Functional Dihydro-1*H*-imidazole Derivatives for MALDI Signal Enhancement of a Lysine-Specific Chemical Modification

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The enhancement of signal sensitivity and quantification of low-abundance proteins is of great importance in proteomic analysis. The preparation of 2-methoxy-4,5-dihydro-1*H*-imidazole (MeO-DHIM) derivatives was accomplished by one-step synthesis of *O*-methyl-isourea. The signal enhancement induced by these attached moieties increases in an order of compound **1** ≈ **4** < **2** < **5** ≈ **6** < **3** in MALDI mass spectrometry. Peptide-compound **3** adduct was approximately 20 times signal enhancement of the unmodified peptide and of 9 times of the peptide-compound **1** adduct. This result demonstrates that the rational designed organic molecules are capable of providing a sensitive tool in the detection of low-abundance proteins in proteomics.

Keywords: MALDI; Dihydroimidazole; Lysine-specific; Guanidinium; Signal enhancement; Mass spectrometry.

The enhancement of detection sensitivity and quantification of low-abundance proteins are of great importance in proteomic analysis.[1-3] Peptide mapping experiments usually employ mass spectrometry;[4-6] for example, peptide mass fingerprinting with MALDI-TOF or ESI-MS/MS tandem mass spectrometry, in combination with other separation technologies such as CE and HPLC, is routinely performed. Although many sophisticated methods, such as 2D gel electrophoresis and multi-dimensional protein identification technology (MudPIT), attempt to resolve the thousands of proteins of living organisms, only some hundreds of proteins can be identified because of large disparities in the concentrations of expressed proteins. Low-abundance proteins are sometimes important biomarkers in disease diagnosis, as well as in the monitoring of disease progression and drug responsiveness; e.g., prostate-specific antigen (PSA), a marker of prostate cancer.[7] Therefore, the detection and analysis of low-abundance proteins is a challenging but vital topic in proteomics.[8,9]

Aside from techniques for the enrichment of target proteins and effective cell-fractionation steps prior to such enrichment, low-abundance proteins may be more readily detected after attachment of a charged species. For instance, an arginine-containing peptide, attached to peptide fragments obtained by trypsin digestion, was found to increase detection sensitivity of MALDI-TOF mass spectrometry by 4–8-folds.[10-14] Recently, significant enhancements in signal sensitivities were achieved using a cyclic guanidinium group as a charge carrier (Fig. 1). In addition, the isotopes were incorporated into the framework of the

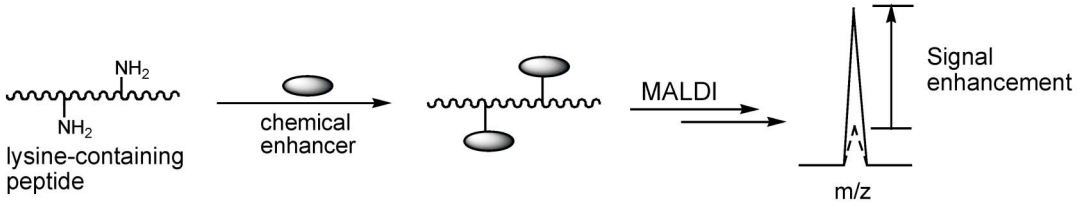

Fig. 1. General concept of signal enhancement in proteomic analysis.

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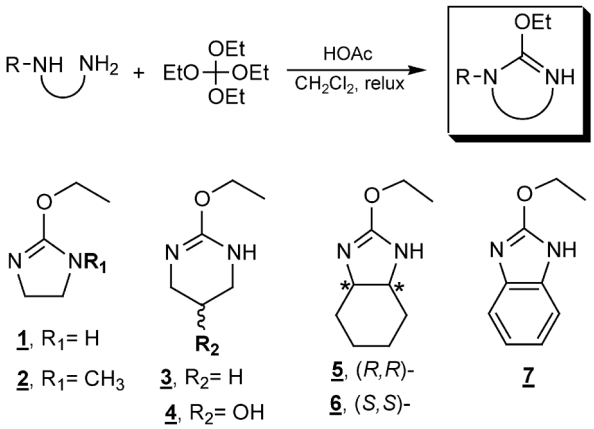
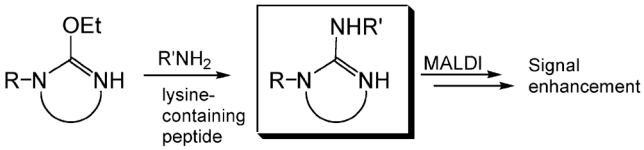
cyclic guanidinium group to act as internal standards for protein quantification. The preparation of 2-methoxy-4,5-dihydro-1*H*-imidazole (MeO-DHIM) was accomplished via the formation of *O*-methyl-isourea.[14-18] The modified guanidinium species coupled to lysine-containing residues in tryptic peptides of horse myoglobin; however, no further details on chemical structures, nor signal enhancements of peptide fragments after coupling, were reported.

Here, we report a one-step ring-closing synthesis for the preparation of a variety of 2-ethoxy-4,5-dihydro-1*H*-imidazoles (EtO-DHIMs), all with functional groups, in reasonable yields. Ethoxy-dihydroimidazole derivatives are capable of substituting a suitable amino group to form a cyclic guanidinium group. Moreover, we show that signal enhancements of tryptic digestion peptides are related to the chemical properties of the enhancers, including basicity and the potential for stabilization of the protonated species. Therefore, a functionalized guanidinium group, used as a conjugated marker, may increase the number of low-abundance proteins that can be detected in and characterized by proteomic techniques.

A typical procedure for the one-step synthesis of EtO-DHIM derivatives was followed. This involved condensation of three equivalents of tetraethyl orthocarbonate[19] and one equivalent of diamine in CH_2Cl_2 in the presence of glacial acetic acid, refluxing at 40 °C for 5 h. The cyclization was quenched with water, followed by extraction with CH_2Cl_2, drying with $MgSO_4$, and evaporation of solvent to obtain desired compounds in reasonable yields (60-85%), as shown in Scheme I. In MALDI mass spectrometry, analyzes are believed to receive a proton from the chemical matrix such as αCHCA to become a positive-charged molecule. Therefore, signal enhancement of a pep-

Scheme I One-step synthesis of 2-ethyl-1,3-dialkylisourea derivatives with various functional groups

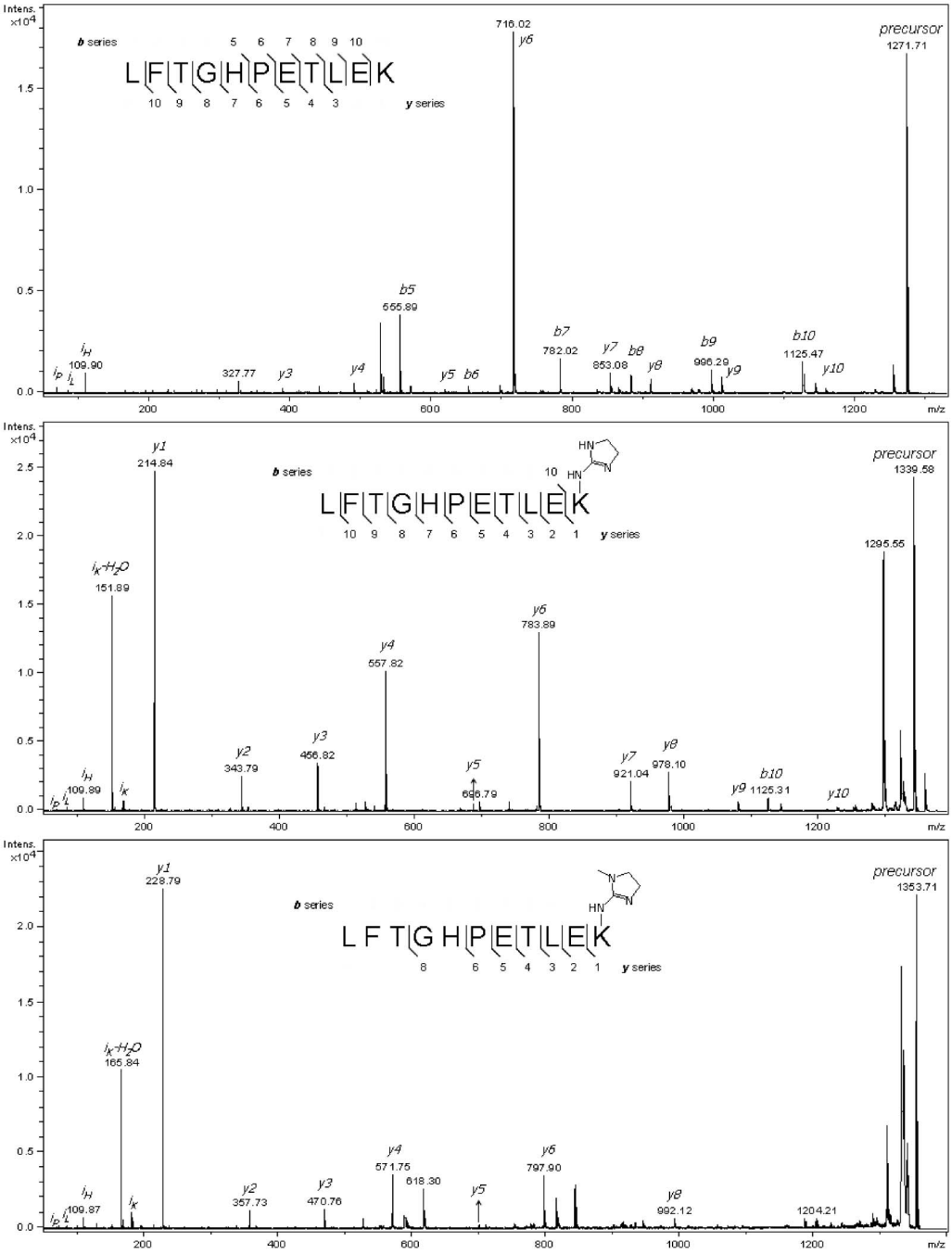




Peptide sequence	Attaching chemical enhancer	Therotical *m/z* (monoisotopic)	Observed *m/z* (monoisotopic)	Error (ppm)
LFTGHPETLEK	-	1271.662996	1271.6562	5.3
LFTGHPETLEK-1	**1**	1339.700444	1339.6938	5
LFTGHPETLEK-2	**2**	1353.716094	1353.7109	3.8
LFTGHPETLEK-3	**3**	1353.716094	1353.7063	7.2
LFTGHPETLEK-4	**4**	1369.711009	1369.7079	2.3
LFTGHPETLEK-5	**5**	1393.747394	1393.7442	2.3
LFTGHPETLEK-6	**6**	1393.747394	1393.7414	4.3

Fig. 2. Observed mass of peptide-DHIM adducts, by MALDI-TOF mass spectrometry, to confirm the formation of cyclic guanidinium moieties. The model peptide with *m/z* 1271.66 was isolated from a tryptic digest of horse myoglobin.

tide fragment in mass spectrometry can be achieved if the analyte were to carry a stable charge or if the analyte showed a high basicity favoring protonation.[20] To this end, methylation of the amino group of Compound **2** was carried out to increase the basicity of DHIM. In addition, six-member-ring analogs, Compounds **3** and **4**, were prepared to offer improved resonance forms after protonation. Compounds **5** and **6** were synthesized to increase hydrophobic characteristics and to introduce chirality, to permit us to explore the influence of chirality on reactivities with peptide fragments. The more hydrophobic Compound **7**[18] was easily prepared by a ring-closing reaction, but had extremely low solubility in water and low reactivity in coupling with a peptide fragment. A typical structural analysis of DHIM derivatives revealed that the ethoxy group appeared at ca. 4.1 ppm as a quartet-splitting peak and at ca. 1.5 ppm as a



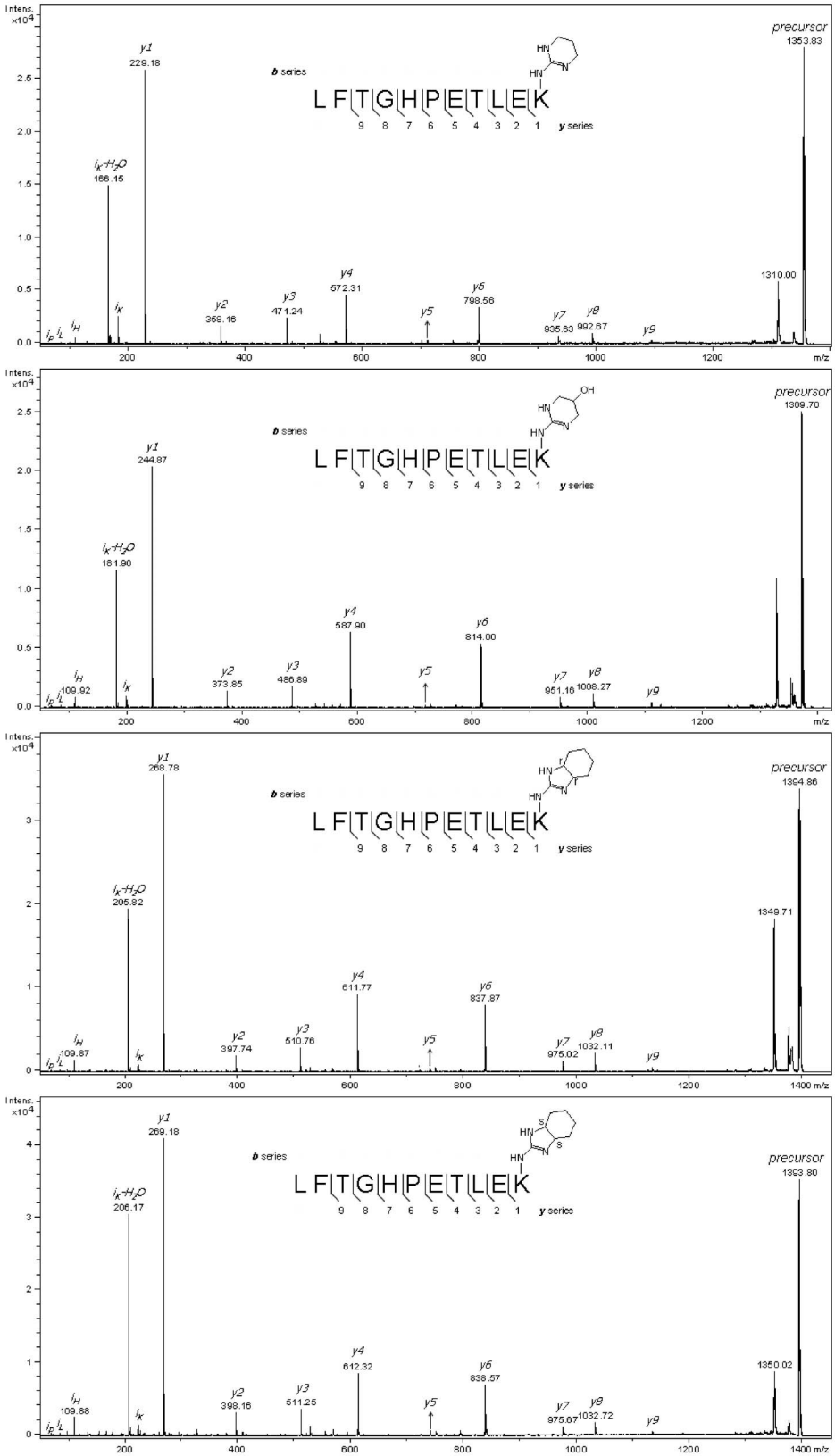


Fig. 3. Fragment analysis of peptide-DHIM adducts in MALDI-TOF/TOF mass spectrometry, to confirm the formation of *y*-ions and lysine-specific attachment.

triplet-splitting peak in NMR spectra.

To explore possible signal enhancements by these compounds in MALDI-TOF mass spectroscopy, a model peptide with 11 amino acid residues (LFTGHPETLEK), purified from a horse myoglobin tryptic digest,[6] was allowed to react with different DHIM derivatives as shown in Fig. 2. Because trypsin specifically[23] cleaves at the C-termini of lysine and araginine, the model peptide with a lysine at one end shows up as a protonated molecular ion, $[M+H]^+$, with a mass-charge ratio (*m/z)* of 1271.66, as a reference peak in MALDI-MS experiments.[12] This model peptide has been well characterized by proteomic analysis after reaction of Compound **1** with trypsin-digested peptides of myoglobin.[11] A peptide-DHIM adduct was prepared by reacting an EtO-DHIM derivative in an aqueous 2.5 M NH_4OH in 50% *v/v* MeOH solution at 40 °C for 16 h, and then purified on a C18 reverse-phase HPLC column to give a yield of 5-77%. The target peptide was chemically modified at the side-chain amino group of lysine using a series of *y*-ions, as confirmed by MALDI-TOF/TOF[21] (see Fig. 3). Compound **1**[11] was found to attach to both the lysine residue and the N-terminal amino group of the peptide fragment; however, Compounds **2-5** reacted with the peptide fragment to yield only lysine-specific adducts without modification of the N-terminus. From MALDI tandem spectra (see Fig. 3), we observed fragment ions form dominant *y* ion series through a C-terminal lysine derivatization. Through the chemical modification of lysine residue, it seems to change ion fragmentation pattern in MALDI-TOF/TOF experiments. A synthesis peptide ($_{NH2}$YKELGFQG$_{amide}$) exhibited the same proclivity for near exclusive formation of *a* ions and especial y^7 and b^2-NH_3 ions while modified lysine residue was near c-terminal of peptide (data not shown). It's found all observed fragment ions contain modified lysine residue. This evidence supports all modified lysine moiety were strong proton carrier, indirectly explained MALDI signal enhancement through proton affinity increased. It also simplified tandem mass spectra, make peptide *denovo* sequencing more easily.

The above results suggest that the introduction of alkyl groups increases steric effects and results in regio-selectivity of the coupling reaction. The chiral sense change between Compounds **5** and **6** did not influence the yields or reactivities of peptide-DHIM adducts. The observed masses of the adducts are shown in Fig. 2. The retention times of the peptide-DHIM adducts of Compounds **1-5**, monitored at 214 nm in HPLC, were 12.02, 12.22, 12.89, 11.39, and 14.36 min (see Fig. 4). It is interesting to note that an attached small (68-122 Da) chemical moiety is capable of dominating the polarity of a large (1270 Da) peptide.

A comparison of signal enhancements by different chemicals requires that the amounts of protein-adducts

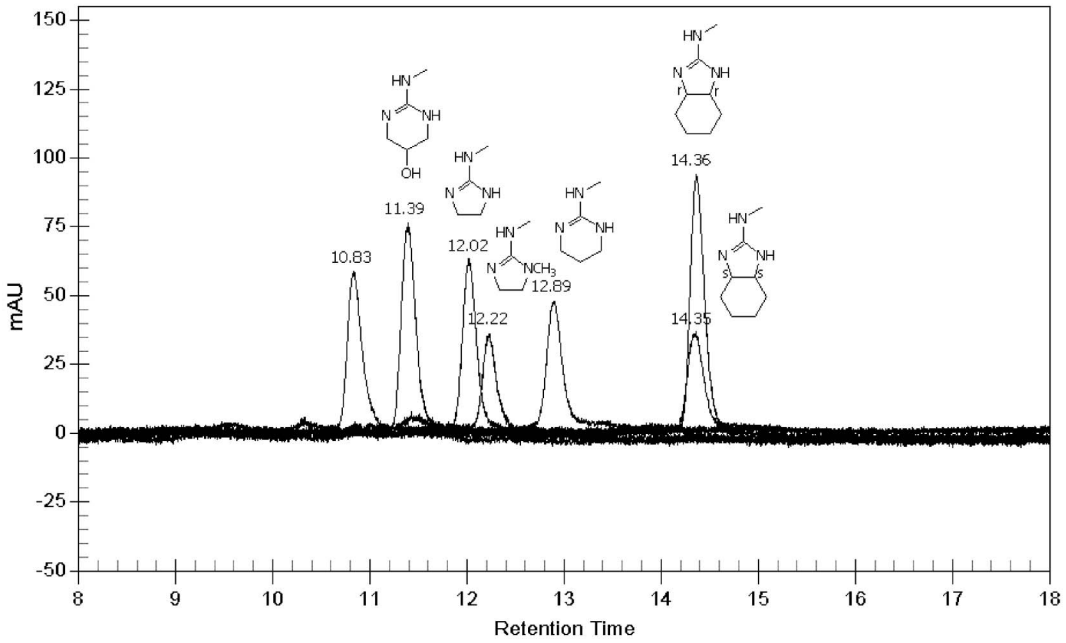


Fig. 4. Reversed-phase HPLC profile of model peptide tethered to different cyclic guanidino moieties.

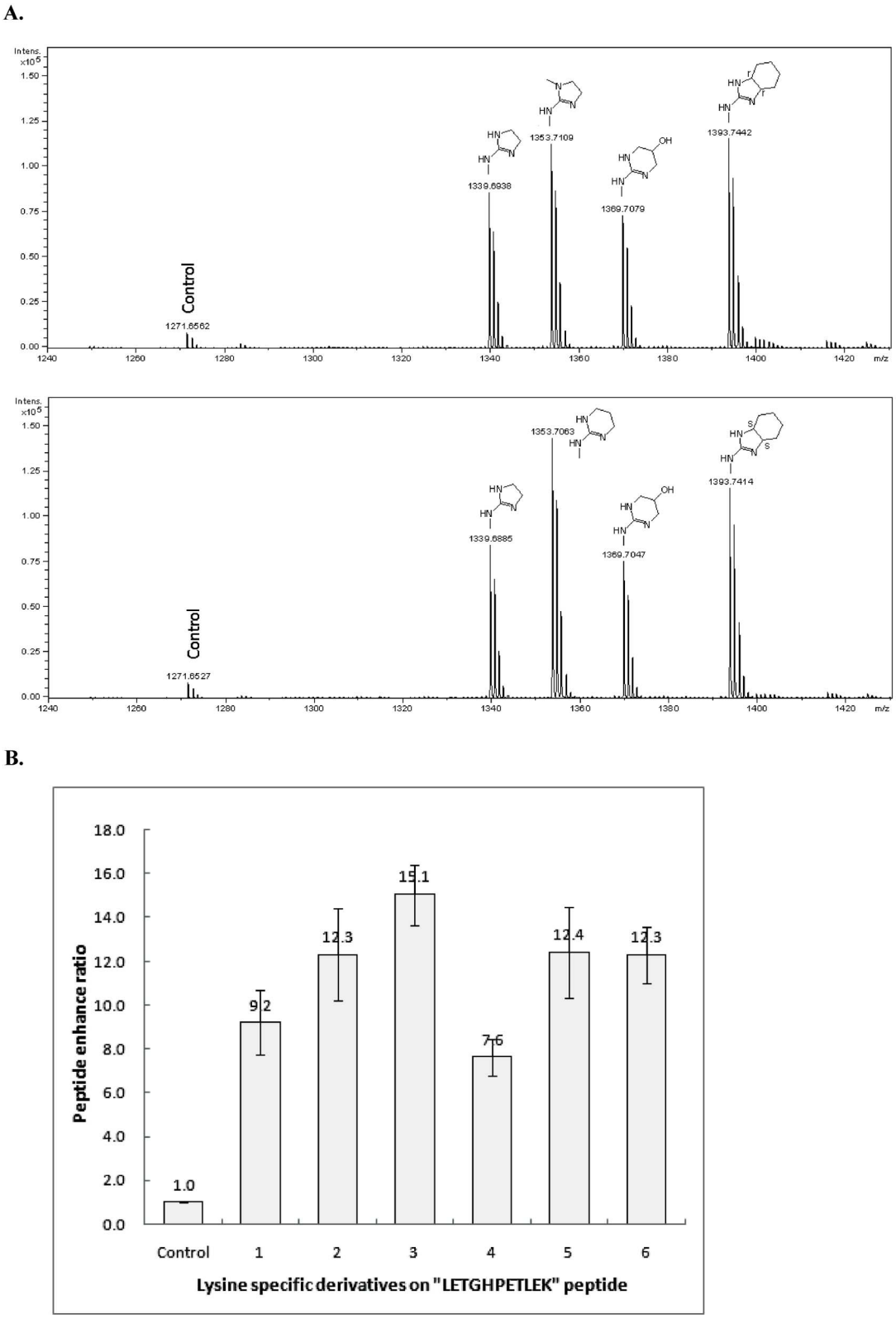


Fig. 5. (A) Relative signal intensities of model peptide tethered to different cyclic guanidinium moieties in MALDI-TOF mass spectrometry. (B) Relative signal intensities in a bar-graph format. These experiments were repeated at least three times.

must be normalized. Because yields after lysine condensation varied, peptide-DHIM adducts were further isolated and purified by HPLC on a reverse-phase C18 column. To quantify the concentration of each isolated adduct, capillary LC with a fixed absorption wavelength at 214 nm was used to assay each sample. Peptide-DHIM adducts were thoroughly mixed at similar concentrations and then subjected, as a mixture, to MALDI-TOF analysis. The procedure was repeated at least three times. Signal enhancements induced by the attached moieties increased in the order of Compound **4** < **1** < **2** ≈ **5** ≈ **6** < **3**, as shown in Fig. 5. The more hydrophobic peptide-DHIM adducts, as indicated by polarity data from HPLC, displayed better signal enhancements. The opposing chiralities of Compounds **5** and **6**[22] did not significantly influence yields from coupling reactions or signal enhancements. The simplicity with which charged species may be formed is of fundamental importance in attempts to increase signal intensities in MALDI-TOF analysis. The proton source is usually supplied by the matrix, such as α-cyano-4-hydroxycinnamic acid (αCHCA) in MALDI experiments. The methylated Compound **2** was synthesized to provide an electron-donating group favoring protonation. As a result, Compound **2**, containing a cyclic guanidinium group, showed better signal enhancement (by 1.5-fold) than did Compound **1**. Thus, increasing basicity favors protonation resulting in signal enhancement.

The signal enhancement of the peptide-Compound **3** adduct was approximately 15.1 times that of the unmodified peptide and 1.6 times that of the peptide-Compound **1** adduct (Fig. 5). The significant enhancement afforded by Compound **3** compared with Compound **1** is ascribed to lower ring strain and better resonance after protonation, to stabilize the positive charge. This enhancement may allow more proteins of low abundance to be detected in peptide mapping experiments. Compound **4** was observed to enhance signals to a level comparable to that seen with use of Compound **1**. The hydroxyl group in the six-membered ring increases the polarity of a peptide-DHIM adduct, as demonstrated by the earlier retention time from HPLC; however, a hydroxyl group is known to interfere with protonation in mass spectrometry, resulting in suppression of signal intensity. This result suggests that signal suppression by a hydroxyl group can be overcome by suitable structural modification. Therefore, modification of the hydroxyl group of Compound **4** may well provide a variety of functional analogs that do not cause signal suppression. For example, O-alkylation is a possible strategy for preparation of a linker to be immobilized on a solid support, where tethering could be monitored using a fluorophore. Such work is ongoing in our laboratory.

In conclusion, signal responses in MALDI mass spectrometry are intrinsic properties of fragment peptides. Low-abundance protein characterization can be peptide-dependent; however, chemical conjugation of a peptide provides a strategy whereby a peptide fragment can gain a charge, permitting mass spectrometry signals to be amplified. Such modifications of peptides may eliminate tedious sample accumulation processes, conventionally required before analysis of low-abundance proteins could commence. In this paper, a simple synthetic route for the preparation of functionalized cyclic diamine compounds is presented. Furthermore, guanidinium species generated by condensation of our compounds with lysine residues of a peptide fragment can enhance signal intensities in MALDI-TOF tests. Modifications of peptides, to increase basicity or to stabilize desirable resonance forms, are important to improve signal intensities in MALDI mass spectrometry experiments. The synthetic method we describe is a simple route for the introduction of other functional groups[24] such as ICAT[25] and ITRAQ,[26] for increasingly sensitive quantification of peptide fragments.[4,5,27]

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