as polycyclic aromatic hydrocarbons (PAHs) and rhodamine fluorophores.[3] Recently, we showed that 6-amidopyrene derivatives were highly detectable in amounts as low as 10 fmol by an LDI MS instrument equipped with a 355 nm laser.[4] To develop new, efficient and precise methods for analyzing the binding modes of target proteins, we planned to use amidopyrene probes for PMF analysis (Fig. 1). After covalent bond formation between amidopyrenes and target proteins and subsequent enzymatic digestion, only amidopyrene-labeled peptide(s) should be detected on LA-LDI MS. If this approach is successful, the purification of labeled peptides should not be necessary, and target–ligand interactions might be analyzed more efficiently. Here we describe a binding position analysis of target proteins with the use of amidopyrene probes as LA-LDI enhancing tags.

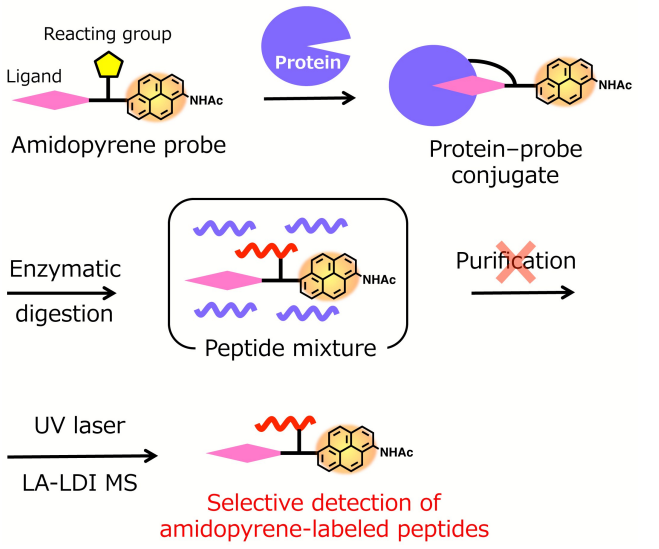


**Fig. 1** Overall strategy for the binding position analysis of target proteins with amidopyrene probes.

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† Electronic Supplementary Information (ESI) available: Text, schemes, and figures that provide all of the experimental procedures, characterization data for all compounds, $^{1}$H and $^{13}$C NMR spectra. See DOI: 10.1039/x0xx00000x

## Results and discussion

### LA-LDI MS analysis of apy-labeled peptides

To explore whether the amidopyrene group can be used in LA-LDI MS, we initially performed a mass analysis of peptide–amidopyrene conjugates. Amidopyrene *N*-hydroxysuccinate (apy–OSu, **1**) was prepared in 66% yield by the condensation of amidopyrene carboxylic acid with NHS (Scheme S1). The NHS group reacts with various nucleophilic functional groups on proteins, such as the ε-amino group of lysine (Lys) and the hydroxy groups of serine (Ser), threonine (Thr), and tyrosine (Tyr). An excess amount of **1** was reacted with actin, a cytoskeletal protein bearing 19 Lys residues, and the conjugate was digested with trypsin to give a mixture of amidopyrene-labeled and unlabeled peptides.

By MALDI MS analysis, both amidopyrene-labeled and unlabeled tryptic peptides were observed with a similar intensity (Fig. 2a, Table S1). In contrast, eight amidopyrene-labeled peptides (prepared from 200 pmol actin) were selectively detected by LA-LDI MS (Fig. 2b). Among them, six peptides (Nos. 1, 4, 8, 11, 15, 16) had internal or *N*-termini Lys residues. In a peptide (No. 3), C-termini Lys residues were labeled with amidopyrene, while trypsin generally does not cleave ε-*N*-acylated Lys residues. The relative intensities of three peptide peaks (Nos. 1, 4, 11) on LA-LDI MS were higher than those on MALDI MS. With some amidopyrene-labeled peptides, characteristic 42 mu-reduced fragment ions were detected in association with their parent ions (Nos. 1, 3, 11), which were assigned as aminopyrenes that had lost ketene ($CH_2$=C=O) from *N*-acetamidopyrene moieties.[4] LA-LDI MS/MS could be used to analyze amino acid sequences and the labeling sites of the amidopyrene moiety (i.e., No. 15, Fig. S1). Meanwhile, two peptides that were not labeled with amidopyrene (Nos. 5 and 10) were slightly detected by LA-LDI MS. Due to the presence of aromatic amino acid residues [i.e., phenylalanine (Phe), Tyr, and tryptophan (Trp)], these peptides might be directly excited by $UV_{355}$ laser irradiation without matrixes. So far, amidopyrene-labeled peptides have been predominantly detected from a mixture of digested peptides by LA-LDI MS, and thus our amidopyrene-labeling method is promising for analysis of the ligand-binding sites of target proteins.

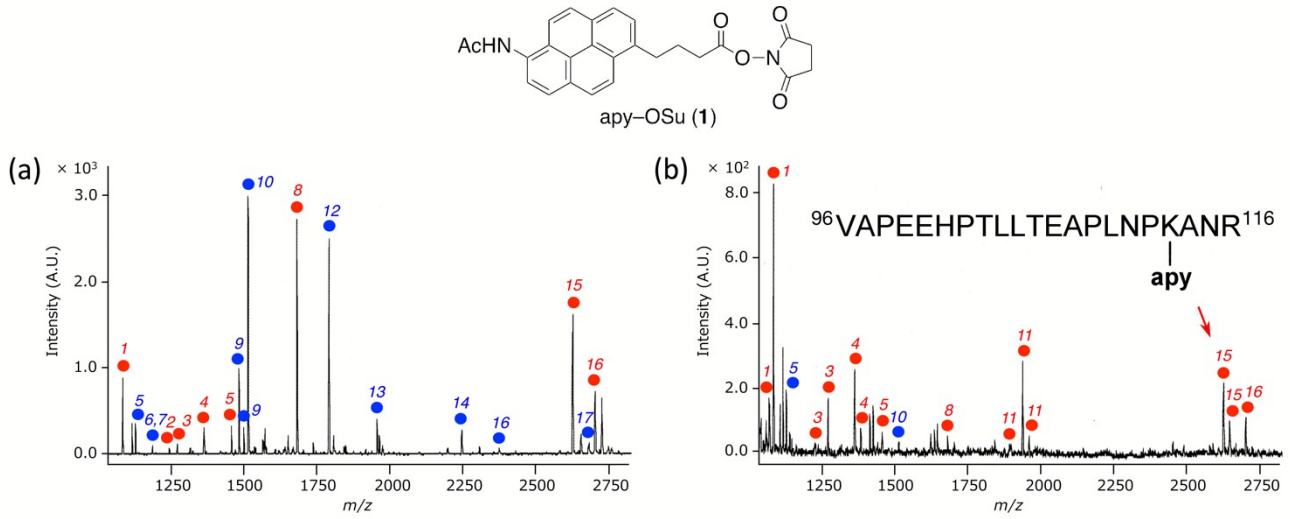


**Fig. 2** Mass spectra of the tryptic peptides of actin unspecifically labeled with apy–OSu (**1**). (a) MALDI and (b) LA-LDI MS. Red and blue circles indicate amidopyrene-labeled and unlabeled actin peptides, respectively. Numerals indicate the positions of tryptic peptides in actin (for details, see Table S1). The structure of an amidopyrene-labeled peptide (No. 15) is shown in (b).

### Labeling of actin with aplyronine A photoaffinity amidopyrene derivative 3

We next performed a binding position analysis of target proteins using amidopyrene probes. Aplyronine A (ApA, **2**) [5] is an antitumor marine macrolide that induces protein–protein interaction between actin and tubulin, two cytoskeletal proteins with dynamic instability (Fig. 3a).[6] It has been shown that the C34 *N*-methyl enamide moiety in aplyronines can be replaced with oxime or hydrazones without a significant loss of activity.[7] We previously synthesized a photoaffinity amidopyrene derivative of aplyronine A (ApA–PaP, **3**), and the structures of its photoreacted products with solvents were established by LA-LDI MS and MS/MS analyses.[4] Since amidopyrene probe **3** exhibits potent actin-depolymerizing activity and inhibits the growth of tumor cells similar to natural ApA (**2**),[4] it might strongly bind to actin to form a 1:1 complex. Here, actin was photolabeled with **3** by $UV_{365}$ irradiation, and this conjugate was analyzed by MALDI-TOF MS, but the results showed that actin was rarely labeled with **3** (Fig. S2). Most of

the carbene species generated from the aryldiazirine moiety in **3** might react with only the solvent (i.e., water molecules) on the surface of actin.

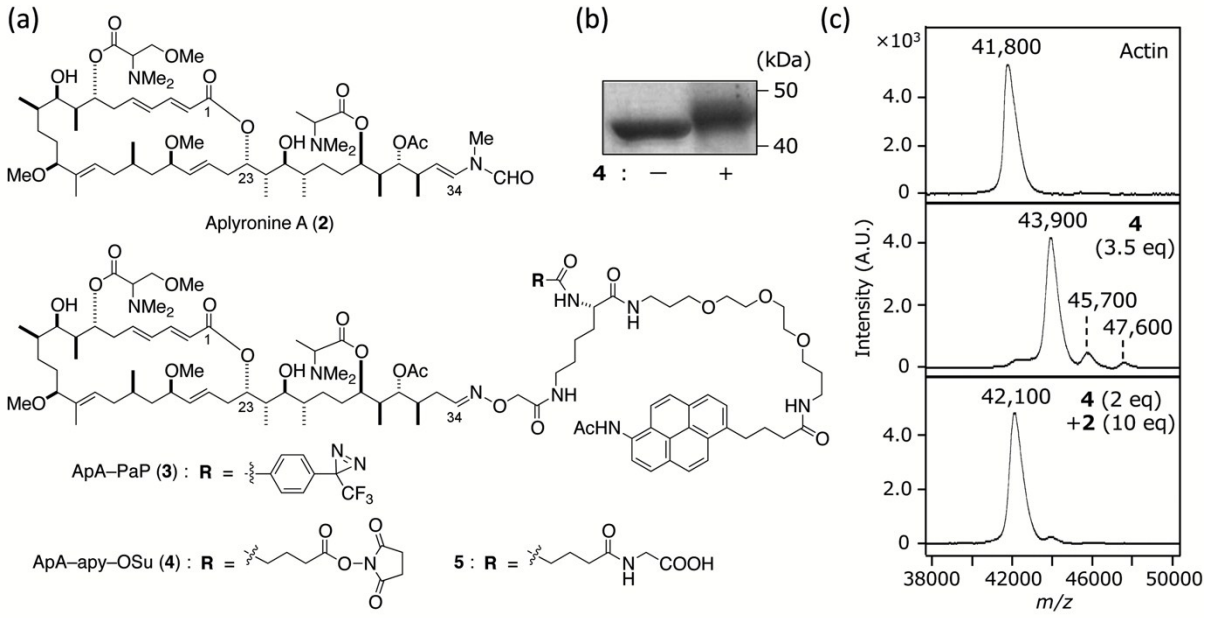


**Fig. 3** Structures of amidopyrene probes and actin-labeling experiments. (a) Structures of aplyronine A (ApA, **2**) and its amidopyrene derivatives. (b) SDS-PAGE analysis of actin labeled with probe **4** (CBB stain). (c) MALDI mass spectra of unreacted actin (top), and actin reacted with **4** in the absence (middle) or presence (bottom) of excess **2**.

**Labeling of actin with aplyronine A amidopyrene NHS ester derivative 4**

To overcome the low protein-labeling efficiency using photoaffinity probe **3**, we next synthesized an ApA amidopyrene probe possessing an NHS ester as a reacting group (ApA–apy–OSu, **4**). ApA C34 aldehyde, prepared by the acidic hydrolysis of ApA (**2**),[8] was sequentially condensed with an alkoxyamine having an amidopyrene moiety and *N*,*N*′-disuccinimidyl glutarate to give **4** (Scheme S1).[9] This compound readily reacted with glycine under basic aqueous conditions to give glycine adduct **5**, which established the formation and high reactivity of **4**.

Next, actin (42 kDa) was reacted with ApA–apy–OSu (**4**) (Fw: 1,977) in sodium bicarbonate buffer with $CaCl_2$ at 4 °C for 3 h. In an SDS-PAGE analysis, an actin–probe conjugate was detected as a CBB-stained band at 44–46 kDa (Fig. 3b). In MALDI-TOF MS of the actin–probe **4** conjugate (calcd. Fw: 43,885 as a 1:1 conjugate), a major peak (*m/z* 43,900 as an average value) was observed along with two minor peaks at *m/z* 45,700 and 47,600 (Fig. 3c). Although an excess amount (3.5 eq) of **4** was used for actin-labeling experiments, almost one molecule bound to monomeric actin. Furthermore, these protein–ligand conjugate mass peaks disappeared in the presence of excess ApA (**2**).[10] These results suggested that probe **4** specifically bound to monomeric actin as with the 1:1 actin–ApA complex.[11]

**MALDI and LA-LDI MS analyses of apy-labeled actin peptides reacted with 4**

The actin that had been labeled with amidopyrene probe **4** was then digested in the combination with trypsin and Glu-C, the latter of which specifically cleaves the C-termini of glutamic acid (Glu) and aspartic acid (Asp) residues. MALDI MS analysis of digested peptides revealed that an amidopyrene-labeled peptide (No. 25, *m/z* 2841.6) was detected as a base peak, along with several unlabeled actin peptides (Fig. 4a, Table S2). In a fluorescent HPLC analysis, the above amidopyrene-labeled peptide was detected as almost a single peak (Fig. S3). These results indicated that actin was highly specifically labeled with probe **4**. In fact, MALDI MS/MS analysis established that the sequence of amidopyrene-containing peptide (No. 25) was $A^{108}$PLNPKANR$^{116}$, in which the NHS ester moiety in **4** specifically reacted with the ε-amino group of K113 (Fig. S4).

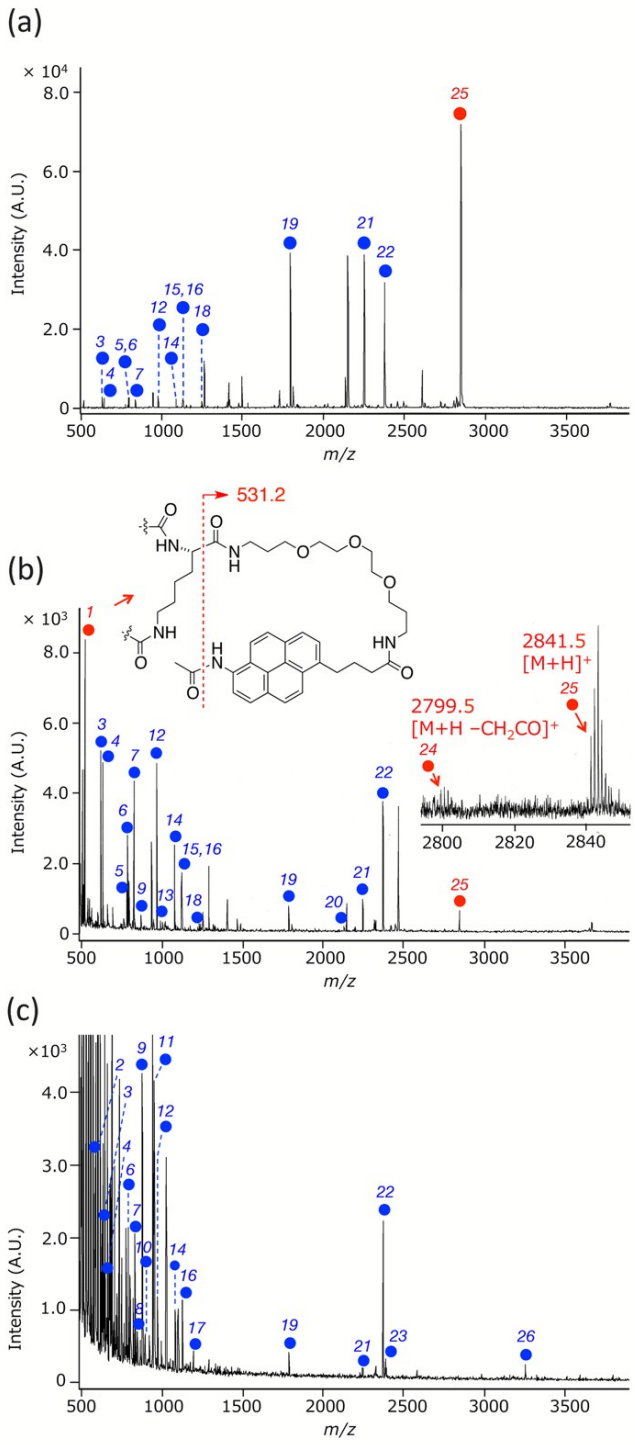


**Fig. 4** Mass spectra of digested (by trypsin + Glu-C) peptide fragments of actin labeled with probe **4**. (a) MALDI and (b) LA-LDI MS. (c) LA-LDI MS of the digested peptides of actin that was not reacted with **4**. Red and blue circles indicate amidopyrene-labeled and unlabeled peptides, respectively. Numerals indicate the positions of digested peptides in actin (for details, see Table S2).

LA-LDI MS analysis of the actin peptides labeled with amidopyrene probe **4** was then conducted (Fig. 4b). As with MALDI MS, a molecular ion peak of amidopyrene-labeled actin peptide was observed at *m/z* 2841.5 (No. 25), but its intensity was very low. Instead, a base peak was observed at *m/z* 531.2 (No. 1), which was formed by cleavage of the C–C bond at the carbonyl α position of the linker part.[4] This fragmentation reduced the detection sensitivity of amidopyrene-labeled peptides, and thus higher amounts of peptides (prepared from 200 pmol actin) were required to detect the molecular ion peak of amidopyrene-labeled peptide by LA-LDI MS. In this condition, several unlabeled peptides, especially those with aromatic amino acid residues (Nos. 10–12, 16, 17, 19, 21–23, 26), were also directly excited by $UV_{355}$ laser irradiation (Fig. 4c). These unlabeled peptides (prepared from 200 pmol actin without **4**, but not those obtained from 1 pmol of actin) were detected by LA-LDI MS. Therefore, the selective detection of amidopyrene-labeled peptides should be possible if the detectable amount of amidopyrene-labeled peptides is reduced to below the order of a picomole.

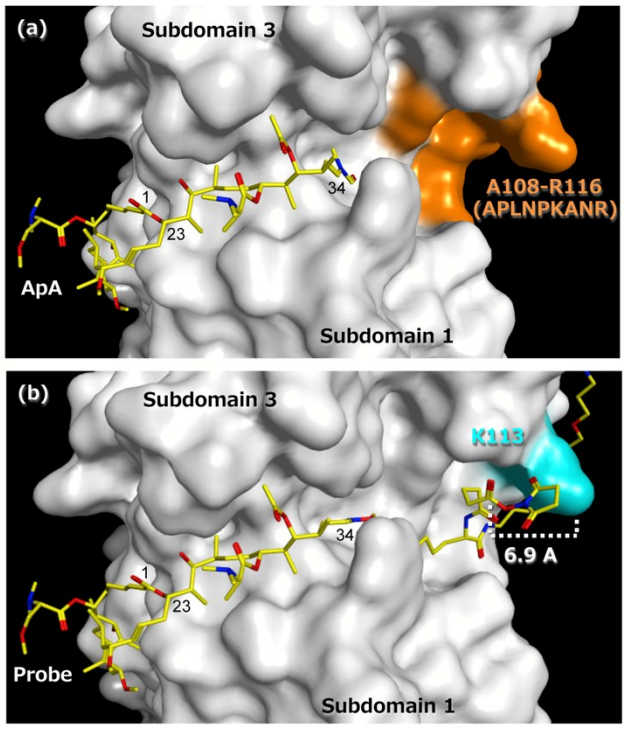


**Fig. 5** Molecular modeling of the actin–probe **4** complex. (a) X-ray structure of the actin–ApA complex (PDB code: 1WUA).[11] Labeled nonapeptide (A108–R116) with **4** is highlighted in orange. (b) The most stable conformer of **4** on actin. The initial structure of the actin–probe **4** complex was constructed by replacing the C24–C34 side-chain part of **2** in (a). A conformational search of **4** was performed using the Amber12-EHT force-field, in which both actin and the ApA moiety (C1–C34) in **4** were fixed. The lysine residue (K113) that reacted with **4** is highlighted in cyan.

**Binding position analysis of probe 4 on actin by using molecular modelling studies**

To examine why the K113 residue of actin was highly efficiently labeled with ApA amidopyrene probe **4**, molecular modeling studies were performed. In the X-ray crystal structure of the actin–ApA complex, A108–R116 residues are located in the hydrophobic cleft between subdomains 1 and 3 of actin, just behind the binding site of the C24–C34 side-chain part of ApA (**2**) (Fig. 5a). A conformational search of amidopyrene probe **4** on actin was then performed, in which both actin and the C1–C34 part in **4** were fixed. As a result, in the most stable conformer, the linker part of **4** faced the A108–R116 residues, and the NHS ester moiety of **4** was closest to the ε-amino group of K113 at 6.9 Å (Fig. 5b). All other Lys residues on actin were too far away to react with the NHS group in probe **4**.

## Experimental section

**General**

NMR spectra were recorded on a Bruker Biospin AVANCE 600 spectrometer (600 MHz for $^{1}$H and 150 MHz for $^{13}$C) or a Bruker Biospin AVANCE 400 spectrometer (400 MHz for $^{1}$H and 100 MHz for $^{13}$C). Chemical shifts are reported in parts per million (ppm) relative to the solvent peaks, $\delta_H$ 3.31 (residual $CHD_2OD$), $\delta_H$ 2.50 (residual $CHD_2S(O)CD_3$) and $\delta_C$ 39.52 for $(CD_3)_2S=O$, respectively. Coupling constants (*J*) were shown in hertz. For the quantification of minute amounts by $^{1}$H NMR analyses, benzene (5–10 mM in $CD_3OD$) was added to the sample solutions as a standard (1:60, v/v). IR spectra were recorded on a JASCO FT/IR-230 spectrometer. High-resolution electrospray ionization mass spectra (HR-ESIMS) were measured on an AccuTOF CS spectrometer (JEOL). All chemicals were used as obtained commercially unless otherwise noted. Organic solvents and reagents for moisture-sensitive reactions were distilled by the standard procedure. Fuji Silysia silica gels BW-820MH and FL60D were used for column chromatography. Merck precoated silica gel 60 F254 plates were used for thin layer chromatography (TLC).

**LDI-MS analysis**

Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS) and its tandem MS analyses were performed using a Bruker UltrafleXtreme spectrometer equipped with a 355 nm Nd:YAG laser, with α-cyano-4-hydroxycinnamic acid (α-CHCA) or sinapinic acid as matrixes. Label-assisted laser desorption/ionization mass spectrometry (LA-LDI MS) and its tandem MS analyses were performed using the same apparatus as for MALDI-TOF MS without matrixes. Samples dissolved in 50% aq. MeOH or MeCN / 0.1–1% TFA were spotted on an MTP384 ground steel target plate and air-dried according to the manufacturer's instructions.

**Labeling of actin with Apy–OSu (1) and triptic digestion**

To remove Tris·HCl from commercially available rabbit muscle actin (Cytoskeleton Inc.), 233 μM (10 mg/mL) actin in G-buffer (8.6 μL, 2 nmol) was diluted to 500 μL with labeling solvent 1 (LS1) [50 mM $NH_4HCO_3$, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol (pH 8.1)], and concentrated to 50 μL by ultrafiltration (14,000 × *g*, 4 °C, 15 min) through a Microcon YM-10 (MWCO 10 kDa, Millipore). This buffer exchange was repeated three times, and diluted with LS1 (300 μL). To the resulting solution (350 μL) was added 5 mM apy–OSu (**1**) in DMSO (20 μL, 100 nmol). After incubation with a rotator for 20 h at room temperature, the solution was exchanged with 50 mM $NH_4HCO_3$ by ultrafiltration (three times as above) to remove unreacted apy–OSu (**1**). To the resulting solution (50 μL) was added 45 mM DTT in 25 mM $NH_4HCO_3$ aq. (1.5 μL), and the solution was incubated at 56 °C for 15 min. The solution was then reacted with 100 mM iodoacetamide in 25 mM $NH_4HCO_3$ aq. (1.5 μL) under shaking at room temperature for 15 min. Sequence-grade modified trypsin (100 ng/μL, 4.3 μL, #V5111, Promega) was added, and the resulting mixture was incubated at 37 °C for 20.5 h. After the addition of 10% TFA aq. (3 μL), the solution was concentrated *in vacuo*. To remove the salts in digestion buffer, samples were lyophilized in water three times. After being dissolved in 50% MeCN containing 0.05% TFA, the peptide mixture was analyzed by LA-LDI and MALDI MS (1/10 amount each).

**Labeling of actin with aplyronine derivatives**

In the photolabeling experiments, 5 mM ApA–PaP (**3**) in DMSO (0.5 μL, 2.5 nmol) was reacted with 8.4 μM actin in G-buffer (23.8 μL, 200 pmol) in a 0.6 mL plastic tube. After incubation with a rotator for 30 min at room temperature, the solutions were cooled on ice and irradiated with UV light (365 nm) for 15 min using a handheld UV lamp (0.8 mW/cm$^2$). The resulting solution was directly analyzed by MALDI-TOF MS with sinapinic acid as a matrix.

In NHS probe experiments, 233 μM actin in G-buffer (43 μL, 10 nmol) was diluted to 500 μL with labeling solvent 2 (LS2) [2 mM $NaHCO_3$, 0.2 mM $CaCl_2$ aq. (pH 8.4)] and concentrated to 50 μL by ultrafiltration (14,000 × *g*, 4 °C, 15 min). This buffer exchange was repeated three times. To the resulting solution (500 μL) was added 2 mM ApA–apy–OSu (**4**) in DMSO (17.5 μL, 35 nmol). For the competition experiments, 2 mM ApA–apy–OSu (**4**) in DMSO (10 μL, 20 nmol) and 10 mM ApA in DMSO (10 μL, 100 nmol) were added simultaneously. After incubation with a rotator for 3 h at 4 °C, 50 mM Tris·HCl (20 μL, pH 8.0) was added to quench the unreacted NHS ester moiety in **4**. The resulting solution was directly analyzed by MALDI-TOF MS with sinapinic acid as a matrix. For SDS-PAGE analysis, labeled actin sample (5 μL) was diluted with water (5 μL), mixed with 2 × SDS buffer (10 μL, Sigma), and boiled at 95 °C for 5 min. SDS-PAGE was performed with a 10% precast polyacrylamide gel (ATTO), and the gel was stained with a Quick-CBB kit (Wako).

**Enzymatic digestion of the actin–probe 4 complex and MS analysis**

The actin–probe **4** complex in LS2 (prepared from 10 nmol of actin, 500 µL) was concentrated to 50 µL by ultrafiltration (14,000 × *g*, 4 °C, 15 min), and the solution was diluted to 500 µL with 25 mM $NH_4HCO_3$ aq. This solvent exchange was repeated three times. To the resulting solution (100 µL) was added 100 mM DTT in 25 mM $NH_4HCO_3$ aq. (5 µL), and the mixture was incubated at 56 °C for 20 min. The solution was reacted with 100 mM iodoacetamide in 25 mM $NH_4HCO_3$ aq. (5 µL) under shaking at room temperature for 20 min, and incubated with a mixture of trypsin (100 ng/µL, 100 µL, as above) and Glu-C (100 ng/µL, 100 µL, #V1651, Promega) at 37 °C for 15.5 h. After 10% TFA (20 µL) was added, the solution was lyophilized in water three times. The resulting reaction mixture was dissolved in 50% aq. MeCN/0.1% TFA, and analyzed by LA-LDI and MALDI MS (1/50 amount each). To purify labeled peptides, samples were loaded on a Develosil RP-AQUEOUS AR-5 HPLC column (1.5 mm I.D. × 150 mm) at 25 °C. A linear gradient of 10% to 100% aq. MeCN containing 0.05 % TFA was applied for 60 min at a flow rate of 100 µL/min, with monitoring of fluorescence ($\lambda_{ex}$ 337 nm and $\lambda_{em}$ 409 nm) to afford an amidopyrene-labeled peptide ($t_R$ = 23.5 min, *m*/*z* 2841.7), which was analyzed by MALDI-TOF MS and MS/MS with $\alpha$-CHCA as a matrix.

#### Molecular modeling

Molecular modeling studies of the actin–probe **4** complex were performed using the Molecular Operating Environment (MOE) 2014.09 program package (Chemical Computing Group Inc.). For docking model studies, water molecules associated with the actin–aplyronine A complex (PDB: 1WUA) were removed, and all protons on the protein and the ligand were complemented. The C34 *N*-methyl enamide moiety in aplyronine A (**2**) was replaced with the amidopyrene linker part of probe **4**. A conformational search was performed using the Amber12:EHT force-field with GB/VI Generalized Born [12] implicit solvent electrostatics ($D_{in}$ = 1, $D_{out}$ = 80). LowModeMD [13] was used for a conformational search, in which both the actin and the ApA moiety (C1–C34) in **4** were fixed.

## Conclusions

To readily analyze the binding mode of protein–ligand interaction, novel chemical probes were developed. Amidopyrene-labeled peptides were selectively detected from a mixture of digested actin peptides by LA-LDI MS. Then actin was labeled with aplyronine A amidopyrene NHS ester derivative **4**. MALDI and LA-LDI MS analyses of a mixture of digested peptides as well as molecular modeling studies established the binding position of aplyronine A on actin. The binding mode of the actin–ApA probe **4** complex was particularly rare from the viewpoints of high labeling efficiency and specificity. In addition, fragmentation at the linker part reduced the detection sensitivity of labeled peptides on LA-LDI MS. Still, by modifying the structures of chemical probes, such as the length, flexibility, and hydrophilicity of the linker part, our LA-LDI MS enhancing tag method with the use of amidopyrene probes should be suitable for the analysis of various target protein–ligand interactions. Our method may contribute to the detailed analysis of binding modes between various ligands and target biomacromolecules that include multiple and weak interactions, such as the actin–ApA–tubulin ternary complex.[6] Further optimization of the amidopyrene probe structures to improve the sensitivity of detection by LA-LDI MS and the development of methods for the efficient analysis of the binding mode of multiple target protein–ligand interactions are in progress.

## Acknowledgements

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9 Due to the instability of the NHS ester moiety, ApA–apy–OSu (**4**) was directly used without purification for the actin-labeling experiments after the reaction with *N*,*N*'-disuccinimidyl glutarate.



 

10 In the competition experiments, the molecular weight of actin increased by ca. 300 Da. This might be due to the non-specific labeling of actin with remaining *N*,*N*'-disuccinimidyl glutarate (Fw = 270) contained in probe **4**.
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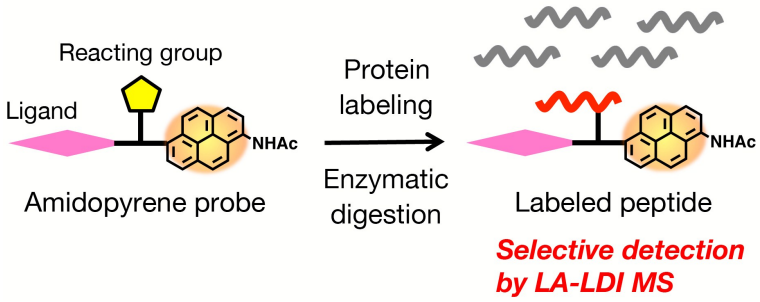
## Graphical abstract




Selective detection of amidopyrene-labeled peptides by LA-LDI MS enabled us to analyze the binding position of ligands on target proteins.