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Synthesis and evaluation of novel photoreactive α-amino acid analog carrying acidic and cleavable functions

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ABSTRACT

A novel photoreactive α-amino acid bearing an acidic residue and a cleavable diazirine was developed. To mimic common acidic α-amino acids, the residue was designed to be *N*-acylsulfonamide that possesses an acidic proton and is able to dissociate under the physiological conditions. The inhibition assay of its biotin-tagged derivative with glutamyl endopeptidase from *Staphylococcus aureus* V8 strain revealed a Ki_{app} value of 162 μM, which is slightly higher than the K_m value of a common substrate. Upon UV irradiation, this derivative specifically photolabeled glutamyl endopeptidase, L-glutamate dehydrogenase, glutamic oxalacetic transaminase, and L-glutamine synthetase, all the enzymes exhibit high affinity toward acidic α-amino acids. In addition, *N*-acylsulfonamide group functioned as a cleavable linker in mild basic solution after a brief *N*-alkylation. Either the multifunctional nature or the simple structure of this acidic α-amino acid surrogate would be useful as versatile photoreactive building block.

Since photoaffinity labeling is a powerful method for the analysis of biological macromolecules, photoreactive α-amino acids have become increasingly important tools for identification and mapping of peptide–protein interactions as well as protein–protein interactions.[1] Introduction of photophores such as aryl azide, benzophenone, and phenyldiazirine into peptide or proteins has been mainly achieved by following two ways: (1) selective chemical modification on the amino acid residues of proteins or peptides, and (2) site-directed incorporation of photoreactive amino acids by using chemical peptide synthesis or transcriptional system in vitro[1a,2] and in vivo.[1b,3] The former method can easily introduce a photophore by the modification of nucleophiles such as NH_2, CO_2H, or SH groups, that often causes the loss of the original nature of the respective residues. The application of latter method is currently limited for the introduction of hydrophobic amino acid analogs.

Herein we report a novel photoreactive and cleavable α-amino acid analog **3** having an anionic residue, which is designed on the basis of *N*-acylsulfonamide chemistry (Fig. 1). The pK_a value of general *N*-acylsulfonamide is close to that of general carboxylic acid, which results in the formation of an anion under the physiological conditions. In fact, *N*-acylsulfonamide is well known as a carboxylic acid bioisostere in medicinal chemistry.[4] It is one of small linkages which exhibits useful features including anionic center generated by deprotonation, possibility to introduce various functions to the acidic residue via acylation, and cleavability by hydrolysis under mild basic condition upon the *N*-alkylation.[5] After cross-linking the interacting molecules, the cleavability would be useful for purification of labeled products as reported before.[6] The cleavability is also essential in the application of photoreactive probes for analyzing protein–protein interactions by label transfer methods.[7]

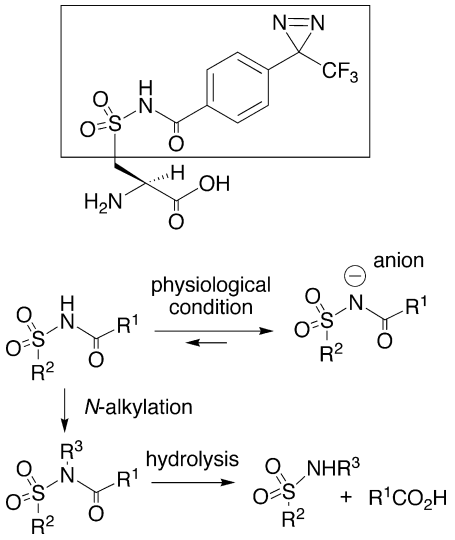

Figure 1. Multifunctional nature of *N*-acylsulfonamide-containing α-amino acid analog.

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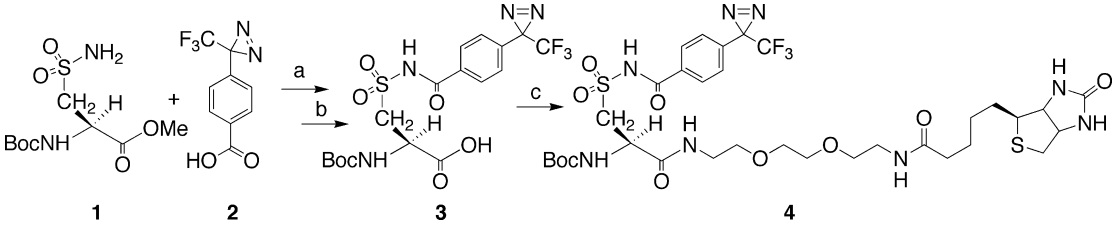
The formation of *N*-acylsulfonamide is usually performed by condensation of a corresponding carboxylic acid with a sulfonamide derivative, or by a reaction of sulfonyl chloride and amide. Other alternatives have been carried out by thio acid/azide amidation,[8] or by reaction of carboxylic acid and *p*-toluenesulfonyl isocyanate.[9] We firstly synthesized an α-amino acid derivative **1** with a sulfonamide group, which was easily prepared from L-cystine,[10] and then coupled with 4-(3-trifluoromethyl-3*H*-diazirin-3-yl)-benzoic acid **2** to give the compound **3** in good yield (Scheme 1). The 3-phenyl-3-trifluoromethyl diazirine derivatives have been recognized to satisfy most of the chemical criteria required for photoaffinity labeling. We also developed an efficient modification on the benzene ring to give a variety of the diazirine derivatives.[1e] A biotin was finally introduced to give the α-amino acid-based photoaffinity probe **4**[11] for the radioisotope-free detection of the labeled protein by chemiluminescence method based on biotin–avidin interactions without any purification of the labeled products.[12] Besides Boc group for *N*-protection, other *N*-protected α-amino acid derivatives (Z and Fmoc) were easily obtained in similar way.

The photoaffinity labeling of glutamyl endopeptidase from *Staphylococcus aureus* V8 strain (V8 protease, 29 kDa, E.C. 3.4.21.19) has been performed with the biotin-tagged photoaffinity probe **4**. This enzyme is a well-known serine protease exhibiting unique specificity to acidic α-amino acid residues (Glu and Asp),[13] and some acylsulfonamide-containing compounds have been used as protease inhibitors.[4b] After incubation of the enzyme with a stoichiometric amount of compound **4** (125 μM) in Tris–phosphate buffer (0.2 M, pH 7.8) at 37 °C for 15 min, the sample solution was irradiated with UV light (360 nm, 15 W ×2) for 40 min on ice and was then subjected to 12% SDS–PAGE after the treatment with SDS sample buffer at room temperature. The proteins were electrophoretically transferred onto a poly(vinylidene difluoride) (PVDF) membrane, and the visualization of the blots on the membrane by chemiluminescence detection was carried out as previously reported.[14] In Figure 2, a single band of protein was detected around 29 kDa, which corresponds to the photolabeled V8 protease (lane 2) whereas no labeled products were observed when the sample was not irradiated (lane 1). The average labeling yield was ca. 5% calculated from the dot-blot analysis using the chemiluminescence detection method compared with the biotinylated albumin (Sigma). The similar value was obtained from density of the band of V8 protease after SDS–PAGE separation. Lanes 3–5 (panel B) and lanes 7–10 (panel C) show the results of label inhibition with *N*-Boc-L-glutamic acid α-amide **5** as a specific glutamyl endopeptidase substrate[15] as well as an analogous derivative of compound **3** without a diazirine moiety **6**, respectively. Depending on the concentration of the competitors, the amount of labeled proteins clearly decreased in both cases. The same inhibition profile was observed when using *N*-Boc-L-aspartic acid α-amide (data not shown) whereas a neutral derivative, *N*-Boc-L-glutamic acid γ-benzyl ester α-amide **7**, did not affect the photolabeling (panel D).

To confirm the specificity of the probe-protein binding, the biotinyl probe **4** was examined in an enzyme assay of V8 protease. The biological activity of compound **4** as a V8 protease inhibitor was spectrophotometrically determined by tracing the production of phenol from a specific substrate, *N*-Boc-L-glutamic acid α-phenyl ester as described.[15] The obtained Ki_{app} value (161.7 ± 2.9 μM) was slightly higher than the K_m value of this substrate (86 μM).

The results of photolabeling and kinetic study described above obviously showed that the photoaffinity probe **4** was specifically recognized by V8 protease, and indicate that the acylsulfonamide could mimic the desired acidic residue. In fact, the probe **4** was confirmed to label other proteins, L-glutamate dehydrogenase (GDH, 50 kDa, EC 1.4.1.2-3),[16] glutamic oxalacetic transaminase (GOT, 65 kDa, EC 2.6.1.1),[17] and L-glutamine synthetase (GSyn, 52 kDa, EC 6.3.1.2).[18] All these enzymes exhibit specificity toward acidic α-amino acids, and the label incorporation was specifically inhibited in the presence of compound **5** (Fig. 3).

To evaluate another attractive characteristics of the *N*-acylsulfonamide-containing photoprobe, its cleavability was examined by the application of our previous scissile biotin protocole.[6] A dot-blot analysis (Fig. 4, panel A) shows the cleavability of the labeled V8



Scheme 1. Synthesis of photoreactive acidic α-amino acid analogs. Reagents and conditions: (a) CDI, DBU in THF, 71%; (b) 1 M aq. NaOH–MeOH, 96%; (c) (+)-biotinyl-3,6-dioxaoctanediamine, HATU, HOAt, DIEA in DMF, 72%.

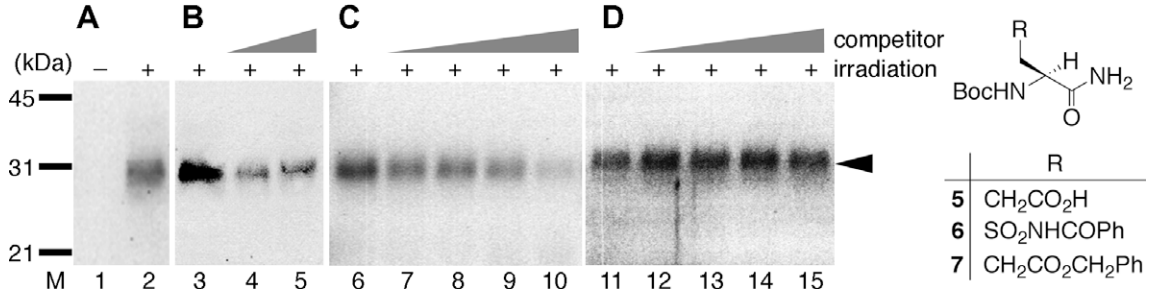


Figure 2. Chemiluminescence detection of V8 protease photolabeled with the biotinyl probe **4**. (A) Lane 1: sample not irradiated, lane 2: sample irradiated. (B) Competitive inhibition of photolabeling of V8 protease with *N*-Boc-glutamic acid α-amide **5**, lanes 3–5: irradiated sample in the presence of 0, 10, and 20 equiv of **5**, respectively. (C) Competitive inhibition with compound **6**, lanes 6–10: samples irradiated in the presence of 0, 0.5, 5, 50, and 100 equiv of **6**, respectively. (D) Competitive inhibition with compound **7**, lanes 11–15: samples irradiated in the presence of 0, 10, 50, 100, and 200 equiv of **7**. M: molecular size marker. Arrow indicates labeled V8 protease.

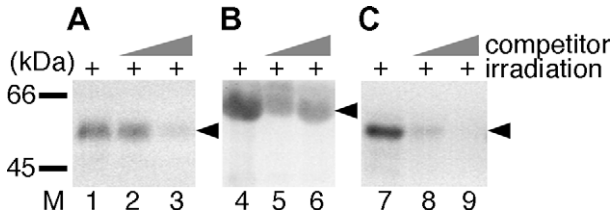


Figure 3. Chemiluminescence detection of proteins photolabeled with the biotinyl probe **4**. The samples containing the enzyme and **4** (1 nmol) were incubated in a 0.1 M potassium phosphate buffer (pH 7.4) with or without the compound **5**, and were subjected to 10% SDS–PAGE. (A) GDH; lanes 1–3: samples irradiated in the presence of 0, 20, and 200 equiv of **5**, respectively; (B) GOT; lanes 4–6: samples irradiated in the presence of 0, 50, and 100 equiv of **5**, respectively; (C) GSyn; lane 7–9: samples irradiated in the presence of 0, 20, and 200 equiv of **5**, respectively. M: molecular size marker. Arrows indicate labeled GDH, GOT, and GSyn, respectively.

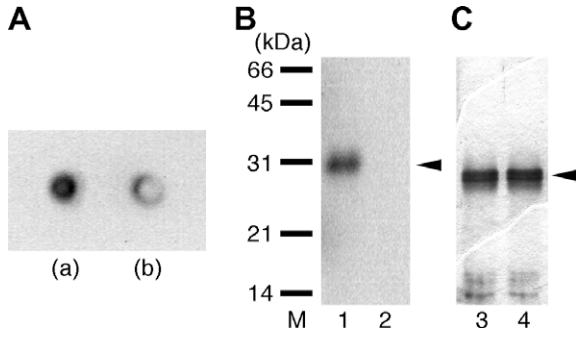


Figure 4. (A) Dot-blot analysis of the cleavage of the biotin-tagged photoaffinity probe **4**: (a) photolabeled sample without any treatment, (b) photolabeled sample after treatment with ICH_2COONa (0.4 M) in a borate buffer (0.1 M, pH 9) for 10 min at room temperature following incubation with NH_4OH (0.6 M) for 60 min at room temperature. (B) Chemiluminescence detection of the photolabeled samples. (C) Silver staining of the samples in (B): lanes 1, 3: photolabeled sample before cleavage; lanes 2, 4: photolabeled sample after treatment with ICH_2COONa and NH_4OH. M: molecular size marker. Arrows indicate labeled V8 protease (B) and non-labeled one (C), respectively.

protease within 30 min at pH 9. Dot (b) is the photolabeled product after the *N*-alkylation using sodium iodoacetate following treatment with ammonium hydroxide solution for 60 min at room temperature. The significant decrease of signal indicates the loss of the biotinyl amino acid moiety from the labeled protein. One of important point is to define the undesired effects of alkylation on proteins. The use of iodoacetic acid sodium salt has the advantage to share the well-established *S*-carboxymethylation conditions[19] for minimizing possible damages on proteins during *N*-alkylation of acylsulfonamide group. To confirm this observation, the labeled samples 'before' and 'after' the cleavage were subjected to SDS–PAGE (lanes 1, 3 and lanes 2, 4, respectively) and chemiluminescence detection (panel B) was performed as well as the silver staining of the gel (panel C). The densitometry of the emission band due to the labeled V8 protease revealed that 10-min alkylation with iodoacetic acid sodium salt was enough for the completion of the cleavage. The silver staining of the gel indicates that no aggregation or degradation of the protein occurred during the cleavage process. The cleavability of acylsulfonamide was already revealed within proteins captured on avidin-matrix.[6]

In summary, a diazirinyl photophore was easily introduced into the sulfonamide side chain of α-amino acid to yield the acidic and cleavable photoprobes. The biotinyl probe has been specifically recognized as a ligand for V8 protease, and has successfully labeled other proteins possessing specificity toward acidic α-amino acid residues. The advantage of *N*-acylsulfonamide group also demonstrated the expected cleavability after a brief *N*-alkylation in mild basic solution. The cleavability should facilitate the fishing-out of biotin-tagged proteins as well as peptides from avidin matrices.[6] Either the simple synthesis or the multifunctional nature of this acidic α-amino acid surrogate would be useful as a versatile photoreactive building block.

Acknowledgments

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11. Compound **4** as a white solid. ^{1}H NMR (400 MHz, CD_3OD, TMS): δ 7.90 (2H, d, J = 7.2 Hz), 7.33 (2H, d, J = 8 Hz), 4.69 (1H, t, J = 7.4 Hz), 4.48 (1H, dd, J = 4.2, 7.8 Hz), 4.28 (1H, dd, J = 4.6, 7.8 Hz), 4.09 (1H, dd, J = 7.2, 14.4 Hz), 3.50–3.76 (13H, m), 3.15–3.25 (1H, m), 2.91 (1H, dd, J = 5.2, 10.2 Hz), 2.69 (1H, d, J = 12.8 Hz), 2.18 (2H, t, J = 7.4 Hz), 1.50–1.80 (4H, m), 1.43 (9H, s), 1.21–1.30 ppm (2H, m); ^{13}C NMR (400 MHz, CD_3OD, TMS): δ 176.0, 168.8, 165.9, 150.7, 137.0, 133.0, 129.5, 129.0, 127.6, 121.7, 80.9, 79.4, 71.3, 70.5, 63.3, 61.6, 56.9, 56.6, 54.8, 41.0, 40.3, 36.7, 30.7, 29.7, 29.4, 28.6, 26.8, 20.8, 14.4 ppm.
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