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Synthesis of a bioprobe for elucidation of target molecule of spongean anti-malarial peroxides

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Abstract—The reactants of an anti-malarial peroxide having a 6-carbomethoxymethyl-3-methoxy-1,2-dioxane moiety treated with $FeSO_4$ were analyzed. For mechanistic study of the anti-malarial peroxide, two biotinylated probes to elucidate the target molecules were designed and synthesized. The two synthesized probes showed potent anti-malarial activity, and one of them was proved to form an irreversible binding with protein in a model experiment.
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Since the discovery of artemisinin, many kinds of cyclic peroxides with anti-malarial activity were reported from synthetic and natural resources.[1] In the course of our continuous investigation in a search for new bioactive principles from marine organisms, the two methyl esters (**1**, **2**) of cyclic peroxides[2] from a marine sponge were shown to exhibit potent anti-malarial activity. Furthermore, a facile construction of the pharmacophore framework, 6-carbomethoxymethyl-3-methoxy-1,2-dioxane, was established.[3] By application of this protocol, readily available peroxides showing not only more potent activity than **1** and **2**[4] but also in vivo efficacy against *Plasmodium berghei* infected mice[5] were disclosed. For the purpose of rational and evidence-based design of new anti-malarials utilizing **1** and **2** as scaffolds, elucidation of the target molecule was intensively required. In this context, we have undertaken to explore a molecular probe to elucidate the target protein of **1** and **2**. This communication deals with a synthesis of biotinylated probes with irreversible binding affinity against protein (Fig. 1).

From a study of the mode of action of artemisinin and its model compounds, the endoperoxide bridge was proved to be degradated by one-electron reduction with

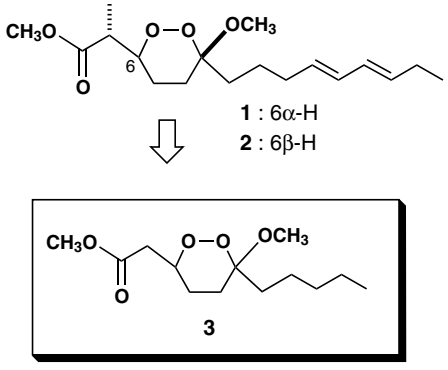

Figure 1.

Fe(II) of heme in the food vacuole, a characteristic organelle in *P. falciparum*, to provide alkyl radicals.[6] The resulting radical species were presumed to form covalent adducts with proteins in the food vacuole to inhibit the proliferation of malaria parasites.[7] Previously, we disclosed that a synthetic peroxide **3** having a 6-carbomethoxymethyl-3-methoxy-1,2-dioxane moiety with a pentyl residue at the C-3 position showed anti-malarial activity with IC_{50} of 0.12 μM against *P. falciparum*.[4] In order to design a bioprobe molecule for studying the mode of action of **3**, the genuine active species of **3** were investigated from the reactants of **3** with Fe(II). Treatment of **3** with $FeSO_4$ in H_2O afforded

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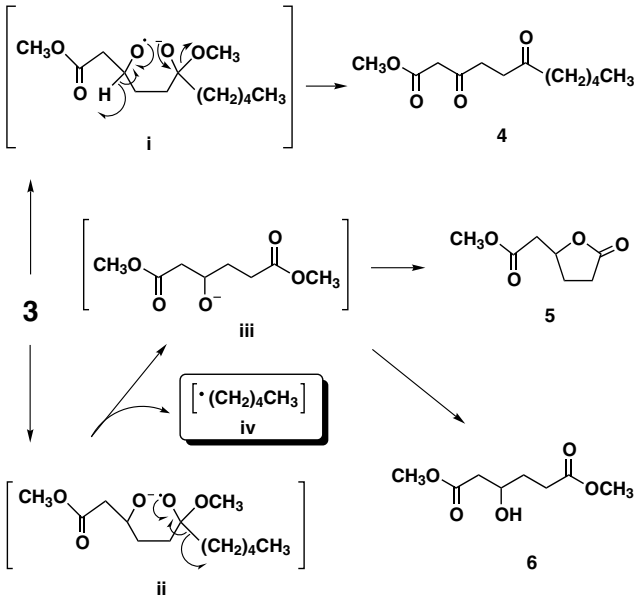
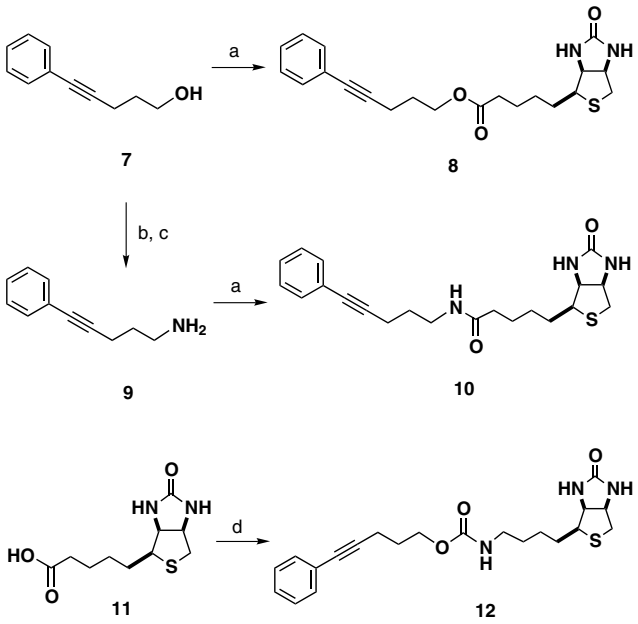


Figure 2. Plausible reaction pathway giving **4**–**6** in the $FeSO_4$ treatment of **3**.



Scheme 1. Reagents and conditions: (a) **11**, EDCI·HCl, pyridine, 93% for **8**, 88% for **10**; (b) MsCl, Et_3N, toluene, then aq NaN_3, Bu_4NBr; (c) *n*-Bu_3P, CH_3CN–H_2O, 2 steps 43%; (d) DPPA, Et_3N, CH_3CN, 55 °C then **7**, 84%.

4, **5**, and **6** in 18%, 28%, and 43% yields, respectively. A plausible reaction pathway from **3** to **4**, **5**, and **6** is depicted in Figure 2. Taking this product distribution into account, a pentyl radical **iv** generated in the formation of a major intermediate **iii** was presumed to be responsible for the anti-malarial activity of **3**. Based on this finding, we planned to introduce biotin, which possesses strong affinity to avidin, at the terminal of the pentyl residue.

For effective labeling of the target protein by the probe molecule and successive isolation of the labeled protein by the tight affinity between biotin and avidin, the linkage between the carbon radical and the biotin residue should be stable during a few hours' exposure in the culture medium. Therefore, the stability of three kinds of linkages (ester, amide, and carbamate) in the culture medium was evaluated as candidate functionalities to connect the carboxylic group in biotin with the C-3 alkyl moiety. For this purpose, three model compounds having 1-phenyl-1-yne chromophore were synthesized as shown in Scheme 1. Condensation of 1-phenyl-1-pentyn-5-ol (**7**) and biotin (**11**) by 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDCI·HCl) in pyridine gave an ester **8** in 93% yield. The mesylate of **7** was subjected to azidation followed by reduction with *n*-Bu_3P to afford a primary amine **9** in 43% yield for two steps. The amine **9** was coupled with biotin (**11**) under the same conditions as for the preparation of **8** to provide an amide **10**. The carbamate **12** was synthesized by the coupling of **7** with an isocyanate, which was generated from biotin (**11**) through diphenylphosphoryl azide (DPPA)-mediated Curtius rearrangement,[8] in 84% yield. Next, the stability in RPMI 1640 medium supplemented with heat-inactivated 10% type A human serum for the three model compounds was evaluated, and the time course of the remains is depicted in Figure 3. As shown in Figure 3, the ester **8** was readily metabolized and

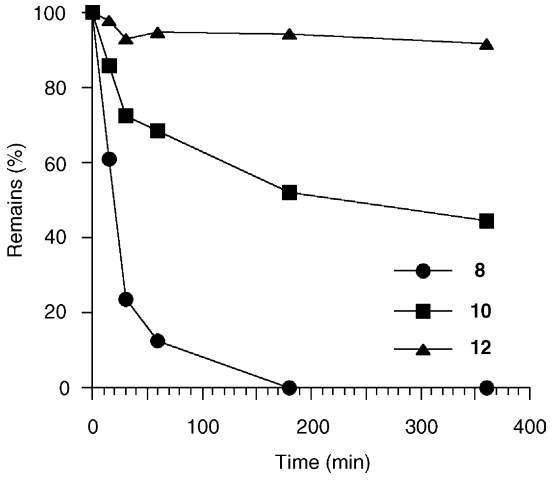
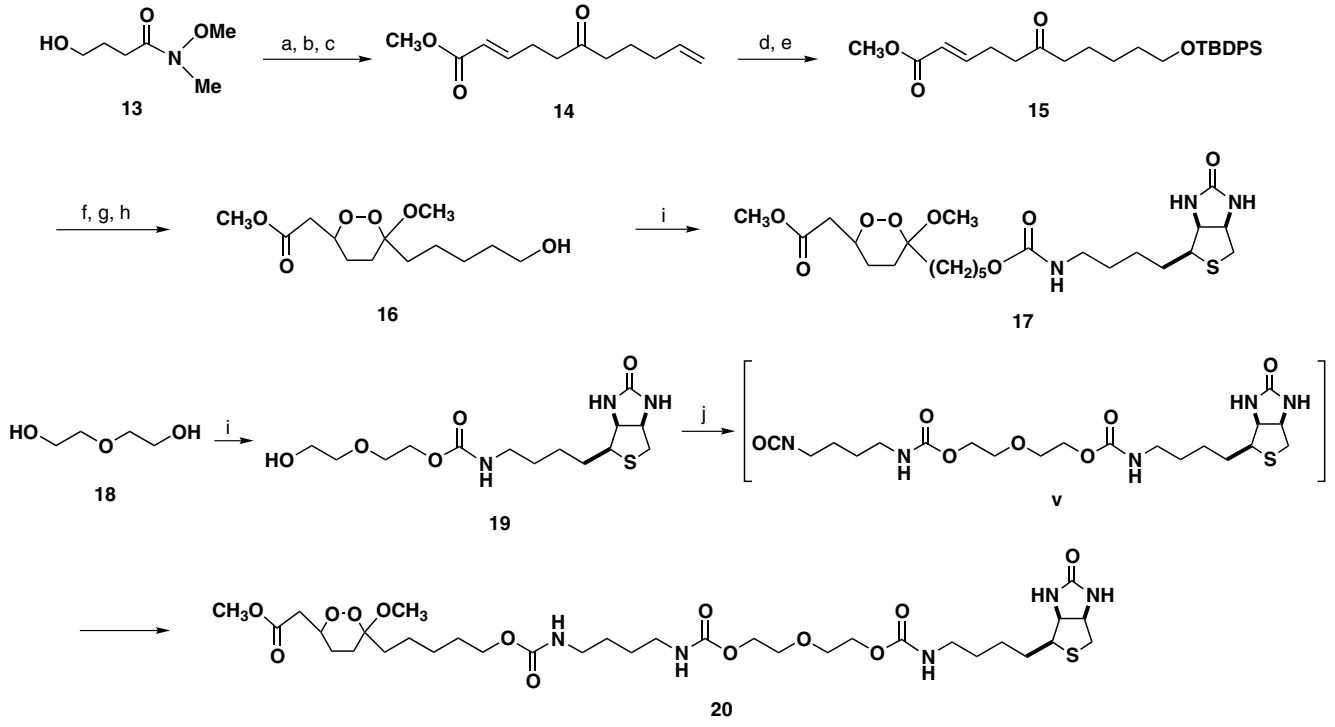


Figure 3. Stability of **8**, **10**, and **12** in the human serum.

disappeared completely within 3 h. The amide **10** showed moderate stability in the human serum. On the contrary, the carbamate **12** was appreciably stable and almost all remained even after 6 h exposure. On the basis of this behavior, the carbamate linkage was chosen for the linkage with biotin.

Synthesis of the biotinylated probe **17** was executed as shown in Scheme 2. Treatment of the Weinreb amide **13**[4] with 1-pentylmagnesiumbromide, which was prepared from 1-bromopentane, afforded a ketoalcohol. Swern oxidation of the ketoalcohol followed by Wittig reaction with (carbethoxymethylene)triphenylphosphorane provided a ketoester **14** in 70% yield for three steps. The ketoester **14** was submitted to hydroboration with



Scheme 2. Reagents and conditions: (a) $CH_2{=}CHCH_2CH_2CH_2MgBr$, THF; (b) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2; (c) $CH_3OCOCH{=}PPh_3$, CH_2Cl_2, 3 steps 70%; (d) 9-BBN, THF, then H_2O_2, aq NaOH, 69%; (e) TBDPSCl, imidazol, CH_2Cl_2, quant.; (f) $H_2O_2{\cdot}H_2NCONH_2$, $Sc(OTf)_3$, MeOH, 52%; (g) Et_2NH, CF_3CH_2OH, 87%; (h) HF–pyridine, THF, quant.; (i) **11**, DPPA, Et_3N, CH_3CN, 75% for **17**, 71% for **19**; (j) 1,4-diisocyanatobutane, pyridine, then **16**, 58%.

9-borabicyclo[3.3.1]nonane (9-BBN) and subsequent oxidation with H_2O_2 to give a primary alcohol, the hydroxyl group of which was protected as a *t*-butyldiphenylsilyl ether to afford **15** in 69% yield. After construction of the 6-carbomethoxymethyl-3-methoxy-1,2-dioxane framework by the $Sc(OTf)_3$ catalyzed peroxyhemiacetalization followed by intramolecular hetero Michael addition, the protecting group was removed by HF-pyridine treatment to provide a peroxide **16** in 45% yield for three steps. Furthermore, biotin (**11**) was connected with **16** through a carbamate linkage in the same fashion as for the preparation of **12** to furnish the desired biotinylated probe **17**[9] in 75% yield.

On the other hand, the adjacent region to the biotin-recognizing domain in avidin was known to consist of hydrophilic amino acid residues.[10] Therefore, introduction of a hydrophilic function was expected to bring about an effective biotin–avidin binding. This presumption directed us to synthesize another biotinylated probe **20** possessing a hydrophilic spacer as illustrated in Scheme 2. The isocyanate derived from biotin (**11**) was treated with diethylene glycol **18** to give a half-carbamate **19** in 71% yield. Coupling of **19** and 1,4-diisocyanatobutane in pyridine and successive treatment of the resulting half-isocyanate **v** with the alcohol **16** in situ furnished the probe **20** in 58% yield.[11]

The anti-malarial activity of both biotinylated probes (**17**, **20**) was assessed. Both compounds inhibited the proliferation of malaria parasite with IC_{50} values of 0.91 μM for **17** and 0.57 μM for **20**, respectively.[4] Since they still preserved moderate anti-malarial potency despite some reduction of activity in comparison with the parent peroxide **3** ($IC_{50} = 0.12\,\mu M$), they were expected to bind to the target protein of the spongean anti-malarial peroxides (**1**, **2**). As a preliminary experiment, we examined the ability of **17** to generate a carbon radical species and label proteins in the presence of $FeSO_4$ under the same acidic medium (acetate buffer, pH 5.2) as the food vacuole in *P. falciparum*. After the probe **17** was incubated with bovine albumin, the reaction mixture was analyzed by avidin bound to horseradish peroxidase on the SDS-page.[12] In only the case of coexistence of **17** and $FeSO_4$, the protein band due to bovine albumin was stained.

From this observation, the biotinylated probe **17** was clarified to generate the expected carbon radical and possess affinity against proteins. Labeling of target proteins in the malaria parasites is currently under investigation in our laboratory.

Acknowledgements

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9. **17**: Colorless powder. *syn*:*anti* = 6.7:1. IR ν_{max} (KBr) cm^{-1}: 1737, 1703, 1256. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.00–2.10 (18H, m), 2.31 (1H, dd, $J = 15.9$, 5.5 Hz, MeO_2CCH_2-a), 2.44 (1H, dd, $J = 15.9$, 7.9 Hz, MeO_2CCH_2-b), 2.66 (1H, d, $J = 12.8$ Hz, SCH_2-a), 2.84 (1H, dd, $J = 12.8$, 3.7 Hz, SCH_2-b), 2.91–3.14 (3H, br s, CHS, $OCONHCH_2$), 3.19 (3H, s, 3-OCH_3, *syn*), 3.21 (3H, s, 3-OCH_3, *anti*), 3.63 (3H, s, CO_2CH_3), 3.97 (2H, t, $J = 6.1$ Hz, CH_2OCONH), 4.27 (1H, br s, NHCONHCH), 4.38–4.48 (1H × 2, m, NHCONHCH, 6-H). FAB-MS: m/z 518 $(M+H)^+$. FAB-HRMS m/z calcd for $C_{23}H_{40}N_3O_8S$: 518.2536, found: 518.2533.
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11. **20**: colorless powder. *syn*:*anti* = 7:1. IR ν_{max} (KBr) cm^{-1}: 1734, 1702, 1260. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.10–1.31, 1.35–1.71 (total 22H, m), 2.31 (1H, dd, $J = 16.0$, 5.9 Hz, MeO_2CCH_2-a), 2.43 (1H, dd, $J = 16.0$, 7.8 Hz, MeO_2CCH_2-b), 2.68 (1H, d, J = ca. 13 Hz, SCH_2-a), 2.86 (1H, dd, J = ca. 13, 4 Hz, SCH_2-b), 3.06–3.16 (7H, m, CHS, 3 × $OCONHCH_2$), 3.19 (3H, s, 3-OCH_3, *syn*), 3.21 (3H, s, 3-OCH_3, *anti*), 3.55–3.68 (8H, m, 2 × OCH_2CH_2O), 3.63 (3H, s, CO_2CH_3), 3.97 (2H, t-like, J = ca. 7 Hz, CH_2OCONH), 4.17–4.24 (1H, br s, NHCONHCH), 4.25–4.48 (1H × 2, m, NHCONHCH, 6-H). FAB-MS: m/z 764 $(M+H)^+$. FAB-HRMS m/z calcd for $C_{33}H_{58}N_5O_{13}S$: 764.3752, found: 764.3750.
12. A solution of albumin (2.5 μg) in acetate buffer (25 μL, pH = 5.2) was treated with a solution of **17** (0.1 mM) in DMSO (5 μL) in the presence or absence of a solution of $FeSO_4 \cdot H_2O$ (0.5 nM) in acetate buffer (20 μL). After standing at 37 °C for 6 h, the whole mixture was treated with SDS-PAGE sample buffer (20 μL, 100 mM Tris–HCl, 4% SDS, 12% mercaptoethanol, 20% glycerol, 0.005% bromophenol blue), and boiled for 5 min. The mixture was subjected to electrophoresis on SDS-polyacrylamide gel (4–20%, gradient), then transferred to a PVDF membrane. The membrane was blocked with PBS buffer containing 5% non-fat dry milk and 0.1% NP-40. After triplicate washing with PBS buffer, the blot was incubated with horseradish peroxidase-conjugated avidin (Bio-Rad) for 1 h. After additional rinses with PBS buffer, the immunoblots were developed using ECL detection kit (Amersham) on Hyperfilm ECL film (Amersham).