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Spontaneous substitution of azulene-derived benzylic alcohols by thiols and its application to labeling/protection of biothiols

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ABSTRACT

By mixing guaiazulene-3-methanol derivatives and thiols at room temperature, benzylic substitution of the alcohol proceeded to yield the corresponding sulfide. Because of the blue color of the guaiazulene derivative, this spontaneous reaction was used for labeling of paper-immobilized biothiols. By treatment with tris(2-carboxyethyl)phosphine hydrochloride, the guaiazulene-3-ylmethyl part of the sulfide could be removed and the original thiol recovered. Based on these findings, a guaiazulene-3-methanol derivative was used as a protective group for the synthesis of cysteine derivatives.

1. Introduction

Azulene is a nonalternant aromatic compound with a characteristic blue color as a result of the small HOMO–LUMO gap compared with its colorless alternant analogue, naphthalene [1–4]. The five-membered part of azulene has cyclopentadienyl-anion-like character due to the formal one π-electron donation from the seven-membered ring part, which results in the acquisition of aromaticity on both rings. Consequently, azulene shows nucleophilicity at its 1- and 3-positions. For the same reason, azulene-1-ylmethyl cations are considerably stabilized by π-delocalization [5], and this makes azulene-1-methanols highly reactive under acidic conditions [6,7]. Moreover, it has been reported that 3-(hydroxymethyl)guaiazulene is even self-reactive in the absence of acid, and gives bis(guaiazulen-3-yl)methane upon storage [8]. This product might be formed via elimination of formaldehyde and the subsequent bimolecular dehydrative condensation, or *vice versa*. Whichever the mechanism, the formation of the dimer indicates the high potential of the hydroxy group as a leaving group in substitutions. Although such an exceptionally high reactivity of azulen-1-ylmethyl alcohol derivatives is quite interesting, only a limited number of functional group transformations of the hydroxy group have been reported: the reaction with ethanol to give the corresponding ethyl ether in the presence of catalytic sulfuric acid [9], and the substitution of the hydroxy group by triphenylphosphine hydrobromide to give a phosphonium salt at an elevated temperature [10]. This situation led us to investigate the reaction of azulene-1-ylmethyl alcohols with other nucleophiles. As a result, we happened to find that the displacement reaction occurred with thiols in the absence of any activators. In this article, we describe this unique reaction, and its potential use for labeling of biomolecules along with utilization as a blue-protective group for thiols in the synthetic reactions.

2. Results and discussion

2.1. Reactivity screening of guaiazulene-derived alcohol and its selective reaction to thiols

For a substrate, we employed 3-(1-hydroxyethyl)guaiazulene (**1a**), which can be readily synthesized according to a reported procedure via Friedel–Crafts acetylation of guaiazulene and reduction of the carbonyl group [11]. We screened the reaction of **1a** with several kinds of nucleophiles in chloroform (50 mM), and found that this compound underwent spontaneous substitution by a thiol (Scheme 1, GA denotes guaiazulen-3-yl group). Other compounds such as methanol, phenol, an amine, a carboxylic acid, and a sulfide did not give any substitution products. Nor did a disulfide show reactivity toward **1a**. In the case of the reaction with the carboxylic acid, a dehydrative self-condensation of **1a** was observed [12].

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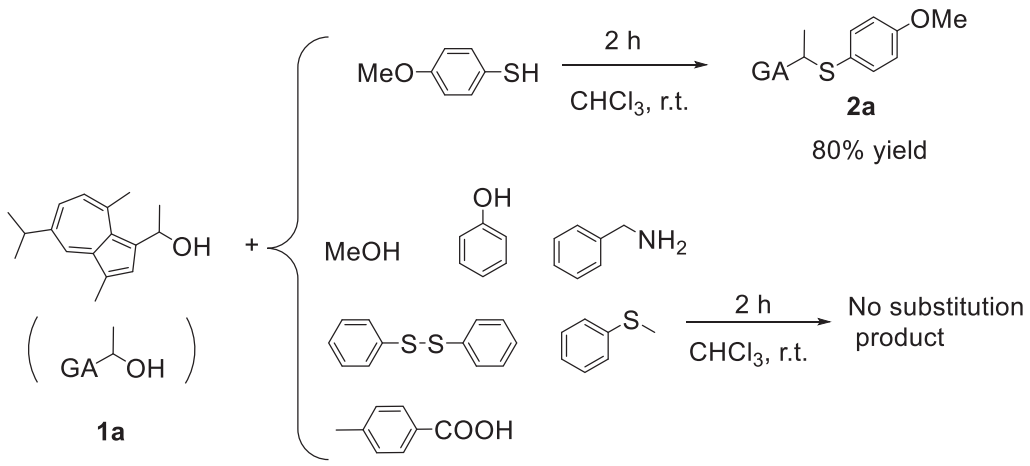
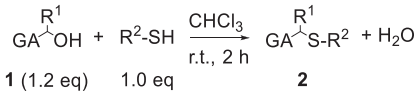


Scheme 1. Reactivity screening of the GA-derived alcohol **1a**.

Table 1
Reaction of GA-derived alcohols **1** with thiols LF.



Entry	Alcohol 1		Thiol		Sulfide 2	
		R^1	R^2	pK_a[a]		Yield [%][b]
1	**1a**	Me	4-MeOC_6H_4-	6.8	**2a**	83 (93)
2	**1a**	Me	Ph$(CH_2)_2$-	10.7[c]	**2b**	80 (80)
3	**1b**	*i*Pr	4-MeOC_6H_4-	6.8	**2c**	80 (89)
4	**1c**	*t*Bu	4-MeOC_6H_4-	6.8	**2d**	80 (85)
5	**1d**	Ph	4-MeOC_6H_4-	6.8	**2e**	81 (82)
6[d]	**1d**	Ph	4-$NO_2C_6H_4$-	4.7	**2f**	0 (13)
7[e]	**1d**	Ph	*t*Bu -	11.1	**2g**	12 (38)

[a] pK_a of thiols taken from Thapa B, Schlegel HB. *J. Phys. Chem. A.* 2016; 120:5726.
[b] The values in the parentheses denote the yield in the presence of 0.3 eq. AcOH.
[c] pK_a value of butanethiol as a reference.
[d] MeOH as a solvent, reaction time is 12 h.
[e] Reaction time is 12 h.

For the substitution of **1a** by the thiol, it was found that the addition of acetic acid accelerates the reaction slightly. By contrast, the reaction was completely hindered in the presence of trimethylamine. This thiol substitution also proceeded in other solvents such as THF and MeOH in the presence of 0.3 eq. acetic acid, meanwhile the process was considerably retarded in DMF (Table S1).

Next, we checked the generality of this substitution with various substrates (Table 1). Besides the aromatic thiols, an aliphatic thiol reacted readily with the guaiazulene-derived alcohol (Entry 2). The substituents on alcohol **1** did not largely affect the reactivity (Entries 1, 3, 4, and 7). By contrast, the progress of the reaction depended significantly on the electronic/steric nature of the thiols; an aryl mercaptan with the electron-withdrawing nitro group showed low reactivity (Entry 6) and the reaction of a sterically hindered thiol was also sluggish (Entry 7). Enhancement of the reaction by the action of acid was observed in most cases. It should be noted that, despite the acidity of aliphatic thiols and phenols are being in a same range, only the former showed the reactivity

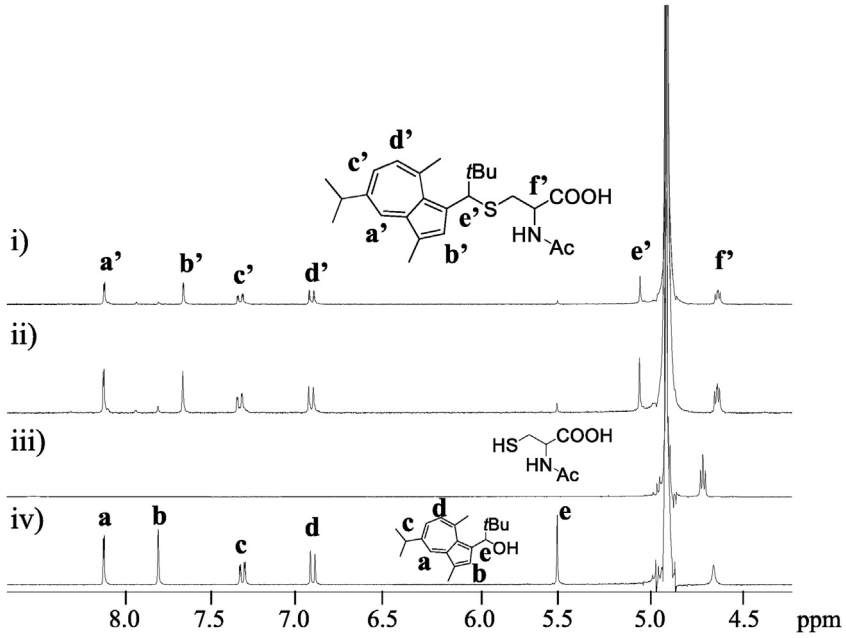
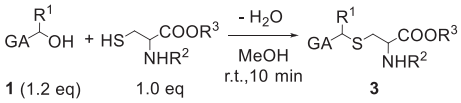


Fig. 1. 400 MHz ^{1}H NMR spectra of the reaction mixture of **1c** and *N*-acetylcysteine along with the starting materials and the isolated product (0.08 M in CD_3OD). I) isolated product; ii) reaction mixture after the addition of *N*-acetylcysteine; iii) *N*-acetylcysteine; iv) the alcohol **1c**.

Table 2
Reaction of **1** with cysteine derivatives LF.



Entry	Alcohol 1		Cysteine deriv.		Sulfide 3	
		R^1	R^2	R^3		Yield [%]
1	**1a**	Me	Ac	H	**3a**	89
2	**1a**	Me	H	Et	**3b**	0 (86[a])
3	**1a**	Me	H	H	**3c**	88[b]
4	**1b**	*i*Pr	Ac	H	**3d**	85
5	**1d**	Ph	Ac	H	**3e**	88

[a] The value in parentheses denotes the result in the presence of 0.5 eq. AcOH and reaction time of 3 h.
[b] Solvent: DMF/H_2O = 1:1; reaction time 2 h.

toward **1a**.

The effect of coexisting acid/base indicates that the protonation of substrate **1** is an important step in the mechanism of this reaction. In the absence of acetic acid, weakly acidic thiols might serve as a proton source. However, it is not clear whether the substitution occurs in an S_N1 or S_N2 manner. It should be mentioned that an attempted addition reaction of 4-methoxybenzenethiol to 3-isobutenylguaiazulene in the presence of acetic acid did not proceed at all. Considering that a protonation of this alkene should give a cationic species, which is the same as that generated by dehydration of protonated **1b**, the intermediacy of the benzylic cation is less likely; hence, the substitution of **1** should proceed in an S_N2-like manner [13]. Incidentally, the coexistence of butylated hydroxytoluene did not affect the thiol substitution of **1**, which indicates that the radical mechanism can be excluded .

There have been several published articles regarding the direct substitution of benzylic/allylic alcohols by thiols under mild reaction conditions; however, they required the assistance of Lewis or Brønsted acids [14–16]. Although there is one example for the acid-free substitution, in that case the reaction was realized only under harsh conditions (100 W microwave, 65 °C) [17]. Closely related to the present result, Ohshima et al. recently reported the substitution of 3-indolyl (hydroxyl)acetates by biothiols such as glutathione or reduced proteins in water [13]. For that reaction, use of acidic buffer (pH 5.4 or lower) was indispensable. In sharp contrast, our reaction does not necessarily require the acidic conditions.

Next, we attempted to apply this reaction to a biothiol derivative, Ac-Cys-OH. As shown in Fig. 1, the ^1H NMR chart of **1c** immediately after the addition of the thiol showed nearly complete disappearance of the peak at 5.5 ppm which indicates conversion of the alcohol, and no further change occurred during a prolonged reaction time. The corresponding substitution product was obtained as a nearly 1:1 mixture of diastereomers in 89% isolated yield. In $CDCl_3$, this reaction also proceeded even at lower concentrations of 5 mM within 10 min.

Reactions proceeded smoothly between alcohol **1** and cysteine derivatives as listed in Table 2. Substitution by cysteine-derived ester with free amino group was sluggish, but the addition of 0.5 equiv. Of acetic acid effectively promoted the process (Entry 2). In addition, the reaction of bare cysteine molecule in an aqueous solvent proceeded despite the need for a longer reaction time; the substitution product was formed as a blue precipitate (Entry 3).

2.2. Coloring of immobilized thiol-containing biomolecules

Encouraged by the results with cysteines, along with the colored nature of the azulene derivatives, we applied this spontaneous reaction to the labeling of immobilized thiol-containing biomolecules. The coloring of the target molecules was conducted with a simple GA-derived alcohol **1a**. As shown in Fig. 2, the labeling of resin-bound Fmoc–cysteine was affected, and resin beads turned blue when using **1a** (Fig. 2). By contrast, beads remained their original color when thiol groups were protected or lacking. Subsequently, this specific coloring of immobilized forms of biothiols was extended in the following work.

Dot blot, a rapid and easy technique for semiquantifying biological target molecules, has been used widely [18]. Biomolecules are immobilized on a membrane [19,20] or paper substrate [21,22], and immersed into a solution of a specific probe. The molecules having particular interactions with the probe can be visualized and estimated semiquantitatively. We attempted the dot blot assay to visualize thiol-containing biomolecules on filter paper.

First, immobilization of glutathione (GSH) demonstrated using the following procedure (Fig. 3a) [20]. GSH was loaded on a piece of filter paper (1 × 4 cm), and this paper was soaked in an ethanolic

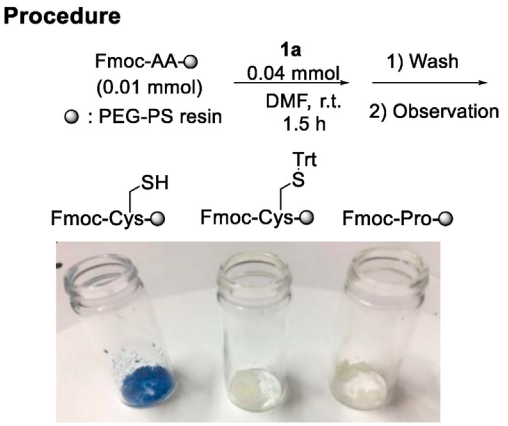


Fig. 2. Labeling of resin-bound Cys with **1a**.

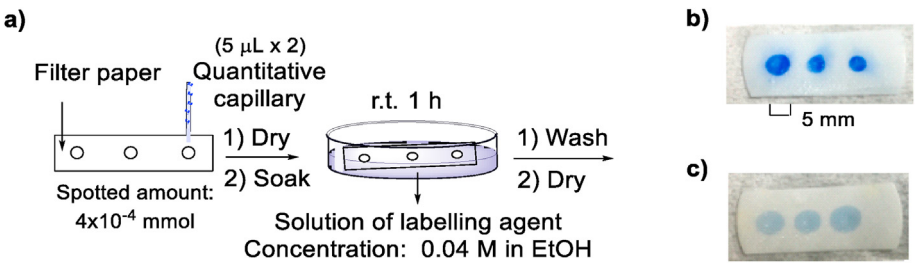


Fig. 3. Immobilization of GSH on filter paper and its detection with CBB and **1a**. A) Dot blot method to label GSH on filter paper; b) Result of using CBB as labeling agent; c) Result of using **1a** as labeling agent.

solution of Coomassie Brilliant Blue R-250 (CBB R-250) [23]. As shown in Fig. 3b, the spotted sites were obviously colored, verifying the immobilization of GSH on the filter paper.

Based on this verification, the labeling of GSH by guaiazulene-derived alcohol **1a** was conducted. The successful coloring of spotted GSH in Fig. 3c indicated that guaiazulene-derived alcohol can be employed to identify thiol-containing biomolecules immobilized on filter paper.

The paper-based staining of biothiol by **1a** could also be applied to the smaller molecule, cysteine (Cys) (Fig. 4). An obvious blue color comparable to that formed with GSH was observed, while non-thiol-containing proline (Pro) did not show any color change.

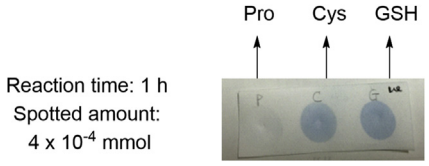


Fig. 4. Staining amino acids on filter paper with **1a**.

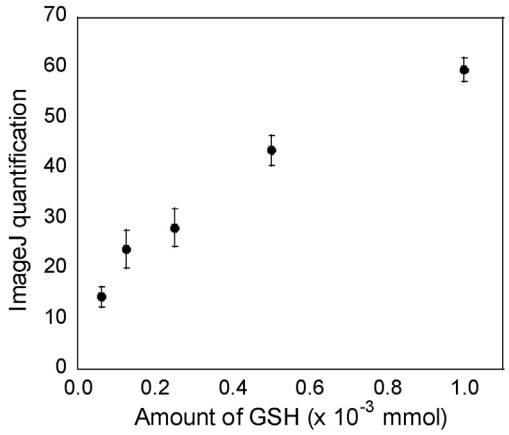


Fig. 5. Relationship between color intensity and the amount of spotted GSH.

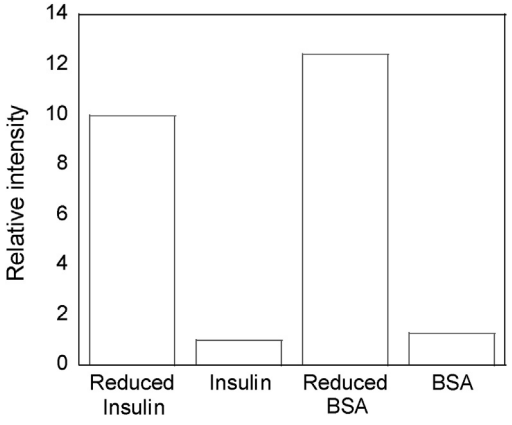


Fig. 6. Relative color intensity of labeled proteins (Insulin: 8.9 μg, BSA: 20 μg).

A control experiment using a dye-bound maleimide [24] resulted in low contrast due to a high level of background and false-positive color change [25,26], which indicates the superiority of **1a**.

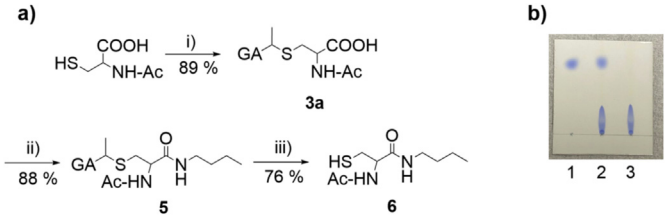
In the next step, a semiquantitative analysis of the loaded GSH was performed [27]. An image of the paper was captured by digital camera and the intensity of the colored spots was measured using NIH ImageJ software (Fig. 5) [28]. This procedure clarified that **1a** is able to detect GSH in the range from 1.25×10^{-4} mmol to 1×10^{-3} mmol.

The generality of current reaction was extended to naturally occurring biomolecules: insulin (5.6 kDa) and bovine serum albumin (BSA) (66.4 kDa). Insulin consists of two polypeptide chains, and has two interchain disulfide bonds and one intrachain disulfide bond [29]. BSA is the most abundant protein in mammalian plasma, which has 17 disulfide bridges and one free thiol group [30]. The disulfide bonds in these molecules were reduced overnight with an excess amount of $NaBH_4$ in water to afford free thiol groups [31,32]. Spotted sites containing the reduced forms of biomolecules showed an obvious color change (Fig. 6). Our method was not able to detect the only existing free Cys in native BSA.

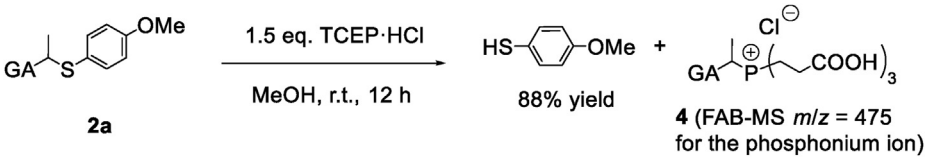
2.3. Utilization of GA-3-methanol derivative as a protective group for thiols

From another point of view, this reaction can be regarded as a protection of thiol, provided that the removal of the guaiazulenylmethyl moiety is realized under certain conditions. Motivated by this idea, we attempted a deprotection reaction using sulfide **2a** (Table S2). As a result, it was found that tris(2-carboxyethyl)phosphine (TCEP) hydrochloride worked well (Scheme 2)

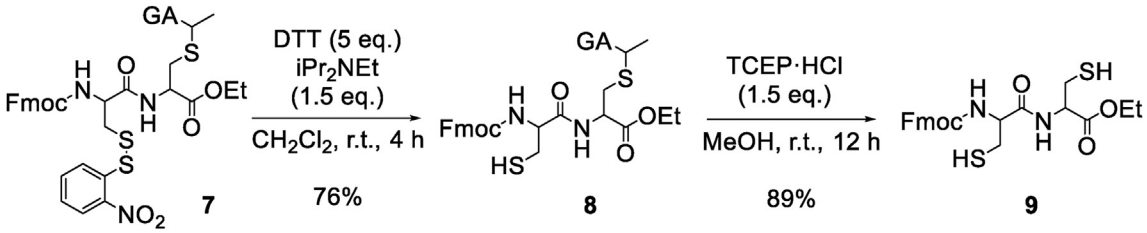
It is noteworthy that the thiol can be readily isolated from the reaction mixture by a simple extraction using chloroform and water; the thiol was found in the organic layer, whereas the blue aqueous layer contained phosphonium salt **4** and unreacted TCEP. The other merit of using TCEP as a deprotecting agent is that it has a reducing power; hence, it is able to inhibit the oxidative formation



Scheme 3. Utilization of GA-derived alcohol as a blue protecting group in the amino acid derivatization. a) i) **1a**, in MeOH, r. t., 20 min; ii) n-butylamine (1.1 eq.), 1-hydroxy-7-azabenzotriazole (1.1 eq.), 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo [4,5-*b*]pyridinium 3-oxide hexafluorophosphate (1.1 eq.), diisopropylethylamine (DIEA, 2.2 eq.) in DMF, r. t., 2 h; iii) TCEP·HCl (1.1 eq.) in MeOH, r. t., 12 h; b) TLC plate (silica gel, no stain required); Lane 1: **1a**; Lane 2: reaction of Ac-Cys-OH with **1a** after 5 min. Lane 3: **3a** (eluent: hexane/ethyl acetate = 1/1 + 1% AcOH).



Scheme 2. Deprotection reaction of sulfide **2a** with TCEP.



Scheme 4. Sequential deprotection of side chains on a Cys-Cys dipeptide **7**.

of a disulfide from the thiol product. TCEP has been used for reductive cleavage of disulfide bonds [33]; however, in the present case, the reagent served as a nucleophile to regenerate the thiol from the sulfide. Other known disulfide-reducing agents such as dithiothreitol (DTT) or $NaBH_4$ did not show any deprotecting ability.

The stability of this protective group was confirmed under weakly basic conditions (trimethylamine or piperidine). By contrast, partial deprotection occurred under acidic conditions (4 eq. TFA in CH_2Cl_2, *ca.* 50% yield).

On the basis of the finding presented above, alcohol **1a** was applied as a protective group to the synthesis of a cysteine derivative (Scheme 3). The progress of the reaction could be easily monitored by TLC as blue spots.

This protective group was further applied for sequential deprotection of a differently protected Cys–Cys derivative (Scheme 4). First deprotection with DTT successfully underwent disulfide bond cleavage, and the subsequent treatment with TCEP gave a fully deprotected dipeptide. This methodology is considered to be useful in the synthesis of Cys-containing peptides. As mentioned, the guaiazulenylmethyl sulfide was stable under Fmoc-deprotecting conditions (20% piperidine in DMF); the peptide **7** might be further elongated further.

Several azulene derived "blue" protective groups have been reported to date; 1) azulen-5-ylethoxycarbonyl group was applied as Fmoc-like protective group for amino group [34]; 2) 2-(azulen-1-yl)-2-oxoacetyl chloride, prepared from azulene and oxalyl chloride *in situ*, has been used to protect hydroxy groups [35]; and 3) azulen-6-ylethoxy group was utilized to protect carboxylic acids [36]. Here we have added another azulene-based protective group that is unique to thiols. The present protection method is based on the high benzylic cation stabilizing nature for the five membered ring part of the azulene, which has not been utilized as a working principle for the protection of nucleophilic function groups.

3. Conclusion

A spontaneous substitution of guaiazulene-3-methanol derivatives by thiols was developed. The reaction proceeded under mild conditions in a range of nonpolar to aqueous solvents and required no activators, albeit slight enhancement by an acid was observed. The click-chemistry-like nature of this reaction could be successfully applied to the blue labeling of immobilized biothiols as detectable by the naked eye. Considering the unreactive nature of alcohol **1** to disulfide, this detection of thiols should be potentially applicable to the instant monitoring of thiol/disulfide-based redox-responsive materials [37,38]. Another merit of this substitution is that the original thiol can be regenerated by treating the **1**-derived sulfide with TCEP. When coupled with the stability toward DTT and piperidine along with the inherent blue color, the guaiazulen-3-ylmethyl group is expected to be applicable to the Fmoc solid-phase synthesis of peptides with multiple Cys residues via an orthogonal protection/deprotection pathway [39].

4. Experimental section

4-1) The typical procedure for the thiol substitution of azulene-derived benzylic alcohol is as follows: To a 30 mL round-bottomed flask containing alcohol **1a** (42.0 mg, 0.173 mmol) in $CHCl_3$ (3 mL) was added 4-methoxybenzenethiol (20.2 mg, 0.144 mmol). The reaction mixture was stirred at rt for 2 h, and then it was partitioned between water and chloroform. The organic layer was dried with $MgSO_4$ and the solvent was removed by evaporation. The crude mixture was purified by preparative thin layer chromatography (hexane: ethyl acetate = 95:5) to afford 43.5 mg of sulfide **2a** as a blue amorphous solid (83% yield).

4-2) The typical procedure for "deprotection", or the formation of the thiol from the sulfide, is as follows: To a 30 mL round-bottomed flask containing a sulfide **2a** (55 mg, 0.15 mmol) in MeOH (3 mL) was added TCEP·HCl (64 mg, 0.22 mmol) and the resulting mixture was stirred for 12 h at rt. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$ for five times. The collected organic layer was dried over $MgSO_4$ and the solvent was removed to give 18.5 mg of 4-methoxybenzenthiol as a colorless oil (88% yield).

The preparation of guaiazulene-3-ylmethyl alcohols, procedures for the synthesis of cysteine derivatives and peptides, the characterization data of isolated compounds, and the ^1H and ^{13}C NMR spectra are presented in Supplementary data.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2021.131998.

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[12] (*E*)-1,3-Bis(guaiazulen-3-yl)but-1-ene was obtained in 40% yield. 1H NMR (400 MHz, CDCl3) δ = 8.071 (d, J = 2 Hz, 1H), 7.931 (d, J = 2 Hz, 1H), 7.697 (d, J = 5.6 Hz, 2H), 7.244 (d, J = 10.8 Hz, 1H), 7.142 (d, J = 10.8 Hz, 1H), 6.971 (d, J = 15.6 Hz, 1H), 6.821 (d, J = 10.4 Hz, 1H), 6.696 (d, J = 10.4 Hz, 1H), 6.315 (dd, J = 15.6, 5.2 Hz, 1H), 4.695 (quintet, J = 6.8 Hz, 1H), 3.050 (s, 3H), 3.016 – 2.933 (m, 2H), 2.703 (s, 3H), 2.632 (s, 3H), 2.56 (s, 3H), 1.622 (d, J = 6.8 Hz, 3H), 1.344 (d, J = 6.8 Hz, 1H), 1.299 (d, J = 6.8 Hz, 1H). This product is considered to be formed via acid-mediated dehydration of **1a** and the subsequent nucleophilic substitution of other **1a** molecule by the action of acid.
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