— Article —

Accurate Molecular Weight Measurements of Cystine Derivatives on FAB-MS

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(Received September 8, 2022; Accepted November 17, 2022)

Summary Cystine derivatives are important biomolecules in living systems. In this study, the accurate molecular weights of various cysteines and S-S bond-containing peptides were obtained by mass spectrometry using a dithiodiethanol (DTDE) matrix and fast atom bombardment (FAB) ionization in conjunction with a double-focusing mass spectrometer, involving the detection of the target compound as $[M+H]^+$. The method is also applied to other sulfur-containing amino acids.

Key words — cystine; molecular weight; FAB-MS; S-S bond; sulfur-containing amino acid

INTRODUCTION

Mass spectrometry (MS) is a commonly used and indispensable tool in organic chemistry. Various sulfur-containing amino acids contribute significantly to the maintenance and integrity of cellular systems by influencing the cellular redox state and the detoxification capacities of these systems with regards to toxic compounds, free radicals, and reactive oxygen species.[1] Cystine (Cys-Cys) is a dimer of cysteine (Cys) linked by a disulfide bond. It is present in many foods, as well as in skin, horns, and hair.[2] Cystine also plays an important role in a variety of cellular functions and is involved in metabolic pathways.[3,4] Cystine present in urine, blood, and other biological samples acts as an important biomarker for various pathological conditions, such as inherited metabolic disorders and cystinosis.[4,5,6] Cystine disulfide bridges help stabilize the biologically active conformation of peptides and proteins.

The disulfide bond of cystines is susceptible to scission under reductive conditions or in the presence of various nucleophiles; it is readily cleaved at high temperatures.[7] Moreover, as cystine derivatives bear amino acid moieties with reactive properties, identification by MS is not always possible. Consequently, the molecular ion $M^{+\cdot}$ peak of cystine could not be detected using conventional electron ionization (EI), confirmed by their absence in popular MS databases.[8-10] Meanwhile, the identification and quantification of cystine in both biomedical and nutritional studies have been conducted by liquid chromatography mass spectrometry (LC-MS) or LC-tandem MS (MS/MS), in which ionization was electrospray ionization (ESI), obtaining protonated molecule-peaks $[M+H]^+$.[5,6,11-22] LC-MS systems are commonly equipped with a compact and economical quadrupole (Q) mass analyzer, which is suitable for the quantitative analysis of various compounds.[23] However, it is difficult to determine accurate molecular weights (MWs)[24] using QMS, only giving nominal mass. Therefore, to acquire accurate MWs, MS spectrometers equipped with time-of-flight (TOF) or Fourier-transform (FT) analyzers such as Orbitrap

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are currently available.[23]

We previously reported that liquid secondary ion (LSI)- or fast atom bombardment (FAB)-ionizations using a triethanolamine (TEOA)-NaCl matrix system were effective for accurate measurements of the MWs of nucleoside phosphoramidites,[25,26] which are common building blocks in oligonucleoside synthesis.[27] FAB-MS is currently equipped at our research facility. In the context, we herein report that FAB-MS measurements using a dithiodiethanol (DTDE) matrix can be used to accurately determine the MWs of cystine derivatives and sulfur-containing amino acids. To our knowledge, FAB has not been used thus far for the ionization of cystine. A double-focusing mass spectrometer with a magnetic-electric sector was used and accurate MWs as $[M+H]^+$ with an average mass error less than 0.5 mDa (error: absolute mass accuracy) were obtained, thus providing the formulae of various cystine derivatives. Furthermore, the present method can be readily applied to oligo- and cyclic peptides linked by S-S bonds.

MATERIALS AND METHODS

Materials

Compounds (**1, 3, 4, 8, 12, 14, 15, 16, 18**), and all matrices for FAB-MS were purchased from TCI. Compound **2** was purchased from Combi-Blocks, while compounds **5, 9**, and **17** were purchased from Watanabe Chemical. Compounds **6** and **13** were purchased from Angene and Nacalai Tesque, respectively. DiNAC (**7**) was prepared via the oxidation of NAC (**13**) with hydrogen peroxide in our laboratory. Oxytocin (**10**) and conotoxin (**11**) were purchased from the Peptide Institute, INC. Ultrapure water for dissolving analytes was obtained using an Elix Advantage water purification system (Merck), and the other solvents were commercially available.

MS analysis

EI-, FAB-, and ESI-MS were performed on a JMS-700(2) double-focusing mass spectrometer (JEOL Ltd., Tokyo, Japan) with a magnetic-electric (*B-E*) sector geometry. There are no special requirements for the standard EI-, FAB-, and ESI-MS procedures or system configuration. Low-resolution (LR) mass spectra were acquired by magnetic scanning (ten average scans), with a resolution of 1000 or 2000 (10% valley definition). High-resolution (HR) mass spectra were obtained using accelerating voltage scans (five average scans), and the resolution was 5000 or 6000 (10% valley definition). For HR-FAB-MS data, polyethylene glycol (PEG) 200, PEG 300, PEG 400, PEG 600, PEG 1000, and their Na^+ adducts were used as references to produce accurate masses, except for conotoxin (**11**). $[(CsI)_nCs]^+$ was used as a reference for **11**. HR-FAB mass spectra were measured using the insertion/removal technique of the target analyte and reference. The LR- and HR-MS were in the positive ion mode. The samples were introduced into the ion source using a direct inlet system using the EI or FAB methods. EI-MS was measured at a chamber temperature of 180 ° C (ionization energy of 40 eV, ionization current of 300 μA) with an accelerating voltage of 8 kV. The FAB samples were prepared as a mixture of each compound (ca. 0.02 mg/0.3 μL) in solvents (MeOH, d HCl, or H_2O) and matrix (ca. 0.4 μL). The mixture placed on the FAB target was ionized by a fast-atom Xe beam with an energy of 6 keV, and the secondary ions were accelerated to 8 kV. ESI experiments were carried out with a 2.0 kV needle voltage at 250 ° C desolvation-temperature using N_2 as the nebulizing and dry gas. The sample (0.1-0.5 mg) in a solvent [1 mL of MeOH, H_2O, MeOH/H_2O (50/50), or d HCl] was infused into the ion source using a syringe-pump technique.

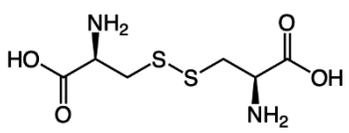
RESULTS

FAB-MS of Cystine and *N,N'*-Di-Boc-cystine

Initially, the mass spectra of cystine (**1**) was measured using EI. As seen in entry 1 of Table 1, only amino-acid fragments were obtained, while a molecular ion

$M^{+\cdot}$ was not present. This is in agreement with current mass databases.[8-10] Next, we focused on FAB, which has been used as a mild and soft ionization technique for various labile materials. The matrix used in FAB-MS analysis plays a significant role as a supporting base (a relatively involatile solvent which holds the sample in position on the sample target, and affects proton donating/ accepting yields) to achieve effective and stable ionizations.[23] As illustrated in Fig. 1, glycerol (G), *m*-nitrobenzyl alcohol (NBA), triethanolamine (TEOA),[25] diethanolamine (DEOA), magic bullet [MB: a 3:1 mixture of dithiothreitol (DTT) and dithioerythritol (DTE)], a 1:1 mixture of DTT and α -thioglycerol (TG), DTDE, and TG are

Table 1. MS conditions for cystine (**1**).



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Entry	Ionization Method	Matrix[c]	RI[a,b](%)			
			$[M+H]^+$	$[M+Na]^+$	$[M+matrix+H]^+$	$[2M+H]^+$
1	EI[d]		–	–	–	–
2	**FAB**[e]	**G**	**100**	sp[f]	5.7	6.3
3		**NBA**	**100**	sp	6.0	sp
4		TEOA	nd[g]	nd	nd	nd
5		TEOA-NaCl	nd	nd	nd	nd
6		DEOA	nd	nd	nd	nd
7		**MB**	**100**	7.0	nd	sp
8		**DTT/TG**	**100**	sp	nd	4.7
9		**DTDE**	**100**	nd	5.5	7.5
10		**TG**	**100**	sp	nd	6.1
11	**ESI**[e]		**100**	12.5	–	7.2

[a]RI: Intensity relative to the base peak ion (100%). [b]High molecular mass ions such as $[M+2Na]^+$, $[M+matrix+Na]^+$, $[2M+Na]^+$, and $[2M+matrix+H]^+$ were omitted. [c]see Fig. 1. [d]see experimental. [e]Cystine was dissolved into d HCl. [f]sp: small peak (< 3%). [g]nd: not detected.

commonly used as matrices for FAB.

FAB-MS using G generated the $[M+H]^+$ of **1** as the base peak (100%), accompanied by $[M+matrix+H]^+$ (5.7%) and $[2M+H]^+$ (6.3%) peaks (Table 1, entry 2). Similarly, the use of NBA, MB, DTT/TG, DTDE, and TG also provided the $[M+H]^+$ of **1** as the base peak (entries 3 and 7-10, respectively), while those of TEOA and DEOA (entries 4 and 6, respectively) did not yield any useful peaks of **1**. Although the TEOA-NaCl system was effective in the previous MS study regarding nucleoside phosphoramidites,[25-27] it could not be applied to the measurement of cystine (**1**) (entry 5). However, as the ESI method has been widely used in both biomedical and nutritional studies, it predicted the $[M+H]^+$ of **1** as the base peak, together with the sodium-ion adduct molecule {$[M+Na]^+$ (12.5%)} and $[2M+H]^+$ (7.2%) (entry 11).

For the chemical synthesis of peptides, a range of protecting groups have been developed to block sensitive moieties, which may either be removed directly or during oxidative folding. Among them, *t*-butyl carbamate is used extensively for amine protection in peptide and heterocyclic synthesis, which is usually cleaved with acid.[28] EI and FAB methods have been investigated for *N*,*N*-bis[(*tert*-butoxy) carbonyl]-L-cystine (**2**, *N*,*N*'-Di-Boc-cystine), which is an *N*-terminal protected synthon for peptide synthesis (Table 2). The EI method detected the desired $M^{+\cdot}$ peak of **2** with a relative intensity of only 0.2% to the base peak ion [$(CH_3)_3C^+$: *m/z* 57] (entry 1). Alternatively, FAB-MS using G, NBA, and TG yielded the $[M+H]^+$ peak of *N*,*N*'-Di-Boc-cystine (**2**) with relative intensities of 15.0, 5.6, and 5.4%, respectively, together with the associated $[M+Na]^+$ peaks with intensities of 18.8, 42.7, and 29.2% (entries 2, 3, and 10), respectively. In the case of TEOA, TEOA-NaCl, DEOA, MB, and DTT/TG matrices (entries 4-8), the $[M+H]^+$ peaks of **2** were not detected (entries 4-6) or were insignificant (entries 7 and 8), however, their $[M+Na]^+$ peaks were observed with relative intensities of 26.7-100% (entries 4,5,7, and 8), except for DEOA (entry 6). The use of DTDE for 2 produced an optimum result, significantly increasing the $[M+H]^+$ peak to 47.1%. This sensitivity was higher than that of the $[M+Na]^+$ peak (15.6%) (entry 9). Based on these results, DTDE is the most suitable matrix for cystine and its derivatives in FAB-MS. Moreover, there is a resemblance between the chemical structures of cystine and DTDE in that they both possess an S-S linker and terminal hydrophilic moieties (Fig. 1). Given that analytes need to mix well

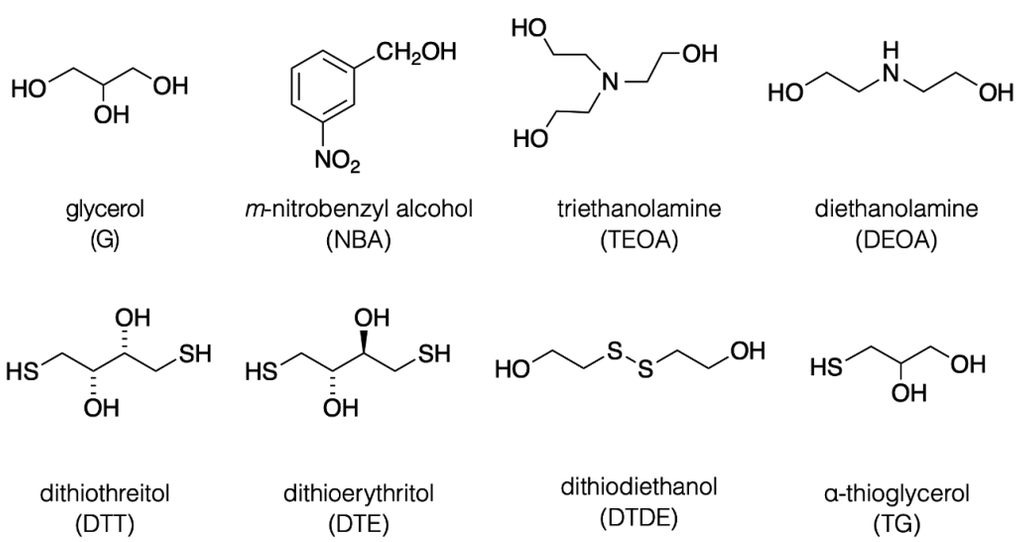
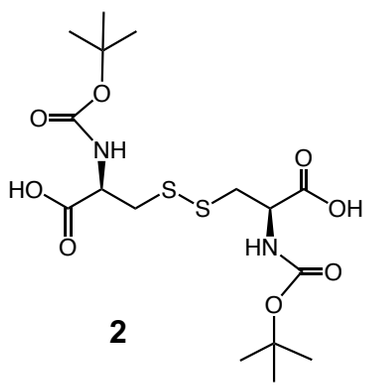


Figure 1. Chemical structures of matrices used for FAB-MS.

with the matrices, this structural similarity could make DTDE an excellent matrix for cystine derivatives.

The FAB-MS spectrum of cystine (**1**) using the DTDE matrix was characterized and the results are displayed in Fig. 2. The fragment ions at *m/z* 152 (**a**) and 122 (**b**) were generated by the cleavage of the S-S bond, and the *m/z* 198 ion corresponds to $[1/2\ (M+DTDE)+H]^+$.

Table 2. MS conditions for *N,N'*-Di-Boc-cystine (**2**).[a]



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Entry	Ionization Method	Matrix	RI (%)		
			$M^{+\cdot}$	$[M+H]^+$	$[M+Na]^+$
1	EI		0.2	–	–
2	FAB[b]	G		15.0	18.8
3		NBA		5.6	42.7
4		TEOA		nd	76.6
5		TEOA-NaCl		nd	100
6		DEOA		nd	nd
7		MB		0.7	66.1
8		DTT/TG		1.5	26.7
9		**DTDE**		**47.1**	**15.6**
10		TG		5.4	29.2

[a]see Table 1.[b]*N,N'*-Di-Boc-cystine (**2**) was dissolved into MeOH.

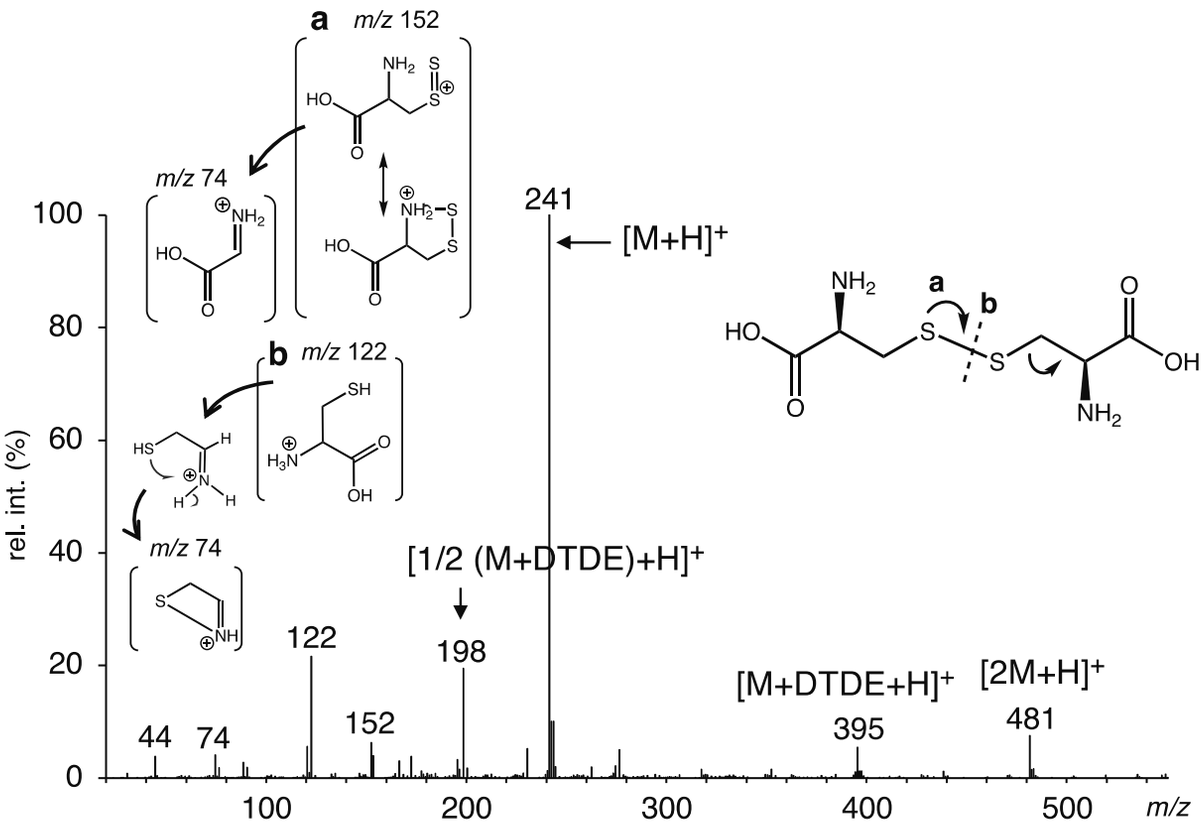

Figure 2. Positive ionization FAB-MS spectrum of cystine (**1**, MW 240) introduced in the DTDE matrix.

Accurate mass measurements of cystine derivatives and S-S bond-containing peptides using the DTDE/FAB-MS method

Accurate mass measurements of a range of cystine derivatives were carried out to study the scope and limitation of the DTDE/FAB-MS method, as shown in Table 3. The measured accurate masses (MAMs) of cystine (**1**) and *N,N'*-Di-Boc-cystine (**2**) appeared at *m/z* 241.0318 and 441.1367, respectively, in the externally calibrated spectrum (polyethylene glycol) by HR-MS (entries 1 and 2), with respective errors of only 0.1 and 0.2 mDa from the expected mass numbers. Consequently, we applied this method to cystine derivatives **3-7**, cysteine derivative **9**, and S-S bond-containing peptides **8**, **10**, and **11**. The structures of **3-11** are shown in Fig. 3, and their observed and theoretical masses are summarized in Table 3.

N,N'-Bis[(benzyloxy)carbonyl]-L-cystine (*N*-Cbz, **3**) is a popular *N*-terminal protected cystine which is cleaved *via* facile hydrogenolysis (H_2/ Pd-C).[28] The MAM of **3** was obtained with an error of only 0.3 mDa (entry 3). Methyl, allyl, and *t*-butyl esters are the most commonly used protecting groups in organic chemistry, specifically for carboxylic acids. *C*-Terminal protective cystines **4**·2HCl (*C*-Me), **5**·TsOH (*C*-allyl), and **6**·2HCl (*C*-^{t}Bu) in Fig. 3 are readily cleaved by various methods, namely, aqueous alkali metal hydroxides for methyl ester **4**, use of transition metal-catalyzed methods including palladium catalysis for allyl ester **5**, and moderate acidic hydrolysis with the release of isobutylene for *t*-butyl ester **6**.[28] Esters **4**, **5**, and **6** were analyzed using MS conditions that provided the respective MAM peaks with small errors (0.1-0.3 mDa) (entries 4-6). *N,N'*-diacetyl-L-cystine (DiNAC, **7**) is the disulfide dimer of *N*-acetylcysteine (NAC) and has immunomodulating properties.[29] It is a potent, orally active modulator of contact sensitivity/delayed hypersensitivity reactions in rodents,[30] and also has

antiatherosclerotic effects in Watanabe-heritable hyperlipidemic rabbits.[31] DiNAC (**7**) was subjected to the FAB-MS conditions to obtain the MAM (error: 0.4 mDa) (entry 7). Glutathione is a tripeptide comprised of three amino acids (cysteine, glutamic acid, and glycine), which plays a central role in signaling pathways and the defense against oxidative damage.[32] Upon oxidation, glutathione (GSH) is transformed into glutathione disulfide (GSSG, **8**) in Fig. 3. The concentrations of GSH and GSSG and their molar ratio are indicators of cell functionality and oxidative stress.[33] GSSG (**8**) was also characterized by the use of the present matrix with excellent mass accuracy (error: 0.5 mDa) (entry 8). Unsymmetrical disulfide **9** [FmocCys(^{t}BuS)OH] is a sulfenyl derivative prepared from cysteine which is protected by fluorenylmethyl carbamate (Fmoc). Although the Fmoc group has excellent acid stability, it is readily cleaved by simple amines.[28] The *t*-butyl group of **9** can be removed by the reduction of thiols such as $HO(CH_2)_2SH$ or $NaBH_4$.[28] The MAM peak for **9** (16.0%) was obtained with a small error (0.4 mDa) (entry 9).

As FAB is mainly applied to analytes up to approximately m/z 3000,[23] we carried out measurements of cyclic peptides with high masses. Oxytocin (**10**, MW 1006) is a typical cyclic peptide in

Table 3. FAB-MS measurements using DTDE matrix: accurate protonated molecule mass.[a]

entry	compound	EI ($M^{+\cdot}$)	FAB $[M+H]^+$	calculated exact mass	measured accurate mass	mass accuracy (mDa)
1	**1**[b] (Cys-Cys)	nd[a]	100	241.0317	241.0318	0.1
2	**2**[c] (*N*-Boc)	0.2	47.1	441.1365	441.1367	0.2
3	**3**[c] (*N*-Cbz)	nd	93.0	509.1052	509.1049	0.3
4	**4**[c] (*C*-Me)	3.4 ($[M+H]^+$)	100	269.0630	269.0627	0.3
5	**5**[c] (*C*-allyl)	–[d]	100	321.0943	321.0944	0.1
6	**6**[c] (*C*-^{t}Bu)	–	100	353.1569	353.1572	0.3
7	**7**[c] (DiNAC)	–	100	325.0528	325.0524	0.4
8	**8**[e] (GSSG)	–	100	613.1598	613.1593	0.5
9	**9**[c] [FmocCys(^{t}BuS)OH]	–	16.0	432.1303	432.1307	0.4
10	**10**[c] (Oxytocin)	–	100	1007.4443	1007.4448	0.5
11	**11**[e] (Conotoxin)	–	100	1351.4917	1351.4921	0.4
12	$(PhS—)_2$	100	–	–	–	–
13	$[CH_3(CH_2)_{11}S—]_2$	100	–	–	–	–

[a]see Table 1. [b]Dissolved into d HCl. [c]Dissolved into MeOH. [d]not measured.
[e]Dissolved into H_2O.

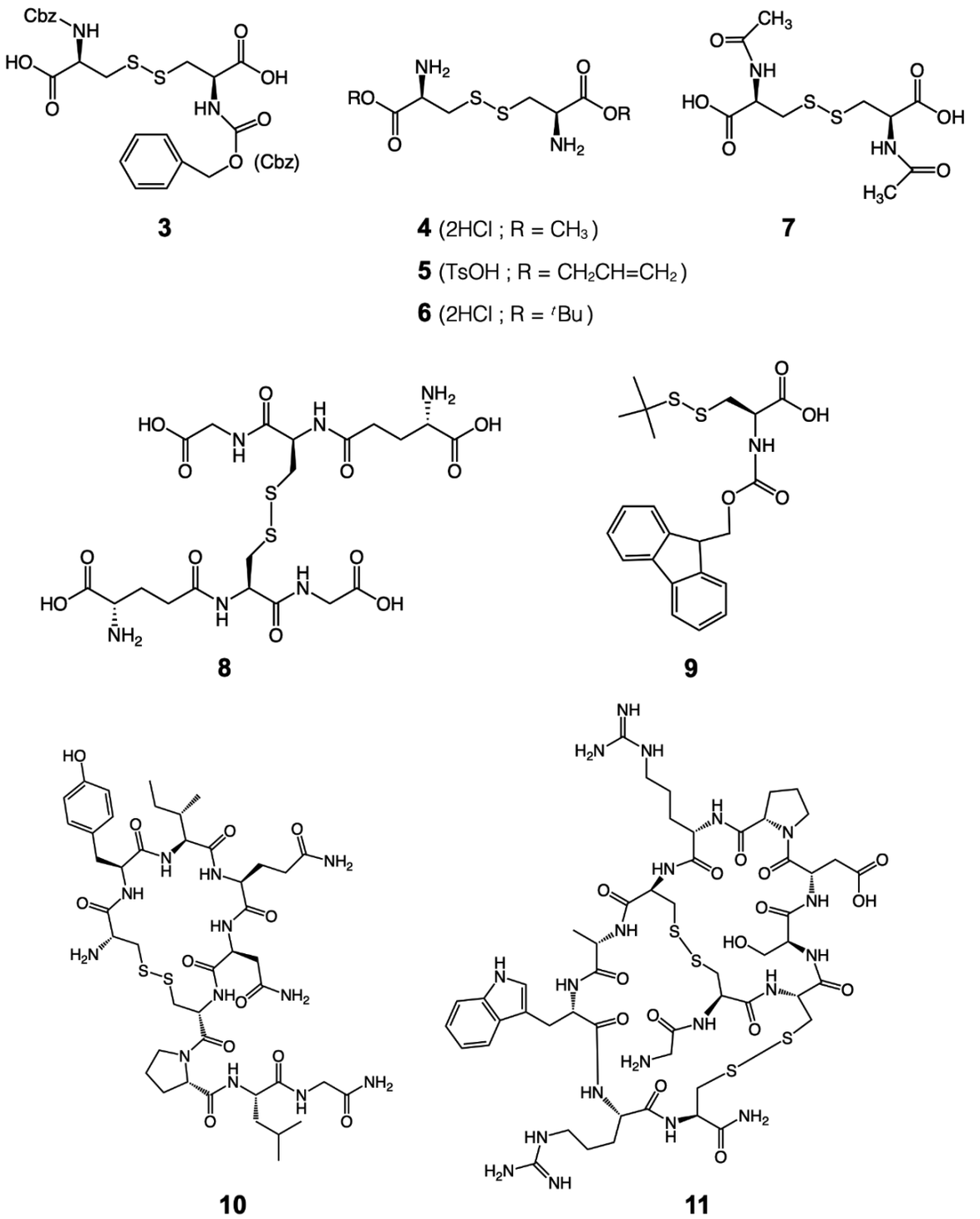

Figure 3. Chemical structures of cystine derivatives **3-11**.

the sequence (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2); its *C*-terminus has been converted to a primary amide and a disulfide joins the cysteine moieties, as illustrated in Fig. 3. Oxytocin is a hormone that acts on organs in the body (including the breast and uterus), functions as a chemical messenger in the brain, controls key aspects of the reproductive system (including childbirth and lactation), and affects aspects of human behavior.[34,35] The MAM peak of **10** (*m/z* 1007.4448; error: 0.5 mDa) (entry 10) indicates the efficiency of DTDE matrix in FAB ionization. α-Conotoxin ImI (**11**, MW 1350),[24] bearing two inner

disulfide bonds [Cys^2-Cys^8, Cys^3-Cys^{12}], is a neuropeptide isolated from the venom of the marine cone snail, *genus Conus* (Fig. 3) which inhibits the nicotinic acetylcholine receptors of nerves and muscles.[36] The MAM with low mDa-level mass accuracy (error: 0.4 mDa) (entry 11) was obtained using the proposed method.

The EIMS spectra of diphenyl disulfide and didodecyl disulfide were measured (entries 12 and 13) for the comparison of cystine derivatives with simple disulfides. The $M^{+\cdot}$ peaks were obtained with 100% intensity. These results emphasize the susceptibility of the scission of the S-S bonds in cystine derivatives bearing amino acid moieties.

Extension to sulfur-containing amino acids

We attempted to extend this method to sulfur-containing amino acids (**12-17**), as illustrated in Fig 4. Cysteine (**12**) [2], NAC (**13**), and GSH (**14**) contain a sulfanyl (SH) group and are reductive forms of cystine (**1**), DiNAC (**7**), and GSSG (**8**), respectively. NAC (**13**) is a medication used for acetaminophen overdose and chronic bronchopulmonary disorders.[29] GSH (**14**) plays a central role in the defense against oxidative damage and in signaling pathways.[32] As shown in Table 4, the MAMs of **12**, **13**, and **14** were easily measured with high $[M+H]^+$ intensities and low errors (<0.2 mDa) using the DTDE/FAB-MS method (Table 4, entries 1-3). Furthermore, MS measurements of two sulfur-containing amino acids, methionine (**15**) and taurine (**16**), were carried out.[1] Methionine is an essential amino acid with a sulfide structure, while taurine (2-aminoethanesulfonic acid) is one of the most abundant amino acids and plays an important role in essential biological processes.[2] The MAM of methionine (**15**) was easily measured as a base peak, and that of taurine (**16**: MW 125) with a low mass was observed,[37] together with a shorter peak-lifetime (20-30 s) than that of the ordinary FAB peaks (> 5 min) (entries 4 and 5). The disodium salt {**17**, [FmocCys(SO_3H)OH]}, which is an Fmoc and *S*-sulfonate derivative of cysteine, is a synthon for peptide synthesis. The sulfonate can be removed by thiols.[28] Significantly, this mild ionization method afforded the MAM of **17** as a disodium salt with minimal errors (0.1 mDa) (entry 6).[38] Furthermore,

Table 4. FAB-MS measurements using the DTDE matrix: accurate protonated molecule mass.[a]

entry	compound	EI ($M^{+\cdot}$)	FAB $[M+H]^+$	calculated exact mass	measured accurate mass	mass accuracy (mDa)
1	**12**[b] (Cys)	4.8	68.1	122.0276	122.0278	0.2
2	**13**[c] (NAC)	–	100	164.0381	164.0380	0.1
3	**14**[b] (GSH)	–	100	308.0916	308.0915	0.1
4	**15**[d] (Met)	–	100	150.0589	150.0591	0.2
5	**16**[b] (Taurine)	–	100[e]	126.0225	126.0227	0.2
6	**17**[b] [FmocCys(SO_3H)OH]	–	4.8[f]	468.0164	468.0165	0.1
7	**18**[d] $(SeCys)_2$	nd	100	336.9208	336.9207	0.1

[a]see Table 1. [b]Dissolved into H_2O. [c]Dissolved into MeOH. [d]Dissolved into d HCl.
[e]see Reference 37. [f]see Reference 38.

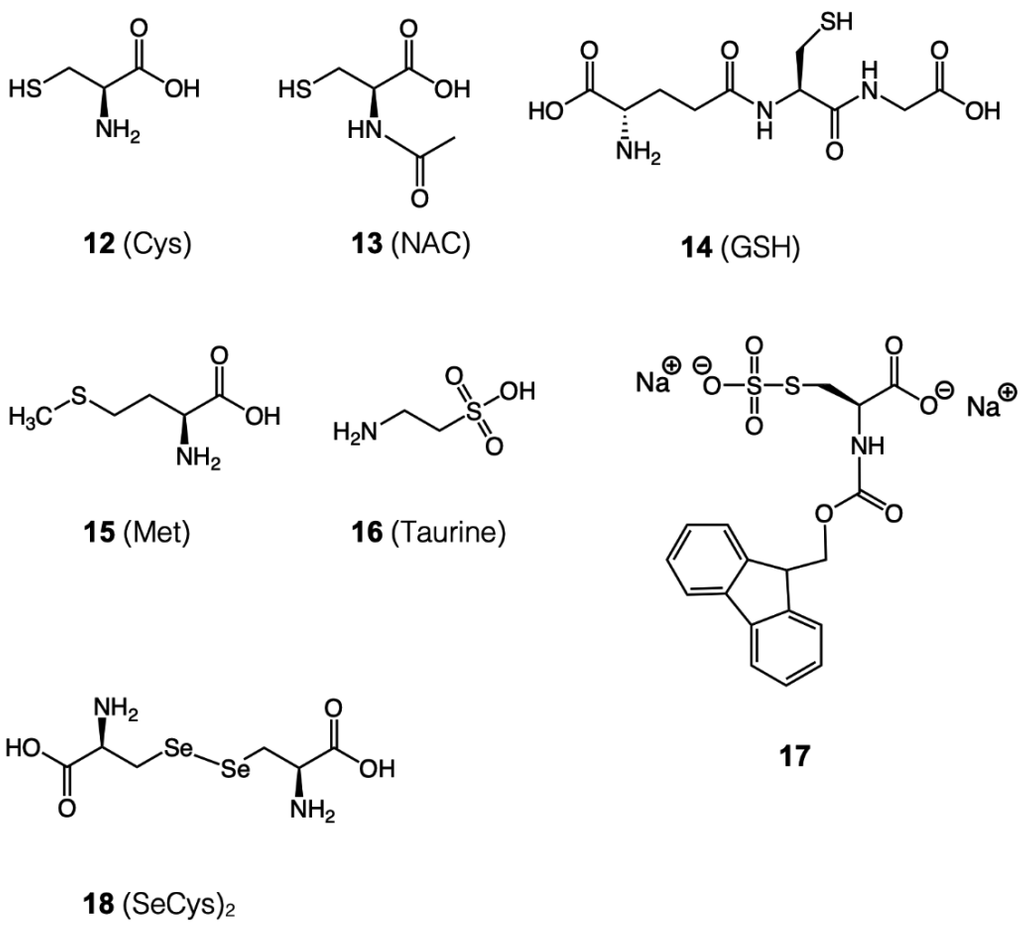

Figure 4. Chemical structures of sulfur-containing amino acids **12-18**.

selenocystine (**18**), with a homologous Se-Se bond, was examined by this method.[39] Selenocystine reduces tumor growth without affecting body weight and promotes antiproliferative activity in melanoma and cervical cancer cells.[40] Although EI of 18 was not detected, the present method provided an MAM of 18 with a high $[M+H]^+$ (100%) species and low error (0.1 mDa) (entry 7).

CONCLUSIONS

The DTDE matrix on FAB-MS equipped with a double-focusing mass spectrometer was useful for the measurement of cystine derivatives and sulfur-containing amino acids. The proposed method rapidly and easily measures the accurate MWs of a variety of cystines as protonated molecules $[M+H]^+$ with an average mass error less than 0.5 mDa. All compounds investigated gave MAMs using the proposed method. This matrix on FAB may be used for other related compounds bearing thiol, sulfide, or diselenide functional groups. These results prove that this method has a broad scope and good functional group tolerance. Hence, the proposed method is beneficial to organic chemists for the characterization of a range of cystine derivatives, sulfur-containing amino acids, and disulfide compounds.

ACKOWLEDGEMENTS

We would like to thank Professor Y. Usami, Dr. H. Yoneyama, and Dr. A. Asano at Osaka Medical and Pharmaceutical University for their helpful discussions and technical support. Both authors are

grateful to Emeritus Pro. T. Shioiri at Nagoya City University for valuable discussions and encouragement. This work was financially supported by the Osaka Medical and Pharmaceutical University.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary data.

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