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Full paper

Activation of Disulfide Bond Cleavage Triggered by Hydrophobization and Lipophilization of Functionalized Dihydroasparagusic Acid

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Concisely synthesized and functionalized dihydroasparagusic acid (DHAA) derivatives were used to show that the introduction of a hydrophobic functional group dramatically reduced air oxidation activity at the dithiol moieties and dominantly activated the cleavage of S-S bonds in proteins, presumably due to the hydrophobization and lipophilization. Notably, the reaction sites of water-reactive dithiol moieties behaved similarly to hydrophobic and lipophilic functional groups, which suggests impersonation of the reaction site.

The disulfide (S-S) unit between two cysteine amino acids in proteins is one of the most significant bonds for the formation of a tertiary structure.[1] Casadio and coworkers predicted that approximately 20% of human proteins contain bonded cysteines.[2] β-Mercaptoethanol (β-ME)[5] and racemic (2*S*,3*S*)-1,4-dimercaptobutane-2,3-diol (dithiothreitol (DTT), Cleland's reagent)[6,7] are widely used as reducing agents for the cleavage of S-S bonds in proteins[3] during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[4] (Figure 1a,b). Compared with DTT, β-ME is relatively stable and storable in solution.[8] However, β-ME has less reducing ability than DTT.[9,10] Although DTT exhibits greater reducing ability due to its intramolecular cyclization, it also undergoes air oxidation more quickly and is therefore difficult to handle.

Recent efforts in our laboratory have focused on controlling reactivity at the reaction site via the introduction of functional groups.[11] In general, an amine absorbent reacts with not only CO_2 but also H_2O (moisture) because of the hydrophilicity of amine functionality. However, in a previous study, we found that the introduction of a hydrophobic arylalkyl group into hydrophilic amines conferred CO_2 selectivity.[11c] In particular, *m*-xylylenediamine (MXDA) absorbed CO_2 only, without water contamination. Therefore, we hypothesized that the water-reactive functional group located near the lipophilic group might masquerade as a hydrophobic group (hydrophobization). It is well known that air oxidation of thiols is highly dependent on pH in the aqueous solution; this fact encouraged us to hypothesize that avoiding contact between H_2O (or OH^-) and -SH might protect against oxidation of the thiol. In addition, an increase in lipophilicity should activate S-S bond cleavage in proteins (lipophilization) because proteins represent lipophilic areas in water (Figure 1c). Here, we report the activation of S-S bond cleavage caused by the hydrophobization and lipophilization of thiol moieties in functionalized dihydroasparagusic acid[9] (DHAA) derivatives.

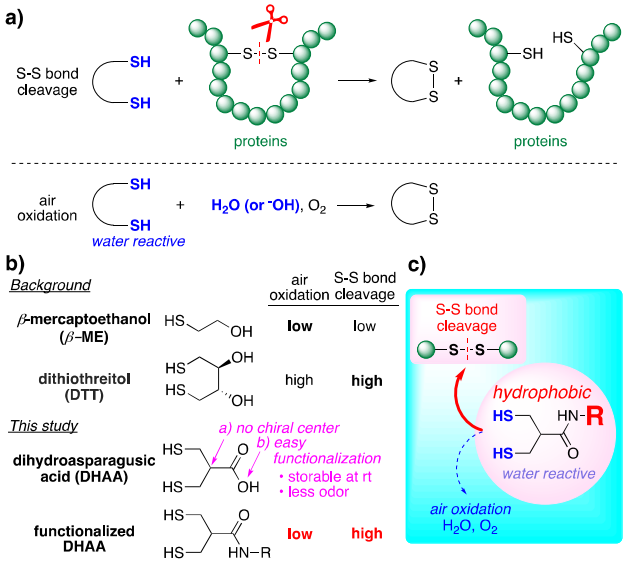

Figure 1. Reactivity concerning thiol-disufide interchange reaction and air oxidation of thiol unit. a) The reaction equations show both S-S bond cleavage and air oxidation of common dithiols. b) General thiols for the SDS-PAGE and developed dithiols based on DHAA are drawn. c) Our conceptual design for the selective S-S bond cleavage of proteins using DHAA derivative is depicted.

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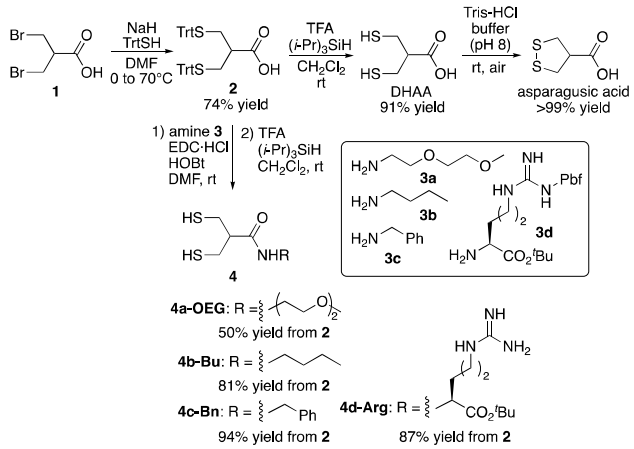
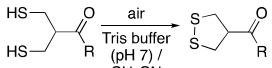


Scheme 1. Concise routes for syntheses of DHAA and its derivatives.

DHAA[12], which has anti-inflammatory effects, antioxidant activity, and tyrosinase inhibitory activity, is the reduced form of asparagusic acid, which can be isolated from several species of asparagus.[13] Oxidation of the propane-1,3-dithiol unit forms the 5-membered ring of 1,2-dithiolanes.[14] Whitesides et al[15] reported that the rate constants of ring closing/opening reactions with propane-1,3-dithiol/5-membered 1,2-dithiolane are greater than those of reactions with butane-1,4-dithiol/6-membered 1,2-dithiane. In addition, the carboxylic acid moiety of achiral DHAA has additional potential for concise transformation. Although several types of synthetic methods for DHAA[16] have been reported, most pathways require basic conditions for deprotecting the *S*-functionalities, which would cause the undesired oxidation of dithiols to form asparagusic acid.[17] Initially, our effort was directed toward the development of concise syntheses of DHAA and its derivatives via the use of acidic conditions during the last deprotection step. For this purpose, a trityl group was chosen for *S*-protection.[18] Commercially available 3-bromo-2-(bromomethyl)propanoic acid (**1**) was reacted with trityl-protected thiol under basic conditions to produce *S*-protected DHAA (**2**) in 74% yield (Scheme 1). After deprotection of the trityl group by the addition of TFA and $(i\text{-Pr})_3SiH$[19], DHAA was observed in 91% yield. On the other hand, atmospheric oxidation of the dithiol easily proceeded in Tris-HCl buffer (pH 8) to form asparagusic acid in quantitative yield.[13] When acidic conditions were used for deprotection of the trityl group, oxidized asparagusic acid was not isolated. Thus, the described pathway is useful for avoiding undesired oxidation. In addition, the carboxylic acid group of the *S*-protected intermediate **2** was readily transformed into the corresponding amides. In fact, the condensation reaction between **2** and 3,6-dioxaheptylamine (**3a**) with EDC and HOBt followed by deprotection of the trityl group under acidic conditions afforded the dithiol amide **4a-OEG** in 50% yield in 2 steps. The other amides **4b-Bu**, **4c-Bn**, and **4d-Arg** were also produced from amines **3b-d** and **2** in high yield. Using this route (TrtSH, **2**, DHAA, and **4**), we detected no unpleasant odors; this lack of odor is a significant advantage of the use of the thiol derivatives as a reducing agent. In addition, DHAA is an extremely stable solid and can be stored for several months at room temperature. Thus, short-step syntheses of various types of DHAA derivatives were achieved.

With syntheses of the desired DHAA derivatives in hand, we next investigated the relative oxidation rates in air. The air oxidation[14] of the dithiol to the disulfide in Tris-HCl buffer (pH 7) at room temperature was monitored by observing the degree of disappearance of dithiol (Figure 2a). When DHAA was exposed to air, the half-life was 15 h. The oxidation rate was greater for **4a-OEG** ($t_{1/2}$ = 9 h) than for DHAA. In contrast, the oxidation rates of **4b-Bu** ($t_{1/2}$ = 23 h) and **4c-Bn** ($t_{1/2}$ = 25 h) were slower than that of DHAA. Thus, increasing the hydrophobicity of the reducing agent provided resistance to air oxidation. Although the reduction agents were fully dissolved in solution, reactivity appeared to be strongly effected by higher hydrophobicity (cf. ClogP: DHAA 0.29, **4a-OEG** 0.40, **4b-Bu** 1.72, **4c-Bn** 1.92).

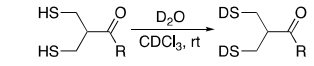
a)



Entry	Dithiol	$t_{1/2}$ (h)[a]
1	**DHAA**	15
2	**4a-OEG**	9
3	**4b-Bu**	23
4	**4c-Bn**	25

[a] Calculated by ^{1}H NMR

b)



DHAA	time	0 h	0.25 h	0.5 h
	relative integral of SH	100%	6%	4%
4c-Bn	time	0 h	1 h	3 h
	relative integral of SH	100%	38%	10%

Figure 2. Comparable experiments of DHAA derivatives for the reactivity of air oxidation a) The half-life of disappearance of dithiols (DHAA, **4a-c**) in Tris buffer (pH 7)/CH_3CN (4:1) due to the air oxidation were calculated from ^{1}H NMR. b) The H/D interchange examinations of DHAA and **4c-Bn** in $CDCl_3$ and D_2O. The relative integrals of SH were monitored by ^{1}H NMR with time.

To our knowledge, the oxidation mechanisms of dithiol in air remain unclear, presumably due to their complexity.[20] However, the effect of H_2O (or OH^-) must be crucial for air oxidation because the oxidation rate is highly dependent on solution pH. To further investigate oxidation resistance, we examined an H/D interchange reaction (Figure 2b). After the addition of D_2O (25 μL) to a solution of dithiol in $CDCl_3$ (20 mM, 0.5 mL), the change from -SH to -SD was monitored using ^{1}H NMR. In the case of DHAA, the thiol proton was almost entirely converted to deuterium after only 30 min. In contrast, >3 h was required for the H/D interchange of **4c-Bn**. This result also suggests that the hydrophobicity of the benzyl group imparts pseudo-hydrophobic properties to thiol moieties.

Our next endeavor focused on the cleavage of peptide S-S bonds using DHAA and its derivatives. For this purpose, we initially applied an excess amount (10 equiv.) of the protected cystine derivative **5** as a simple mimic of the target molecule (Figure 3a). When 1 equiv. of DHAA was treated with **5** in Tris buffer (pH 7) /CH_3CN (2:5) at room temperature for 1.5 h, the desired thiol **6** was hardly observed. After changing the pH of Tris buffer from 7 to 8, 54% yield (calculated from dithiol) of the thiol product **6** was obtained (entry 1). Thus, same solution (Tris buffer (pH 8) /CH_3CN (2:5)) was applied for this preliminary examination. Although the yield was slightly lower

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for **4a-OEG** (48%) than for DHAA, **4b-Bu** (60%) and **4c-Bn** (78%) afforded better yields than DHAA (entries 2-4). In all cases, 80-85% yields of unreactive disulfide **5** (calculated from starting material **5**) were recovered, which would indicate the stoichiometric reduction by dithiol. Thus, the introduction of a hydrophobic group into DHAA tended to activate S-S bond cleavage.

We continued our investigation by conducting additional comparative experiments to examine S-S bond cleavage in antibodies.[21] The disulfides in the immunoglobulin (Ig) monomer, which is a basic functional unit of antibodies, are essential for connecting the two heavy chains and two light chains. After adding the reducing agents (1.0 mM) to mouse IgG2$_a\kappa$ isotype ctrl antibody solution in Tris buffer/CH_3CN (4:1) and incubating for 15 min at room temperature, we quenched the reactions by adding iodoacetamide and processed the samples for SDS-PAGE to analyze disulfide bonds cleavage.[22] Images obtained after staining with Coomassie Brilliant Blue dye are shown in Figure 3b. In this experiment, intact antibody (H_2L_2) was detected near 250 kDa as seen in the sample without thiol. After the treatment with lipophilic thiols **4b-Bu** and **4c-Bn**, the fully cleaved heavy chain (H) and light chain (L) were observed around 50 kDa and 25 kDa, respectively, as main bands. In contrast, hydrophilic thiols such as DTT, DHAA, **4a-OEG**, and **4d-Arg** (cf. ClogP 0.30) produced no or only faint H and L bands, indicating much less efficient S-S bond cleavage of antibody. The quantitative data from three independent experiments revealed that the appearance of the heavy chain and the light chain was 9.3- and 2.8-fold higher, respectively, by the most efficient lipophilic dithiol **4c-Bn**, compared with DHAA (Figure 3c).

The S-S bonds in polypeptides are crucial structures for maintaining their essential function. Loss of S-S bonds from polypeptides would change their activity and has a high potential for the next generation of pharmaceutical targets. To check this possibility, we tested for the antigen-binding activity of IgG after the treatment with various dithiol derivatives, by using a Human CRP ELISA Kit[23]. As shown in Figure 3d, DTT treatment significantly reduced the antigen-binding ability of anti-CRP antibody (52% inhibition), indicating that the S-S bond cleavage leads to the loss of antibody activity. In the case of DHAA (68%) and **4a-OEG** (72%), slightly higher inhibitory activities were observed compared with DTT. Moreover, the dithiols **4b-Bu** (87%) and **4c-Bn** (90%) containing hydrophobic groups exhibited the highest class of inhibition, whereas the amino acid-like **4d-Arg** (52%) and the phosphine TCEP[24,25] (63%), a well-known alternative reduction reagent for disulfide, showed only moderate inhibitory activity. These results suggest that the lipophilicities of the reduction agents were strongly associated with their inhibitory activities, which might be applicable to the functional modulation of antibodies or proteins containing S-S bonds.

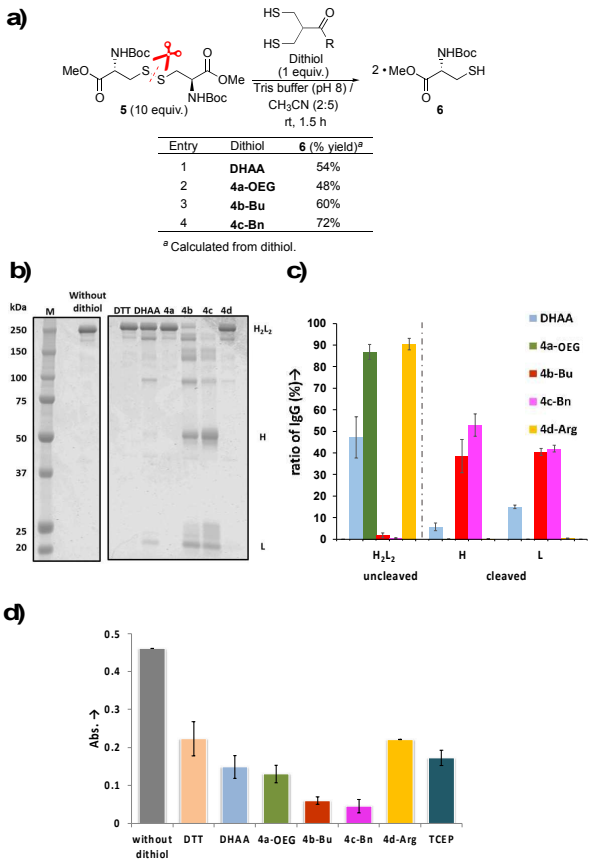

Figure 3. Activity for S-S bond cleavage of reduction agents. a) S-S bond cleavage of cystine derivative **5** using DHAA and its derivatives **4a-c** was described. b) The gel images of SDS-PAGE followed by Coomassie staining after the S-S bond cleavage of mouse IgG2$_a\kappa$ isotype ctrl antibody using DTT and DHAA derivatives are shown. c) The quantitative data of the bands corresponding to H_2L_2, H, and L in SDS-PAGE analyses (n = 3) as shown in b). d) Inhibitory activities of DTT, DHAA, **4a-d**, and TCEP based on the Human CRP ELISA experiments. The immobilized antibody specific for human CRP from a Human CRP ELISA Kit was treated with 0.1 M reducing agents (DTT, DHAA, **4a-d**, or TCEP) in Tris buffer (pH 7)/CH_3CN (4:1) or buffer alone for 1 h at 37 °C. After washing, the immobilized antibody was reacted with human CRP standard. The subsequent reactions were conducted according to a standard protocol of the Human CRP ELISA kit and the absorbance was measured at 450 nm (n = 3).

In summary, we developed a concise and effective synthetic method for dihydroasparagusic acid (DHAA) and its derivatives in which the final stage can be performed under acidic conditions to avoid the formation of undesired disulfides. Air oxidation tests and H/D interchange analyses using functionalized DHAA derivatives showed that the introduction of a hydrophobic functional group reduced air oxidation activity based on the observed decrease in the reaction rate between the thiol moiety and water (as indicated by comparing DHAA and **4a-OEG** with **4b-Bu** and **4c-Bn**). Nevertheless, the hydrophobic dithiol moieties had relatively

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high potential for inducing S-S bond cleavage in proteins, as indicated by the SDS-PAGE and ELISA assays. These contradictory reactivities might be concluded that the reaction sites of water-reactive dithiol moieties behaved similarly to hydrophobic and lipophilic functional groups, which suggests impersonation of the reaction site (*reaction site masquerade*). Further investigation of this objective is currently ongoing.

Conflicts of interest

There are no conflicts to declare.

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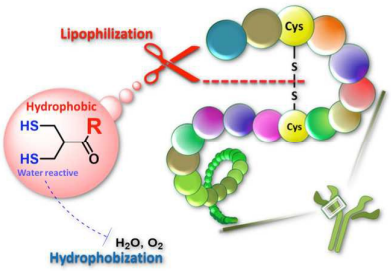
Introduction of hydrophilic group into dihydroasparagusic acid (DHAA) indicated higher reduction ability of disulfide in protein and lower air oxidation.



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