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Enhancing Electrospray Ionization Efficiency of Peptides by Derivatization

Hamid Mirzaei and Fred Regnier*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

With the advent of electrospray ionization mass spectrometry, the world was given a new way to look at complex peptide mixtures. Identification of proteins via their signature peptides requires ionization of a representative portion of the peptides derived from proteins by proteolysis. Unfortunately, matrix effects prohibited electrospray ionization of many peptides. This paper describes the development of a new labeling reagent that simultaneously adds a permanent positive charge to peptides and increases their hydrophobicity to enhance their ionization efficiency. The labeling agent is preactivated with *N*-hydroxysuccinimide to react with primary amines to form a peptide bond. In the most dramatic case, ionization efficiency of the peptide ADRDQYELLCLDNTRKPVDEYK increased 500-fold after derivatization as opposed to other peptides where ionization efficiency was impacted little. Ionization efficiency of peptides was enhanced roughly 10-fold in general by derivatization. Peptides of less than 500 Da experienced the greatest increase in ionization efficiency by derivatization. Poor ionization efficiency of native peptides was found to be due more to their inherent structural properties than the matrix in which ionization occurs.

Protein identification in proteomics is achieved in several ways. One is to tryptic digest a proteome and after several dimensions of chromatographic fractionation peptide cleavage fragments are identified by electrospray ionization mass spectrometry (ESI-MS).[1,2] Because a tryptic digest of even a simple proteome can contain several hundred thousand peptides, chromatographic fractions being introduced into an ESI-MS can contain hundreds to thousands of components.[3,4] This causes a problem in ESI-MS. As the complexity of samples being introduced into the instrument increases, many peptides fail to ionize because of a phenomenon known as matrix suppression of ionization.[5]

Passage of peptides from droplets formed during the electrospray process into the gas phase is the result of peptide desolvation.[6] Peptide hydrophobicity seems to play a role in desolvation, probably because hydrophobicity dictates the rate at which peptides migrate to the surface of droplets.[7−13] But for these gas-phase species to be detected they need to acquire charge. Obviously basic peptides are more likely to absorb a proton and ionize. Attaching a quaternary amine to peptides enhances ionization by providing a permanent positive charge. It has been noted that when hydrophobicity and good gas-phase proton affinity are combined in hydrophobic, cationic peptides they ionize more readily along with suppressing the ionization of other peptides.[14,15] It is interesting that addition of tetramethylammonim bromide to a solution of peptides being electrosprayed suppresses the ionization of all peptides.[16] This means that surfactantlike species can saturate the droplet surface and push peptides toward the interior of droplets. It also explains how hydrophobic peptides derived from more abundant proteins suppress ionization. They diminish or eliminate droplet surface area for other peptides to be protonated and pass into the gas phase.

The hypothesis being tested in this paper is that by tagging peptides with a derivatizing agent that contains both a quaternary amine and an alkyl group it will be possible to increase peptide migration to the surface of droplets and concomitantly electrospray ionization efficiency. The derivatization process used in these studies was similar to that used in stable isotope coding for relative quantification.[17]

EXPERIMENTAL SECTION

Materials. Synthetic peptides Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH, H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH, H-Ala-Phe-Pro-Leu-Glu-Phe-OH, H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH, H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-OH, H-Tyr-Gly-Gly-Phe-Met-Lys-OH, H-Pro-His-Pro-Phe-His-Phe-His-

* Corresponding author. E-mail: fregnier@purdue.edu.

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Phe-Phe-Val-Tyr-Lys-OH, H-Ala-Leu-Gly-OH, H-Ala-Gly-Gly-OH, H-Ala-Met-OH, and H-Ala-Ser-OH were purchased from Bachem Biosience Inc. (King of Prussia, PA). Trifluoroacetic acid (TFA) was purchased from Pierce Co. (Rockford, IL). Dithiothreitol (DTT), trypsin, *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), apotransferrin (human), 4-iodobutyric acid, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), 4-aminobutanoic acid, potassium bicarbonate, methyl iodide, chloroform, and *N*,*N*-dimethyl-*N*-octylamine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile (ACN) and urea were purchased from Mallinckrodt. (St. Louis, MO). 218TP54 reversed-phase C_{18} columns were purchased from Vydac (W. R. Grace & Co.-Conn. Columbia, MD). DE44H10426 Zorbax reversed-phase C_{18} column (0.5 × 150 mm) and DE45C00085 Zorbax reversed-phase C_8 column (0.3 × 150 mm) were purchased from Agilent Technologies, Inc. (Palo Alto, CA). Reversed-phase chromatography (RPC) analyses were done on a BioCAD 20 Microanalytical Workstation (PE Biosystems, Framingham, MA). The LC system used in conjunction with the mass spectrometer was an Agilent 1100 series instrument. LC/MS mass spectral analyses were done using a Sciex QSTAR hybrid LC/MS/MS Quadrupole TOF mass spectrometer (Applied Biosystems, Foster City, CA). All spectra were obtained in the positive ion mode.

Methods. Synthesis of 4-Iodo(2,5-dioxopyrrolidin-1-yl) Butyrate. This reagent was synthesized according to the procedure of Taran et al.[18] 4-Iodobutyric acid (1 g, 467 mmol), NHS (0.54 mg, 4.69 mmol), and DCC (0.96 g, 4.66 mmol) were dissolved in 10 mL of AcOEt. The reaction mixture was stirred at room temperature for 16 h. The dicyclohexyl urea formed was discarded by filtration over Celite, and the crude material was concentrated and chromatographed on silica gel (petroleum ether/AcOEt, 1/1). The resultant yellow powder was recrystalized from petrolumn ether/EtOH(9/1) yielding activated ester 4-iodo-(2,5-dioxopyrrolidin-1-yl) butyrate.

Synthesis of [3-(2,5)-Dioxopyrrolidin-1-yloxycarbonyl)-propyl]dimethyloctylammonium (C_8-QAT). This reagent was synthesized according to the procedure by Taran et al. with slight modifications.[18] *N*,*N*-Dimethyl-*N*-octylamine (280 mg, 1.8 mmol), activated ester 4-iodo[2,5-dioxopyrrolidin-1-yl] butyrate (550 mg, 1.8 mmol), and silver trifluoromethanesulfonate (460 mg, 1.8 mmol) were dissolved in 2 mL of anhydrous acetone. The solution was stirred for 16 h at room temperature. The silver iodide formed was discarded by filtration over Celite, and the solvent was evaporated. The resultant orange oil was dissolved in 1 mL of ACN, and the final product was precipitated by addition of 10 mL of EtOAc.

Synthesis of (3-Carboxypropyl)trimethylammonium Chloride. $(CH_3)N^+(Cl)CH_2CH_2CH_2(C{=}O)OH$ was prepared according to the method of Sioma et al.[15] A 10.0-g (0.097 mol) aliquot of 4-aminobutanopic acid, 48.0 g (0.497 mol) of $KHCO_3$, and 30 mL (0.482 mol) of CH_3I were stirred in 1 L of MeOH for 24 h. The MeOH was removed under vacuum and the crude residue slurried with 200 mL of $CHCl_3$, then filtered, and acidified with 50 mL of concentrated HCl. The H_2O was removed under vacuum and the remaining residue extracted with 400 × 2 mL of anhydrous acetone, concentrated under reduced pressure, slurried with 200 mL of THF, and filtered to yield the final product.

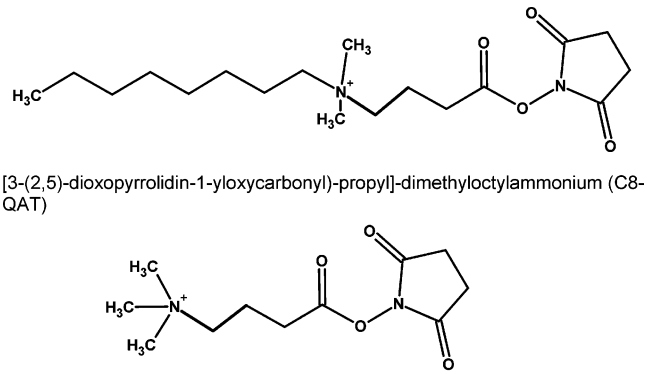

Figure 1. Structures of the QAT and C_8-QAT reagents. The C_8-QAT reagent is more hydrophobic than QAT and will convey greater hydrophobicity to labeled peptides.

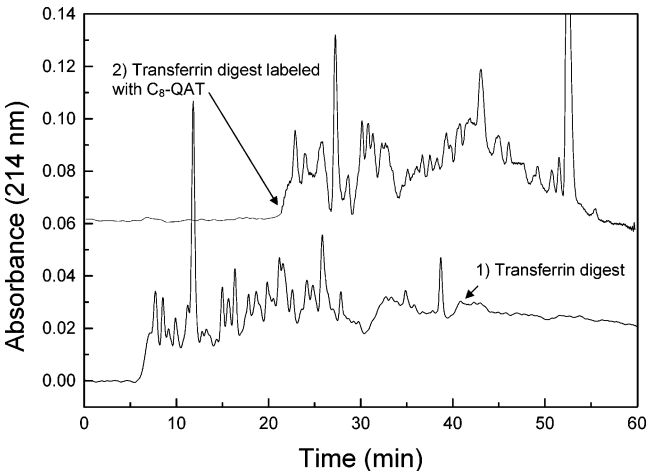

Figure 2. Reversed-phase chromatograms for (1) unlabeled and (2) C_8-QAT-labeled transferrin digest separated on a C_{18} column. The digests were separated on a Vydac C_{18} column using a 60-min gradient from 99.5% buffer A (0.01% TFA in dI H_2O) to 60% buffer B (95% ACN/0.01% TFA in dI H_2O). There is a 15-min delay in retention time caused by increased hydrophobicity of the labeled peptides. The labeled digest also shows a higher level of complexity and longer gradient elution due to retention of very hydrophilic peptides that did not retain before labeling. The labeling reagent peaks were removed by subtraction of labeling reagent only chromatogram from the labeled digest's.

Synthesis of [3-(2,5)-dioxopyrrolidin-1-yloxycarbonyl)-propyl]trimethyllammonium (QAT). This reagent was prepared according to the method of Sioma et al.[15] A 13.2-g (0.073 mol) aliquot of (3-carboxypropyl)trimethylammonium chloride, 8.4 g(0.073 mol) of NHS, and 16.5 g (0.080 mol) of DCC were dissolved in ACN. Once dissolved, the resulting solution was refrigerated for 24 h. The formed 1,3 dicyclohexylurea was filtered off, and the ACN was evaporated under vacuum. The crude residue was slurried with 200 mL of THF, filtered, and recrystallized from ACN/THF to yield the final product.

Derivatization of Model Peptides with QAT and C_8-QAT Reagent. Model peptides were dissolved in 50 mM Hepes, pH 8.00, at a final concentration of 1 mg/mL. A 50-fold molar excess of each derivatization reagent was added individually to the peptide

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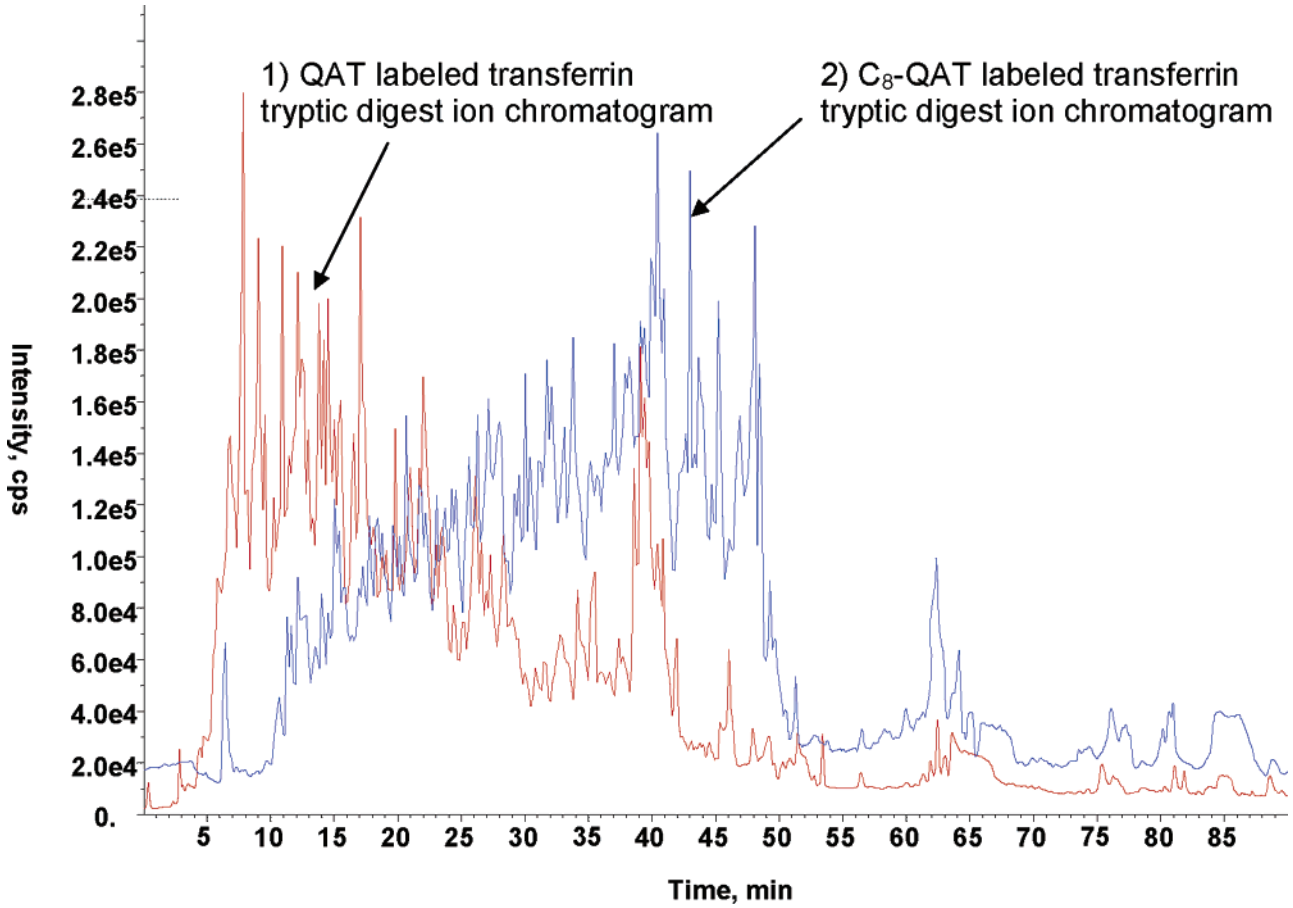

Figure 3. Ion chromatograms for (1) QAT-labeled and (2) C_8-QAT-labeled transferrin digest separated on a C_8 reversed-phase column. Separation of transferrin digests on a less hydrophobic reversed-phase column such as C_8 allows the hydrophobic effect to become more dominant. The retention time delay as a result of C_8-QAT labeling is reduced to 5 min and elution of C_8-QAT-labeled peptides at lower organic percentage enhances the hydrophobic effect. Some QAT-labeled peptides (5%) do not retain on C_8 column, but overall there is 1.25-fold increase in ionization efficiency. The C_8-QAT-labeled peptides are also distributed more evenly across the chromatogram. Decrease in local concentration of peptides in droplets also decreases the ionization suppression.

solutions, and the reaction was allowed to proceed for 2 h at room temperature. After the reaction was finished, *N*-hydroxylamine was added in excess, and the pH was adjusted to 11−12 with sodium hydroxide to hydrolyze esters. The reaction was allowed to proceed for 10 min, and then the pH was adjusted back to 7−8 with 1−2 drops of glacial acetic acid.

Proteolysis. To denature, reduce, and alkylate transferrin, urea and DTT were added to a final concentration of 6 M and 10 mM, respectively. Mixtures were incubated for 1 h at 37 °C, iodoacetamide was then added to a final concentration of 20 mM, and the reaction allowed to proceed for an additional 30 min at 4 °C. Cysteine was then added to a final concentration of 10 mM to quench extra iodoacetamide. Samples were diluted 6-fold with 50 mM HEPES, pH 8.0, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Sequence grade trypsin was added (2%), and the reaction mixture was incubated at 37 °C for at least 8 h. Proteolysis was stopped by adding TLCK (trypsin/TLCK ratio of 1:1 (w/w)).

Derivatization of Model Peptides with QAT and C_8-QAT Reagent. A 50-fold molar excess of each derivatization reagent was added to the tryptic digest peptide, and the reaction was allowed to proceed for 2 h at room temperature. After the reaction was finished, *N*-hydroxylamine was added in excess and the pH was adjusted to 11−12 with sodium hydroxide to hydrolyze esters. The reaction was allowed to proceed for 10 min, and then the pH was adjusted back to 7−8 with 1−2 drops of glacial acetic acid.

LC/MS Analysis. Derivatized and nondrivatized model peptides as well as transferrin digest were separated on an Agilent Zorbax C_8 (0.3 × 150) and C_{18} column (0.5 × 150 mm) using an Agilent 1100 series instrument (Agilent Technologies, Inc.) at 4 μL/min. Solvent A was 0.01% TFA in deionized H_2O (dI H_2O), and solvent B was 95% ACN/0.01% TFA in dI H_2O. The flow from the column was directed to the Q-STAR workstation (Applied Biosystems, Framingham, MA) equipped with an ESI source. Flow from the HPLC was diverted to waste for 10 min after sample injection at 100% solvent A to remove salts, remaining derivatizing reagent APTA, and weakly adsorbed peptides. The Q-STAR was then reconnected and peptides were separated in a 15-min linear gradient (from 0 to 60% B). MS spectra were obtained in the positive ion mode at a sampling rate of one spectrum/s.

LC Separation of Tryptic Peptides. Peptides from the transferrin tryptic digest were separated on a Vydac C_{18} column (4.6 × 250 mm) using an BioCAD 20 Micro-Analytical Workstation (Applied Biosystems, Framingham, MA) at 1 mL/min. Solvent A was 0.1% TFA in dI H_2O and solvent B was 95% ACN/0.1% TFA in dI H_2O. Peptides were separated in a 60-min linear gradient (from 0 to 60% B).

Table 1. List of Model Peptides Used To Study the Effect of QAT and C_8-QAT Labeling on Ionization Efficiency[a]

	peptide	native	area	QAT	area	C_8-QAT	area	R_i based ratio native:QAT:C_8-QAT
1	Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH (HP: −24.5)	$(715.89)^{2+}$	0	$(844.50)^{2+}$	203	$(942.11)^{2+}$	0	1:12:20
		$(477.60)^{3+}$	260	$(563.34)^{3+}$	1364	$(628.41)^{3+}$	2519	
		$(358.95)^{4+}$	0	$(422.75)^{4+}$	1663	$(471.56)^{4+}$	2471	
2	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH (HP: −1.3)	$(1296.69)^{1+}$	2362	$(1423.78)^{1+}$	25	$(1521.89)^{1+}$	24	1:0.82:0.21
		$(648.89)^{2+}$	30491	$(712.39)^{2+}$	18204	$(761.45)^{2+}$	14058	
		$(432.89)^{3+}$	25886	$(475.26)^{3+}$	30599	$(507.49)^{3+}$	5175	
3	H-Ala-Phe-Pro-Leu-Glu-Phe-OH (HP: 6.1)	$(723.37)^{1+}$	26652	$(850.47)^{1+}$	23236	$(948.58)^{1+}$	11548	1:1:0.56
		$(362.19)^{2+}$	724	$(425.73)^{2+}$	3190	$(474.79)^{2+}$	3372	
		$(241.79)^{3+}$	0	$(284.16)^{3+}$	0	$(316.86)^{3+}$	296	
4	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH (HP: −10.7)	$(1299.55)^{1+}$	1470	$(1426.65)^{1+}$	208	$(1524.76)^{1+}$	111	1:1.91:0.83
		$(650.28)^{2+}$	21985	$(713.83)^{2+}$	15123	$(762.88)^{2+}$	11110	
		$(433.85)^{3+}$	6631	$(476.22)^{3+}$	42773	$(508.59)^{3+}$	14162	
5	H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-OH (HP: −5.1)	$(967.43)^{1+}$	5797	$(1094.53)^{1+}$	686	$(1192.64)^{1+}$	50	1:0.14:0.34
		$(484.21)^{2+}$	4160	$(554.27)^{2+}$	893	$(596.82)^{2+}$	3691	
		$(323.14)^{3+}$	0	$(369.84)^{3+}$	0	$(398.22)^{3+}$	0	
6	H-Tyr-Gly-Gly-Phe-Met-Lys-OH (HP: −1.3)	$(701.32)^{1+}$	0	$(957.53)^{1+}$	339	$(1153.75)^{1+}$	28938	1:6.67:33.3
		$(351.16)^{2+}$	1656	$(479.27)^{2+}$	9766	$(576.87)^{2+}$	28603	
		$(234.78)^{3+}$	0	$(319.51)^{3+}$	0	$(384.92)^{3+}$	0	
7	H-Pro-His-Pro-Phe-His-Phe-His-Phe-Phe-Val-Tyr-Lys-OH (HP: −2.6)	$(1318.67)^{1+}$	771	$(1445.77)^{1+}$	53	$(1543.88)^{1+}$	46	1:2:1
		$(659.84)^{2+}$	9579	$(723.39)^{2+}$	4056	$(772.44)^{2+}$	5569	
		$(440.22)^{3+}$	12440	$(482.59)^{3+}$	6724	$(515.29)^{3+}$	17173	
8	H-Ala-Leu-Gly-OH (HP: 5.2)	$(259.15)^{1+}$	3082	$(387.26)^{1+}$	2687	$(485.37)^{1+}$	47781	1:1:16.67
		$(130.07)^{2+}$	0	$(194.14)^{2+}$	0	$(243.18)^{2+}$	0	
9	H-Ala-Gly-Gly-OH (HP: 1)	$(203.09)^{1+}$	0	$(331.19)^{1+}$	3170	$(429.31)^{1+}$	2781	1:16.67
		$(102.16)^{2+}$	0	$(166.10)^{2+}$	0	$(215.15)^{2+}$	10251	
10	H-Ala-Met-OH (HP: 3.7)	$(220.08)^{1+}$	355	$(348.19)^{1+}$	10989	$(446.30)^{1+}$	43094	1:31.25:125
		$(110.54)^{2+}$	0	$(174.60)^{2+}$	0	$(223.65^{2+}$	0	
11	H-Ala-Ser-OH (HP: 1)	$(176.07)^{1+}$	155	$(304.18)^{1+}$	2156	$(402.29)^{1+}$	2304	1:20:20
		$(130.07)^{2+}$	0	$(152.50)^{2+}$	0	$(201.65)^{2+}$	0	

[a] All peptides were labeled with QAT and C_8-QAT. Native, QAT-labeled and C_8-QAT-labeled forms of each peptide were mixed in 1:1:1 ratio and separated on a C_{18} column. The injection volume was 5 μL in all cases. The area under the peak for all charge states of each peptide were summed and used as a total ionization measure. The ratios were calculated by dividing the total peak areas for each peptide by the total peak area of the native peptide.

RESULTS

Derivatizing Agent Architecture. The strategy for enhancing electrospray ionization of peptides through derivatization was to use a derivatizing agent that would (1) have both quaternary amine and alkyl groups, (2) N-acylate primary amines, (3) be preactivated to form peptide bonds, and (4) acylate peptides in water. The reagent [3-(2,5)-dioxopyrrolidin-1-yloxycarbonyl)propyl]dimethyloctylammonium (Figure 1) was developed to meet these criteria. Peptides derivatized with this reagent will be designated as having a C8-quaternary amine tag (C_8-QAT). When activated with NHS, this tagging agent will be referred to as C_8-QAT-NHS. The synthesis of this derivatizing agent is very similar to that of [3-(2,5)-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethyllammonium (Figure 1) which has been previously described.[15] Peptides derivatized with this quaternary amine tag (QAT) have been shown to have improved ionization efficiency.[19]

Peptide Derivatization. The C_8-QAT-NHS labeling agent was added to peptide mixtures in at least a 50-fold molar excess and incubated at room temperature overnight. The reaction was terminated by addition of excess *N*-hydroxylamine and the pH adjusted to 11−12 with a few drops of saturated sodium hydroxide solution. Hydrolysis of C_8-QAT-NHS and any O-acylation products was achieved in 10 min after the addition of base. The pH was then adjusted back to 7−8 with 1−2 drops of glacial acetic acid.

(19) Sioma, C. S., 2003.

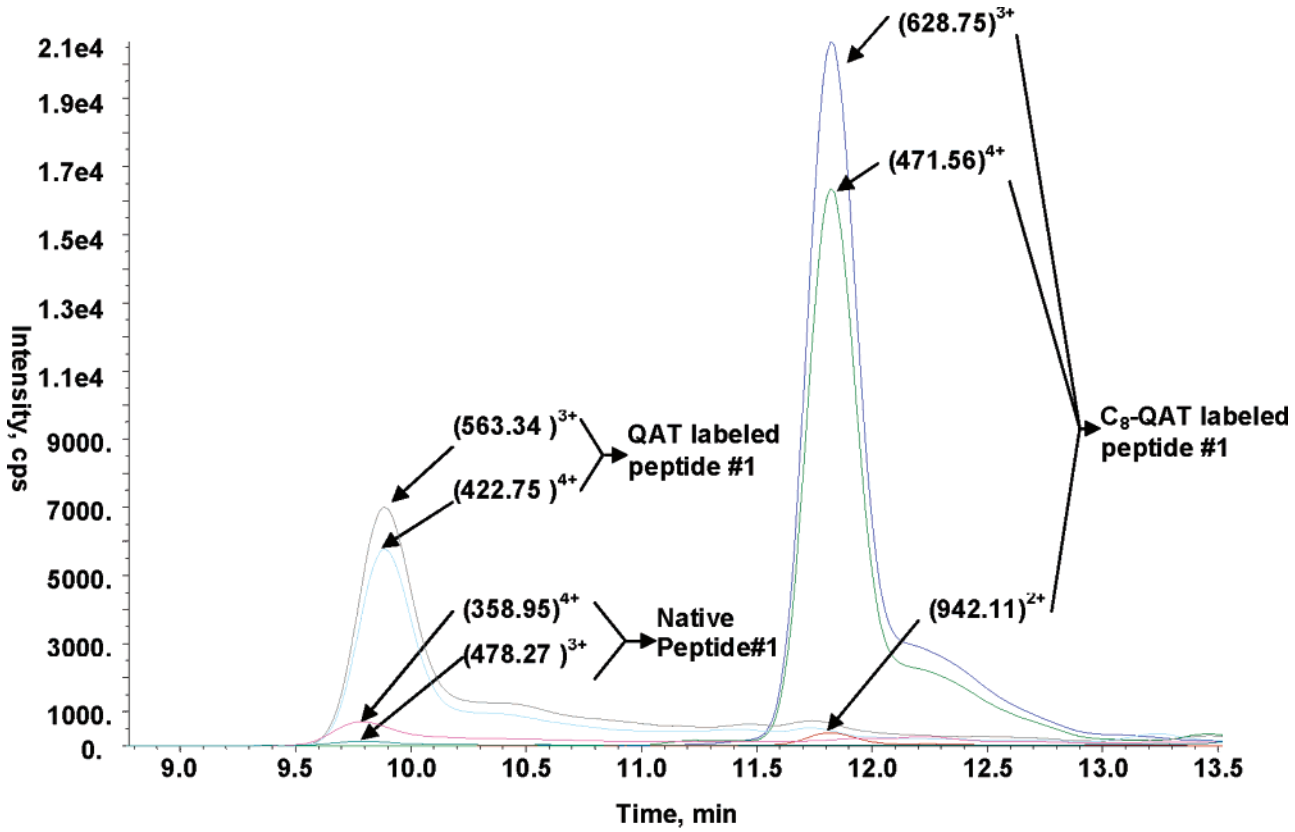

Figure 4. Extracted ion chromatograms for native, QAT-, and C8-QAT-labeled model peptide Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH. All the charge states for different forms of the peptide were extracted from the total ion chromatogram. The total ion chromatogram was obtained by separation of a mixture of native, QAT-, and C8-QAT-labeled peptide mixed in 1:1:1 ratio on a C_{18} column. The area under the peak for each charge state of each labeled peptides was calculated and then summed. The summation of peak areas of all charge states of a peptide form was used as a representative of total ionization for that peptide.

Reactivity of the C_8-QAT-NHS was found to be the same as that of the previously described QAT-NHS reagent (Figure 1).

Effect of C_8-QAT Labeling on Reversed-Phase Chromatographic Retention Time. The impact of C_8-QAT derivatization on peptide retention in RPC was studied using a transferrin tryptic digest. Native peptides and those tagged with C_8-QAT were prepared and fractionated in separate runs on a Vydac C_{18} column using a 60-min linear gradient starting from solvent A to 60% solvent B. Figure 2 shows overlaid chromatograms of the labeled and unlabeled tryptic peptide digests. C_8-QAT labeling induced roughly a 20-min increase in the retention time of peptides. The relative increase in retention of small hydrophilic peptides was greater than that of larger hydrophobic peptides.

Analyte retention in reversed-phase chromatography (RPC) is generally accepted to be due to a solvophobic effect driven by the surface tension of a polar mobile phase. According to solvophobic theory, polar solvents force hydrophobic molecules to interact with molecules that are similarly hydrophobic to minimize their contact area with the solvent.[20,21] Although peptides have a large number of polar groups, the side chains of many amino acids in peptides are hydrophobic. The solvophobicity of these hydrophobic groups in polar mobile phases drives peptides to interact with the solvophobic surface of RPC columns. But not all peptides are retained by RPC columns. Those that are not lack sufficient hydrophobicity to interact with the stationary phase. As observed, the hydrophobic C_8-QAT would be expected to increase the retention of a small hydrophilic peptide in RPC more than that of a large hydrophobic peptide that already interacts strongly with the stationary phase.

Although increasing the retention of hydrophilic peptides beyond the solvent front is desirable, a 20-min increase in retention is greater than needed. The QAT and C_8-QAT derivatized transferrin digests were also examined with an octylsilane (C_8 Zorbax) column (Figure 3). Comparing the chromatograms in Figures 2 and 3, it is seen that retention of peptides tagged with the C_8-QAT reagent is substantially shorter on the octyl (C_8) than the octadecyl (C_{18}) RPC column. The reduced hydrophobicity of the C_8 Zorbax column compensates for the increased hydrophobicity of peptides derivatized with the C_8-QAT reagent. Directing the nonretained peak from the C_8 column into a C_{18} column showed that 5% of the peptides captured by the C_{18} column were not retained on the C_8 column. Peptides not retained by the C_8 column are generally small hydrophilic peptides of minimal value in protein identification (data not shown).

Effect of C_8-QAT Labeling on Electrospray Ionization of Model Peptides. Tagging peptides with C_8-QAT alters them in two important ways that might impact electrospray ionization. One is the introduction of a quaternary amine, giving them a permanent positive charge. The second is to make them more hydrophobic. The relative contribution of quaternization was studied by derivatizing model peptides with the QAT reagent. As noted in Figure

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Table 2. List of Model Peptides Used to Study the Effect of QAT and C_8-QAT Labeling on Ionization Efficiency[a]

	peptide	native	area	QAT	area	C_8-QAT	area	R_i based ratio native:QAT:C_8-QAT
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		$(478.27)^{3+}$	591	$(563.34)^{3+}$	3798	$(628.41)^{3+}$	7621	
		$(358.95)^{4+}$	1587	$(422.75)^{4+}$	2189	$(471.56)^{4+}$	7154	
2	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH (HP: −1.3)	$(1296.69)^{1+}$	305	$(1423.78)^{1+}$	59	$(1521.89)^{1+}$	78	1:1:2
		$(648.89)^{2+}$	7259	$(712.39)^{2+}$	3845	$(761.45)^{2+}$	13672	
		$(432.89)^{3+}$	5865	$(475.26)^{3+}$	6388	$(507.49)^{3+}$	11320	
3	H-Ala-Phe-Pro-Leu-Glu-Phe-OH (HP: 6.1)	$(723.37)^{1+}$	32622	$(850.47)^{1+}$	29211	$(948.58)^{1+}$	19766	1:1:1
		$(362.19)^{2+}$	0	$(425.73)^{2+}$	3661	$(474.79)^{2+}$	13012	
		$(241.79)^{3+}$	559	$(284.16)^{3+}$	138	$(316.86)^{3+}$	1076	
4	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH (HP: −10.7)	$(1299.55)^{1+}$	43	$(1426.65)^{1+}$	20	$(1524.76)^{1+}$	229	1:1.46:7.69
		$(650.28)^{2+}$	2286	$(713.83)^{2+}$	2525	$(762.88)^{2+}$	22748	
		$(433.85)^{3+}$	2848	$(476.22)^{3+}$	4787	$(508.59)^{3+}$	15638	
5	H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-OH (HP: −5.1)	$(967.43)^{1+}$	2310	$(1094.53)^{1+}$	1121	$(1192.64)^{1+}$	928	1:0.2:2
		$(484.21)^{2+}$	3148	$(554.27)^{2+}$	0	$(596.82)^{2+}$	9955	
		$(323.14)^{3+}$	0	$(369.84)^{3+}$	0	$(398.22)^{3+}$	0	
6	H-Tyr-Gly-Gly-Phe-Met-Lys-OH (HP: −1.3)	$(701.32)^{1+}$	0	$(957.53)^{1+}$	668	$(1153.75)^{1+}$	576	1:6.8:20
		$(351.16)^{2+}$	1316	$(479.27)^{2+}$	8674	$(576.87)^{2+}$	26563	
		$(234.78)^{3+}$	0	$(319.51)^{3+}$	00	$(384.92)^{3+}$	0	
7	H-Pro-His-Pro-Phe-His-Phe-His-Phe-Phe-Val-Tyr-Lys-OH (HP: −2.6)	$(1318.67)^{1+}$	188	$(1445.)^{1+}$	129	$(1543.88)^{1+}$	0	1:0.33:6.67
		$(659.84)^{2+}$	3958	$(723.39)^{2+}$	921	$(772.44)^{2+}$	9056	
		$(440.22)^{3+}$	2437	$(482.59)^{3+}$	1112	$(515.29)^{3+}$	33390	
8	H-Ala-Leu-Gly-OH (HP: 5.2)	$(259.15)^{1+}$	129	$(387.26)^{1+}$	2002	$(485.37)^{1+}$	50151	1:13.3:333
		$(130.07)^{2+}$	0	$(194.14)^{2+}$	0	$(243.18)^{2+}$	0	
9	H-Ala-Gly-Gly-OH (HP: 1)	$(203.09)^{1+}$	0	$(331.19)^{1+}$	0	$(429.31)^{1+}$	6598	∞
		$(102.16)^{2+}$	0	$(166.10)^{2+}$	0	$(215.15)^{2+}$	0	
10	H-Ala-Met-OH (HP: 3.7)	$(220.08)^{1+}$	0	$(348.19)^{1+}$	0	$(446.30)^{1+}$	12507	∞
		$(110.54)^{2+}$	0	$(174.60)^{2+}$	0	$(223.65^{2+}$	0	
11	H-Ala-Ser-OH (HP: 1)	$(176.07)^{1+}$	0	$(304.18)^{1+}$	189	$(402.29)^{1+}$	3524	1:20
		$(89.04)^{2+}$	0	$(152.50)^{2+}$	0	$(201.65)^{2+}$	0	

[a] All peptides were labeled with QAT and C_8-QAT. Native, QAT-labeled, and C_8-QAT-labeled forms of each peptide were mixed in 1:1:1 ratio and separated on a C_{18} column. The injection volume was 20 μL in all cases (4 times the amount used in previous table). The area under the peak for all charge states of each peptide were summed and used as a total ionization measure. The ratios were calculated by dividing the total peak areas for each peptide by the total peak area of the native peptide (R_i). In higher concentration, all C_8-QAT-labeled peptides show higher ionization efficiency than other forms of the same peptide (native and QAT labeled).

1, the QAT and C_8-QAT reagents are identical in structure with the exception of the substitution of an octyl group for a methyl group on the quaternary amine. It has been previously reported that some peptides tagged with the (QAT) reagent experience a moderate increase in electrospray ionization efficiency.[19]

The relative ionization of native, QAT-labeled, and C_8-QAT-labeled peptides was studied using 11 model peptides varying in size, hydrophilic to hydrophobic amino acid ratio, and number of cationic amino acids (Table 1). Hydrophobicity (HP) of native peptides was calculated using relative hydrophobicity of their amino acids. All the model peptides were labeled separately with both the QAT and C_8-QAT reagents and mixed with native peptides in a 1:1:1 ratio (See Figure 4). The mixture (5-μL injection volume) was separated on a Zorbax C_{18} column using a 60-min gradient starting from 99.5% buffer A (0.01% TFA in dI H_2O) to 60% buffer B (95% ACN/0.01% TFA in dI H_2O). Relative ionization (R_i) of peptides was calculated using the formula

$$R_i = \sum A_i^{+n} / \sum A_{\text{native}}^{+n}$$

where $\sum A_{\text{native}}^{+n}$ is the sum of the area under the curves of all charge states of the native peptide and $\sum A_i^{+n}$ is the sum of the area under the curves for all the charge states of the peptide for which R_i is being calculated (peptide i). Since R_i is normalized against a native peptide, the value of R_i for the native peptide is always 1.

It is seen in Table 1 that for the peptide Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH that R_i for the C_8-QAT peptide is 20 while R_i for the QAT derivatized form of the peptide is 12.

Table 3. List of Native and C_8-QAT-Labeled Transferrin Peptides Identified by LC/MS/MS Analysis[a]

no.	native peptide	*m/z*	RT	labeled peptide	*m/z*	RT	R_i based ratio native: C_8-QAT
1	AD*RD*QYE*LLCLD NTRKPVDEYK	$(917.44)^{3+}$	13	A*DRD*QYE*LLCLDNT RK*PVDEYK*	$(852.48)^{4+}$	52	1:500
2	CD*E*WSVNSVGK	$(633.87)^{2+}$	40	*CD*E*WSVNSVGK*	$(860.47)^{2+}$	45	1:72
3	CLKD*GAGD*VAFVK	$(683.82)^{2+}$	18	*CLK*DGAGDVAFVK*	$(1001.56)^{2+}$	54	1:2
4	CQSFRDHM*K	$(584.27)^{2+}$	22	*CQSFRD*HM*K*	$(821.34)^{2+}$	44	1:8
5	CQSFRD*HMK	$(1173.96)^{2+}$	18	*CQSFRD*HMK*	$(813.46)^{2+}$	38	1:43
6	DCHLAQVPSHTVVAR	$(817.01)^{2+}$	30	*DCHLAQVPSHTVVAR	$(929.97)2^{+}$	49	1:453
7	D*CHLAQVPSHTVVAR*	$(839.04)^{2+}$ $(559.67)^{3+}$	40	*D*CHLAQVPSHTVVAR°	$(951.61)^{2+}$ $(634.75)^{3+}$	54	1:19
8	D*SGFQM*NQLRGK	$(710.01)^{2+}$	27	*D*SGFQM*NQLRGK*	$(936.06)^{2+}$	55	1:21
9	EDLIWELLNQAQEHFGK	$(1035.52)^{2+}$	49	*EDLIWELLNQAQEH FGK*	$(1261.84)^{2+}$ $(841.56)^{3+}$	70	1:1.6
10	E*D*PQTFYYAVAVVKK	$(901.43)^{2+}$	20	*ED*PQTFYYAVAVVK*K*	$(820.04)^{3+}$ $(1229.56)^{2+}$	27	1:29
11	HSTIFENLANK	$(637.32)^{2+}$	20	*HSTIFENLANK*	$(863.43)^{2+}$	51	1:14
12	SASDLTWDNLK	$(625.31)^{2+}$	27	*SASDLTWDNLK*	$(851.38)^{2+}$	57	1:79
13	SASDLTWD*NLK	$(636.43)^{2+}$	43	*SASD*LTWDNLK*	$(862.54)^{2+}$	50	1:37
14	TVRWCAVSEHEATK	$(539.68)^{3+}$ 1008	37	*TVRWCAVSE*HE*ATK*	$(1057.02)^{2+}$	57	1:2.2
15	VPPRMD*AK	$(468.25)^{2+}$	29	*VPPRMD*AK*	$(694.41)^{2+}$	47	1:8
16	RLAVGALLVCAVLGL LAVPD*KTVR	$(858.41)^{3+}$	47	*RLAVGALLVCAVLGL CLAVPD*K*TVR*	$(1008.92)^{3+}$	69	1:8
17	AIAANEADAVTLDAG VYDAYLAPNNLKPVVA EFYGSK	$(989.28)^{4+}$	46	*AIAANEADAVTLDAG LVYDAYLAPNNLK*PV VAEFYGSK*	$(1158.95)^{4+}$	70	1:3
18	KCSTSSLLE*ACTFRRP	$(921.94)^{2+}$	24	*K*CSTSSLLE*ACTF RRP°	$(765.76)^{3+}$ $(1148.13)^{2+}$	61	1:20
19	WCAVSEHE*ATKCQSFR	$(952.50)^{2+}$	38	*WCAVSEHE*ATK*C QSFR	$(786.07)^{3+}$ $(1178.58)^{2+}$	48	1:14

[a] The average increases in ionization efficiency of these peptides was calculated to be 70-fold. The area under the peak for all charge states of each peptide were summed and used as a total ionization measure. The area under the peak for all charge states of each peptide were summed and used as a total ionization measure. The ratios were calculated by dividing the total peak areas for each peptide by the total peak area of the native peptide. Key: *AA, C8QAT-labeled N-terminal; E*, sodiated glutamic acid; D*, sodiated aspartic acid M*, oxidized methionine to methionine sulfoxide; K*, C8QAT labeled; AA°, sodiated C-termial.

This peptide has two primary amine groups and was derivatized twice. A similar behavior is seen in the peptide H-Tyr-Gly-Gly-Phe-Met-Lys-OH, which is labeled twice. Ionization efficiency of the C_8-QAT derivative is 33 times that of the native peptide and 5 times greater than the QAT derivative. The most dramatic differences in ionization efficiency were seen with the peptides H-Ala-Gly-Gly-OH and H-Ala-Met-OH, which were singly labeled. Ionization of native H-Ala-Gly-Gly-OH was not detectable relative to the C_8-QAT derivative while that of native H-Ala-Met-OH was 125 times lower than that of the C_8-QAT tagged peptide. Ionization efficiency of the C_8-QAT derivative was also much larger than that of the QAT derivative in the case of these peptides. The small peptide H-Ala-Ser-OH also experienced a 20-fold increase in ionization efficiency after C_8-QAT derivatization. Although the QAT tag increased the ionization efficiency of these peptides, derivatization with the C_8-QAT group clearly had a much more dramatic impact on ionization efficiency of these peptides.

In contrast, derivatization of the peptides H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH, H-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-OH, H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-OH, and H-Ala-Phe-Pro-Leu-Glu-Phe-OH with the QAT or C_8-QAT labels either decreased ionization efficiency slightly or had little effect. The single derivatizable primary amine in these peptides was at the amino terminus. Derivatization had an equally small impact on the ionization efficiency of H-Pro-His-Pro-Phe-His-Phe-His-Phe-Phe-Val-Tyr-Lys-OH, which was singly labeled at the C-terminal lysine.

When the same 11 model peptides along with the QAT and C_8-QAT derivatives were examined at 4 times higher concentration, a different picture of ionization efficiency emerged (Table 2). Increases in ionization efficiency of the peptides Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH and H-Tyr-Gly-Gly-Phe-Met-Lys-OH after C_8-QAT derivatization were 7 and 20, respectively, at the higher concentration as opposed to 20 and 33 at the lower concentration. There was also a 1−7-fold increase in the ionization efficiency of other peptides greater than 500 Da in the mixture. Only with peptides of lower than 500 Da was there an increase in ionization efficiency greater than 300.

Effect of C_8-QAT Labeling on Electrospray Ionization of Tryptic Peptides. The objective in the experiments described below was to determine the impact of C_8-QAT labeling on electrospray ionization of typical tryptic peptides. This was achieved using native and C_8-QAT-labeled transferrin digests with an equimolar distribution of peptides at a concentration of 10^{-6} M. The labeled and unlabeled digests were mixed in a 1 to 1 ratio and separated on the C_{18} Zorbax column as described above before electrospray ionization and introduction into the mass spectrometer. MASCOT was used for peptide identification after new masses for the tagged amine groups at the N-terminus and on lysine were included as a variable modification. Peptides identified by this procedure are listed in Table 3. R_i values were calculated and used to determined differences in ionization efficiency.

The average increase in ionization was estimated to be 70-fold. A 500-fold increase in ionization efficiency was observed for the peptide ADRDQYELLCLDNTRKPVDEYK. This miss-cleaved peptide was labeled on three different amino acids, i.e., the N-terminus and both lysine residues. But the enhancement of

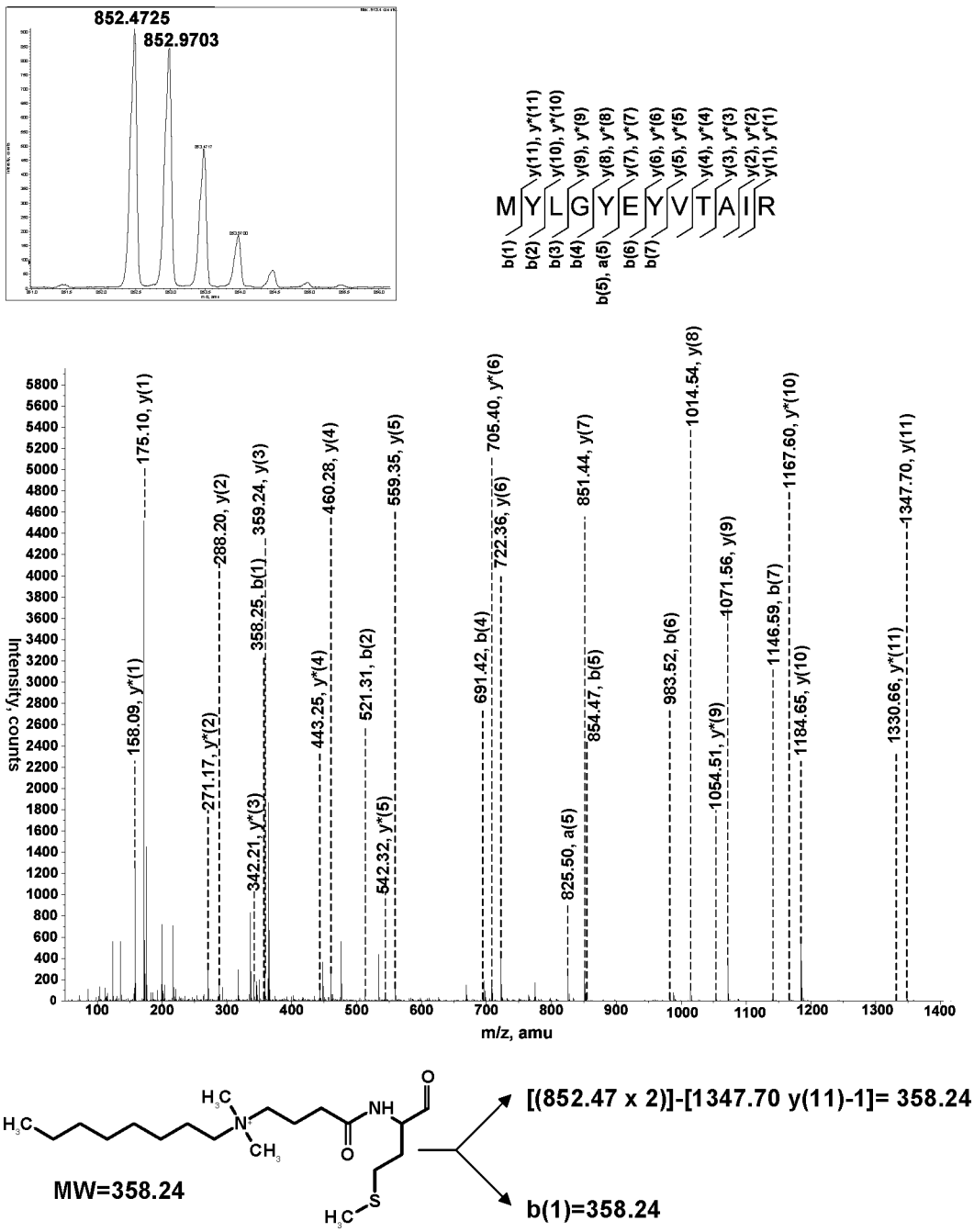

Figure 5. MS/MS spectrum for the peptide MYLGYEYVTAIR. All the y ions and seven b ions were found for this peptide and mapped on the spectrum. The mass of methionine labeled with C_8-QAT reagent was calculated using both y and b ions.

ionization seen with this peptide was probably not due to being derivatized at multiple sites. Ionization of the peptide DCHLAQVPSHTVVAR with a single tag was enhanced 453-fold. The number of sites tagged seemed not to correlate with increases in ionization efficiency throughout this work. For example, the peptide CLKDGAGDVAFVK is triply tagged (at N-terminus and both lysine residues) but showed only a 2-fold increase in ionization efficiency.

MS/MS Analysis of C_8-QAT-Labeled Peptides from Transferrin. The effect of C_8-QAT labeling on CID-induced fragmentation was examined using peptides from the transferrin digest. The MS/MS spectrum and the peak map for MYLGYEYVTAIR is shown in Figure 5, showing that all the y ions and seven b ions were found. The mass of C_8-QAT-labeled methionine at the N-terminus of the peptide can be calculated using either the b(1) ion or the difference between the precursor ion mass and the mass of y(11)fragment ion → $(852.45)^{2+} - 1347.70$ y(11) − 1.0079. A proton is subtracted from the mass of y(11) ion because this fragment has absorbed a proton to be positively charged following loss of the quaternary amine tag. All the peptides in Table 3 were identified from their MS/MS spectra. A total of 25 peptides were identified using manual sequencing (data not shown).

Discussion. Electrospray ionization of peptides is governed by two major factors. One is the ability of the peptide to acquire charge (generally a proton) before or during escape from the surface of electrospray droplets. The second is their surface activity, i.e., the ease with which the peptide migrates to the

droplet surface. The rationale in the design of the C_8-QAT labeling agent was that introduction of a permanent positive charge into peptides and addition of an adjacent aliphatic hydrocarbon tail would augment both of these processes. Data presented here and in previous work[7] indicate that in most cases quaternization does in fact improve ionization efficiency. It is also clear that adding an octyl side chain to the quaternary amine can have a very substantial impact on ionization efficiency.

Ideally it would be possible to look at the structures of a family of peptides and predict their relative ionization efficiency before and after derivatization with a reagent such as the C_8-QAT. That is not possible from the data presented here. Generally the ionization efficiency of peptides that ionize well in native form was not improved substantially by derivatization. In contrast, ionization of many peptides that ionized poorly was improved 1 order of magnitude or more through derivatization with the C_8-QAT. One of the more predictable observations was that the ionization efficiency of peptides under 500 Da would improve 1 order of magnitude or more with derivatization. Actually, a 300-fold increase in ionization efficiency was seen in one case. Another observation of major importance was that a relatively minor change in peptide concentration can change the relative ionization efficiency of peptides in a mixture. Tagging peptides with C_8-QAT moderated this effect.

The lack of correlation between ionization efficiency and (1) peptide size above 500 Da, (2) the presence of cationic amino acids such as histidine and arginine in peptides, (3) number of C_8-QAT tags added to a peptide, and (4) peptide hydrophobicity was surprising. A dramatic 500-fold increase in ionization efficiency following C_8-QAT tagging was seen in a peptide of almost 1000 Da. In another case a similar peptide showed a 2-fold increase.

The presence of cationic amino acids such as arginine and histidine is generally thought to be very desirable in peptide ionization because of the ability to acquire protons during the electrospray process. Surprisingly, the number of cationic amino acids in a peptide was not a good indicator of ionization efficiency. This is a clear indicator that the electrospray ionization process depends on more than acquiring a protein to become ionized.

Based on the rationale that peptide hydrophobicity could impact migration to the droplet surface, it was surprising that neither the number of hydrophobic C_8-QAT groups added to peptides nor the net hydrophobicity of native peptides was useful in predicting relative changes in ionization efficiency follow tagging. One contributor to this anomaly could be that peptides entering the electrospray inlet have just left a RPC column. RPC effluent can contain up to 60% acetonitrile. This will seriously diminish solvophobicity toward hydrophobic peptides in electrospray droplets. A high content of organic solvent in the droplet would suppress the surface activity of the hydrophobic tags as well by decreasing surface tension in the droplet. Peptide conformation could be another that would contribute to the surface activity of peptides, particularly with larger peptides It is conceivable that hydrophobic regions of a peptide could be shielded from a polar solution by polar amino acid side chains. Derivatization could either enhance or diminish this shielding.

CONCLUSION

Based on data presented in this paper, it is concluded that addition of a tag containing a quaternary amine with an adjacent *n*-octyl chain to primary groups in peptides can increase the electrospray ionization efficiency of many peptides by 10-fold or more. This enhancement of ionization efficiency occurs most consistently with peptides under 500 Da but can have an equally large impact on larger peptides as well. The most dramatic increases in ionization efficiency following derivatization are likely to occur with peptides that are difficult to ionize in the native state. Smaller degrees of augmentation are likely in peptides that ionized well in the untagged form. It is further concluded that improvements in ionization efficiency following C_8-QAT tagging are most likely due to increased accumulation of peptides at droplet surfaces by imparting surfactant properties to peptides.

Finally it is concluded that derivatization of peptides with reagents such as C_8-QAT can improve detection sensitivity of many peptides while minimizing differences in ionization efficiency. This may be particularly useful in the analysis of complex peptide mixtures such as those derived from tryptic digests of a proteome.

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