Dichloromaleimide (diCMI): A Small and Fluorogenic Reactive Group for Use in Affinity Labeling

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Chemical probes comprising a ligand moiety, a reactive group (e.g. epoxide, haloacetyl or photoreactive group) and a tag unit (e.g. fluorophore or radioisotope) are widely used in affinity labeling to identify the target proteins of bioactive molecules. However, design and synthesis of highly functionalized chemical probes are often time-consuming. In this paper, we propose a simple design strategy for chemical probes bearing a small 2,3-dichloromaleimide (diCMI) unit, which serves as a combined reactive group and tag unit by reacting with a nucleophilic lysine residue near the ligand-binding site of the target protein to generate the 2-amino-3-chloromaleimide fluorophore. Model ligand–protein experiments confirmed that the diCMI unit has suitable reactivity and fluorogenic capability for efficient affinity labeling.

Key words affinity labeling; dichloromaleimide; protein modification

Results and Discussion
Molecular Design, Model Reaction and Spectrometric Analysis

The maleimide motif is a highly reactive Michael acceptor and has been widely used for chemical modification of thiols of biomolecules.\textsuperscript{5--7} For instance, fluorophore-conjugated maleimides have been employed for the modification of cysteine residues.\textsuperscript{8,9} Recently, 2-bromomaleimide and 2,3-dibromomaleimide were developed as novel cysteine-labeling reagents, which react with thiol in an addition-elimination sequence (nucleophilic substitution) to afford thiomaleimide.\textsuperscript{10--18} In an organic solvent such as tetrahydrofuran (THF), 2,3-dibromomaleimide also reacts with amine, affording 2-amino-3-bromomaleimide as an amine-conjugation product. Interestingly, 2-aminoimaleimides were recently reported to exhibit strong fluorescence at >400 nm with large Stokes shifts (>100 nm).\textsuperscript{19} We thus initially expected that 2,3-dibromomaleimide would be available as a fluorogenic reactive group for lysine residues. However, to our knowledge, reaction of 2,3-dibromomaleimide and amine in aqueous media (or alcohols) has not been reported. On the other hand, 2,3-dichloromaleimides react with amine in alcohols.\textsuperscript{20} Therefore, we selected diCMI as a candidate fluorogenic reactive group for use in affinity labeling.

To evaluate the reactivity of the diCMI unit, we first examined the reaction of N-n-butyl-2,3-dichloromaleimide (1) with n-propylamine in H\textsubscript{2}O–THF (1:2) (Table 1, entry 1). Nearly 50% of the starting material was converted to the desired product 2a within 1 h, and 3a was obtained in 17% yield. Importantly, this result revealed that the diCMI unit is stable in aqueous media and exhibits moderate reactivity with amine. It is noteworthy that this reaction was carried out at high concentration (0.10 M), which mimics a reaction promoted by ligand–target interaction. To our surprise, dichloromaleimide 1 showed no reactivity with 1-propanethiol even in the presence of excess thiol (entries 2, 3). We further tested these reactions in phosphate buffer (pH 7.0)/THF considering the possible pH effect (entries 4, 5). As a result, amine adduct 2a and thiol adduct 2b were obtained in 12 and 73%, respectively. This means that dichloromaleimide reacts with both amine and thiol under neutralized aqueous conditions.

Next, UV-Vis and fluorescence properties of 1, 2a and 2b were evaluated (Figs. 2a, b). As shown in Fig. 2a, the amine adduct 2a was found to show a maximum absorbance at ca. 375 nm similar to a reported 2-aminoimaleimide fluorophore, 2-bromo-3-n-butyramino-N-methylmaleimide (4).\textsuperscript{19} As expected, 2a exhibited strong fluorescence (Fig. 2b, $\lambda_{ex}$: 374 nm, $\lambda_{em}$: 482 nm in 1,4-dioxane), whereas 1 showed no significant fluorescence (data not shown). Moreover, the thiol adduct 2b was found to show weak fluorescence ($\lambda_{ex}$: 372 nm, $\lambda_{em}$: 471 nm). These fluorescence data, as well as the above reactivity data, indicate that the diCMI unit would be suitable for fluorogenic labeling of amines and thiols, with much higher fluorescence intensity compared to 2-amino-3-chloromaleimide fluorophore. The fluorescence spectra and quantum yields of 1, 2a and 2b are shown in Fig. 2 and Table 1, respectively.

Note

Affinity labeling is a powerful method to label and visualize target proteins of bioactive molecules.\textsuperscript{1,2} This method generally utilizes a chemical probe composed of a ligand moiety, a reactive group and a tag unit (Fig. 1a). The reactive group serves to form a covalent bond with the target protein. For efficient and specific target labeling, the reactive group should be stable in aqueous media, have low reactivity towards non-target molecules, and react appropriately with the target protein after ligand–target binding. The tag unit serves to visualize the target protein. Fluorophores are often used as a tag unit because of the ease of detection. However, a major obstacle to affinity labeling is often synthesis of the highly functionalized chemical probe. More importantly, the ligand must retain its binding affinity after introduction of these two functional groups (reactive group and tag unit). For these reasons, a small alkyne tag is often used in affinity labeling, since it can be subsequently conjugated with a detection unit (e.g. azide-fluorophore, azide-biotin), despite the inconvenience it can be subsequently conjugated with a detection unit (e.g. azide-fluorophore, azide-biotin). For these reasons, a small alkyne tag is often used in affinity labeling, since it can be subsequently conjugated with a detection unit (e.g. azide-fluorophore, azide-biotin).
Fig. 1. Schematic Representation of Affinity Labeling

(a) General affinity labeling using a reactive group and a tag unit. AA means an amino acid residue near the ligand-binding site. (b) Simple affinity labeling method using the small diCMI unit. The diCMI unit reacts with a nucleophilic lysine (Lys) residue near the ligand-binding site to afford the 2-amino-3-chloromaleimide fluorophore.

Table 1. Reaction of 1 with n-Propylamine/I-Propanethiol

| Entry | Solvent | X      | Yield (%) | 1  |
|-------|---------|--------|-----------|----|
| 1     | A       | NH     | 47 (2a)   | 17 (3a) | 21 |
| 2     | A       | S      | —         | —     | 98 |
| 3     | A       | S      | —         | —     | 93 |
| 4     | B       | NH     | 12 (2a)   | Trace(3a) | 81 |
| 5     | B       | S      | 73 (2b)   | —     | —  |

* a Solvent A: H2O–THF (1:2), solvent B: phosphate buffer (0.6 M, pH 7.0)–THF (1:2). b This compound was observed in the 1H-NMR spectrum of crude material. Reaction conditions: 1 (0.10 M (entries 1–3), 20 mM (entries 4, 5)), n-propylamine/I-propanethiol (0.10 or 1.0 M (entries 1–3), 20 mM (entries 4, 5)), r.t., and 1 h.

Fig. 2. Spectral Analysis of 1, 2a and 4

2-Bromo-3-n-butylamino-N-methylmaleimide (4) was used as a standard.19) (a) UV-Vis spectra of 1, 2a, b and 4. Measurement conditions: 100µM compound in 1,4-dioxane. The molar extinction coefficients were calculated from the maximum absorption (εmax: 3400 M−1 cm−1 at 375 nm for 2a, 1501 M−1 cm−1 at 372 nm for 2b and 3500 M−1 cm−1 at 376 nm for 4).19) (b) Fluorescence spectra of 2a, b and 4. Measurement conditions: 100µm compound in 1,4-dioxane. The standard compound 4 was excited at 374 nm as reported. The relative fluorescence quantum yields (ϕf) of 2a and b were found to be 0.40 (λex: 375 nm) and 0.018 (λex: 372 nm), respectively.
intensity of the amine adduct. The relative fluorescence quantum yields ($\phi_f$) of 2a and b were found to be 0.40 and 0.018, respectively, by comparison with 4 ($\phi_f=0.20$).20) 2-Amino-3-chloromaleimide fluorophores are likely to have better fluorescence properties than 2-amino-3-bromomaleimides because of the intramolecular heavy atom effect (Cl versus Br), as seen in 7-(diethylamino) coumarin derivatives.21) We also note that 2a shows a solvatochromic character (data not shown), like 2-aminomaleimide.19)

Probe Design and Synthesis

To confirm the usefulness of the diCMI unit for affinity labeling, we selected biotin–streptavidin as a model ligand–target protein because of their ready availability. Biotin–diCMI probes were designed based on the reported X-ray crystal structure of biotin–streptavidin complex (PDB ID: 1STP, monomer state is shown22) (Fig. 3). It is well known that the carboxylic acid end of biotin has a low impact on the binding affinity. Therefore, the diCMI unit was attached to biotin at this position via a linker unit (Fig. 3b). The distances between the diCMI unit and the amides of 5a and b were calculated to be 6.7 and 12.6 Å, respectively. Since lysine 119 (K119), the nearest lysine residue to the ligand-binding site, is located 12.9–17.6 Å from the carboxylic acid end of biotin, 5b was expected to react more efficiently than 5a with streptavidin. We note that streptavidin has no cysteine residues; thus this labeling takes place without involving thiol species. Importantly, the synthesis of the designed biotin–diCMI probes was achieved in only three steps from biotin; conjugation of a linker unit, followed by deprotection of the tert-butoxycarbonyl (Boc) group, and reaction with dichloromaleic anhydride to afford the probes 5a and b (Chart 1).

Evaluation of Streptavidin Labeling

We next evaluated fluorescence labeling of streptavidin, the model target protein, by using 2,3-dichloromaleimides 1, 5a and b (Fig. 4). The labeling reaction was performed in phosphate buffer (pH 7.0) at 0°C. After the reaction, sodium dodecyl sulfate (SDS) sample buffer was added and each sample was subjected to polyacrylamide gel electrophoresis (PAGE). Labeled streptavidin was visualized using an in-gel fluorescence imager and then stained with Coomassie Brilliant Blue (CBB). As shown in Fig. 4a, both 5a and b were found to label the target streptavidin (lanes 3, 4). The probe 5b showed stronger fluorescence than 5a, which probably means that the diCMI unit of 5b had better access to K119. In the case of compound 1 (diCMI without the ligand unit), almost no labeling took place under the same conditions (lane 2). Furthermore, the labeling of streptavidin by 5b was inhibited by addition of biotin as a competitor (Fig. 4b). These results indicate that the labeling reaction was accelerated by the biotin–streptavidin interaction. We then examined the target selectivity of diCMI probe molecules in the presence of non-target proteins, bovine serum albumin (BSA) and carbonic anhydrase II (CAII). Under these conditions, both 5a and b labeled the target streptavidin (lanes 1, 3), but sufficient target-selectivity was observed with the designed 5b. Although non-target BSA was also labeled in some degree, competition analysis importantly revealed that the target streptavidin was specifically labeled by...
5a or b through ligand–target interactions (lanes 2, 4). This result demonstrated that the diCMI unit has suitable reactivity for target-specific labeling.

**Conclusion**

We have examined the utility of the diCMI unit as a fluorogenic reactive group for affinity labeling. diCMI was stable in aqueous media and showed sufficient reactivity with amine. Spectrometric analysis revealed that 2-amino-3-chloromaleimide has strong fluorescence. Model experiments with the biotin-streptavidin system confirmed that the diCMI unit has excellent characteristics for target-specific labeling. Notably, diCMI-based chemical probes are simple to design and synthesize. Since the diCMI unit is small, the biological activity of the ligand is likely to be well retained. Thus, the diCMI unit is expected to be useful for affinity labeling.

**Experimental**

**Chemistry**

**Synthetic Procedures**

Chemicals were purchased from Sigma-Aldrich Co. LLC (U.S.A.), Kanto Chemical Co., Inc. (Japan), Tokyo Chemical Industry Co., Ltd. (Japan), Acros Organics (U.S.A.) or Wako Pure Chemical Industries, Ltd. (Japan), and used without further purification. Reactions were monitored by TLC (Merck silic​a gel 60F254 plate). Bands were visualized using UV light or appropriate reagents followed by heating. Flash chromatography was carried out with silic​a gel (Silica gel 60N, 40–50 µm particle size) purchased from Kanto Chemical Co., Inc. NMR spectra were recorded on a JEOL JNM-GX500 or JNMECA-500 spectrometer, operating at 500 MHz for 1H-NMR and at 125 MHz for 13C-NMR. High-resolution mass spectra (HR-MS) were obtained using a BRUKER micrOTOF II mass spectrometer.

\[ \text{N-n-Butyl-2-chloro-3-(n-propylamino)maleimide (2a)} \]

\[ \text{N'-n-Butyl-2,3-dichloro-N'-propylmaleimide (3a)} \]

n-Propylamine (8.20 µL, 0.100 mmol) was added to a solution of dichloromaleic anhydride (250 mg, 1.50 mmol) in acetic acid (1.00 mL) and the mixture was stirred at room temperature for 30 min, and then refluxed for 4 h. After cooling to room temperature, the mixture was added saturated aq. NaHCO₃. The whole was extracted with AcOEt. The combined organic layer was dried over Na₂SO₄ and concentrated. The residue was purified by flash silica-gel chromatography (AcOEt–n-hexane=1:20) to afford 1 (313 mg, 1.41 mmol, 94%) as a colorless oil.

\[ 1 \text{H-NMR (CDCl₃): } \delta = 0.86 (3H, t, J=7.4 Hz), 1.25 (2H, tq, J=7.4, 7.4 Hz), 1.53 (2H, tt, J=7.5, 7.5 Hz), 3.53 (2H, t, J=7.2 Hz). \]

\[ 13C-NMR (CDCl₃): \delta = 13.6, 20.0, 30.6, 39.3, 133.3, 163.2. \]

HR-MS (electrospray ionization (ESI)) m/z: 276.0170 (Calcd for C₉H₈Cl₂NO₂ (M+Na⁺): 276.0170).

N-n-Butyl-2-chloro-3-(n-propylamino)maleimide (2a)

n-Propylamine (8.20 µL, 0.100 mmol) was added to a solution of 1 (22.2 mg, 0.100 mmol) in H₂O–THF (0.900 mL, 1:2) at room temperature, and the mixture was stirred for 1 h. After the reaction, the mixture was immediately concentrated under reduced pressure. The residue was purified by flash silica-gel chromatography (AcOEt–n-hexane=1:15 to 1:2) to afford 2a (4.80 mg, 47.0 µmol, 47%) as a yellow solid and 3a (4.80 mg, 17.1 µmol, 17%) as a white solid.

\[ 1H-NMR (CDCl₃): \delta = 0.82 (3H, t, J=7.2 Hz), 0.92 (3H, t, J=7.4 Hz), 1.23 (2H, tq, J=7.5, 7.5 Hz), 1.48 (2H, tt, J=7.5, 7.5 Hz), 1.61 (2H, tq, J=7.2, 7.2 Hz), 3.41 (2H, t, J=7.2 Hz), 3.49 (2H, dt, J=7.2, 7.2 Hz), \]

\[ \lambda_{em} > 540 \text{ nm) } \]

\[ \lambda_{ex}: 440–500 \text{ nm} \]
3.53 (1H, br s), 6.77 (1H, br s). 13C-NMR (CDCl 3) δ: 156.7, 164.3, 173.8. HR-MS (ESI) m/z: 25.6, 28.1, 28.4, 35.9, 39.1, 40.3, 40.5, 55.8, 60.2, 60.3, 61.8, 61.9, 69.9, 70.0, 70.1, 79.3, 156.2, 173.0, 173.8. HR-MS (ESI) m/z: 497.2401 Calcd for C_{15}H_{33}N_{5}O_{5}S (M+ Na^+): 497.2404.

3-Biotinylamino-proplyamine (7a)

Compound 7a was prepared differently from the reported procedure.23 Trifluoroacetic acid (TFA) (739 µL, 9.60 mmol) was added dropwise to a solution of 6a (384 mg, 0.960 mmol) in CHCl 3 (20.0 mL) at 0°C, and the mixture was stirred at room temperature for 3h. The solvent was then removed under reduced pressure to afford 7a (463 mg, TFA adduct, quant.) as an orange oil. This compound was used for the next reaction without further purification. 7a: δ-H-NMR (CD 3 OD): δ: 1.40 (2H, tt, J = 7.4, 7.4 Hz), 1.52–1.71 (4H, m), 1.80 (2H, tt, J = 6.9, 6.9 Hz), 2.19 (2H, t, J = 7.4 Hz), 2.66 (1H, d, J = 12.6 Hz), 2.87–2.90 (3H, m), 3.16 (1H, dt, J = 4.9, 9.3 Hz), 3.23 (2H, dt, J = 2.0, 6.8 Hz), 4.26 (1H, dd, J = 4.5, 7.5 Hz), 4.46 (1H, dd, J = 5.0, 7.5 Hz). HR-MS (ESI) m/z: 323.1512 Calcd for C_{15}H_{33}N_{5}O_{5}S (M+ Na^+): 323.1512.

2-(2-(Biotinylamino)ethoxy)ethoxylethylamine (7b)

Compound 7b was prepared differently from the reported procedure.23 TFA (200 µL, 2.60 mmol) was added dropwise to a solution of the compound 6b (121 mg, 0.260 mmol) in CHCl 3 (2.00 mL) at 0°C, and the mixture was stirred at room temperature for 6h. The solvent was then removed under reduced pressure to afford 7b (129 mg, TFA adduct, quant.) as a yellow oil. 7b: δ-H-NMR (CD 3 OD): δ: 1.35–1.37 (4H, m), 1.47–1.67 (4H, m), 2.14 (2H, t, J = 7.2 Hz), 2.63 (1H, d, J = 12.6 Hz), 2.84 (1H, dd, J = 4.6, 12.6 Hz), 3.04 (2H, brs), 3.12–3.15 (1H, m), 3.29 (2H, t, J = 4.5 Hz), 3.47 (2H, t, J = 5.0 Hz), 3.57 (4H, brs), 3.62 (2H, t, J = 4.5 Hz), 4.24 (1H, brs), 4.43 (1H, brs). HR-MS (ESI) m/z: 397.1872 Calcd for C_{15}H_{33}N_{5}O_{5}S (M+ Na^+): 397.1880.

N-(3-Biotinylamino)propyl-2,3-dichloromaleimide (5a)

A solution of 2,3-dichloromaleic anhydride (22.0 mg, 0.130 mmol) and 7a (39.5 mg, 0.130 mmol) in acetic acid (1.00 mL) was stirred at room temperature for 15 min under an Ar atmosphere. The mixture was then stirred at 120°C for 3h and the solvent was removed under reduced pressure. The residue was purified by flash silica-gel chromatography (CHCl 3 –MeOH=20:1 to 15:1) to afford 5a (14.3 mg, 0.0318 mmol, 24%) as a white solid. 5a: δ-H-NMR (CD 3 OD): δ: 1.35 (2H, tt, J = 7.7, 7.7 Hz), 1.49–1.65 (4H, m), 1.72 (2H, tt, J = 7.0, 7.0 Hz), 2.10 (2H, J = 7.0 Hz), 2.60 (1H, d, J = 12.6 Hz),
2.83 (1H, dd, J = 5.2, 12.6 Hz), 3.08 (2H, t, J = 6.9 Hz), 3.12 (1H, dt, J = 5.8, 8.5 Hz), 3.51 (2H, t, J = 7.2 Hz), 4.22 (1H, dd, J = 4.3, 7.8 Hz), 4.39 (1H, dd, J = 4.8, 7.8 Hz). 13C-NMR (CDCl3) δ: 26.8, 29.1, 29.5, 29.7, 36.8, 37.5, 37.9, 41.1, 57.0, 61.6, 63.3, 134.2, 164.5, 166.1, 176.2. HR-MS (ESI) m/z: 471.0630 Calcd for C30H27Cl2N4O4S (M+Na+) : 471.0631.

N-(2-(2-(2-(Biotinylamino)ethoxy)ethoxy)ethyl)-2,3-dichloromaleimide (5b)
A solution of 2,3-dichloromaleic anhydride (8.90 mg, 0.0534 mmol) and 7b (20.0 mg, 0.0534 mmol) in acetic acid (0.200 mL) was stirred at room temperature for 15 min under an Ar atmosphere. The mixture was then stirred at 120°C for 4 h and the solvent was removed by air-flow. The residue was purified by flash silica-gel chromatography (CHCl3-MeOH=20:1 to 15:1) to afford 5b (15.8 mg, 0.0302 mmol, 57%) as a white solid. 5b: 1H-NMR (CDCl3) δ: 1.39 (2H, tt, J = 7.6, 7.6 Hz), 1.58–1.71 (4H, m), 2.22 (2H, t, J = 6.4 Hz), 2.85 (1H, dd, J = 4.9, 12.9 Hz), 3.10 (1H, dt, J = 4.5, 7.3 Hz), 3.36 (2H, t, J = 4.6 Hz), 3.46 (2H, t, J = 5.2 Hz), 3.49–3.50 (2H, m), 3.53–3.55 (2H, m) 3.61 (2H, t, J = 7.8 Hz), 4.39 (1H, dd, J = 4.9, 7.7 Hz), 6.68 (2H, brs). 13C-NMR (CDCl3) δ: 25.7, 28.1, 28.3, 35.8, 38.7, 39.3, 40.5, 55.7, 60.7, 62.2, 67.6, 69.9, 70.1, 133.5, 163.2, 164.2, 173.9. HR-MS (ESI) m/z: 545.1005 Calcd for C20H18Cl2N4O4S (M+Na+): 545.0999.

Spectrometric Analysis
Methanol for spectrochemical analysis was purchased from Wako Pure Chemical Industries, Ltd. and 1,4-dioxane for spectrochemical analysis was purchased from Kanto Chemical Co., Inc. The absorption spectra were recorded on a Shimadzu UV-2400PC spectrometer equipped with a 50 W halogen lamp, and a deuterium lamp (slit width: 0.2 nm). The fluorescence measurements were performed on a JASCO FP-6500 equipped with a 150 W xenon lamp (slit width: 3 nm). A 1.0 cm quartz cell was used for the measurements.

Biological Assay
Material Preparation
Streptavidin was purchased from Wako Pure Chemical Industries, Ltd. (streptavidin from Streptomyces avidinii, >90%, 10–20 units/mg). BSA was purchased from Sigma-Aldrich Co., LLC (Bovine Serum Albumin, 96–99%), CAII was also purchased from Sigma-Aldrich Co., LLC (Carbonic Anhydrase Isozyme II from bovine erythrocytes, ≥3000 W-A units/mg protein). The proteins were used without further purification. Streptavidin, BSA and CAII were dissolved in MilliQ water, and the concentrations were determined by measuring the absorbance at 280 nm. The HEK-293 cell lysate was prepared as follows: HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2. The cell pellet was lysed with solubilization buffer (20 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) sodium salt (Hepes- Na) (pH 7.0), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, and complete mini (Roche, Switzerland)) for 15 min on ice and centrifuged at 15000×g for 10 min at 4°C. The resulting supernatant was collected and used as HEK-293 cell lysate.

General Labeling Procedure
Streptavidin was incubated with test compound in phosphate buffer (100 µL, pH 7.0) for 30 min at 0°C. In the competition analysis, streptavidin was pre-incubated with biotin for 30 min at 0°C before addition of test compound, and the excess probes were removed by Amicon Ultra-0.5 mL (Merck Millipore Ltd., Germany), after the labeling reaction. The resulting sample was mixed with 5x SDS-PAGE loading buffer, heated for 20 min at 120°C, and subjected to SDS-PAGE. The gel was visualized with an in-gel fluorescence imager (ATTO Ez-Capture II) (diaphragm: 3.0, exposure time: 5 s) equipped with WSE-5500 VarioRays (λex': 440–500 nm, peak: 470 nm) and stained with Commissaire Brilliant Blue (see Pico™ CBB Stain Kit).

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Conflict of Interest
The authors declare no conflict of interest.

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