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Multivalent Photoaffinity Probe for Labeling Small Molecule Binding Proteins

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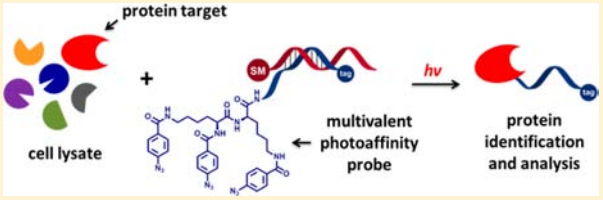
Supporting Information

ABSTRACT: Characterization of small molecule (SM)−protein interaction is of high importance in biomedical research such as target identification and proteomic profiling. Photo-cross-linking is a powerful and straightforward strategy to covalently capture SM's binding proteins. The DNA-based photoaffinity labeling method is able to capture SM's protein targets with high specificity but suffers low cross-linking efficiency, which limits its utility for low abundance and low affinity proteins. After screening a variety of cross-linkers, by utilizing the multivalency effect, the cross-linking efficiency was improved by nearly 7-fold without compromising probe specificity. The generality and performance of multivalent photoaffinity probes have been validated with a variety of SM-protein pairs in the complexity of cell lysates.



INTRODUCTION

Small molecules (SM) are important tools in exploring biology and drug discovery.[1−3] On the molecular level, it is of great importance to accurately and specifically characterize SM−protein interactions. For instance, phenotypic assays evaluate small molecule holistic biological effects and provide opportunities for new target discovery and clues to explore unknown pathways;[4] therefore, effective affinity probes are instrumental in revealing small molecules' binding targets and to illuminate the underlying molecular mechanisms.[5] In another aspect, small molecule-based activity probes are also widely used in proteomic profiling of protein compositions and functions in cells and organisms within therapeutically relevant contexts (activity-based protein profiling, ABPP).[6−13]

Classical affinity pull-down, a method using an immobilized or tagged small molecule as bait to isolate its binding proteins from cell lysates, has succeeded in identifying the targets of a number of important bioactive molecules and historic drugs.[14−17] However, since these probes are usually limited to high abundance and high affinity proteins due to the dynamic, noncovalent nature of most SM−protein interactions, affinity probes equipped with additional chemical or photoreactive groups were developed to covalently capture, purify, and identify protein targets (Figure 1a). Since first developed in 1994,[18] this type of probe has received extensive utility in numerous studies.[10−12,19−31]

Recently, our group developed a method named DNA-programmed affinity labeling (DPAL).[32] In DPAL, functions of protein binding, cross-linking, and tagging that usually exist in a single probe are decoupled to two complementary DNA probes (binding probe and capture probe, abbreviated as BP and CP).

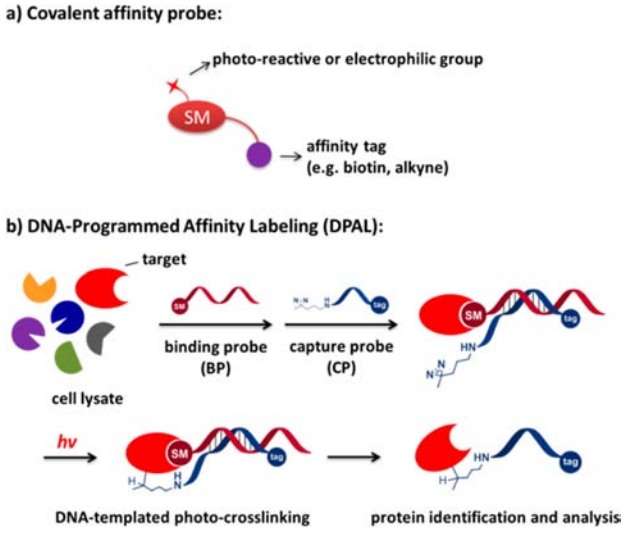


Figure 1. (a) Covalent affinity probe is usually equipped with a reactive group for protein capture and an affinity tag for protein purification and identification after the capture. (b) DNA-programmed affinity labeling (DPAL). DPAL uses a dual-probe system to capture target proteins from cell lysates. Previously an alkyl diazirine group was used in CP as the photo-cross-linker.

After SM binds to the target, the DNA tag in BP guides CP hybridization, which places the diazirine in proximity to the

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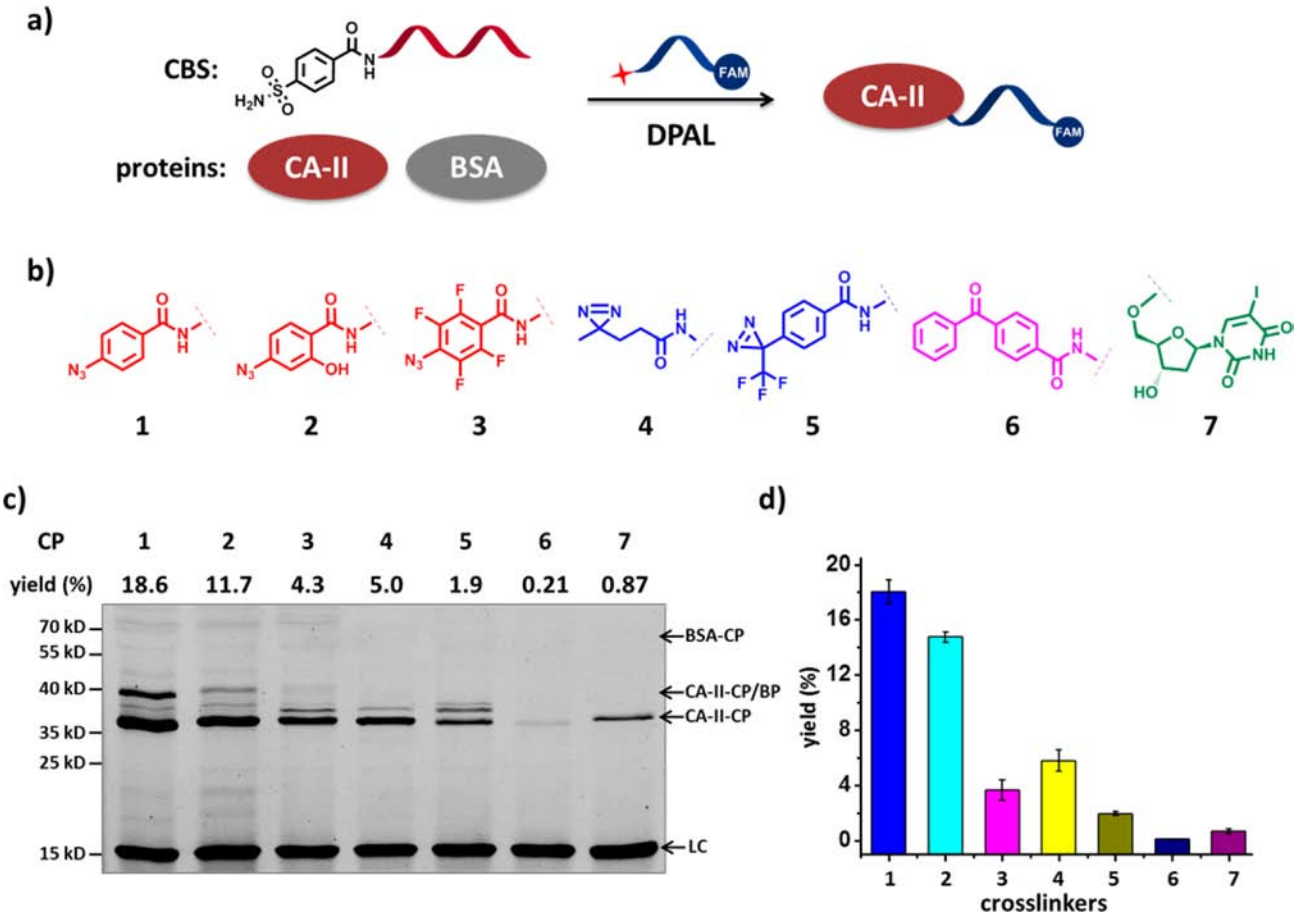

Figure 2. (a) Target CA-II capture mediated by CBS-BP and FAM-CP in the presence of nonspecific protein BSA. Red star represents the photo-cross-linker on FAM-CP. (b) Various photo-cross-linkers used in this study. (c) Representative SDS-PAGE result, monitored by FAM fluorescence. CPs used and corresponding labeling yields are marked on top of the gel image. Yields were calculated by comparing FAM fluorescence with the internal fluorescence standard. CA-II and BSA: 5 μM. BP and CP: 10 μM each. Irradiation: 365 nm, 0 °C for 30 min. LC: loading control, a 66-nt, 5′-FAM-labeled DNA. Expected positions of protein−DNA conjugates are marked by arrows. CA-II-CP/BP: the duplex of CA-II-CP and BP. (d) Comparison of the CA-II labeling yields of different photo-cross-linkers. Estimated uncertainty (error bars) is based on three replicates of each experiment.

protein for cross-linking under light irradiation (Figure 1b). DPAL probes have shown excellent generality, specificity, and sensitivity in identifying proteins in the complexity of cell lysates and the introduction of DNA also enables multiplexed protein labeling.

However, DPAL suffers low cross-linking yield (2−8% in most cases), which is sufficient for the purpose of protein identification, but brings significant difficulties for studying low abundance/low affinity proteins (e.g., very large quantities of cell lysates are required). Moreover, it limits DPAL from being used as a detection method (e.g., through polymerase chain reaction amplification of the DNA tag).[33−35] It is well-known that the carbene intermediate generated from diazirine under light irradiation is highly reactive, but it can also be rapidly quenched by solvent molecules or by going through intramolecular rearrangements;[36,37] therefore, although diazirine usually exhibits less nonspecific labeling, its cross-linking efficiency is relatively low.[38,39] In order to address this issue, the performance of DNA-based affinity probes was optimized in this study. First, a variety of photoreactive groups were screened for the one with higher cross-linking yield. Second, in order to increase the effective molarity of the cross-linker to the protein, multivalent affinity probes were designed and the cross-linking yield can be improved by nearly 7-fold. Finally, we validated that the improved efficiency does not compromise probe specificity by testing a variety of SM−protein pairs in the complexity of cell lysates.

RESULTS AND DISCUSSION

Comparing Cross-Linking Efficiencies of Different Photoreactive Groups. Besides the alkyl diazirine used in DPAL, a number of different photoreactive groups have been incorporated into various affinity probes in many applications.[25,40,41] Among them, iodouracil (I-dU), aryldiazirines, arylketones, and arylazides are the most frequently used types. Their reactivities and selectivities are often discussed and compared in the literature, all exhibiting different advantages and disadvantages.[26,40,42−44] Therefore, seven representative examples covering all four types of cross-linkers were selected and compared for their performances in parallel DPAL experiments (Figure 2a). The *m*-hydroxyphenyl azide (**2**) was selected due to its improved propensity to interact with protein surface.[45] The pentafluorophenyl azide (**3**) is known to have significantly lower rate of rearrangement to the much less reactive dehydroazepine.[46] Similarly, comparing with the alkyl diazirine (**4**), the 3-trifluoromethyl-3-phenyl diazirine (**5**) also

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avoids the rearrangement to the less reactive diazo species.[41,44,47]

First, CBS, a known binder of carbonic anhydrase II (CA-II) with a moderate affinity (K_d: 3.2 μM),[48] was coupled to the 5′-amine of a 24-nt DNA as the BP (Figure 2a). Then photoreactive groups **1**−**6** were conjugated to the 3′-amine of a 15-nt DNA modified with a 5′-carboxyfluorescein (FAM) group as the CP, respectively. I-dU (**7**) was directly incorporated in CP as a special nucleobase via automated DNA synthesis (Figure 2b and see the Supporting Information). Next, the mixture of CBS-BP and FAM-CP was subjected to DNA-programmed photo-cross-linking procedure in the presence of target CA-II. Additionally, equimolar BSA (bovine serum albumin), a nonspecific protein, was added as a background to examine the specificity of these probes. These cross-linkers can be activated under a wide range of irradiation wavelengths; however, for better comparison, the same nonprotein/DNA-damaging irradiation condition (365 nm) was applied to all experiments. Reactions were heat-denatured and analyzed by SDS-PAGE. Photo-cross-linking yields were determined by measuring FAM fluorescence of the formed CA-II-CP conjugates, calibrated by an internal fluorescence standard (Figure 2c). Additional bands formed above the CA-II-CP band are considered to be the DNA duplex formed between the CA-II-CP and CBS-BP since complementary DNAs may renature during SDS-PAGE, a phenomenon observed previously and confirmed experimentally.[32]

As shown in the comparison chart of Figure 2d, arylazides **1** and **2** showed distinctively higher cross-linking yields (18.0% and 14.7%, respectively). Interestingly, arylazide **3** and aryldiazirine **5** gave significantly lower yields than their nonfluorinated counterparts (**1** and **4**; Figure 2d). Formerly these "no-arrangement" cross-linkers were developed to reduce nonspecific labeling from the less reactive but longer-lived rearrangement products (dehydroazepine and diazo species).[40,42] In DPAL, nonspecific labeling was suppressed effectively with a salt effect;[32] therefore we postulate that, in DPAL, the rearrangement products from cross-linkers **1** and **4** may have also contributed to the cross-linking reaction and therefore resulted in higher labeling yields.[43,46] No significant BSA labeling was observed for all cross-linkers, proving the specificity of these probes. Based on this result, the simple, unsubstituted phenylazide **1** (**AZ-1**) was selected as the cross-linker in subsequent studies.

Determining the Optimal BP/CP Hybridization Site. DPAL's dual-probe design provides unique modularity; CP can hybridize at different sites on BP to tune the labeling performance.[32] A series of phenylazide-conjugated CPs (**AZ-1**-CP) complementary to different sites on the CBS-BP (different "n" values; Figure 3) were prepared and compared for their CA-II labeling efficiencies in DPAL experiments. Results show that highest labeling yield (~20%) was achieved with the typical "side-by-side" (n = 0) hybridization format; in general, CPs with negative n values have higher efficiency than positive ones (Figure 3). We hypothesize that a recessed CP may present the phenylazide with higher effective molarity to CA-II due to the large size of the protein, while a recessed BP may cause more steric hindrance for CA-II binding.

Improving Cross-Linking Efficiency through Multivalent Cross-Linkers. Previously, Hamachi and co-workers reported that the introduction of multiple organocatalytic DMAP groups in affinity probes is able to improve the cross-linking yield.[49] We reason that this multivalency effect may also

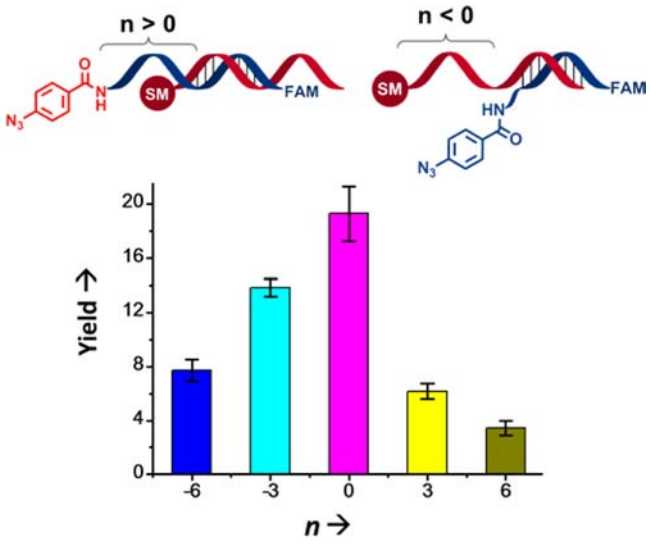

Figure 3. Effect of BP/CP hybridization site (different n values) on the labeling yield with the phenylazide **AZ-1** as the cross-linker. SM: CBS. target protein: CA-II. Reaction setup and conditions are the same as in Figure 2. Labeling yields were determined by an internal FAM-labeled 66-nt DNA. Estimated uncertainty (error bars) are based on three replicates of each experiment.

be applicable to DPAL;[50,51] incorporation of multiple cross-linkers in a single CP should be a straightforward way to improve the labeling yield. First, a series of carboxylic acids with multiple phenylazides were prepared by using the lysine scaffold and then coupling them to the CP DNA. One CP having two phenylazides and two CPs having three phenylazides but different spatial arrangements were prepared (**diAZ-8**, **triAZ-9**, and **triAZ-10**; Scheme 1). These multivalent CP probes were mixed with CBS-BP and subjected to DPAL conditions in the presence of target CA-II and background BSA. As shown in Figure 4, multivalent cross-linkers were able to significantly improve probes' labeling yields (Figure 4b and c). The trivalent **triAZ-9** and **triAZ-10** gave labeling yields of 32.8% and 38.3%, respectively, which is approximately 7-fold over the average yield of diazirine-based CP (**DZ-4** in Figure 2). Importantly, no noticeable nonspecific BSA labeling was observed for all multivalent probes. Moreover, a trivalent diazirine CP (**triDZ-11**, Figure 4d) was also prepared and similar improvement was observed (Figure 4e and f). Collectively, these results have demonstrated that indeed the probe efficiency can be improved with the multivalency strategy. It is worth noting that the introduction of multiple cross-linkers significantly increases the probe size, which usually is detrimental for conventional affinity probes; however, due to DPAL's modularity, modifications made on CP are unlikely to affect protein−BP interactions.

Affinity Labeling of Proteins in the Background of Cell Lysate with Multivalent Probes. Next, in order to validate the generality and performance of these multivalent probes, along with CBS-CA-II, we also studied two additional SM−protein pairs (Figure 5a): AP1497-FK506 binding protein 12 (FKBP12, K_d: 25.7 nM)[52] and the weakly binding chymostatin-papain (IC_{50}: 14 μM).[53] BPs of these SMs were combined with CPs either containing a single phenylazide (**AZ-1**) or three phenylazides (**triAZ-10**) and their labeling performances were compared in the background of HeLa cell lysate supplemented with BSA. Results show that the

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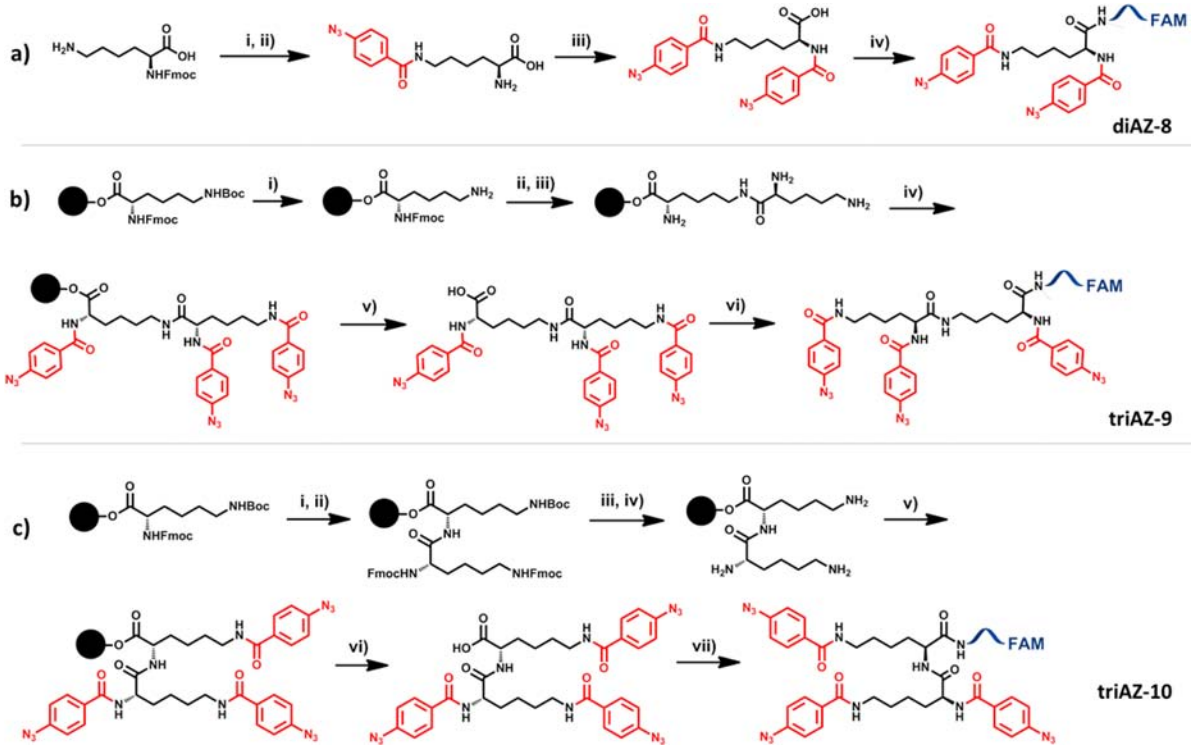
Scheme 1. Synthesis of Multivalent Capture Probes diAZ-8, triAZ-9, and triAZ-10[a]



[a]Reagents and conditions: (a) (i) DIPEA, *N*-hydroxysuccinimidyl-4-azidobenzoate, DMF, r.t., overnight; (ii) 3% DBU in DMF, r.t., 1 h; (iii) DIPEA, *N*-hydroxysuccinimidyl-4-azidobenzoate, DMF, r.t., 2 h, 49.5%; (iv) DCC, NHS, FAM-DNA, DMF, and phosphate buffer, r.t., 2 h. (b) (i) 1,4-dioxane: concentrated H_2SO_4 = 9:1 (v/v), 8 ± 2 °C, 2.5 h; (ii) Fmoc-Lys(Fmoc)-OH, HOAt, HATU, DIPEA, DMF, r.t., 1 h, repeated 3 times; (iii) 20% piperidine in DMF, r.t., 5 min, repeated 3 times; (iv) 4-azidobenzoic acid, HOAt, HATU, DIPEA, DMF, r.t., 1 h, repeated 3 times; (v) TFA:H_2O:TIPS = 95:2.5:2.5, r.t., 2 h, 35.9%; (vi) DCC, NHS, FAM-DNA, DMF, and phosphate buffer, r.t., 2 h. (c) (i) 20% piperidine in DMF, r.t., 5 min, repeated 3 times; (ii) Fmoc-Lys(Fmoc)-OH, HOAt, HATU, DIPEA, DMF, r.t., 1 h, repeated 3 times; (iii) 1,4-dioxane:concentrated H_2SO_4 = 9:1, (v/v), 8 ± 2 °C, 2.5 h; (iv) 20% piperidine in DMF, r.t., 5 min, repeated 3 times; (v) 4-azidobenzoic acid, HOAt, HATU, DIPEA, DMF, r.t., 1 h, repeated 3 times; (vi) TFA:H_2O:TIPS = 95:2.5:2.5, r.t., 2 h, 45.0%; (vii) DCC, NHS, FAM-DNA, DMF, and phosphate buffer, r.t., 2 h; Filled circles: Wang resin.

multivalent **triAZ 10** probe has significantly improved the labeling yields for all three SM−protein pairs, and importantly, very little labeling of cellular proteins and nonspecific BSA were observed (Figure 5b, c, and d), indicating that the improvement does not compromise probe specificity.

CONCLUSION

In summary, we have optimized the cross-linking efficiency of DPAL probes. Simple, unsubstituted phenylazide was found to be the optimal photo-cross-linker, presumably due to its tendency to generate longer-lived reactive intermediates. By further introducing multiple phenylazide cross-linkers into the probe, labeling yields of multivalent probes have been significantly improved. One advantageous feature of DPAL is that CP modification does not affect SM−protein binding; therefore, in principle, four or even more cross-linkers could be incorporated to further improve the labeling efficiency. Importantly, the probe specificity is not compromised despite the presence of multiple cross-linkers. This type of multivalent probe may have wide application in studying small molecule−protein interactions, such as a method for sensitive protein detections when coupled with PCR amplification.

EXPERIMENTAL PROCEDURES

Materials and Methods. All reagents and solvents were purchased from commercial sources. Purified proteins were purchased from Sigma-Aldrich (CA-II, papain) and Sino Biological Inc. (BSA, FKBP12). Small molecules were purchased from J&K Scientific Ltd. and Beijing Ouhe Technology Co. Ltd. 5,6-FAM was purchased from Beijing Fanbo Biochemicals. Amino acids and resin were purchased from GL Biochem (Shanghai) Ltd. Water was purified with a Thermo Scientific Barnstead Nanopure system. Proteins and protein−DNA conjugates were analyzed by SDS-PAGE (12% or 15%). All gel images were captured by a Bio-Rad Chemidoc system. Photo-cross-linking experiments were conducted by a UVP CL-1000L Ultraviolet Cross-linker at 365 nm wavelength with an intensity of approximately 100 $\mu J/cm^2$.

DNA Synthesis. DNA oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer using standard phosphoramidite protocols and were purified by C18 reverse-phase HPLC with aqueous 0.1 M triethylammonium acetate (TEAA)/CH_3CN gradient on Agilent 1200 HPLC systems (Eclipse-XDB C18, 5 μM, 9.4 × 200 mm or 4.6 × 150 mm). After solid-phase synthesis, oligonucleotides were cleaved by AMA (55 °C, 55 min) over a dry bath, then concentrated and neutralized (2.0 M TEAA) before HPLC purification. For all nonstandard phosphoramidites, coupling time was modified to 999 s. Nonstandard phosphoramidites were either purchased or prepared in our laboratory as described in the Supporting Information. Oligonucleotides with a 3′-amino group were synthesized using the 3′-amino-modifier C7 CPG. 5′-Amine

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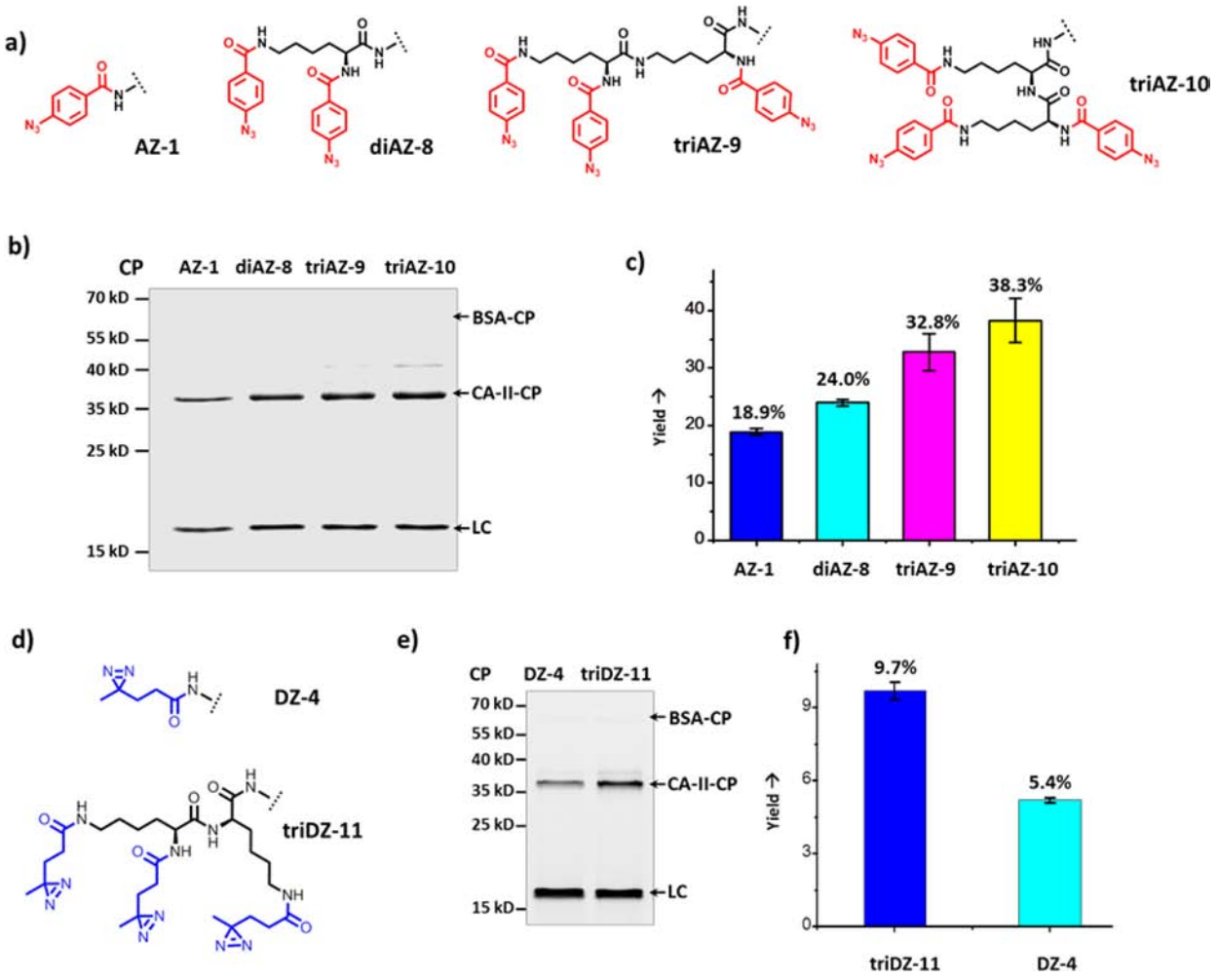

Figure 4. (a) A series of arylazide cross-linkers were compared for their efficiencies in labeling CBS-BP's target CA-II in the BSA background. (b) Representative SDS-PAGE analysis results, monitored by FAM fluorescence. (c) Comparison of CA-II labeling yields. (d) Two different diazirine cross-linkers were also compared for their CA-II-labeling efficiencies. (e) Representative SDS-PAGE analysis result, monitored by FAM fluorescence. (f) Comparison of the CA-II labeling yields of **DZ-4** and **triDZ-11**. Reaction setup and experimental conditions are the same as in Figure 2. Labeling yields were determined by an internal FAM-labeled 66-nt DNA. Estimated uncertainty (error bars) are based on three replicates of each experiment.

was incorporated using the 5′-amino-modifier 5. Oligonucleotides were quantitated by a BioTek Epoch UV−vis spectrometer based on extinction coefficients at 260 nm.

Binding Probe (BP) Synthesis. After solid phase DNA synthesis, the 5′-MMT amino protecting group was removed by 80% acetic acid. Carboxylic acids of small molecules were conjugated to the 5′-amine of the BP DNA via amidation as described in detail in the Supporting Information. Typically, small molecules can either be activated by EDCI/NHS in situ or in the form of *N*-hydroxysuccinimidyl ester by DCC/NHS.

Capture Probe (CP) Synthesis. After solid phase DNA synthesis but without AMA cleavage, 5′-MMT amino protecting group was removed by 3% TCA directly on CPG. 5,6-FAM was activated by HOBt, HBTU, and DIPEA in DMF. After incubation at room temperature for 1 h, the activation mixture was added to the CPG of CP DNA. The suspension was agitated at 37 °C overnight. After washing the CPG with DMF and CH_3CN, 5′-FAM-modified CP DNA was cleaved by AMA. The cleaved DNA was desalted by a NAP-10 column (GE Pharmacia) and then purified by reverse-phase HPLC. Photo-cross-linkers were conjugated to the 3′-amine of CP DNAs with the same method as in BP labeling (see the Supporting Information for details). As for the CP containing I-dU (7), the commercially available I-dU phosphoramidite was used as the first base in automated DNA synthesis. In order to match the length of the 3′-amine modifier C7 in other CPs, an additional triethylene glycol phosphoramidite was coupled immediately after the I-dU incorporation (see the Supporting Information). Final product CP DNAs were obtained by HPLC purification.

DNA Sequences. In Figures 2, 4, and 5: BP, 5′-GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, 5′-AGG GCT TTG GAA TTC-3′. In Figure 3: BP, n = 0, 5′-GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, n = 0, 5′-AGG GCT TTG GAA TTC-3′; BP, n = −3, 5′-CCT GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, n = −3, 5′-AGG GCT TTG GAA TTC-3′; BP, n = −6, 5′-GTA CCT GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, n = −6, 5′-AGG GCT TTG GAA TTC-3′; BP, n = 3, 5′-GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, n = 3, 5′-AGG GCT TTG GAA TTC TTT-3′; BP, n = 6, 5′-GAA TTC CAA AGC CCT CAC AAT CCC-3′; CP, n = 6, 5′-AGG GCT TTG GAA TTC TTT GCA-3′.

Solid-Phase Synthesis of Multivalent Photo-Cross-Linkers. Fmoc-Lys (Boc)-Wang resin was initially swelled with DMF for 20 min. Fmoc was deprotected by 20% piperidine in DMF (room temperature, 5 min, repeated 3 times). Then the

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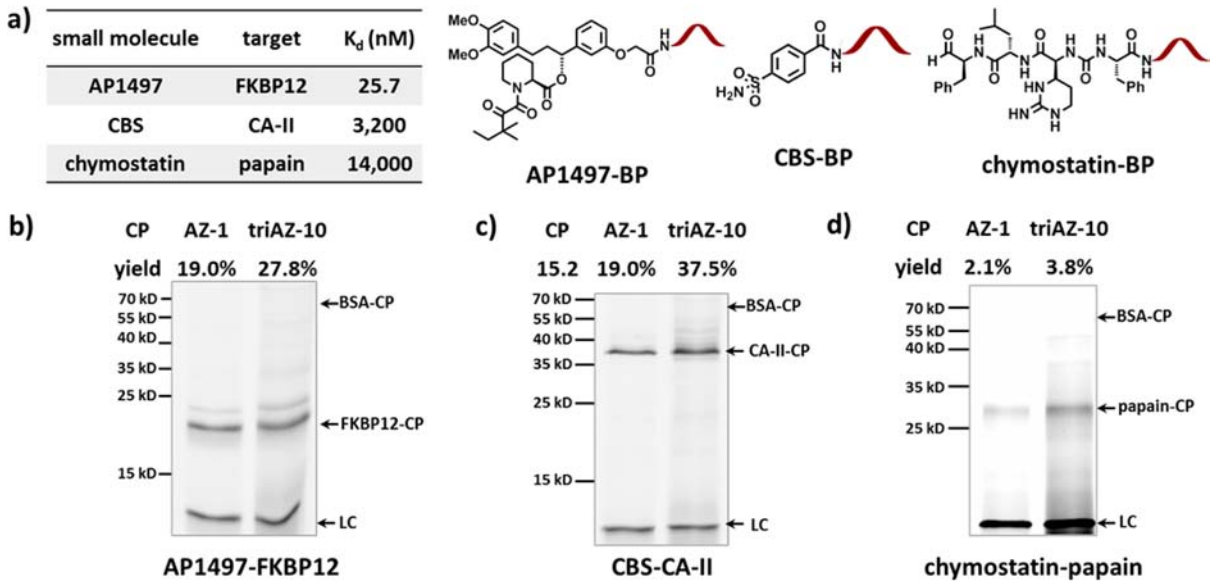

Figure 5. Test of generality and labeling specificity of multivalent probes. (a) Structures of SMs, their respective protein targets and binding affinities. SDS-PAGE analysis results for (b) AP1497-FKBP12, (c) CBS-CA-II, and (d) chymostatin-papain, monitored by FAM fluorescence. Corresponding labeling yields are marked on top of gel images. Reaction setup and conditions are the same as in Figure 2 except 5 μM BSA and 10 μg (1.0 mg/mL) HeLa cell lysate were used as the nonspecific background. Labeling yields were determined by an internal FAM-labeled 50-nt DNA.

resin was washed with 5× DMF, 5× DCM, and 5× DMF. Coupling step was carried out by pouring a solution of carboxylic acid of the photo-cross-linker (0.4 M in DMF, 6.0 equiv) with 5.7 equiv HBTU, 6.0 equiv HOBt, and 16.0 equiv DIPEA to the resin. For single couple, after 2 h, the resin was washed with 5× DMF, 5× DCM, and 5× DMF. For multiple couples, reaction time was shortened to 30−60 min. Coupling efficiency was checked with Kaiser's test. Cleavage was performed with a cocktail of TFA/H_2O/TIPS (95%/2.5%/2.5%). The mixture was added to the dry resin prewashed with DCM. After 2 h at r.t., the resin was washed with an equal volume of TFA once. Combined eluents were concentrated by blowing with argon. The crude peptides were obtained by precipitating with cold ether and centrifugation at 5000 rpm/min for 5 min. The residue was dissolved in 0.1% TFA containing cosolvent of acetonitrile (1:1), purified by preparative HPLC, and lyophilized. Alternatively, the crude product can be purified by column chromatography. Boc-protected Wang resin (15 g) was deprotected by a prechilled (8 ± 2 °C) mixture of concentrated H_2SO_4/1,4-dioxane (1:9 v/v, 120−125 mL). The suspension was stirred at 8 ± 2 °C for 2.5 h and filtered. The resin was washed with 10% v/v DIPEA in DMF (3× 150 mL) and then with CH_2Cl_2 (4× 180 mL).

(*S*)-2,6-Bis(4-azidobenzamido)hexanoic Acid (diAZ-8). Fmoc-Lys-OH (184.0 mg, 0.50 mmol) and DIPEA (148.6 mg, 1.15 mmol) were added to 5 mL DMF. *N*-Hydroxysuccinimidyl-4-azidobenzoate (169.0 mg, 0.65 mmol) was added to the solution. The reaction was stirred at r.t. overnight and monitored by TLC. After completion, 150 μL DBU (3% in DMF) was added to deprotect Fmoc (1 h, r.t.). Then *N*-hydroxysuccinimidyl-4-azidobenzoate (169.0 mg, 0.65 mmol) was added to the solution again. After 2 h, DMF was removed under vacuum. Water and chloroform were added to the residue for phase separation and the aqueous phase was extracted twice by chloroform. Organic fractions were combined and washed by saturated NH_4Cl solution and brine. The organic phase was dried by Na_2SO_4, concentrated, and purified by column chromatography (DCM:MeOH = 40:1 to 20:1) to afford the product as a white solid (49.5%). ^{1}H NMR (500 MHz, DMSO) δ 12.54 (s, 1H), 8.54 (d, J = 7.7 Hz, 1H), 8.43 (t, J = 5.6 Hz, 1H), 7.94−7.90 (m, 2H), 7.88−7.83 (m, 2H), 7.20−7.14 (m, 4H), 4.36 (ddd, J = 9.5, 7.7, 5.0 Hz, 1H), 3.25 (dd, J = 13.2, 6.6 Hz, 2H), 1.89−1.75 (m, 2H), 1.55 (pd, J = 13.3, 6.5 Hz, 2H), 1.48−1.35 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 173.76 (s), 165.55 (s), 165.14 (s), 142.35 (s), 142.00 (s), 131.26 (s), 130.55 (s), 129.34 (s), 128.99 (s), 118.77 (d, J = 1.9 Hz), 52.55 (s), 38.90 (s), 30.23 (s), 28.65 (s), 23.28 (s). ESI-MS (positive) m/z 435.15336 (calcd. for $C_{20}H_{20}N_8O_4$: 435.15347).

(*S*)-2-(4-Azidobenzamido)-6-((*S*)-2,6-bis(4-azidobenzamido) hexanamido)hexanoic Acid (triAZ-9). Fmoc-Lys(Boc)-Wang resin (1.0 g, 0.28 mmol) was added to a peptide synthesis vessel. (i) Deprotection: Boc group was removed as described above. (ii) First coupling: Fmoc-Lys(Fmoc)-OH (992.3 mg, 1.68 mmol), HATU (606.8 mg, 1.60 mmol), HOAt (227.0 mg, 1.68 mmol), DIPEA (0.78 mL, 4.48 mmol), r.t., 1 h, repeated 3 times. (iii) Deprotection: 20% piperidine in DMF (15 mL), r.t., 5 min, repeated 3 times. (iv) Second coupling: 4-azidobenzoic acid (274.0 mg, 1.68 mmol), HATU (606.8 mg, 1.60 mmol), HOAt (227.0 mg, 1.68 mmol), DIPEA (0.78 mL, 4.48 mmol), r.t., 1 h, repeated 3 times. (v) Cleavage: TFA/H_2O/TIPS (95/2.5/2.5) 10 mL, r.t., 2 h. The crude precipitation formed in cold ether was purified by column chromatography using DCM/MeOH (30:1 to 10:1). The product was obtained as a white solid (35.9%). ^{1}H NMR (500 MHz, DMSO) δ 12.35 (s, 1H), 8.54 (d, J = 7.6 Hz, 1H), 8.41 (t, J = 5.5 Hz, 1H), 8.34 (d, J = 7.9 Hz, 1H), 7.95−7.90 (m, 5H), 7.85 (d, J = 8.6 Hz, 2H), 7.19 (d, J = 8.6 Hz, 2H), 7.15 (dd, J = 8.5, 1.3 Hz, 4H), 4.43−4.28 (m, 2H), 3.21 (td, J = 13.0, 6.3 Hz, 2H), 3.05 (dtd, J = 25.2, 12.8, 6.5 Hz, 2H), 1.75 (tt, J = 22.6, 7.2 Hz, 4H), 1.58−1.27 (m, 8H). ^{13}C NMR (126 MHz, DMSO) δ 173.75 (s), 171.66 (s), 165.57 (s), 165.37 (s), 165.11 (s), 142.37 (s), 142.20 (s), 141.99 (s), 131.25 (s), 130.76 (s), 130.54 (s), 129.39 (d, J = 6.4 Hz), 128.98 (s), 118.77 (d, J = 6.3 Hz), 118.66 (s), 53.48 (s), 52.66 (s), 38.92 (s), 38.21 (s), 31.36 (s), 30.19 (s), 28.72 (s), 28.65 (s), 23.19

(d, J = 4.4 Hz). ESI-MS (positive) m/z 710.28874 (calcd. for $C_{33}H_{35}N_{13}O_6$: 710.29060).

(*S*)-6-(4-Azidobenzamido)-2-((*S*)-2,6-bis(4-azidobenzamido)hexanamido)hexanoic Acid (triAZ-10). Fmoc-Lys(Boc)-Wang resin (1.5 g, 0.42 mmol) was added to a peptide synthesis vessel. (i) Fmoc deprotection: 20% piperidine in DMF (15 mL), r.t., 5 min, repeated 3 times. (ii) First coupling: Fmoc-lys(Fmoc)-OH (1488.5 mg, 2.52 mmol), HATU (910.2 mg, 2.39 mmol), HOAt (340.5 mg, 2.52 mmol), DIPEA (1.17 mL, 6.72 mmol), r.t., 1 h, repeated 2 times. (iii) Boc deprotection: same as described above. (iv) Fmoc deprotection: same as in (i). (v) Second coupling: 4-azidobenzoic acid (441.0 mg, 2.52 mmol), HATU (910.2 mg, 2.39 mmol), HOAt (340.5 mg, 2.52 mmol), DIPEA (1.17 mL, 6.72 mmol), r.t., 1 h, repeated 3 times. (vi) Cleavage: TFA/H_2O/TIPS (95/2.5/2.5) 15 mL, r.t., 2 h. The crude precipitation formed in cold ether was purified by column chromatography using eluent DCM/MeOH (30:1 to 10:1). The product was obtained as a white solid (45.0%). ^{1}H NMR (500 MHz, DMSO) δ 12.38 (s, 1H), 8.43 (t, J = 5.5 Hz, 2H), 8.38 (d, J = 8.0 Hz, 1H), 8.10 (d, J = 7.6 Hz, 1H), 7.95−7.83 (m, 6H), 7.22−7.12 (m, 6H), 4.47 (td, J = 8.7, 5.3 Hz, 1H), 4.17 (tt, J = 15.9, 7.9 Hz, 1H), 3.27−3.18 (m, 4H), 1.83−1.68 (m, 3H), 1.57−1.47 (m, 4H), 1.44−1.31 (m, 4H). ^{13}C NMR (126 MHz, DMSO) δ 173.54 (s), 171.93 (s), 165.42 (s), 165.12 (s), 142.25 (s), 142.00 (d, J = 2.1 Hz), 131.26 (s), 130.75 (s), 129.37 (s), 129.00 (d, J = 3.6 Hz), 118.95−118.53 (m), 53.20 (s), 51.89 (s), 45.65 (s), 40.14 (s), 31.26 (s), 30.77 (s), 28.79 (s), 28.71 (s), 23.14 (s), 22.90 (s). ESI-MS (positive) m/z 710.28887 (calcd. for $C_{33}H_{35}N_{13}O_6$: 710.29130).

(*S*)-2-((*S*)-2,6-Bis(3-(3-methyl-3*H*-diazirin-3-yl)-propanamido)hexanamido)-6-(3-(3-methyl-3*H*-diazirin-3-yl)propanamido)hexanoic Acid (triDZ-11). Fmoc-Lys-(Boc)-Wang resin (1.0 g, 0.28 mmol) was added to a peptide synthesis vessel. (i) Fmoc deprotection: 20% piperidine in DMF (15 mL), r.t., 5 min, repeated 3 times. (ii) first coupling: Fmoc-Lys(Fmoc)-OH (992.3 mg, 1.68 mmol), HATU (606.8 mg, 1.60 mmol), HOAt (227.0 mg, 1.68 mmol), DIPEA (0.78 mL, 4.48 mmol), r.t., 1 h, repeated 2 times. (iii) Boc deprotection: same as described above. (iv) Fmoc deprotection: 20% piperidine in DMF (15 mL), r.t., 5 min, repeated 3 times; (v) second coupling: 3-(3-methyl-3*H*-diazirin-3-yl)-propanoic acid (215.3 mg, 1.68 mmol), HATU (606.8 mg, 1.68 mmol), HOAt (227.0 mg, 1.68 mmol), DIPEA (0.78 mL, 4.48 mmol), r.t., 1 h, repeated 3 times. (vi) Cleavage: TFA/H_2O/TIPS (95/2.5/2.5) 15 mL, r.t., 2 h. The crude precipitation formed in cold ether was purified by reverse-phase preparative HPLC as a white solid. (24.7%). ^{1}H NMR (500 MHz, DMSO) δ 12.47 (s, 1H), 8.01 (d, J = 7.7 Hz, 1H), 7.94 (d, J = 8.2 Hz, 1H), 7.82−7.74 (m, 2H), 4.26 (td, J = 8.5, 5.2 Hz, 1H), 4.11 (td, J = 8.6, 5.0 Hz, 1H), 3.04−2.94 (m, 4H), 2.04 (tq, J = 14.8, 7.4 Hz, 2H), 1.96−1.90 (m, 4H), 1.73−1.65 (m, 2H), 1.58−1.52 (m, 6H), 1.41−1.33 (m, 4H), 1.27 (td, J = 14.4, 6.9 Hz, 6H), 0.98 (d, J = 1.1 Hz, 9H). ^{13}C NMR (126 MHz, DMSO) δ 173.45 (s), 171.77 (s), 170.69 (s), 170.47 (s), 170.43 (s), 52.10 (s), 51.69 (s), 38.40 (s), 38.24 (s), 31.74 (s), 30.56 (s), 29.90 (s), 29.84 (s), 29.73 (s), 29.58 (s), 28.78 (s), 28.60 (s), 25.74 (d, J = 2.6 Hz), 22.76 (s), 22.61 (s), 19.29 (s), 19.23 (s). ESI-MS (positive) m/z 605.35103 (calcd. for $C_{27}H_{44}N_{10}O_6$: 605.35181).

DNA-Programmed Affinity Labeling. Specific conditions (probe concentration, reaction temperature, reaction time, irradiation condition, etc.) for individual experiments have been described in respective figure captions. BP, protein, and/or lysate were incubated at 4 °C for 2 h before CP was added. The mixture (typically the total reaction volume is 20 μL) was maintained for another 30 min before irradiation at 365 nm for 30 min over ice. The sample was heat-denatured at 95 °C for 10 min and then analyzed by SDS-PAGE, visualized and quantitated by UV trans-illumination and densitometry. For all experiments monitored by FAM fluorescence, a filter at 365 nm was used for detection and quantitation. An FAM-labeled 66-nt or 50-nt DNA (10 μM, 2 μL) was used as an internal fluorescence standard to calculate the cross-linking yield.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed DNA synthesis, sequences, modification, and probe structures, characterization data, and other experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AcOH, acetic acid; AMA, 1:1 (v:v) aqueous methylamine (40% wt.):aqueous ammonium hydroxide (30% wt.); Boc, *tert*-butoxycarbonyl; BP, binding probe; BSA, bovine serum albumin; CA-II, carbonic anhydrase II; CP, capture probe; CPG, controlled pore glass; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPEA, *N*,*N*′-diisopropylethylamine; DMF, *N*, *N*′-dimethylformamide; DPAL, DNA-programmed affinity labeling; EDCI, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide, hydrochloride; FAM, carboxyfluorescein; Fmoc, fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′ *N*′-tetramethyluronium hexafluorophosphate; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOAt, *N*-hydroxy-7-azabenzotriazole; HOBt, *N*-hydroxyl-benzotriazole; PAGE, polyacrylamide gel electrophoresis; NHS, *N*-hydroxysuccinimide; SM, small molecule; TCA, trichloroacetic acid; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

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