Specific protein-labeling and ligand-binding position analysis with amidopyrene probes as LDI MS tags

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PAPER

Specific protein-labeling and ligand-binding position analysis with amidopyrene probes as LDI MS tags †

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To readily analyze the binding mode of protein–ligand interactions, we developed ligand-bound-type and ligand-dissociation-type probes having 6-amidopyrene (apy) as a detecting group. Matrix- and label-assisted laser desorption/ionization mass spectrometry (MALDI and LA-LDI MS) analyses and a covalent docking simulation using these probes precisely determined the binding position of the ligand biotin on the target protein avidin (RMSD = 0.786 and 0.332 Å). Our apy-probe-labeling method may be useful for determining the unknown ligand-binding sites of various target proteins.

Introduction

Chemical probes, in which ligands (small bioactive compounds) are conjugated with reacting and detecting groups, have been used to identify target biomacromolecules (proteins, nucleotides, glycans, etc.) and analyze their binding positions.[1] Among the detecting groups, pyrene, a polyaromatic hydrocarbon, has unique properties as a fluorescent dye and mass-detection tag. Pyrene-conjugated compounds have been shown to be selectively detected by label-assisted laser desorption/ionization mass spectrometry (LA-LDI MS) without matrices.[2] We previously showed that 6-amidopyrene (apy) derivatives were highly detectable in amounts as low as 10 fmol by an LDI MS instrument equipped with a 355 nm laser.[3]

In previous chemical-probe studies on the antitumor macrolide aplyronine A,[4] the ligand and apy moiety were connected to the ε-amino group and the C-terminus of an L-lysine (Lys) linker, respectively.[5] To introduce the ligands having an amino group, here we designed a ligand-bound-type probe, in which the positions of apy and ligand were replaced (Fig. 1a). As for the reacting group, *N*-hydroxysuccinimidyl (NHS) ester was connected to the α-amino group of the Lys linker, as in previous studies. To improve the detection sensitivity of apy-labeled products, we also designed a ligand-dissociation-type probe, in which the reacting NHS ester group was inserted between the ligand and apy moiety (Fig. 1b). If the covalent bonds between ligands and reacting groups are cleaved by the nucleophilic attack of target molecules, only apy groups would remain on the near ligand-binding sites of the protein surface. Several efficient protein-labeling methods have been developed, such as ligand-directed tosylate and acyl imidazole chemistry [6] and SEAlide reagents.[7] Meanwhile, we previously reported that actin was non-specifically labeled with amidopyrene *N*-hydroxysuccinate (apy–OSu, **3**), and eight apy-labeled actin peptides were predominantly detected by LA-LDI MS (from 200 pmol actin).[5] To develop new, efficient and precise methods for analyzing the binding modes of target proteins, here we investigated apy probes having an NHS ester for use in peptide-mass fingerprint (PMF) analysis in combination with molecular modeling studies.

Results and discussion

Based on the concepts as mentioned above, we designed two amidopyrene biotin derivatives, apy–biotin–OSu (**1**) and apy–OSu–biotin (**2**) as ligand-bound-type and ligand-dissociation-type probes, respectively. Biotin (vitamin B7) is a co-enzyme for multiple carboxylase enzymes including avidin, a basic protein with 128 amino acid residues as a monomer (Fig. S1). In the crystal structure of the avidin–biotin complex (PDB: 2avi),[8] among the eight Lys residues, K135 is closest to the biotin-binding site. The distance between the K135 ε-amino group and the biotin carboxyl group is 15.9 Å. The longest calculated distances between the NHS ester and biotin amide carbon atoms in **1** and **2** are 28.0 and 20.2 Å, respectively, which might be long enough to reach the inside ligand-binding pocket on the avidin–probe complexes. A polyethylene glycol (PEG) linker was used between the ligands and the NHS ester moieties in both **1** and **2**, since it might increase the solubility and flexibility of apy derivatives to more efficiently interact with target molecules.

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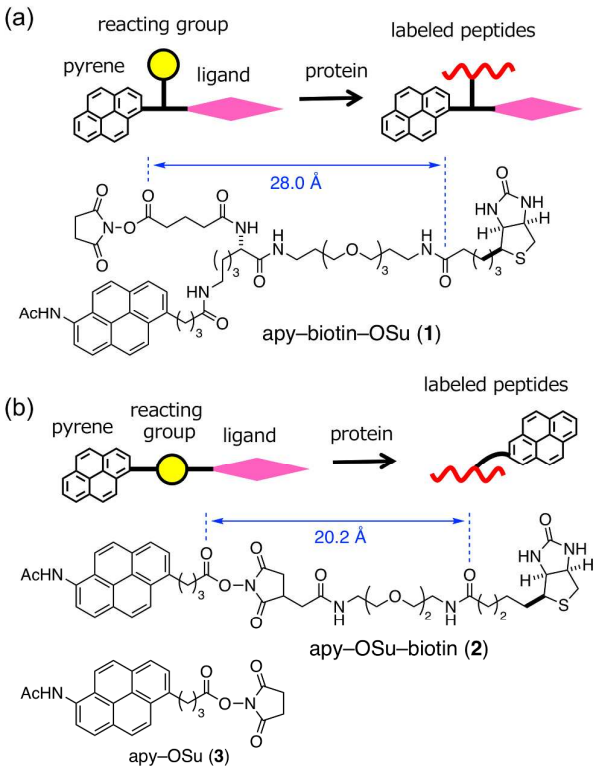
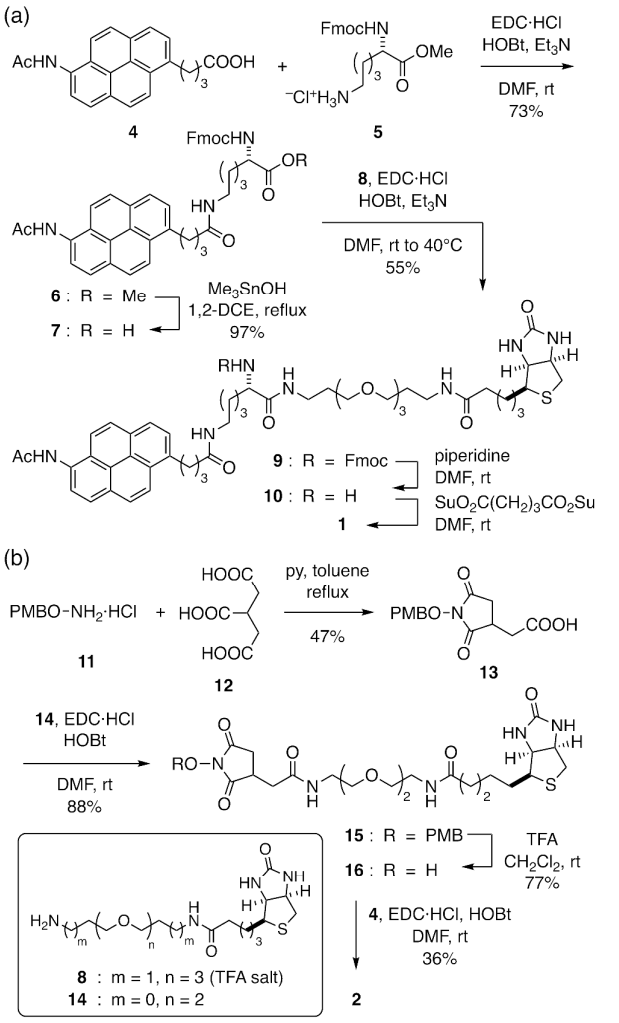


Figure 1 Design concept and structures of apy-biotin probes. (a) ligand-bound-type. (b) ligand-dissociation-type.

Scheme 1a summarizes the synthesis of ligand-bound-type probe **1**. Amidopyrene carboxylic acid **4** [3] was condensed with commercially available Fmoc–Lys–OMe·HCl (**5**) using EDC and HOBt to give amide **6** (73%). Deprotection of methyl ester in **6** proceeded with the use of trimethyltin hydroxide [9] at 75 °C in 1,2-dichloroethane to afford **7** (97%). Coupling of **7** with a PEG-linked biotin amine (**8**) [10] in the presence of EDC and HOBt yielded apy–biotin conjugate **9** in moderate yield (55%). Subsequent Fmoc deprotection in **9** with piperidine in DMF afforded secondary amine **10**, which was condensed with *N*,*N*′-disuccinimidyl glutarate in DMF to provide probe **1**.[11]

Next, ligand-dissociation-type apy-biotin probe **2** was synthesized (Scheme 1b). Dehydrating condensation of *p*-methoxybenzyloxyamine hydrochloride (**11**) [12] and 1,2,3-propanetricarboxylic acid (**12**) with pyridine [13] yielded PMB-protected alkoxyimide **13** (47%). Subsequent condensation of the carboxylic acid in **13** with a commercially available PEG-linked biotin amine (**14**) using EDC and HOBt gave amide **15** (88%). Removal of the PMB group in **15** using TFA in CH_2Cl_2 gave *N*-hydroxyimide **16** (77%), which was condensed with **4** to afford probe **2** in a moderate yield (36%).

With apy-probes in hand, we labeled avidin with **1**–**3** in 2 mM sodium bicarbonate / phosphate buffered saline (PBS) at room temperature for 48 h. On MALDI MS, native avidin was detected as several peaks (major: 16.0 kDa) due to multiple *N*-glycosylation at an asparagine (Asn41) residue (Fig. 2d). As for the avidin–probe **1** conjugate, the intensity of the desired conjugate peak (average *m/z* 17,000) was very low (3~5% conversion, for three runs) (Fig. 2a). Along with unreacted avidin, one major peak and one minor peak (*m/z* 16,200 and 17,200) were observed, which corresponded to the adducts of *N,N′*-disuccinimidyl glutarate with native avidin and the avidin–probe **1** conjugate. This might be due to the presence of succinimidyl reagent in impure **1**. With probe **2**, a major peak (*m/z* 16,300) corresponding to the adduct of one apy molecule was detected along with native avidin (35~40% conversion, based on the mass peak intensity, for three runs) (Fig. 2b). In both mass spectra, labeled avidins were detected as multiple peaks, due to the different glycosylation patterns of native avidin. With excess **3** (20 eq.), adducts of 1–3 apy molecules were detected (*m/z* 16,300, 16,600, and 16,900) (Fig. 2c). These results suggested that probe **2** specifically reacted with avidin in a 1:1 ratio, despite the use of an excess amount (5 eq.) for labeling experiments.



Scheme 1 Synthesis of amidopyrene biotin probes **1** and **2**.

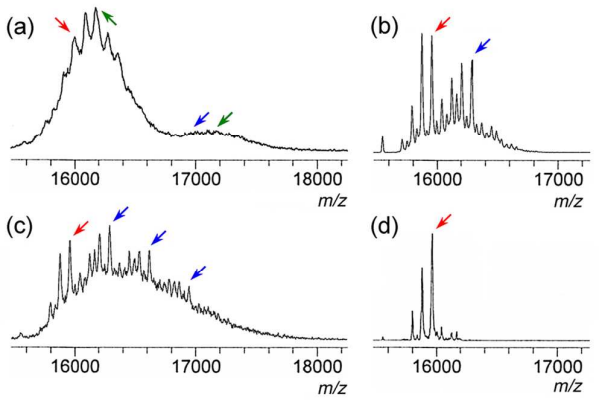


Figure 2 MALDI MS of avidin reacted with apy-probes **1** (a), **2** (b), **3** (c), and unreacted avidin (d). Red and blue arrows indicate native avidin (15,960 Da, a major peak) and probe-labeled avidin (997 Da increase in (a) and 327 Da increase per apy molecule in (b) and (c)), respectively. Green arrows indicate that the products reacted with *N,N'*-disuccinimidyl glutarate (212 Da increase per molecule). Due to multiple *N*-glycosylation of native avidin, Arrows were only shown for the representative ones for each mass spectrum.

Since both native and apy-labeled avidins were highly stable, strong denaturing conditions (6 M guanidine·HCl at 95 °C for 15 min) were required for effective digestion. Subsequent reductive *S*-alkylation (DTT / iodoacetamide) and trypsin digestion (diluted to 1 M guanidine) afforded apy-labeled avidin peptides. To remove the high concentration nonvolatile salts from tryptic peptide solution, several desalting methods were examined. With a conventional reversed-phase ODS tip column (ZipTip® C_{18}), apy-labeled peptides were detected by MALDI MS, but their intensities were fairly low compared with non-labeled peptides (Fig. S2). Other desalting methods, including ZipTip® C_4, dialysis from 0.1% TFA, and reversed-phase HPLC (C_4, C_{18}, and C_{30}), were also unsuccessful due to the significant loss of labeled peptides. With a TSK-G3000S polystyrene gel batch column, however, we were pleased to find that 1 M guanidine solution was successfully desalted. With this gel, non-labeled avidin peptides were eluted between 0 and 75% aq. MeOH fractions. In contrast, due to the high affinity of amidopyrene moiety for polystyrene gel, apy-labeled peptides were selectively eluted in the 75% aq. MeOH fraction (Fig. 3). Such a high alcohol concentration of the eluate might prevent the aggregation and insolubilization of apy-labeled peptides to improve their recovery and detection sensitivity on LDI MS.

As for the ligand-bound-type probe **1**, a probe-conjugated peptide (No. 16, *m/z* 2561.2) was detected by MALDI MS as a sole labeled product, along with several non-labeled avidin peptides (Fig. 3a and Table S1). The sequence of apy-labeled peptide was established to be S^{125}SVNDIGDDWKATR^{138} by MALDI MS/MS analysis, in which the NHS ester moiety in **1** reacted with the ε-amino group of K135 (Fig. S3). With ligand-dissociation-type probe **2**, an apy-labeled peptide (No. 9, *m/z* 1890.7) was detected as a base peak, and its relative intensity was >30 times higher than that of the second largest peak (No. 11, *m/z* 1921.8) on MALDI MS (Fig. 3b). Based on the MS/MS analysis, the sequence of major apy-labeled peptide (No. 9) was determined to be S125–R138, in which the K135 residue was labeled as with **1** (Fig. S4). Furthermore, this apy-labeled peptide (No. 9, *m/z* 1890.6) was also detected by LA-LDI MS using 350 pmol amount of avidin, along with a non-labeled peptide (No. 4) (Fig. 3e). In contrast, with probe **3**, five (six) apy-labeled avidin peptides (Nos. 1, 2, (5), 9, 11, and 20) were detected (Figs. 3c and S2c). Five of the eight Lys residues in avidin (K27, K69, K82, K135, and K151) were unspecifically labeled (Table S1). Thus, avidin-labeling by **1** and **2** was found to be highly site-specific.

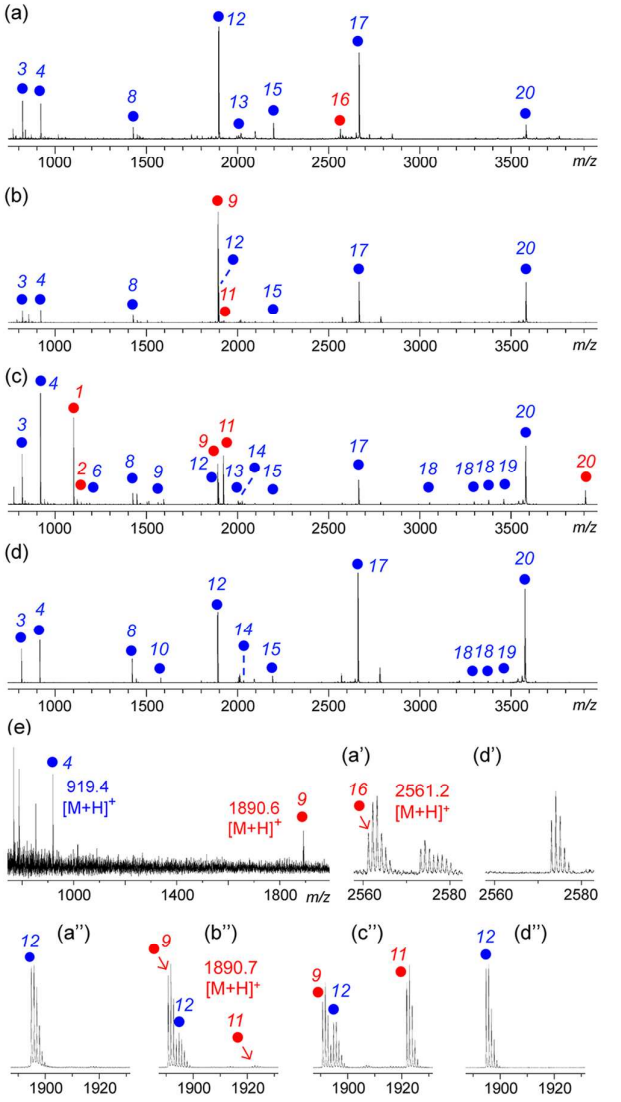


Figure 3 LDI-MS analysis desalted with TSK-G3000S resin. (a)–(d) MALDI MS of the tryptic peptides of avidin (2 pmol) labeled with **1** (a), **2** (b), or **3** (c), and control (d). Magnified spectra are shown in (a'), (d') and (a'')–(d''). (e) LA-LDI MS of the tryptic peptides of avidin (350 pmol) labeled with **2**. Red and blue circles indicate apy-labeled and unlabeled peptides, respectively. Numerals indicate the positions of tryptic peptides in avidin (for details, see Table S1).

To understand why the K135 residue of avidin was selectively labeled with apy-probes **1** and **2**, a docking simulation was performed, in which the biotin pocket was specified as the ligand-bound site (Fig. S5a). In the most stable conformers, both of the biotin moieties in **1** and **2** highly overlapped that in the avidin–biotin complex (Figs. 4a and 4b). The RMSD values of the biotin 15 atoms (bicyclic structure except for H) were 0.463 Å for **1** and 0.378 Å for **2**, respectively. In these complexes, NHS ester moieties in **1** and **2** were closest to the K135 ε-amino group (8.5 and 8.6 Å), and too far away to react with other Lys residues (>17.6 Å) (Table S2). Furthermore, all of the biotin moieties of five lowest energy conformers (within 1.75 and 1.72 kcal/mol) were located in similar positions as in the original, and their NHS ester moieties were also close to K135 (4.4~14.1 Å) (Table S3). These results suggested that the specific labeling of K135 by **1** and **2** was reliable.

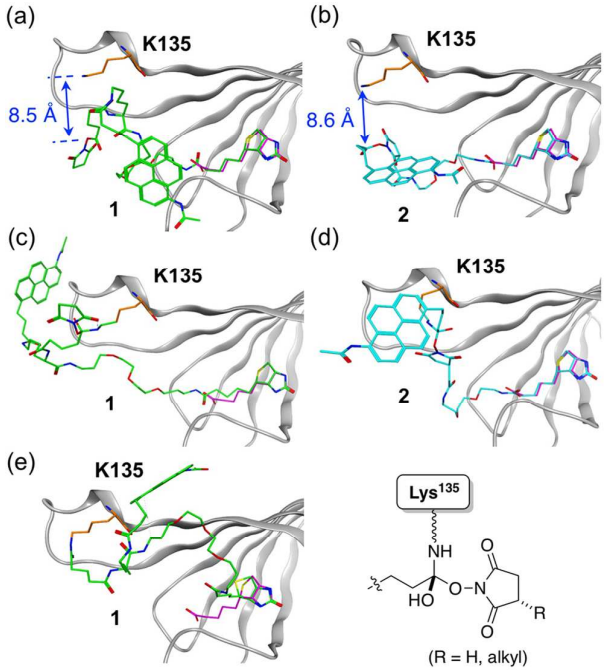


Figure 4 Molecular modeling of the avidin–probe complexes. (a, b) Docking simulations of **1** and **2** with avidin. (c, d) Covalent docking simulations of the hemiacetal intermediate by the reaction of NHS esters in **1** and **2** with the K135 ε-amino group of avidin (right bottom). (e) Covalent docking simulation for the amide conjugate of **1**. The most stable conformers of **1** and **2** on avidin are shown in green and cyan. The ligand biotin on the original complex and the K135 residue are highlighted in magenta and orange, respectively.

In general, target molecules have multiple ligand-binding sites (hydrophobic pockets), and it is difficult to determine the binding position of ligands on targets only by the docking simulations without limiting where the ligands bind. To precisely predict the ligand-binding sites based on LDI-MS analysis, covalent docking simulations were performed, in which no ligand-binding sites were specified. In the case of **2**, the ligand moiety dissociates during protein-labeling. Thus, we supposed the hemiacetal intermediates that were formed by the reactions of NHS esters with the K135 ε-amino group of avidin. In the most stable conformers of the covalent docking models, both of the biotin moieties of **1** and **2** highly overlapped the original (RMSD = 0.786 and 0.332 Å) (Figs. 4c and 4d). The ligand biotin has a ureido ring fused with a tetrahydrothiophene ring, which interacts with six residues of avidin (N36, S40, Y57, T59, T101, and N142) (Fig. S6a). The biotin moieties in the most stable conformers of **1** and **2** interacted with five (except for T59) or all six residues of the biotin-binding site (Fig. S6b). Among the five lowest-energy conformers (within 2.11 and 2.62 kcal/mol), those with the lowest energies accounted for high proportions (72.9 and 83.0%) (Table S4). We examined all of the calculations that the hemiacetal carbon and the α carbon of *N*-alkoxyimide were settled as *R* and *S*, respectively. Differences in these stereogenic centers did not significantly affect the heat of formations and binding modes of the most stable avidin–probe complexes (data not shown). For comparison, covalent docking simulations were also conducted with other Lys residues on avidin (i.e., K69 and K95, see Table S2) for **1** and **2**, but none of the biotin ligands in the calculated conformers were located close to the original ligand-binding site (RMSD >13 Å, data not shown).

Furthermore, another covalent docking simulation was performed for the amide conjugate of **1** (Fig. 4e). For the most stable conformer, the biotin moieties of **1** well-overlapped the original (RMSD = 0.952 Å for the bicyclic nine atoms except for H), while the valeric acid moiety had a rather different conformation (Table S4). Thus, the positions of the ligand biotin moiety in **1** and **2** were precisely determined by covalent docking simulations in combination with LDI-MS analysis. Especially in the case of **2**, the calculated biotin ligand position well-coincided with that in the original, due to the more suitable linker length and simpler structure than those of **1**.

Conclusions

In conclusion, to readily analyze the binding mode of protein–ligand interactions, we developed amidopyrene probes. MALDI and LA-LDI MS analyses of digested peptides as well as a docking simulation established the binding position of the ligand biotin on the target protein avidin. While the detection sensitivity of apy-labeled peptides by LA-LDI MS has not been improved, our apy-probe-labeling method may be useful for determining unknown ligand-binding sites of various target proteins. Further optimization of pyrene structures for LDI MS tags and the development of efficient methods for the analysis of multiple target protein–ligand interactions are in progress.

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, δ_H 7.26 (residual C<u>H</u>Cl$_3$), δ_H 3.30 (residual C<u>H</u>D$_2$OD), δ_H 2.50 (residual C<u>H</u>D$_2$S(O)CD$_3$), δ_C 77.0 (<u>C</u>DCl$_3$), δ_C 49.0 (<u>C</u>D$_3$OD), and δ_C 39.5 [(<u>C</u>D$_3$)$_2$S=O], respectively. Coupling constants (*J*) are shown in hertz. 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) was performed using a Bruker UltrafleXtreme or Ultraflex III spectrometer equipped with a 355 nm Nd:YAG laser (Smartbeam 1000 or 200 MHz), with α-cyano-4-hydroxycinnamic acid (α-CHCA) or sinapinic acid as matrices. Label-assisted laser desorption/ionization mass spectrometry (LA-LDI MS) was performed using the same apparatus as for MALDI-TOF MS without matrices. 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. Tandem MALDI MS/MS analysis was performed using an ABI SCIEX 5800 MALDI-TOF/TOF 5800 analyzer equipped with a Nd:YAG laser (1000 Hz).

Labeling of avidin with amidopyrene biotin probes and trypsin digestion.

To a solution of avidin (85 μg, 5 nmol) in PBS(–) and 2 mM $NaHCO_3$ (240 μL) was added 1.67 mM apy–biotin–OSu (**1**) (10 μL, 15 nmol), 1.67 mM apy–biotin–OSu (**2**) (10 μL, 15 nmol), 10 mM apy–OSu (**3**) (10 μL, 100 nmol), or DMSO (10 μL). After incubation for 48 h at room temperature, the resulting solution containing apy-labeled proteins (1 μL) was desalted using a pipette-attached tip column (Zip-Tip C_4, Millipore) for protein MS analysis (Fig. 2). The remaining solution was lyophilized, dissolved in 6 M guanidine·HCl in 50 mM NH_4HCO_3 (60 μL), and heated at 95°C for 15 min. To the resulting denatured protein was added 45 mM DTT in 50 mM NH_4HCO_3 aq. (4 μL), and the solution was incubated at 50 °C for 15 min. The solution was then reacted with 100 mM iodoacetamide in 50 mM NH_4HCO_3 aq. (4 μL) at room temperature for 15 min. After the resulting solution was diluted with 50 mM NH_4HCO_3 (512 μL) (to avoid deactivation of trypsin at a high guanidine concentration), sequence-grade modified trypsin (100 ng/μL, 20 μL, #V5111, Promega) was added, and the resulting mixture was incubated at 37 °C for 20 h. Hydrolysis was stopped by the addition of 10% TFA aq. (15 μL).

Desalting and LDI MS analysis of apy-labeled peptides.

Desalting with the use of gel-permeation resin was performed as follows: 1/100 amount (6.2 μL) of the above tryptic peptide solution was diluted with water (44 μL) and mixed well with a TSK-G3000S polystyrene gel resin (Tosoh Co., 10 μL). After the filtrates were removed using centrifuge filter units, the resin was washed with 0%, 25%, 50%, 75%, and 100% aq. MeOH (in order) (50 μL each). Apy-labeled peptides were mainly eluted in the 75% MeOH fraction, and 1/25 amount (2 μL, 2 pmol based on the original avidin protein) was used for MALDI MS analysis (Figs. 3a–d). Similarly, 7/100 amount (43 μL, 350 pmol) of the tryptic peptide solution was desalted with a TSK-G3000S resin, and the 75% MeOH-eluted sample was concentrated and directly loaded to an LDI-MS plate without matrices (Fig 3e). Notably, no apy-labeled peptides were detected on LA-LDI MS with the use of any other desalting methods (even with >5 nmol amount of avidin). For comparison, 1/100 amount (6.2 μL) of the above tryptic peptide solution was used for desalting using a pipette-attached tip column (Zip-Tip C_{18}, Millipore), and 1/25 amount (2 μL, 2 pmol) was used for MALDI MS analysis (Fig. S2).

Molecular modeling.

Molecular modeling studies of the avidin–probe complexes were performed using the Molecular Operating Environment (MOE) 2018.0101 program package (Chemical Computing Group, Inc.). For docking model studies, all water molecules in the avidin–biotin complex (PDB: 2AVI) were removed except water molecules near the ligand, and all protons on the proteins and ligands in the complex were complemented. A conformational search was performed using the Amber14:EHT force-field with GB/VI Generalized Born [14] implicit solvent electrostatics (D_{in} = 1, D_{out} = 80) and with LowModeMD.[15] In the docking simulations of apy–biotin probes with avidin, the biotin pocket generated by the Site-Finder mode was settled as the ligand-binding site, which included 23 residues (N36, L38, S40, Y57, T59, V61–T64, W94, F96–S99, T101, F103, W121, L123–S126, R138, and N142). For the covalent docking simulations, conformational searches of the hemiacetal intermediates or the amide products that react with the ε-amino group of K135 were performed, in which all atoms of the protein surface were settled as the ligand-binding sites.

Synthesis of amidopyrene biotin probes.

Amide 6. To a stirred solution of 6-amidopyrene carboxylic acid **4** [3] (161 mg, 0.46 mmol) and Fmoc–Lys–OMe·HCl (**5**) (220 mg, 0.52 mmol) in dry DMF (8 mL) were added *N*-(3-

dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) (135 mg, 0.71 mmol), 1-hydroxybenzotriazole (HOBt) (119 mg, 0.88 mmol), and Et_3N (0.10 mL, 0.71 mmol) under a nitrogen atmosphere. After being stirred for 24 h at room temperature, the resulting mixture was diluted with $CHCl_3$ (12 mL), washed with sat. NH_4Cl and brine, dried and azeotropically concentrated with toluene. The crude material was purified with a SiO_2 column chromatography (20 g, $CHCl_3$/acetone = 20/1 to 1/1) to give amide **6** 241 mg (73%) as a light yellow solid. **6**: R_f = 0.74 ($CHCl_3$/MeOH = 4/1); mp. 180.0–182.2 ºC; $[\alpha]_D^{21}$ +15 (*c* 0.10, $CHCl_3$/MeOH = 4/1); ^{1}H NMR (400 MHz, DMSO-d_6); δ 10.28 (s, 1H), 8.32–8.14 (m, 7H), 7.97 (d, *J* = 8.1 Hz, 2H), 7.95 (br s, 1H), 7.87 (d, *J* = 7.4 Hz, 2H), 7.70 (d, *J* = 8.4 Hz, 2H), 7.42–7.38 (m, 2H), 7.30 (dd, *J* = 7.6 Hz, 7.6 Hz, 2H), 4.27 (d, *J* = 6.7 Hz, 2H), 4.23–4.21 (m, 1H), 4.18 (t, *J* = 6.8 Hz, 1H), 3.62 (s, 3H), 3.36 (t, *J* = 7.7 Hz, 2H), 2.28 (t, *J* = 7.3 Hz, 2H), 2.27 (s, 3H), 2.00 (tt, *J* = 7.7, 7.3 Hz, 2H), 1.96–1.95 (m, 2H), 1.77–1.76 (m, 2H), 1.77–1.09 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 172.3, 169.1, 156.1, 143.9 (2C), 140.1 (2C), 136.6, 131,8, 129,2, 128.3, 127.9, 127.7, 127.6 (2C), 127.1 (2C), 127.0 (2C), 125.1 (2C), 124.7 (2C), 124.6, 124.4, 123.9, 123.3, 122.6, 121.5, 120.1 (2C), 65.1, 52.0, 51.7, 46.8, 34.6, 32.2, 30.5, 29.6, 28.9, 27.4, 23.5, 22.7; IR ($CHCl_3$) 3432, 3017, 2954, 1717, 1675, 1520, 847 cm^{-1}; HRMS (ESI) *m/z* 732.3044 (calcd for $C_{44}H_{43}N_3NaO_6$ $[M+Na]^+$, Δ –0.5 mmu).

Carboxylic acid 7. Amide **6** (220 mg, 0.31 mmol) was dissolved in 1,2-dichloroethane (9 mL) followed by addition of trimethyltin hydroxide [9] (328 mg, 1.82 mmol) under a nitrogen atmosphere. After the stirring for 9 h at 75 °C, the reaction mixture was concentrated *in vacuo*, and the residue was suspended in $CHCl_3$ (6 mL) and 10 mM aq. $KHSO_4$ (9 mL). After the stirring for 30 min at room temperature, the reaction mixture was filtered, and the residue was washed with 10 mM aq. $KHSO_4$ to give carboxylic acid **7** (208 mg, 97%) as a colorless powder. **7**: R_f = 0.49 ($CHCl_3$/MeOH = 4/1); mp. 215.5–220.1 °C; $[\alpha]_D^{21}$ +46 (*c* 0.68, $CHCl_3$/MeOH = 4/1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 8.31 (d, *J* = 9.1 Hz, 1H), 8.25–8.13 (m, 6H), 7.92 (d, *J* = 7.8 Hz, 1H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.83 (br s, 1H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.59 (br s, 1H), 7.39 (dd, *J* = 7.5 Hz, 7.5 Hz, 2H), 7.30 (dd, *J* = 7.5 Hz, 7.5 Hz, 2H), 4.24 (d, *J* = 7.0 Hz, 2H), 4.16 (t, *J* = 6.9 Hz, 1H), 4.03–4.10 (m, 1H), 3.37 (t, *J* = 7.1 Hz, 2H), 2.28 (t, *J* = 7.1 Hz, 2H), 2.27 (s, 3H), 1.99 (tt, *J* = 7.6 Hz, 7.1 Hz, 2H), 1.75–1.69 (m, 2H), 1.69–1.57 (m, 2H), 1.38–1.29 (m, 4H) [–COO*H* signal was not observed]; ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.0 (2C), 169.0, 156.0, 143.9 (2C), 140.7 (2C), 136.7, 131.7, 129.1, 128.3, 127.9, 127.7, 127.5 (2C), 127.1 (2C), 127.0 (2C), 125.1 (2C), 124.7 (2C), 124.5, 124.3, 123.9, 123.3, 122.6, 121.4, 120.0 (2C), 65.1, 52.1, 46.7, 34.8, 32.2, 30.9, 29.0, 27.5, 26.5, 23.5, 22.8; IR ($CHCl_3$) 3286, 3024, 2942, 1685, 1653, 1541, 1523, 847 cm^{-1}; HRMS (ESI) *m/z* 718.2899 (calcd for $C_{43}H_{41}N_3NaO_6$ $[M+Na]^+$, Δ +1.1 mmu) .

Fmoc-apy–biotin 9. To a stirred solution of carboxylic acid **7** (4.2 mg, 6.0 μmol) and biotin–$(PEG)_3$–amine TFA salt **8** [16] (8.2 mg, 15 μmol) in dry DMF (1 mL) were added EDC·HCl (8.2 mg, 43 μmol), HOBt (5.6 mg, 41 μmol), and Et_3N (5.8 μL, 42 μmol) under a nitrogen atmosphere. After being stirred for 22 h at room temperature and for 40 °C for 7 h, the resulting mixture was diluted with $CHCl_3$ (10 mL), washed with sat. NH_4Cl and brine, dried and azeotropically concentrated with toluene. The crude material was purified with a SiO_2 column chromatography (1.4 g, $CHCl_3$/MeOH = 20/1 to 1/1) to give Fmoc–apy–biotin **9** (3.7 mg, 55%) as a light yellow powder. **9**: R_f = 0.76 ($CHCl_3$/MeOH = 4/1); mp. 76.5–80.2 °C; $[\alpha]_D^{21}$ +6.1 (*c* 0.07, $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 8.35–8.11 (m, 7H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.87 (d, *J* = 7.6 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.65–7.55 (br s, 2H), 7.48 (br s, 1H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.30 (dd, *J* = 8.0 Hz, 7.0 Hz, 2H), 7.09 (br s, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.32–4.07 (m, 6H), 3.48–3.33 (m, 14H), 3.10–3.02 (m, 5H), 2.95 (m, 2H), 2.79 (dd, *J* = 12.4, 5.0 Hz, 1H), 2.56 (d, *J* = 12.4 Hz, 1H), 2.35–2.24 (m, 5H), 2.08–1.95 (m, 4H), 1.63–1.20 (m, 16H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.9, 171.8, 171.7, 167.0, 162.7, 156.1, 143.9 (2C), 140.7 (2C), 131.7, 131.6, 129.1, 128.6, 128.3, 127.6, 127.5 (2C), 127.1 (2C), 126.9 (2C), 125.1 (2C), 124.7 (2C), 124.5, 124.3, 123.9, 123.3, 122.6, 121.4, 120.1 (2C), 69.8, 69.5, 68.1, 68.0, 67.7, 67.4, 65.1 61.0, 59.2, 55.4, 52.5, 46.7, 38.1, 35.7 (2C), 35.2, 34.8, 31.8, 29.8, 29.4, 29.3, 29.1, 29.0, 28.3, 28.2, 28.0, 25.3, 23.2, 22.4; IR ($CHCl_3$) 3464, 3027, 3006, 2977, 1716, 1654, 1557, 1542, 1523, 1507, 1456, 849 cm^{-1}; HRMS (ESI) *m/z* 1146.5366 (calcd for $C_{63}H_{77}N_7NaO_{10}S$ $[M+Na]^+$, Δ +2.1 mmu).

Apy–biotin–OSu (1). A solution of amide **9** (1.4 mg, 1.2 μmol) in a 1:1 mixture of dry DMF and piperidine (1 mL) was stirred at room temperature for 4.5 h. The reaction mixture was azeotropically concentrated with toluene to give amine **10** (quant. monitored by TLC analysis), which was immediately used for the next step without further purification. To a solution of the above amine **10** in dry DMF (0.4 mL) was added 74 mM *N,N'*-disuccinimidyl glutarate solution in dry DMF (80 μL, 5.9 μmol). After being stirred at room temperature for 50 min, the reaction mixture was azeotropically concentrated with toluene. The resulting mixture was dissolved in $CHCl_3$ (8 mL), washed with sat. $NaHCO_3$ aq. and brine, dried and concentrated. The crude material (1/10 amount) was partially purified with an ODS column chromatography (0.2 g, MeOH) to give amidopyrene NHS biotin probe **1**, which was dissolved in DMSO (24 μL) and used for the labeling experiments without further purification. **1**: R_f = 0.71 ($CHCl_3$/MeOH = 4/1); HRMS (ESI) *m/z* 1135.5159 (calcd for $C_{57}H_{76}N_8NaO_{13}S$ $[M+Na]^+$, Δ +1.4 mmu).

Carboxylic acid 13. A mixture of *p*-methoxybenzyloxyamine hydrochloride (**11**) [12] (78 mg, 0.40 mmol) and 1,2,3-propanetricarboxylic acid (**12**) (70 mg, 0.40 mmol) in dry pyridine (30 μL) and toluene (9 mL) was stirred at reflux

temperature under a nitrogen atmosphere for 6 h with continuous removal of water with a Dean–Stark apparatus. The resulting mixture was quenched with 1 M HCl (3 mL), and extracted with EtOAc (10 mL × 10). The combined extracts were washed with brine, dried, and concentrated. The crude material was purified with a SiO_2 column chromatography (2 g, $CHCl_3$/acetone = 20/1 to 5/1) to give carboxylic acid **13** (55.2 mg, 47%) as a colorless oil. **13**: R_f = 0.14 (4:1 $CHCl_3$/MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (d, *J* = 8.7 Hz, 2H), 6.92 (d, *J* = 8.7 Hz, 2H), 4.99 (s, 2H), 3.80 (s, 3H), 3.00 (m, 1H), 2.84 (dd, *J* = 9.0, 17.7 Hz, 1H), 2.73 (dd, *J* = 6.1, 17.0 Hz, 1H), 2.66 (dd, *J* = 4.5, 17.0 Hz, 1H), 2.48 (dd, *J* = 4.4, 17.7 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 176.5, 174.8, 173.8, 162.4, 133.2 (2C), 127.8, 115.4 (2C), 79.7, 56.2, 35.2, 35.0, 33.2; IR ($CHCl_3$) 3510, 3028, 2957, 2841, 1727, 1614, 1516, 1388, 1304, 1253, 1176, 1061, 1036, 991, 827 cm^{-1}; HRMS (ESI) *m/z* 316.0784 (calcd for $C_{14}H_{15}NNaO_6$ $[M+Na]^+$, Δ –0.7 mmu).

PMB–OSu–biotin (15). To a stirred solution of carboxylic acid **13** (5.9 mg, 20 μmol) and EZ-Link Amine-PEG_2-Biotin (**14**) (7.5 mg, 20 μmol, Thermo #21346) in dry DMF (0.3 mL) were added EDC·HCl (7.7 mg, 40 μmol) and HOBt (7.0 mg, 50 μmol) under a nitrogen atmosphere. After being stirred for 24 h at room temperature, the resulting mixture was diluted with $CHCl_3$ (5 mL), washed with sat. NH_4Cl and brine, dried and concentrated. The crude material was purified with a SiO_2 column chromatography (0.5 g, $CHCl_3$/MeOH = 19/1 to 4/1) to give amide **15** 11.4 mg (88%) as a colorless oil. **15**: R_f = 0.62 (4:1 $CHCl_3$/MeOH); $[\alpha]_D^{24}$ +19 (*c* 0.97, MeOH); IR ($CHCl_3$) 3667, 3441 (br), 3298, 3006, 2937, 1724, 1700, 1662, 1613, 1516, 1460, 1253, 1098, 827, 771 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (d, *J* = 8.8 Hz, 2H), 6.92 (d, *J* = 8.8 Hz, 2H), 4.99 (s, 2H), 4.58 (br s, 1H), 4.66 (br dd, *J* = 8.0, 4.7 Hz, 1H), 4.28 (dd, *J* = 7.8, 4.5 Hz, 1H), 3.80 (s, 3H), 3.60 (br s, 4H), 3.54 (t, *J* = 5.5 Hz, 2H), 3.53 (t, *J* = 5.4 Hz, 2H), 3.34 (m, 3H), 3.18 (ddd, *J* = 8.8, 5.9, 4.7 Hz, 1H), 3.07 (ddd, *J* = 9.0, 5.9, 4.5 Hz, 1H), 2.90 (dd, *J* = 12.6, 4.9 Hz, 1H), 2.83 (dd, *J* = 17.7, 9.1 Hz, 1H), 2.76–2.65 (m, 3H), 2.45 (dd, *J* = 17.7, 4.4 Hz, 1H), 2.21 (t, *J* = 7.3 Hz, 2H), 1.78–1.52 (m, 4H), 1.48–1.38 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 173.6, 171.1, 169.8, 160.4, 153.3, 131.6 (2C), 125.7, 113.8 (2C), 78.2, 77.2, 70.1, 70.0, 69.8, 61.7, 60.2, 55.5, 55.3, 43.0, 40.5, 39.2, 39.1, 35.8, 35.5, 33.9, 31.9, 28.0, 25.5; HRMS (ESI) *m/z* 672.2691 (calcd for $C_{30}H_{43}N_5NaO_9S$ $[M+Na]^+$, Δ +1.8 mmu).

HOSu–biotin (16). A solution of amide **15** (8.0 mg, 12 μmol) in a 1:1 mixture of CH_2Cl_2–TFA (1 mL) was stirred at room temperature under a nitrogen atmosphere for 30 min. The resulting mixture was azeotropically dried with toluene, and purified with a SiO_2 column chromatography (0.5 g, $CHCl_3$/MeOH = 19/1 to 4/1) to give HOSu–biotin (**16**) (5.0 mg, 77%) as a colorless oil. **16**: R_f = 0.18 (4:1 $CHCl_3$/MeOH); $[\alpha]_D^{26}$ +140 (*c* 0.51, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 4.49 (br dd, *J* = 7.8, 4.1 Hz, 1H), 4.30 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.61 (s, 3H), 3.54 (t, *J* = 5.6 Hz, 2H), 3.53 (t, *J* = 5.6 Hz, 2H), 3.36 (m, 4H), 3.20 (ddd, *J* = 8.8, 5.9, 4.4 Hz, 1H), 3.11 (m, 1H), 2.92 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.89 (m, 1H), 2.86 (dd, *J* = 17.8, 8.9 Hz, 1H), 2.72–2.67 (m, 3H), 2.48 (dd, *J* = 17.8, 4.4 Hz, 1H), 2.22 (t, *J* = 7.4 Hz, 2H), 1.79–1.53 (m, 4H), 1.50–1.39 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 176.8, 176.2, 174.1, 172.4, 166.1, 71.3 (2C), 70.6, 70.5, 63.4, 61.6, 57.0, 41.0, 40.3, 40.3, 36.8, 36.1, 35.1, 32.5, 29.7, 29.5, 26.8; IR ($CHCl_3$) 3453, 3323, 3019, 2929, 2863, 1711, 1674, 1525, 1459, 1139 cm^{-1}; HRMS (ESI) *m/z* 552.2107 (calcd for $C_{22}H_{35}N_5NaO_8S$ $[M+Na]^+$, Δ +0.9 mmu).

apy–OSu–biotin (2). To a stirred solution of amidopyrene carboxylic acid **4** (3.6 mg, 10 μmol) and HOSu–biotin (**16**) (5.5 mg, 10 μmol) in dry DMF (0.3 mL) were added EDC·HCl (7.7 mg, 40 μmol) and HOBt (7.0 mg, 50 μmol) under a nitrogen atmosphere. After being stirred for 40 h at 30 °C, the resulting mixture was diluted with $CHCl_3$ (5 mL), washed with brine, dried and azeotropically concentrated with toluene. The crude material was purified with a SiO_2 column chromatography (0.5 g, $CHCl_3$/MeOH = 19/1 to 4/1) to give apy–OSu–biotin (**2**) 3.2 mg (36%) as a lightyellow oil. **2**: R_f = 0.47 (4:1 $CHCl_3$/MeOH); ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.31 (s, 1H), 8.36 (d, *J* = 9.4 Hz, 1H), 8.32 (s, 1H), 8.28–8.15 (m, 5H), 8.13 (t, *J* = 5.8 Hz, 1H), 7.97 (d, *J* = 7.8 Hz, 1H), 7.84 (t, *J* = 5.3 Hz, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.29 (m, 1H), 4.11 (m, 1H), 3.53–3.35 (m, 6H), 3.22–3.16 (m, 4H), 3.17 (t, *J* = 5.3 Hz, 2H), 3.08 (m, 1H), 3.01 (m, 1H), 2.87 (t, *J* = 7.1 Hz, 1H), 2.80 (m, 1H), 2.64 (m, 1H), 2.57 (t, *J* = 6.2 Hz, 2H), 2.54 (m, 2H), 2.39 (t, *J* = 7.3 Hz, 1H), 2.27 (s, 3H), 2.15–2.09 (m, 2H), 2.08–2.00 (m, 2H), 1.62–1.55 (m, 2H), 1.52–1.41 (m, 4H), 1.34–1.21 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 174.4, 172.6, 172.2, 172.1, 169.6, 169.1, 162.7, 135.8, 131.9, 129.4, 128.4, 127.9, 127.8, 127.4, 127.2, 124.9, 124.7, 124.7, 124.4, 123.9, 123.4, 122.5, 121.6, 79.2, 69.5 (2C), 69.2, 69.0, 61.0, 59.2, 55.5, 40.1, 38.6, 38.5, 38.4, 35.1, 33.3, 31.6, 30.2, 28.2, 28.1, 26.8, 25.3, 23.6; HRMS (ESI) *m/z* 857.3550 (calcd for $C_{44}H_{53}N_6O_{10}S$ $[M+H]^+$, Δ +1.1 mmu).

Conflicts of interest

There are no conflicts to declare.

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entry text

Ligand-dissociation type amidopyrene probes, being useful for LDI MS, were developed for Specific protein-labeling and ligand-binding position analysis