Tetrahedron 72 (2016) 5612–5619

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

# Design and synthesis of new mass tags for matrix-free laser desorption ionization mass spectrometry (LDI-MS) based on 6,11-dihydrothiochromeno[4,3-*b*]indole

Nana Kang, Jung-Min Lee, Aeran Jeon, Han Bin Oh, Bongjin Moon *

*Department of Chemistry, Sogang University, Seoul 04107, South Korea*

ARTICLE INFO

*Article history:*
Received 23 June 2016
Received in revised form 16 July 2016
Accepted 19 July 2016
Available online 20 July 2016

*Keywords:*
Matrix-free LDI-MS
Photocleavable mass tag
Thiochromene
Pummerer rearrangement
MALDI-TOF-MS

ABSTRACT

We have rationally designed and synthesized new mass tags that are heterolytically cleavable upon UV-irradiation; these tags are based on a 6,11-dihydrothiochromeno[4,3-*b*]indole skeleton. Unless exposed to UV light, the dithioacetal group maintained its stability under normal conditions. After chemical conjugation of the mass tags with biomolecules of interest, such as proteins, the resulting conjugates efficiently and selectively generated the corresponding mass-tag fragment ions without the aid of a matrix under laser desorption/ionization (LDI) conditions. We envision that these new dithioacetal-based tags would provide a new platform of the so-called matrix-free laser desorption ionization mass spectrometry (LDI-MS), that would allow multiple detection of biomarkers with high sensitivity and selectivity. The limit of detection (LOD) of these tags was measured to be 5 fmol in the case of non-conjugated mass tag themselves and 2.8 fmol in the case of mass-tag-conjugated myoglobin.



## 1. Introduction

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) is a powerful tool for analyzing large biomolecules[1] and has been used in various clinical diagnostic applications.[2] Although the MALDI method enables direct ionization of high-molecular-weight biomolecules and their subsequent identification by mass spectrometry, the technique suffers from the inherent limitation arising from the required use of a matrix. Typically, the matrix consists of small molecules, such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid),[3] α-cyano-4-hydroxycinnamic acid (CHCA),[4] and 2,5-dihydroxybenzoic acid (DHB),[5] that assist the ionization of analytes upon UV laser irradiation. The matrix can thereby contaminate the mass signals in the low-molecular-weight region. Furthermore, because the use of the matrix ensures that all heavy biomolecules in the sample are ionized, the mass spectrum not only shows the mass information of the specific biomolecules of interest but also may contain those of other heavy contaminants unless the sample is highly pure (see Fig. 1a). To circumvent this problem, a new technique called matrix-free laser desorption ionization mass spectrometry (LDI-MS) was suggested, where heterolytically photocleavable mass tags were employed under matrix-free conditions (see Fig. 1b). In 1999, Shchepinov and co-workers have firstly reported the use of trityl group-based LDI mass tags for encoding in combinatorial oligonucleotide synthesis.[6] Since then, trityl type LDI tags have been employed in many applications such as mass spectrometry imaging,[7] mass tagging in solid phase synthesis of oligonucleotides,[8] the

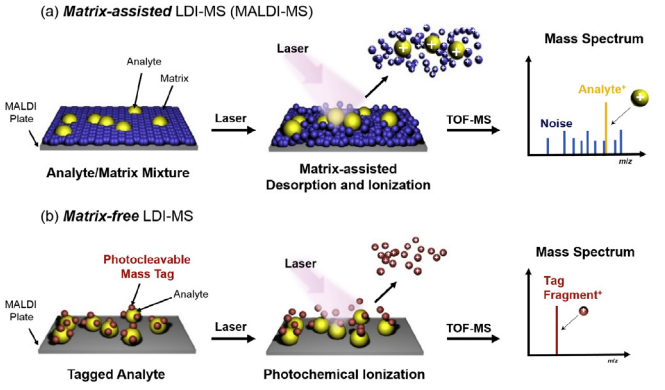



**Fig. 1.** Difference between the detection principles of (a) conventional MALDI-MS and of (b) matrix-free LDI-MS. (a) In MALDI-MS, background contamination from the matrix is the inherent limitation; (b) by contrast, in LDI-MS, background contamination is avoided because no matrix is used.

* Corresponding author. Fax: +82 2 701 0967; e-mail address: bjmoon@sogang.ac.kr (B. Moon).

http://dx.doi.org/10.1016/j.tet.2016.07.052
0040-4020/

detection of single nucleotide polymorphism,[9] calibration of mass spectrometers,[10] signal amplification in MALDI-MS,[11] and immunoassays for detecting intact protein antigens using a MALDI-MS instrument.[12] Recently, Topolyan and co-workers reported a new mass tag based on another stable carbocation species, triphenylcyclopropenylium ion, and demonstrated its high sensitivity in both in ESI and LDI mass spectrometry.[13]

Another type of photocleavable mass tag for LDI-MS was developed by Maki and co-workers in 2007, where *p*-alkoxybenzoate was employed as the core group. Chemical conjugation of this tag to an analyte allowed the generation of the negative ion bearing the analyte and enabled detection of the analyte by matrix-free LDI-MS.[14] It is noteworthy that while trityl group-based mass tags generate positive ions, *p*-alkoxybenzoate group-based tags generate negative ions.

*Ortho*-nitrobenzyl (ONB) group is a well-known photo-cleavable group and has been introduced to several mass tags for MALDI-MS.[15] However, the role of ONB group in these tags was simply homolytic chemical bond cleavage upon UV-irradiation. The use of matrix was still required to promote the ionization of the photochemically cleaved tags.

One of the most promising future applications of LDI-MS mass tags represents multiple detection of biomarkers via immunoassays. With respect to the preparation of ideal mass tags for multiplex analysis to detect many different biomarkers, preparing a library of mass tags with a 'mass-variation group' should be easy. Fig. 2 describes the working principle of the mass-tagged antibody-based multiple detection of biomarkers using the matrix-free LDI-MS technique. The overall procedure is similar to that of conventional antibody-based biomarker detection, where a surface-immobilized capture antibody and a signaling detection antibody are used to detect a target antigen (biomarker). If the detection antibodies are conjugated with the photocleavable mass tags with specific molecular masses, the matrix-free LDI should immediately generate a set of detectable ions and the TOF MS of the ions should specify the identity and amount of each detection antibody. When a larger number of mass tags are employed, an increased number of biomarkers can be detected simultaneously. Therefore, further efforts to design improved tag molecules for multiplexing are certainly desired.

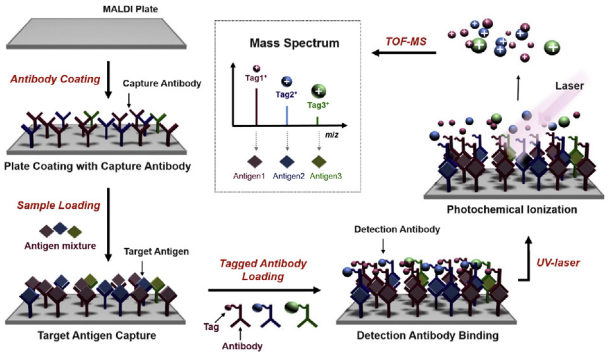



**Fig. 2.** Schematic of the multiplex detection strategy using three different photocleavable mass tags by the matrix-free LDI-MS method.

In this article, we wish to report new mass tags for LDI-MS based on a new chemical scaffold; 6,11-dihydrothio-chromeno[4,3-*b*]indole. The tags were rationally designed and synthesized considering synthetic flexibility, photochemical property, and improved solubility under bio-conjugation conditions. The sensitivity of the tags under the LDI-MS conditions was satisfactory enough so that they can be used for biomarker detection in the future.

## 2. Results and discussion

### 2.1. Photocleavable LDI-MS mass tag design

The design principle of the mass tag is shown in Fig. 3. Most importantly, the tag should have a photocleavable part that favorably cleaves in heterolytic fashion so that the resulting ions can be detected by MS. To promote this phenomenon, the tag should contain an appropriate chromophore that can absorb UV light and direct its energy toward bond cleavage. A mass variation group should also be present in the ion for detection, and the other ionic side should bear a reactive group[16] that allows the conjugation of the tag with biomolecules. With these design principles in mind, we chose the 2-(alkylthio)-2*H*-thiochromene skeleton (**1**) as the UV chromophore (see Fig. 3). We envision that UV absorption by the 2*H*-thiochromene core could induce heterolytic cleavage of C–S bonds, resulting in thiochromenium ion (**2**) and alkyl thiolate ($RS^-$), because this process is facilitated by the aromatization of 2*H*-thiochromene into thiochromenium ion. Furthermore, we conjectured that the UV absorbance of **1** could be tuned by modifying the fused aromatic group (Ar). Notably, thiochromene dithioacetal **1** should be sufficiently stable under physiological conditions so that it is not ionized until it is subjected to the LDI conditions.

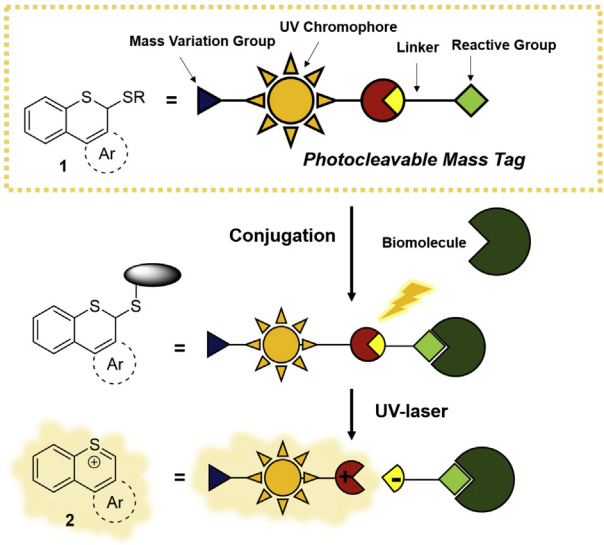



**Fig. 3.** Design principle of a heterolytically photocleavable LDI mass tag reagent.

Initially, two thiochromene dithioacetals, **3** and **4** (Fig. 4), were prepared via modified versions of procedures reported in the literature;[17] these dithioacetals were subsequently subjected to LDI-MS. Whereas **4** exhibited a weak mass signal under LDI conditions, **3** did not exhibit any signal under the same conditions (see Fig. S1 and S2 in Supplementary data). The *para*-OMe group on the aryl ring appeared to enhance the stability of the thiochromenium ion. On the basis of this observation, we attempted to stabilize the thiochromenium ion by introducing an indole ring, as shown in **5a** (Fig. 4). By altering the benzene ring with an indole ring, we anticipated that both the UV absorption at 355 nm, which is the typical UV laser wavelength used in a MALDI instrument, and the stability of the resulting thiochromenium ion should be enhanced. Indeed, the absorbance of **5a** at 355 nm was 8-fold greater than that of **4**, whereas those of **3** and **4** were negligible, as shown in the UV–vis spectra (see Fig. 5). To our delight, **5a** exhibited 75-fold higher photocleavage efficiency than **4** under matrix-free LDI-MS conditions (vide infra).

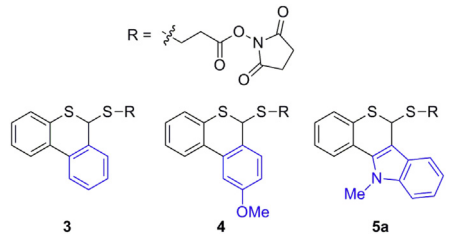



**Fig. 4.** Structures of mass tags **3**, **4**, and **5a** in which fused aryl groups are varied.

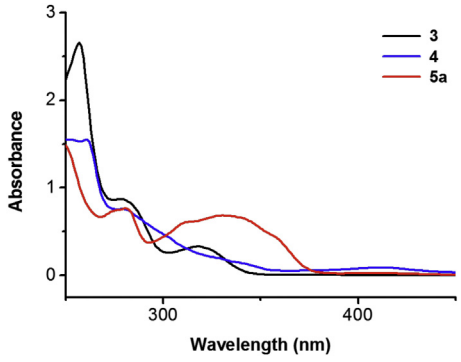
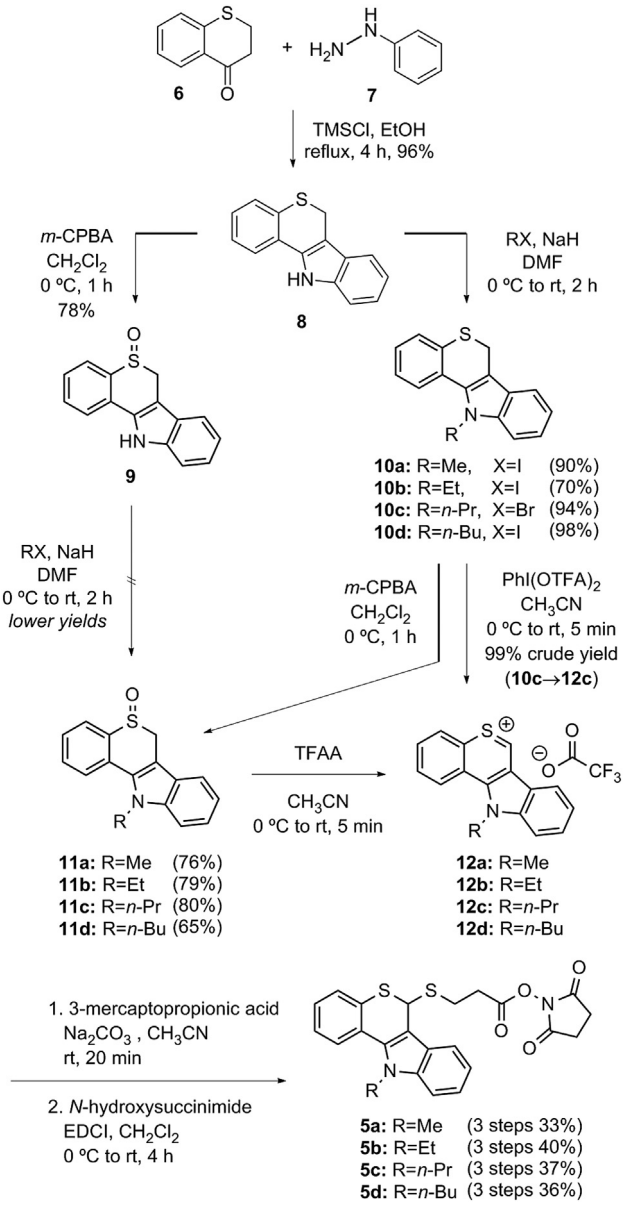



**Fig. 5.** UV–vis spectra of compounds **3**, **4**, and **5a** in $CH_2Cl_2$ solution ($5.00\times10^{-5}$ M). Molar extinction coefficients (ε) at 355 nm are; 149 $M^{-1}$ $cm^{-1}$ (**3**), 1200 $M^{-1}$ $cm^{-1}$ (**4**), and 9430 $M^{-1}$ $cm^{-1}$ (**5a**), respectively.

## 2.2. Synthesis of photocleavable LDI-MS mass tags

The syntheses of **5a** and its mass-varied derivatives **5b**–**d** are described in Scheme 1.[18] The indole moiety was conveniently introduced via the Fischer indole synthesis reaction between ketone **6** and phenyl hydrazine (**7**).[18b] In this step, trimethylsilyl chloride (TMSCl) was added to the equimolar mixture of **6** and **7** in EtOH to generate anhydrous HCl in situ, and the mixture was heated at reflux to give 6,11-dihydro-thiochromeno[4,3-*b*]indole (**8**) in good yield (96%). *m*-CPBA oxidation of **8** afforded sulfoxide **9** in 78% yield. In contrast to our expectation, the subsequent N-alkylation attempt of **9** provided undesired α-alkylation products in addition to the desired N-alkylation products, likely because of the α-proton of the sulfoxide becoming more acidic than that of the sulfide. Therefore, N-alkylation was performed prior to S-oxidation. N-Alkylation of indole **8** was carried out with various alkyl halides using sodium hydride as a base to give *N*-alkylated thiochromenoindole derivatives **10a**–**d** in excellent yields (70–98%). In this case, we chose four alkyl groups with lower masses, i.e., methyl, ethyl, propyl, and butyl groups, to minimize the increase in hydrophobicity. Because a large number of alkyl halides are commercially available and are relatively inexpensive, mass variation of the mass tags can be easily extended to a wide range of molecules. Sulfides **10a**–**d** could be easily oxidized to sulfoxides **11a**–**d** with *m*-chloroperbenzoic acid (*m*-CPBA) in good yields. In the next step, conversion of 11-alkyl-6,11-dihydro-thiochromeno[4,3-*b*]indole 5-oxide (**11**) into thiochromenium salt **12** by the Pummerer rearrangement[19] was carried out using trifluoroacetic anhydride (TFAA). Treatment of sulfoxides **11a**–**d** with TFAA (3 equiv) in acetonitrile provided the thiochromenium ion pair **12a**–**d** in 5 min. In this case, at least 3 equiv of TFAA was required to complete the reaction. After excess TFAA and solvent were removed under reduced pressure, anhydrous diethyl ether was added to the mixture to obtain thiochromenium salts **12a**–**d** as deep-yellow solids; these products were subjected to the next reaction without further purification. Some hypervalent iodine reagents, such as $PhI(OTFA)_2$ and PhI(CN)OTf, are known to promote the Pummerer rearrangement starting from the sulfide substrate because of their thiophilic 'soft' character.[20] For example, oxidation and the concomitant rearrangement of **10c** to **12c** could be achieved in a single step in high yield by treating with $PhI(OTFA)_2$. The impurities in the crude thiochromenium salts were removed by washing with diethyl ether, and the resulting residues were sufficiently pure for the next reaction. Thiochromenium salts **12a**–**d** were then trapped with 3-mercaptopropionic acid in acetonitrile in the presence of $Na_2CO_3$. The use of a base such as $Na_2CO_3$ was critical in this case. Otherwise, the reaction did not proceed to completion even when excess thiol was employed. We suspect that the reaction appears to be reversible under acidic conditions in which dithioacetals are re-protonated by the residual trifluoroacetic acid (TFA) and subsequent C–S bond re-cleavage occurs. The resulting mixture was simply purified by being washed with $H_2O$ and aq $NH_4Cl$; these products were then subjected to the next step without further purification. Finally, the carboxylic acid groups were transformed into *N*-hydroxysuccinimide active esters (NHS ester) **5a**–**d** by the Steglich esterification[21] using *N*-(3-dimethyl aminopropyl)-*N*'-ethyl-carbodiimide hydrochloride (EDCI).




**Scheme 1.** Synthetic routes for new photocleavable mass tags **5a**–**d**.

### 2.3. LDI-MS study of the photocleavable mass tags

After obtaining the mass tags **5a**–**d**, we tested their photocleavage efficiencies under matrix-free LDI-MS conditions (See Fig. 6). To our delight, the LDI-MS signals exhibited single *m*/*z* peaks that correspond to the masses of thiochromenium ions $\mathbf{5a}^{+}$–$\mathbf{d}^{+}$ with high signal to noise (S/N) ratios at 100 pmol loading. To check the feasibility of multiple detection with these mass tags, we also subjected an equimolar mixture of **5a**–**d** to LDI-MS (see Fig. 7). Interestingly, heavier mass tags were observed to exhibit greater LDI-MS abundance. In repeated experiments, this propensity was reproducibly observed; however, the reason for this greater abundance is not yet fully understood. Nevertheless, we believe that the unequal LDI-MS sensitivity of the mass tags could be compensated through the use of isotope-labeled internal standards that will be introduced in the future applications. Because a 14 Da mass difference can be sufficiently resolved by TOF MS analysis in this mass region, multiplex analyses should be achievable. The sensitivity of the mass tags was also tested, and the limit of detection (LOD) of mass tag **5d**, which has the highest efficiency, was measured to be 5 fmol (see Fig. S3 in Supplementary data). In order to compare the relative sensitivity of our tag to that of trityl group based LDI-MS tags, we have prepared an analog of **5** (**Thc-Tag**) in which the alkylthio group is replaced with a phenylthio group and subjected it to LDI-MS with equimolar amount of tris(4-methoxyphenyl)methyl phenyl thioether (**Trit-Tag**). In this experiment, **Thc-Tag** exhibited ~2.2 fold higher signal intensity than **Trit-Tag** (see Fig. S4 in Supplementary data).

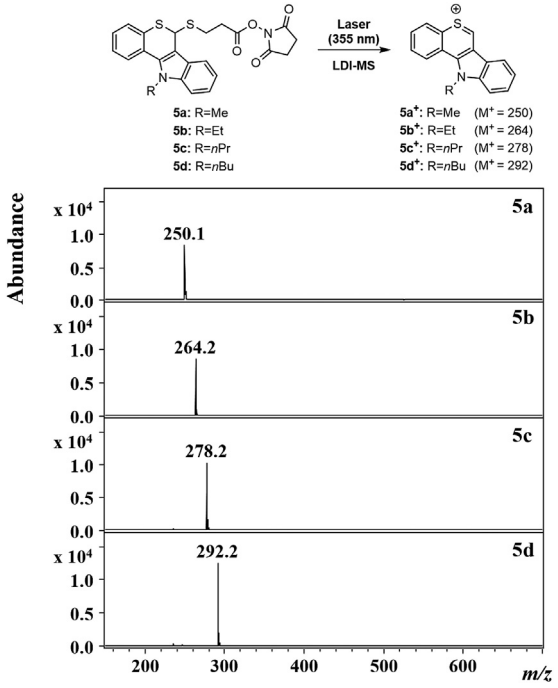



**Fig. 6.** LDI-MS spectra of mass tags **5a**–**d** (100 pmol loading).

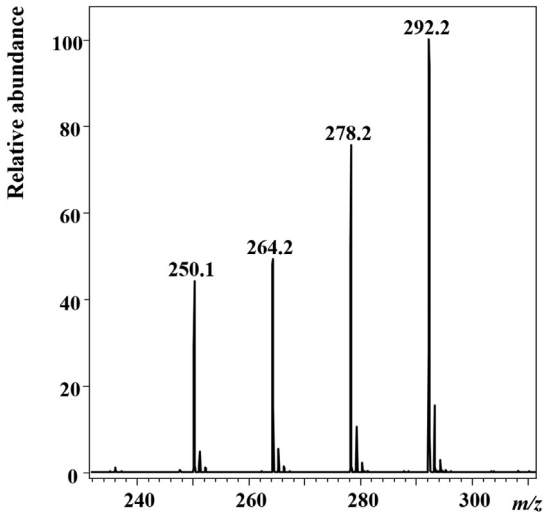



**Fig. 7.** LDI-MS spectrum of an equimolar mixture of **5a**–**d**.

### 2.4. Demonstration of protein detection by LDI-MS

We next examined the use of mass tags **5a**–**d** for bioconjugation of proteins and subsequent detection by LDI-MS. Myoglobin from equine skeletal muscle (17.6 kDa) was chosen as a test substrate. To a TEAB buffer solution (triethylammonium bicarbonate 50 mM, 70 μL, pH=8.0) were added a solution of myoglobin (142 μM, 20 μL, 2.8 nmol) in TEAB buffer and a solution of mass tag **5d** (20 mM in DMSO, 10 μL, 202 nmol). The resulting mixture was incubated for 1 h at 25 °C. The insoluble precipitates were removed by centrifugation. To scavenge the residual unreacted tags, a commercially available polymer-supported amine [tris(2-aminoethyl)amine, polymer-bound, 4.0–5.0 mmol/g N loading, 30 mg, 105–150 μmol] was added to the supernatant. After removing the resin by a simple centrifugation, the supernatant was diluted 10-fold with the mixture of TEAB buffer solution (50 mM):DMSO=9:1 and 1 μL of the solution [2.8 pmol (~50 ng) of myoglobin] was loaded onto a MALDI plate. Subjection of the sample to LDI-MS resulted in a mass spectrum with a single peak at 278 Da; this peak corresponds to the thiochromenium ions cleaved from the tagged myoglobin without any interference peaks, as shown in Fig. 8. To verify that the peaks originated only from the tags bound to myoglobin and not from the residual non-reacted mass tags, we

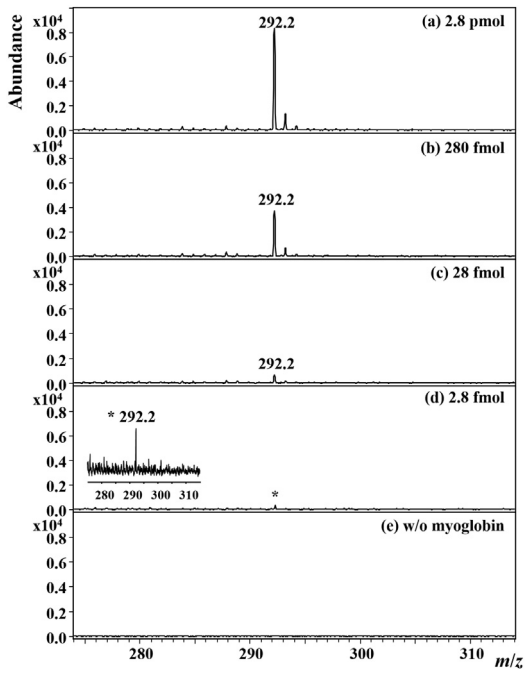



**Fig. 8.** Sensitivity test of **5d**-mass-tagged myoglobin under LDI-MS conditions; (a) 2.8 pmol, (b) 280 fmol, (c) 28 fmol, and (d) 2.8 fmol loading; (e) LDI-MS spectrum of a negative control experiment performed in the absence of myoglobin to ensure that all of the excess mass tags were removed by the purification method.

conducted a negative control experiment. When the experiment was performed in the same manner except but without myoglobin, no peaks were observed in the spectrum, as shown in Fig. 8e. In this protein conjugation and LDI-MS detection experiment, the LOD for myoglobin was determined to be lower than 2.8 fmol (~50 pg).

## 3. Conclusion

In conclusion, we have successfully designed and synthesized new mass tags based on dithioacetal derivatives of 6,11-dihydrothiochromeno[4,3-*b*]indole; these tags are easily ionisable upon UV irradiation in a MALDI-MS instrument, without the use of a matrix. The tags were readily synthesized in five or six steps and in reasonably high overall yields (16–27%) starting from commercially available starting materials. The dithioacetal structure was selectively photocleavable upon UV irradiation while maintaining its stability under normal dark conditions. The introduction of the indole moiety to the tag was critical to induce greater photoionization efficiency by increasing both the absorbance of UV light and the stability of the resulting thiochromenium ion. Additionally, easy mass variation of the tags was also possible through simple N-alkylation of the indole moiety, which is critical for the application of these tags to the future multiple detection of biomarkers. The protein tagging and purification procedures were optimized and demonstrated using myoglobin as a model protein. The detection limit of the mass tags under normal LDI conditions was estimated to be ~5 fmol level, whereas that of tagged myoglobin was ~50 pg (2.8 fmol), comparable to the lower limits for other conventional bioassay techniques.[22] Currently, we are attempting to apply these tags to multiplex detection of a set of biomarkers by employing antibody-based assays; the results will be reported in due course.

## 4. Experimental section

### 4.1. General methods

All commercially obtained solvents and reagents were used without further purification unless noted below. All reactions were performed in oven dried glassware. Anhydrous diethyl ether ($Et_2O$) and tetrahydrofuran (THF) were purified by refluxing with, and distilling from sodium under $N_2$ atmosphere. Anhydrous $CH_2Cl_2$ were purified by the same techniques from $CaH_2$. *N,N*-dimethylformamide (DMF) was distilled over anhydrous $MgSO_4$ under $N_2$ atmosphere. Triethylammonium bicarbonate buffer (1.0 M, pH=8.0, TEAB buffer), myoglobin from equine skeletal, and tris(2-aminoethyl)amine; polymer-bound (4.0–5.0 mmol/g N loading) were purchased from Sigma–Aldrich. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 nm Merck silica gel plates (60F254). The spots were visualized by UV light irradiation and ceric ammonium molybdate staining. Flash chromatography was performed by using hand-packed columns of Merck silica gel (230–400 mesh). $^1H$ NMR and $^{13}C$ NMR spectra were obtained using a Varian 400-Mercury INOVA 400 (400 MHz for $^1H$ spectrometer and 100 MHz for $^{13}C$ spectrometer). Chemical shifts were reported relative to tetramethylsilane ($\delta$ 0.00) and DMSO-$d_6$ peak ($\delta$ 2.50) for $^1H$ NMR spectra and DMSO-$d_6$ peak ($\delta$ 39.52) for $^{13}C$ NMR spectra. IR spectra were obtained using a Thermo-Nicholet Avartar-330 IR spectrometer with a single-bounce ATR mode using a ZnSe crystal (Smart MIRacle). HRMS were obtained using a Varian Ion Trap Mass Spectrometer 500 3D-ION. UV–vis spectra were obtained using a JASCO V-660 UV–vis spectrophotometer. Elemental analyses were performed at the Organic Chemistry Research Center (OCRC) using a Thermo Flash EA 1112 elemental analyzer. All matrix-free assisted laser desorption ionization time-of-flight mass (LDI-TOF MS) spectra were obtained using Autoflex Speed series of Bruker Daltonics (Leipzig, Germany) equipped with 355 nm laser pulse. An MTP 384 ground steel plate was used for sample loading. A flexControl software was used as a data acquisition system to transfer mass spectra to hpZ4000 computer.

### 4.2. Synthesis

*4.2.1. 6,11-Dihydrothiochromeno[4,3-b]indole (**8**).* Thiochro-man-4-one (0.700 g, 4.26 mmol) and phenyl hydrazine (0.461 g, 4.26 mmol) were diluted with EtOH (7 mL) and to the solution was added trimethylsilyl chloride (0.463 g, 4.26 mmol) in one portion. The reaction mixture was heated at reflux for 4 h and cooled to room temperature. The solution was basified with saturated $NaHCO_3$ aqueous solution and diluted with EtOAc (10 mL). The organic layer was separated and the aqueous layer was extracted with ethyl acetate three times (20 mL×3). The combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The resulting solid was dispersed in EtOAc:$Et_2O$=1:8 (*v*/*v*) solution, filtered, and dried under reduced pressure to give **9** as an ivory solid (0.961 g, 4.08 mmol, 96%): mp=162–165 °C; $R_f$=0.33 (EtOAc:hexane=1:4); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.58 (s, 1H), 7.71 (d, *J*=7.2 Hz, 1H), 7.53 (d, *J*=8.0 Hz, 1H), 7.39 (d, *J*=8.0 Hz, 1H), 7.34 (d, *J*=7.6 Hz, 1H), 7.24 (dd, *J*=8.0, 7.2 Hz, 1H), 7.13–7.17 (m, 2H), 7.04 (dd, *J*=7.2, 7.0 Hz, 1H), 4.28 (s, 2H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 136.7, 132.4, 131.8, 127.5, 127.4, 127.2, 125.84, 125.78, 122.9, 122.4, 119.4, 118.5, 111.4, 105.9, 22.7; IR (ZnSe-ATR) 3336 (w), 1957 (w), 1915 (w), 1871 (w), 1785 (w), 1453 (w), 1440 (w), 1416 (w), 1312 (w), 1276 (w), 1177 (w), 1006 (w), 1036 (w), 918 (w), 864 (w), 761 (m), 733 (vs), 670 (w) $cm^{-1}$; Anal. Calcd for $C_{15}H_{11}NS$: C, 75.92; H, 4.67; N, 5.90; S, 13.51. Found: C, 75.92; H, 4.62; N, 5.91; S, 13.63.

*4.2.2. 6,11-Dihydrothiochromeno[4,3-b]indole 5-oxide (**9**).* 6,11-Dihydrothiochromeno[4,3-*b*]indol (**8**, 0.480 g, 2.02 mmol, 1.0 equiv) was dissolved in distilled $CH_2Cl_2$ (15 mL) and to the solution was added 3-chloroperbenzoic acid (69%, 0.556 g, 2.23 mmol) at 0 °C. After 1 h, to the solution was added 20% sodium thiosulfate aqueous solution (4 mL) to quench the reaction. The organic layer was separated and the aqueous layer was extracted with ethyl acetate three times (30 mL×3). The combined organic layers were sequentially washed with saturated $Na_2CO_3$ aqueous solution and brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The crude was dispersed in cold $CH_2Cl_2$ and then insoluble solid was filtered and washed with cold $CH_2Cl_2$ to give **10** as a light green solid in 78% yield; mp=223–226 °C dec; $R_f$=0.41 (EtOAc:hexane=1:1+MeOH 10%); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.98 (s, 1H), 7.89 (d, *J*=7.6 Hz, 1H), 7.84 (d, *J*=7.6 Hz, 1H), 7.70 (dd, *J*=8.0, 7.6 Hz, 1H), 7.66 (d, *J*=8.0 Hz, 1H), 7.51 (dd, *J*=8.0, 7.6 Hz, 1H), 7.47 (d, *J*=8.0 Hz, 1H), 7.20 (dd, *J*=7.6, 7.2 Hz, 1H), 7.09 (dd, *J*=7.6, 7.2 Hz, 1H), 4.66 (d, *J*=15.6 Hz, 1H), 4.40 (d, *J*=15.6 Hz, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 137.2, 137.1, 132.3, 130.9, 128.9, 128.2, 127.4, 126.7, 123.2, 123.0, 119.8, 118.9, 111.9, 101.0, 43.8; IR (ZnSe-ATR) 3140 (w br), 1592 (w), 1444 (w), 1349 (w), 1321 (w), 1282 (w), 1211 (w), 1112 (w), 1037 (w), 1009 (m), 951 (w), 824 (w), 788 (w), 758 (m), 742 (vs), 731 (s), 656 (w). $cm^{-1}$; HRMS (ESI) *m*/*z* calcd for $C_{15}H_{11}NNaOS$ ($[M+Na]^+$) 276.0454, found 276.0456.

*4.2.3. General procedure for preparation of 11-alkyl-6,11-dihydrothiochromeno[4,3-b]indoles (**10a–d**).* To the mixture of 6,11-dihydrothiochromeno[4,3-*b*]indol (**8**, 1.50 g, 6.32 mmol, 1.0 equiv) and NaH (60%, dispersion in mineral oil, 0.556 g, 12.6 mmol, 2.0 equiv) was added anhydrous DMF (10 mL) at 0 °C under $N_2$ atmosphere. The reaction mixture was stirred at 0 °C. After 30 min, to the solution was added alkyl halide (12.6 mmol, 2.0 equiv) under $N_2$ atmosphere and the reaction mixture was

stirred at room temperature. After 2 h, $H_2O$ (10 mL) and EtOAc (30 mL) were sequentially added to the solution. The organic layer was separated and the aqueous layer was extracted with ethyl acetate three times (40 mL×3). The combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The crude was purified by flash column chromatography on silica gel (EtOAc:hexane=1:15) to give the desired product **10a**–**d** as an ivory solid.

*4.2.3.1. 11-Methyl-6,11-dihydrothiochromeno[4,3-b]indole (**10a**).* Yield: 90%; mp=94–97 °C; $R_f$=0.32 (EtOAc:hexane=1:15); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.83 (d, $J$=7.6 Hz, 1H), 7.62 (d, $J$=8.0 Hz, 1H), 7.50–7.54 (m, 2H), 7.33 (dd, $J$=7.6, 6.8 Hz, 1H), 7.21–7.25 (m, 2H), 7.11 (d, $J$=7.6, 7.2 Hz, 1H), 4.15 (s, 2H), 3.95 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 138.5, 134.5, 133.7, 128.6, 127.8, 127.1, 126.2, 125.0, 124.1, 122.5, 119.7, 118.7, 110.3, 109.7, 32.8, 22.8; IR (ZnSe-ATR) 2887 (w), 1908 (w), 1875 (w), 1834 (w), 1470 (w), 1426 (w), 1360 (w), 1275 (w), 1235 (w), 1219 (w), 1121 (w), 1164 (w), 1082 (w), 1046 (w), 943 (w), 821 (w), 737 (vs), 714 (m), 675 (w) cm$^{-1}$; Anal. Calcd for $C_{16}H_{13}NS$: C, 76.46; H, 5.21; N, 5.57; S, 12.76. Found: C, 76.49; H, 5.22; N, 5.43; S, 12.64.

*4.2.3.2. 11-Ethyl-6,11-dihydrothiochromeno[4,3-b]indole (**10b**).* Yield: 70%; mp=122–126 °C; $R_f$=0.36 (EtOAc:hexane=1:15); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.68 (d, $J$=7.6 Hz, 1H), 7.63 (d, $J$=8.4 Hz, 1H), 7.51–7.56 (m, 2H), 7.35 (dd, $J$=7.6, 7.2 Hz, 1H), 7.21–7.26 (m, 2H), 7.12 (dd, $J$=7.6, 7.2 Hz, 1H), 4.40 (q, $J$=7.0 Hz, 2H), 4.13 (s, 2H), 1.39 (t, $J$=7.0 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 137.7, 133.8, 133.7, 128.7, 127.7, 127.0, 126.4, 124.4, 124.3, 122.6, 119.8, 118.8, 118.7, 110.2, 22.7, 15.5, 15.4; IR (ZnSe-ATR) 2970 (w), 1936 (w), 1847 (w), 1477 (w), 1457 (w), 1363 (w), 1340 (w), 1284 (w), 1208 (w), 1162 (w), 1131 (w), 1086 (w), 1040 (w), 1102 (w), 784 (w), 746 (vs), 712 (w), 670 (w) cm$^{-1}$; Anal. Calcd for $C_{17}H_{15}NS$: C, 76.94; H, 5.70; N, 5.28; S, 12.08. Found: C, 76.88; H, 5.71; N, 5.29; S, 12.07.

*4.2.3.3. 11-Propyl-6,11-dihydrothiochromeno[4,3-b]indole (**10c**).* Yield: 94%; mp=75–80 °C; $R_f$=0.40 (EtOAc:hexane=1:15); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.69 (d, $J$=8.0 Hz, 1H), 7.62 (d, $J$=7.6 Hz, 1H), 7.56 (d, $J$=8.4 Hz, 1H), 7.51 (d, $J$=7.6 Hz, 1H), 7.35 (dd, $J$=7.6, 7.2 Hz, 1H), 7.20–7.25 (m, 2H), 7.11 (dd, $J$=8.0, 7.2 Hz, 1H), 4.34 (t, $J$=7.2 Hz, 2H), 4.13 (s, 2H), 1.73 (tq, $J$=7.2, 7.2 Hz, 2H), 0.82 (t, $J$=7.2 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 138.2, 133.9, 133.8, 128.8, 128.0, 127.0, 126.4, 124.30, 124.25, 122.6, 119.8, 118.8, 110.6, 110.4, 46.1, 23.3, 22.7, 11.0; IR (ZnSe-ATR) 3056 (w), 2970 (w), 1475 (w), 1460 (w), 1414 (w), 1348 (w), 1204 (w), 1158 (w), 1039 (w), 1012 (w), 754 (m), 738 (vs), 669 (w) cm$^{-1}$; Anal. Calcd for $C_{18}H_{17}NS$: C, 77.38; H, 6.13; N, 5.01; S, 11.47. Found: C, 77.38; H, 6.17; N, 4.95; S, 11.35.

*4.2.3.4. 11-Butyl-6,11-dihydrothiochromeno[4,3-b]indole (**10d**).* Yield: 98%; mp=86–90 °C; $R_f$=0.47 (EtOAc:hexane=1:15); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.71 (d, $J$=7.6 Hz, 1H), 7.62 (d, $J$=8.0 Hz, 1H), 7.55 (d, $J$=8.4 Hz, 2H), 7.52 (d, $J$=8.0 Hz, 1H), 7.34 (dd, $J$=7.6, 7.2 Hz, 1H), 7.20–7.25 (m, 2H), 7.11 (dd, $J$=7.6, 7.2 Hz, 1H), 4.38 (t, $J$=7.6 Hz, 2H), 4.12 (s, 2H), 1.68 (tt, $J$=7.6, 7.6 Hz, 2H), 1.25 (tq, $J$=7.6, 7.2 Hz, 2H), 0.85 (t, $J$=7.2 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 138.1, 134.0, 133.8, 128.7, 127.9, 127.0, 126.3, 124.4, 124.3, 122.5, 119.8, 118.8, 110.6, 110.5, 44.3, 31.9, 22.7, 19.4, 13.5; IR (ZnSe-ATR) 2962 (w), 2921 (w), 1469 (w), 1454 (w), 1360 (w), 1347 (w), 1196 (w), 1120 (w), 1042 (w), 819 (w), 757 (m), 743 (vs), 681 (w) cm$^{-1}$; Anal. Calcd for $C_{19}H_{19}NS$: C, 77.77; H, 6.53; N, 4.77; S, 10.93: Found: C, 77.74; H, 6.55; N, 4.81; S, 10.98.

*4.2.4. General procedure for preparation of 11-alkyl-6,11-dihydrothiochromeno[4,3-b]indole 5-oxide (**11a**–**d**).* 11-Alkyl-6,11-dihydrothiochromeno[4,3-*b*]indole (**9a**–**9d**, 1.0 equiv) was dissolved in distilled $CH_2Cl_2$ and to the solution was added 3-chloroperbenzoic acid (69%, 1.1 equiv) at 0 °C. After 1 h, to the solution was added 20% sodium thiosulfate aqueous solution to quench the reaction. The organic layer was separated and the aqueous layer was extracted with ethyl acetate three times. The combined organic layers were sequentially washed with saturated $Na_2CO_3$ aqueous solution and brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The crude was purified using the method specified below.

*4.2.4.1. 11-Methyl-6,11-dihydrothiochromeno[4,3-b]indole 5-oxide (**11a**).* The crude was purified by filtration to give **11a** as a yellow solid in 76% yield; mp=180–183 °C; $R_f$=0.29 (EtOAc:hexane=1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.89 (d, $J$=7.6 Hz, 1H), 7.83 (d, $J$=7.2 Hz, 1H), 7.68–7.71 (m, 2H), 7.58–7.61 (m, 2H), 7.27 (dd, $J$=7.6, 7.2 Hz, 1H), 7.15 (dd, $J$=7.6, 7.2 Hz, 1H), 4.55 (d, $J$=14.2 Hz, 1H), 4.47 (d, $J$=14.2 Hz, 1H), 4.03 (s, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 140.3, 138.6, 133.1, 131.5, 128.4, 127.0, 126.4, 125.9, 125.1, 123.2, 120.2, 119.0, 110.6, 100.7, 44.2, 32.3; IR (ZnSe-ATR) 2962 (w), 1581 (w), 1524 (w), 1468 (w), 1423 (w), 1356 (w), 1367 (w), 1261 (w), 1227 (w), 1123 (w), 1070 (m), 1048 (s), 1035 (s), 1025 (m), 1016 (m), 817 (w), 759 (vs), 667 (w) cm$^{-1}$; HRMS (ESI) $m/z$ calcd for $C_{16}H_{13}NNaOS$ ([M+Na]$^+$) 290.0610, found 290.0610.

*4.2.4.2. 11-Ethyl-6,11-dihydrothiochromeno[4,3-b]indole 5-oxide (**11b**).* The crude was purified by filtration to give **11b** as a yellow solid in 79% yield; mp=210–213 °C; $R_f$=0.33 (EtOAc:hexane=1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.83 (d, $J$=7.6 Hz, 1H), 7.70–7.75 (m, 3H), 7.59–7.62 (m, 2H), 7.27 (dd, $J$=7.6, 7.2 Hz, 1H), 7.15 (dd, $J$=7.6, 7.2 Hz, 1H), 4.43–4.57 (m, 4H), 1.41 (t, $J$=7.0 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 140.6, 137.7, 132.3, 131.7, 128.5, 127.0, 126.4, 126.2, 124.4, 123.2, 120.3, 119.2, 110.6, 101.1, 44.1, 15.32; IR (ZnSe-ATR) 2974 (w), 1585 (w), 1459 (w), 1339 (w), 1159 (w), 1133 (w), 1075 (w), 1024 (m), 831 (w), 768 (w), 750 (vs), 737 (m), 700 (w), 668 (w) cm$^{-1}$; HRMS (ESI) $m/z$ calcd for $C_{17}H_{15}NNaOS$ ([M+Na]$^+$) 304.0767, found 304.0768.

*4.2.4.3. 11-Propyl-6,11-dihydrothiochromeno[4,3-b]indole 5-oxide (**11c**).* The crude was purified by flash column chromatography on silica gel (EtOAc:hexane=1:1) to give **12c** as a yellow solid in 80% yield; mp=128–132 °C; $R_f$=0.27 (EtOAc:hexane=1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.82 (d, $J$=8.0 Hz, 1H), 7.69–7.77 (m, 3H), 7.58–7.64 (m, 2H), 7.26 (dd, $J$=7.6, 7.2 Hz, 1H), 7.15 (dd, $J$=7.6, 7.2 Hz, 1H), 4.56 (d, $J$=14.0 Hz, 1H), 4.42–4.46 (m, 3H), 1.77 (tq, $J$=7.4, 7.0 Hz, 2H), 0.84 (t, $J$=7.4 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 140.6, 138.3, 132.6, 131.6, 128.4, 126.8, 126.6, 126.1, 124.4, 123.2, 120.3, 119.2, 110.9, 101.2, 45.8, 44.1, 23.2, 11.0; IR (ZnSe-ATR) 2958 (w), 1581 (w), 1481 (w), 1455 (w), 1410 (w), 1362 (w), 1209 (w), 1077 (m), 1056 (m), 1036 (m), 1024 (m), 902 (w), 763 (s), 753 (vs), 730 (m) cm$^{-1}$; HRMS (ESI) $m/z$ calcd for $C_{18}H_{17}NNaOS$ ([M+Na]$^+$) 318.0923, found 318.0925.

*4.2.4.4. 11-Butyl-6,11-dihydrothiochromeno[4,3-b]indole 5-oxide (**11d**).* The crude was purified by flash column chromatography on silica gel (EtOAc:hexane=1:1) to give **12d** as a yellow solid in 65% yield; mp=64–68 °C; $R_f$=0.33 (EtOAc:hexane=1:1); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.82 (d, $J$=7.2 Hz, 1H), 7.77 (d, $J$=7.6 Hz, 1H), 7.69–7.71 (m, 2H), 7.58–7.62 (m, 2H), 7.26 (dd, $J$=7.8, 7.6 Hz, 1H), 7.15 (dd, $J$=7.8, 7.6 Hz, 1H), 4.41–4.57 (m, 4H), 1.72 (tt, $J$=7.2, 7.2 Hz, 2H), 1.27 (tq, $J$=7.2, 7.2 Hz, 2H), 0.85 (t, $J$=7.2 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 140.7, 138.2, 132.6, 131.6, 128.5, 126.8, 126.6, 126.1, 124.3, 123.2, 120.3, 119.2, 110.9, 101.2, 44.2, 44.1, 31.8, 19.4, 13.5; IR (ZnSe-ATR) 2950 (w), 1585 (w), 1482 (w), 1456 (w), 1432 (w), 1349 (w), 1266 (w), 1131 (w), 1079 (m), 1052 (m), 1027 (m), 737

(s), 668 (w) $cm^{-1}$; HRMS (ESI) *m/z* calcd for $C_{19}H_{19}NNaOS$ ($[M+Na]^+$) 332.1080, found 332.1080.

*4.2.5. General procedure for preparation of 2,5-dioxopyrrolidin-1-yl 3-((11-alkyl-6,11-dihydrothiochromeno [4,3-b]indol-6-yl)thio)propanoate (**5a**–**d**) from **11a**–**d**.* 11-Alkyl-6,11-dihydrothiochromeno [4,3-*b*]indole 5-oxide (**11a**–**d**, 1.0 equiv) was dispersed in $CH_3CN$ and to the suspension was added trifluoroacetic anhydride (3.0 equiv) at 0 °C under $N_2$ atmosphere. After 5 min, the deep yellow solution was concentrated in vacuo and the residue was precipitated in anhydrous $Et_2O$ at 0 °C. The resulting yellow solid was filtered, washed with cold anhydrous $Et_2O$, and dried under reduced pressure to give the thionium salt (**12a**–**d**) as a deep yellow solid, which was used without further purification.

The freshly prepared thionium salt (**12a**–**d**, 1.0 equiv) was dissolved in $CH_3CN$ and to the solution was added 3-mercaptopropionic acid (1.0 equiv), followed by $Na_2CO_3$ (1.0 equiv). The reaction mixture was stirred at room temperature until it turns to colorless and diluted with EtOAc. $H_2O$ was added to the mixture until all the solid had dissolved. The organic layer was separated and the aqueous layer was extracted with EtOAc three times. The combined organic layers were wash with saturated $NH_4Cl$ aqueous solution, followed by brine, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The resulting solid was used without further purification. The crude carboxylic acid and *N*-hydroxysuccinimide (1.1 equiv) were dissolved in anhydrous $CH_2Cl_2$ and to the solution was added *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (3.0 equiv) in $CH_2Cl_2$ via cannula at 0 °C under $N_2$ atmosphere. The reaction mixture was stirred at room temperature for 4 h and diluted with $CH_2Cl_2$. The resulting solution was washed twice with $H_2O$, dried over anhydrous $Na_2SO_4$, filtered, and concentrated in vacuo. The crude was purified by flash column chromatography on silica gel (EtOAc:hexane=1:1) to give the desired product as a white solid.

*4.2.5.1. 2,5-Dioxopyrrolidin-1-yl 3-((11-methyl-6,11-dihydrothiochromeno[4,3-b]indol-6-yl)thio)propanoate (**5a**).* Yield: 49%; mp=90–96 °C dec; $R_f$=0.52 (EtOAc:hexane=1:1); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.98 (d, *J*=8.0 Hz, 1H), 7.71 (d, *J*=8.0 Hz, 1H), 7.59 (d, *J*=7.6 Hz, 1H), 7.56 (d, *J*=8.4 Hz, 1H), 7.42 (dd, *J*=8.0, 7.2 Hz, 1H), 7.33 (dd, *J*=7.6, 7.2 Hz, 1H), 7.27 (dd, *J*=8.4, 7.2 Hz, 1H), 7.15 (dd, *J*=7.6, 7.2 Hz, 1H), 6.23 (s, 1H), 4.00 (s, 3H), 3.10–3.24 (m, 2H), 2.96–3.03 (m, 1H), 2.75–2.82 (m, 5H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 170.2, 168.0, 138.2, 134.7, 130.5, 129.7, 127.7, 126.8, 126.7, 125.1, 123.0, 122.7, 120.2, 118.7, 110.7, 110.4, 42.9, 33.0, 31.5, 25.7, 25.5; IR (ZnSe-ATR) 1811 (w), 1782 (w), 1732 (s), 1470 (w), 1426 (w), 1361 (w), 1201 (m), 1064 (m), 824 (w), 743 (s), 668 (w) $cm^{-1}$; Anal. Calcd for $C_{23}H_{20}N_2O_4S_2$: C, 61.04; H, 4.45; N, 6.19; S, 14.17. Found: C, 61.14; H, 4.48; N, 6.10 S, 14.04.

*4.2.5.2. 2,5-Dioxopyrrolidin-1-yl 3-((11-ethyl-6,11-dihydrothiochromeno[4,3-b]indol-6-yl)thio)propanoate (**5b**).* Yield: 40%; mp=82–87 °C dec; $R_f$=0.32 (EtOAc:hexane=1:1); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.81 (d, *J*=8.0 Hz, 1H), 7.71 (d, *J*=8.0 Hz, 1H), 7.60 (d, *J*=7.2 Hz, 1H), 7.58 (d, *J*=8.0 Hz, 1H), 7.44 (dd, *J*=7.6, 7.6 Hz, 1H), 7.33 (dd, *J*=7.6, 7.6 Hz, 1H), 7.27 (dd, *J*=7.6, 7.6 Hz, 1H), 7.16 (dd, *J*=7.6, 7.2 Hz, 1H), 6.21 (s, 1H), 4.44 (q, *J*=7.0 Hz, 2H), 3.08–3.23 (m, 2H), 2.96–3.03 (m, 1H), 2.75–2.82 (m, 5H), 1.42 (t, *J*=7.0 Hz, 3H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 170.1, 167.9, 137.5, 133.9, 130.6, 129.8, 127.6, 126.9, 126.7, 124.4, 123.1, 123.0, 120.3, 118.8, 111.2, 110.4, 42.83, 42.76, 31.5, 25.7, 25.5, 15.4; IR (ZnSe-ATR) 1810 (w), 1781 (w), 1733 (s), 1459 (w), 1426 (w), 1345 (w), 1203 (m), 1066 (m), 1044 (m), 991 (w), 813 (w), 745 (vs) $cm^{-1}$; Anal. Calcd for $C_{24}H_{22}N_2O_4S_2$: C, 61.78; H, 4.75; N, 6.00; S, 13.74. Found: C, 61.83; H, 4.79; N, 5.97 S, 13.91.

*4.2.5.3. 2,5-Dioxopyrrolidin-1-yl 3-((11-propyl-6,11-dihydrothiochromeno[4,3-b]indol-6-yl)thio)propanoate (**5c**).* Yield: 61%; mp=77–82 °C dec; $R_f$=0.36 (EtOAc:hexane=1:1); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.83 (d, *J*=8.0 Hz, 1H), 7.70 (d, *J*=8.0 Hz, 1H), 7.59 (d, *J*=7.6 Hz, 2H), 7.43 (dd, *J*=7.6, 7.6 Hz, 1H), 7.32 (dd, *J*=7.6, 7.6 Hz, 1H), 7.26 (dd, *J*=8.0, 7.2 Hz, 1H), 7.15 (dd, *J*=8.0, 7.2 Hz, 1H), 6.21 (s, 1H), 4.31–4.46 (m, 2H), 3.08–3.21 (m, 2H), 2.96–3.03 (m, 1H), 2.75–2.82 (m, 5H), 1.67–1.82 (m, 2H), 0.83 (t, *J*=7.2 Hz, 3H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 170.1, 167.9, 138.0, 134.1, 130.5, 129.8, 127.5, 126.9, 124.3, 123.0, 122.9, 120.23, 120.20, 118.8, 111.5, 110.7, 46.2, 42.7, 31.4, 25.7, 25.5, 23.1, 10.9; IR (ZnSe-ATR) 2961 (w), 1812 (w), 1783 (w), 1733 (s), 1460 (w), 1424 (w), 1348 (m), 1201 (m), 1065 (m), 1045 (m), 908 (w), 810 (w), 744 (vs) $cm^{-1}$; Anal. Calcd for $C_{25}H_{24}N_2O_4S_2$: C, 62.48; H, 5.03; N, 5.83; S, 13.34. Found: C, 62.42; H, 5.03; N, 5.82 S, 13.40.

*4.2.5.4. 2,5-Dioxopyrrolidin-1-yl 3-((11-butyl-6,11-dihydrothiochromeno[4,3-b]indol-6-yl)thio)propanoate (**5d**).* Yield: 51%; mp=76–81 °C dec; $R_f$=0.35 (EtOAc:hexane=1:1); $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.85 (d, *J*=8.0 Hz, 1H), 7.70 (d, *J*=8.0 Hz, 1H), 7.58–7.60 (m, 2H), 7.43 (dd, *J*=8.0, 8.0 Hz, 1H), 7.32 (dd, *J*=8.0, 7.2 Hz, 1H), 7.26 (dd, *J*=7.6, 7.2 Hz, 1H), 7.15 (dd, *J*=7.6, 7.2 Hz, 1H), 6.21 (s, 1H), 4.40–4.46 (m, 2H), 3.08–3.21 (m, 2H), 2.96–3.03 (m, 1H), 2.77–2.82 (m, 5H), 1.65–1.75 (m, 2H), 1.21–1.29 (m, 2H), 0.86 (t, *J*=7.0 Hz, 3H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): $\delta$ 170.1, 167.9, 137.9, 134.2, 130.5, 129.9, 127.6, 126.9, 126.8, 124.3, 123.02, 122.96, 120.2, 118.8, 111.5, 110.6, 44.5, 42.8, 31.8, 31.5, 25.8, 25.5, 19.3, 13.5; IR (ZnSe-ATR) 2954 (w), 2925 (w), 1811 (w), 1783 (w), 1735 (s), 1459 (w), 1422 (w), 1359 (w), 1200 (m), 1066 (m), 1042 (w), 810 (w), 745 (s), 669 (m) $cm^{-1}$; Anal. Calcd for $C_{26}H_{26}N_2O_4S_2$: C, 63.14; H, 5.30; N, 5.66; S, 12.96. Found: C, 63.14; H, 5.30; N, 5.69 S, 12.90.

*4.2.6. General procedure for preparation of 11-alkyl-11H-thiochromeno[4,3-b]indol-5-ium (**12c**) from **9c**.* To the mixture of 11-propyl-6,11-dihydrothiochromeno[4,3-*b*]indole (**9c**, 0.200 g, 0.716 mmol) and bis(trifluoroacetoxy)iodobenzene (0.369 g, 0.859 mmol) was added anhydrous $CH_3CN$ (1 mL) at 0 °C under $N_2$ atmosphere. After 5 min, the deep yellow solution was concentrated in vacuo and the residue was precipitated in anhydrous $Et_2O$ (4 mL) at 0 °C. The resulting yellow solid was filtered, washed with cold anhydrous $Et_2O$, and dried under reduced pressure to give the thionium salt **12c** (277 mg, 0.709 mmol) as a deep yellow solid, which was used without further purification.

### 4.3. General procedure for the bioconjugation of mass tags to myoglobin

In the conjugation procedure, in order to ensure that mass tags are not photo-degraded, the glass-tubes containing mass tags or conjugated myoglobin were wrapped with aluminum foil. The mass tag (**5d**, 2,5-dioxopyrrolidin-1-yl 3-((11-butyl-6,11-dihydrothiochromeno[4,3-*b*]indol-6-yl)thio)propanoate, 0.50 mg, 1.0 μmol) was dissolved in DMSO (dimethylsulfoxide, 50 μL) to prepare the stock solution at 20 mM. A stock solution of myoglobin (142 μM in TEAB buffer), which was used as a representative protein for conjugation, was prepared by dissolving myoglobin (17.6 kDa, 2.5 mg, 140 nmol) in TEAB buffer solution (pH=8.0, 50 mM, 1 mL). For conjugation of mass tag with myoglobin, the myoglobin stock solution (142 μM in TEAB buffer, 20 μL, 2.8 nmol) was diluted with TEAB buffer (70 μL, 50 mM), and then the solution was reacted with the stock solution of mass tag (20 mM in DMSO, 10 μL, 202 nmol) (The resulting concentration of myoglobin was 28 μM). In the case of a negative control sample (without myoglobin), TEAB buffer (90 μL, 50 mM) was mixed with the stock solution of mass tag (10 μL, 202 nmol). The reaction mixture was vortexed for 1 h at 25 °C. Subsequently, the

mixtures were subjected to centrifugation (7000 rpm, 5 min, rt). To the supernatant (90 μL) was added tris(2-aminoethyl)amine, polymer-bound (Sigma Aldrich, 4.0–5.0 mmol/g N loading, 30 mg, 105–150 μmol) to scavenge the residual unreacted tags. The reaction mixture was vortexed for 30 min at 25 °C. Again, the mixture solution was centrifuged (7000 rpm, 5 min, rt) to remove the polymer resins and the supernatant solution was subjected to LDI-TOF mass spectrometry analysis.

### 4.4. Matrix-free LDI-TOF MS conditions

*4.4.1. Sample preparation.* For LDI-MS analyses, 0.5 μL of the final supernatant solution was diluted 10 times with the mixture of TEAB buffer solution (50 mM):DMSO=9:1 and then 1 μL of the solution (myoglobin: ca. 50 ng, 2.8 nmol) was loaded onto the MTP 384 ground steel plates. The loaded plated was vacuum-dried before MS analysis.

*4.4.2. Mass spectrometry.* Mass spectra were acquired using Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), using a 355 nm Nd:YAG laser in positive ion mode. The following experimental parameters were used: laser power, 60–95%; reflectron voltage, +21 kV; ion source voltage, +19 kV; and delay time, 140 ns; number of laser shots, 5000 shots.

### Acknowledgements

This research was supported by a grant from the Emerging Industrial Technology Development Program funded by the Ministry of Trade, Industry& Energy of Korea (10044352).

### Supplementary data

Supplementary data (The LDI-MS spectra of compounds **3** and **4**. The sensitivity test of mass tags (**5d**) and photocleavage efficiency comparison between **Thc-Tag** and **Trit-Tag** in LDI-MS. The $^{1}H$ and $^{13}C$ NMR spectra of the compounds **5a**–**d, 8**, **9, 10a**–**d**, and **11a**–**d**) associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2016.07.052.

### References and notes

1. (a) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T.; Matsuo, T. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151–153; (b) El-Aneed, A.; Cohen, A.; Banoub, J. *Appl. Spectrosc. Rev.* **2009**, *44*, 210–230; (c) Fenselau, C. *Anal. Chem.* **1997**, *69*, 661A–665A.
2. (a) Sparbier, K.; Wenzel, T.; Dihazi, H.; Blaschke, S.; Müller, G.; Deelder, A.; Flad, T.; Kostrzewa, M. *Proteomics* **2009**, *9*, 1442–1450; (b) Merlos Rodrigo, M. A.; Zitka, O.; Krizkova, S.; Moulick, A.; Adam, V.; Kizek, R. *J. Pharm. Biomed. Anal.* **2014**, *95*, 245–255; (c) Clark, A. E.; Kaleta, E. J.; Arora, A.; Wolk, D. M. *Clin. Microbiol. Rev.* **2013**, *26*, 547–603; (d) Ng, E. W. Y.; Wong, M. Y. M.; Poon, T. C. W. *Top. Curr. Chem.* **2014**, *336*, 139–175.
3. (a) Beavis, R. C.; Chait, B. T.; Fales, H. M. *Rapid Commun. Mass Spectrom.* **1989**, *3*, 432–435; (b) Beavis, R. C.; Chait, B. T.; Standing, K. G. *Rapid Commun. Mass Spectrom.* **1989**, *3*, 436–439.
4. Beavis, R. C.; Chaudhary, T.; Chait, B. T. *Org. Mass Spectrom.* **1992**, *27*, 156–158.
5. Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 89–102.
6. (a) Shchepinov, M. S.; Chalk, R.; Southern, E. M. *Nucleic Acids Symp. Ser.* **1999**, *1*, 107–108; (b) Shchepinov, M. S.; Chalk, R.; Southern, E. M. *Tetrahedron* **2000**, *56*, 2713–2724.
7. (a) Thiery, G.; Shchepinov, M. S.; Southern, E. M.; Audebourg, A.; Audard, V.; Terris, B.; Gut, I. G. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 823–829; (b) Thiery, G.; Anselmi, E.; Audebourg, A.; Darii, E.; Abarbri, M.; Terris, B.; Tabet, J.; Gut, I. G. *Proteomics* **2008**, *8*, 3725–3734; (c) Thiery, G.; Mernaugh, R. L.; Yan, H.; Spraggins, J. M.; Yang, J.; Parl, F. F.; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 1689–1696; (d) Yang, J.; Chaurand, P.; Norris, J. L.; Porter, N. A.; Caprioli, R. M. *Anal. Chem.* **2012**, *84*, 3689–3695; (e) Thiery-Lavenant, G.; Zavalin, A. I.; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 609–614; (f) Dai, C.; Cazares, L. H.; Wang, L.; Chu, Y.; Wang, S. L.; Troyer, D. A.; Semmes, O. J.; Drake, R. R.; Wang, B. *Chem. Commun.* **2011**, 10338–10340.
8. Bernad, J. P. L.; Khan, S.; Korshun, V. A.; Southern, E. M.; Shchepinov, M. S. *Chem. Commun.* **2005**, 3466–3468.
9. Birikh, K. R.; Korshun, V. A.; Bernad, P. L.; Malakhov, A. D.; Milner, N.; Khan, S.; Southern, E. M.; Shchepinov, M. S. *Anal. Chem.* **2008**, *80*, 2342–2350.
10. Shchepinov, M. S.; Southern, E. M. U.S. Patent 0175839, **2004**.
11. Shchepinov, M. S.; Bernad, P. U.S. Patent 0065196, **2011**.
12. Lorey, M.; Adler, B.; Yan, H.; Soliymani, R.; Ekström, S.; Yli-Kauhaluoma, J.; Laurell, T.; Baumann, M. *Anal. Chem.* **2015**, *87*, 5255–5262.
13. Topolyan, A. P.; Strizhevskaya, D. A.; Belyaeva, M. A.; Brylev, V. A.; Ustinov, A. V.; Formanovsky, A. A.; Korshun, V. A. *Analyst* **2016**, *141*, 3289–3295.
14. (a) Maki, T.; Ishida, K. *J. Org. Chem.* **2007**, *72*, 6427–6433; (b) Maki, T. *Biol. Pharm. Bull.* **2012**, *35*, 1413–1416.
15. (a) Olejnik, J.; Ludemann, H.; Olejnik, E. K.; Berkenkamp, S.; Hillenkamp, F.; Rothschild, K. J. *Nucleic Acids Res.* **1999**, *27*, 4626–4631; (b) Lemaire, R.; Stauber, J.; Wisztorski, M.; Van Camp, C.; Desmons, A.; Deschamps, M.; Proess, G.; Rudlof, I.; Woods, A. S.; Day, R.; Salzet, M.; Fournier, I. *J. Proteome Res.* **2007**, *6*, 2057–2067.
16. El-Faham, A.; Albericio, F. *Chem. Rev.* **2011**, *111*, 6557–6602.
17. Banzatti, C.; Melloni, P.; Salvadori, P. *Gazz. Chim. Ital.* **1987**, *117*, 259–261.
18. (a) Young, T. E.; Scott, P. H. *J. Org. Chem.* **1965**, *30*, 3613–3617; (b) Zhang, X.; Li, X.; Allan, G. F.; Musto, A.; Lundeen, S. G.; Sui, Z. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3233–3237.
19. (a) Feldman, K. S. *Tetrahedron* **2006**, *62*, 5003–5034; (b) Smith, L.; Coote, S.; Sneddon, H.; Procter, D. *Angew. Chem., Int. Ed.* **2010**, *49*, 5832–5844.
20. (a) Tamura, Y.; Yakura, T.; Shirouchi, T.; Haruta, J. *J. Chem. Pharm. Bull.* **1986**, *34*, 1061–1066; (b) Feldman, K. S.; Vidulova, D. B. *Tetrahedron Lett.* **2004**, *45*, 5035–5037.
21. Neises, B.; Steglich, W. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 522–524.
22. Shen, J.; Li, Y.; Gu, H.; Xia, F.; Zuo, X. *Chem. Rev.* **2014**, *114*, 7631–7677.