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RESEARCH GROUP REPORTS

Strategies for the Synthesis of Labeled Peptides

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Labeled peptides synthesized by core facilities are frequently used by researchers for following trafficking of a peptide, for binding studies, to determine substrate specificity, and for receptor cross-linking studies. The membership of the Association of Biomolecular Resource Facilities was asked to participate in a study focusing on synthesis of a biotin-labeled peptide, and it was suggested that a new strategy, using Rink amide 4-methylbenzhydrylamine resin coupled with Fmoc-Lys(Dde)-OH, be used. This strategy can be used for addition of a variety of labels other than biotin and should prove useful to core facilities. Comparison of the new strategy to other strategies was performed. Biotin labeling has long been assumed to be routine and specific. Despite the assumed routine nature of synthesizing biotinylated peptides, 9 of the 34 samples submitted did not contain any of the correct product. Although synthesis using Fmoc-Lys(Dde)-OH plus biotin generally gave the highest yields, other approaches also yielded a high percentage of the correct product. Therefore, the various strategies are generally comparable. The major advantage of this new approach is that other labels such as fluorescein, dansyl groups, methyl coumarin, and potentially fluorophores and quenchers used for fluorescence resonance energy transfer (FRET) can be directly incorporated into peptides. (J Biomol Tech 2000;11:155–165)

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Two approaches can be used to generate labeled peptides: the peptide can be synthesized using labeled amino acids, or the label can be added after peptide synthesis has been completed. When a peptide is labeled after synthesis, there occasionally are problems with the location of the label, depending on the composition of the peptide. The use of labeled amino acids during synthesis usually insures the correct positioning of the label.

The ABRF Peptide Synthesis Research Group (PSRG) of the Association of Biomolecular Resource Facilities (ABRF) conducts annual studies to help member laboratories assess their peptide synthesis capabilities.[1] Concurrently, through careful design and synthesis of the test sequence, these studies also serve as an avenue to introduce new techniques to member laboratories. This study focused on the synthesis of side-chain–labeled peptides—specifically, a peptide with a C-terminal biotin-labeled lysine. Participating laboratories were asked to construct the following peptide:

H-Ala-Glu-Lys-Gly-Lys-Leu-Arg-Phe-Lys(biotin)-NH_2

Although use of Fmoc-Lys(biotin)-OH was an obvious choice for direct assembly of this sequence,

one goal of the study was to acquaint member laboratories with a flexible strategy that could be used for introduction of a variety of different labels other than biotin. To make this endeavor especially valuable for core laboratories, it was suggested that the new approach, based on utilization of a side-chain protected lysine residue orthogonal to Fmoc/*t*-Bu that employs Rink amide MBHA resin coupled with Fmoc-Lys(Dde)-OH be used.[2] In this approach, first the peptide is synthesized, then the label is added for coupling to the Lys, followed by cleavage and deprotection. Participating laboratories were asked to submit the requested peptide without purification. The PSRG members used amino acid analysis, capillary electrophoresis, reverse-phase high-performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and electrospray ionization mass spectrometry (ESI/MS) to analyze all peptides submitted from participating laboratories in addition to a test peptide synthesized by the PSRG in preparation for the study. In this report, the results from analysis of the PSRG test peptide and submitted peptides are presented, and the method used by the PSRG for synthesis of the test peptide is described.

METHODS

Synthesis of the Reference Peptide

For testing by the PSRG, H-Ala-Glu-Gly-Lys-Leu-Arg-Phe-Lys(biotin)-NH_2 was synthesized on an AB 431A synthesizer (Applied Biosystems, Foster City, CA). Rink amide MBHA resin was coupled with Fmoc-Lys(Dde)-OH using HBTU/HOBt/DIEA activation.[3] All remaining residues except the *N*-terminal Ala were incorporated as Fmoc-amino acids with the same activation chemistry, using Pbf for Arg, Boc for Lys, and O*t*Bu for Glu as the side-chain protecting groups. The *N*-terminal Ala was incorporated as *t*-Boc-Ala. After completion of the synthesis, the resin was washed twice with *N,N*-dimethylformamide (DMF). The Dde side-chain protection was removed by treatment with 2% hydrazine (Sigma, St. Louis, MO) in DMF (7 mL/0.1 mmole of the protected peptide resin; 2 times, 5 minutes each). The resin was then washed 3 times with DMF followed by 2 washes with DMF:dimethyl sulfoxide (DMSO; 1:1, v/v). A 10-fold molar excess of biotin (Aldrich, Milwaukee, WI) was dissolved in 5 mL of DMF:DMSO (1:1). It was necessary to warm the mixture and vortex it for several minutes to dissolve the biotin completely. The biotin solution was then treated with 2.1 mL of 0.45-M HBTU/HOBt in DMF followed by 0.3 mL of diisopropylethylamine (DIEA). The activated biotin solution was added to the resin, and the mixture was stirred overnight. The resin was washed with DMF:DMSO (1:1; 3 times) followed by dichloromethane:methanol (1:1; 2 times). After the resin was thoroughly dried, the peptide was cleaved and deprotected with Reagent-K.

Amino Acid Analysis

Crude peptide samples were hydrolyzed for 24 hours at 110°C using vapor-phase hydrolysis in 6-N HCl containing 2% saturated phenol/water. The samples were then analyzed by Waters AccQ*Tag chemistry using a Waters Alliance HPLC system (Milford, MA) equipped with a fluorescence detector.

High-Performance Liquid Chromatography

Analyses were conducted on a Waters HPLC system consisting of two model 600 solvent delivery systems, a Wisp model 712 automatic injector, a model 490 programmable wavelength ultraviolet detector, and a DEC model 860 Networking Computer to control system operation and collect data. HPLC conditions were as follows: column, Delta Pak C18 (Waters), 3.9 × 150 mm, 5 μm, 100 Å; buffer A, 0.1% trifluoroacetic acid (TFA) in water; buffer B, 0.1% TFA in acetonitrile; linear gradient, 5% B to 60% B in 45 minutes; flow rate, 1.0 mL/minute; detector wavelengths, 220 and 279 nm.

Capillary Electrophoresis

Peptides were analyzed on a PACE-MDQ CE system (Beckman Instruments, Palo Alto, CA) using an amine-coated capillary and acetate buffer, pH 4.5. The capillary was preconditioned with an amine regenerator and a buffer rinse. Samples were applied by a 5-second pressure injection (2 psi) and separated at 30 kV for 10 minutes using reversed polarity.

Electrospray Ionization Mass Spectrometry

ESI/MS analyses were conducted on a ThermoFinnigan LCQ ion trap mass spectrometer (San Jose, CA). Samples were dissolved in 1 mL of water and diluted at least 100-fold in 70% aqueous acetonitrile/0.5% acetic acid. Peptide samples were introduced into the electrospray interface by flow injection at a rate of 5 μL/minute. MS conditions were as follows: capillary temperature, 200°C; spray voltage, 5 kV; capillary voltage, 30 V; tube lens offset, 18 V; sheath gas, 30 L/min. For MS^n, the relative collision energy was 35%. Spectral averaging for 10 scans was used prior to data acquisition.

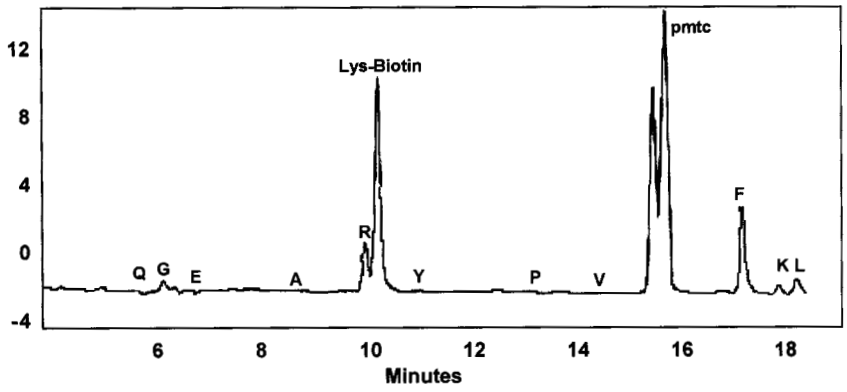

FIGURE 1

Elution position of PTH-Lys(biotin) by Edman sequence analysis. The Lys(biotin) is well resolved from other amino acids and can easily be identified.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

One microliter of a sample solution of approximately 10 to 50 pmole/μL was combined with 1 μL of 4-hydroxy-alpha-cyano-cinnamic acid matrix solution (Hewlett-Packard, Palo Alto, CA), and the mixture was deposited on a stainless steel target (2 spots/sample). MALDI-TOF analyses were performed on either a Voyager Elite or a Voyager DE-STR mass spectrometer (Applied Biosystems) in reflectron mode with delayed extraction. A two-point external calibration based on angiotensin II (m/z 1046.54, MH^+) and adrenocorticotropic hormone (ACTH) 18 to 39 Clip peptide (m/z 2465.2, MH^+) was used for the molecular mass determinations. Post-source decay (PSD) experiments were performed on the Voyager DE-STR. Data were acquired in 10 consecutive frames, lowering the reflectron voltage by 25% at each step. Air was used as collision gas while focusing on the low mass region ($m/z < 200$). The data were smoothed to yield mostly average masses. ACTH-Clip peptide was used for PSD calibration.

Sequence Analysis

Selected peptides were sequenced by Edman degradation using an Applied Biosystems model 494 protein sequencer and an Applied Biosystems model 120A HPLC analyzer fitted with a reverse-phase (RP) HPLC column.

RESULTS AND DISCUSSION

Analysis of the Test Peptide

The integrity of the reference peptide was confirmed by amino acid analysis, capillary electrophoresis, Edman degradation, RP-HPLC, MALDI-TOF/MS, and ESI/MS. All analyses gave the anticipated results. As shown in Figure 1, PTH-Lys(biotin) was well resolved from the other amino acids during Edman degradation analysis.

The ESI mass spectrum of the test peptide is shown in Figure 2. Three charge states of the peptide were readily detected: m/z 1173.7, $[M+H]^+$, m/z 587.5, $[M+2H]^{2+}$, and m/z 392.1, $[M+3H]^{3+}$. The tandem mass spectrometry (MS/MS) spectrum obtained by collision-induced dissociation of the 2+ ion confirmed the expected sequence (Fig. 3).

Analysis of Submitted Peptides

Thirty-four peptides were submitted in crude form by participating laboratories. Eight different methods were used for the synthesis. The best crude material was found to be 96% pure by RP-HPLC. A summary of the analytical data for the submitted peptides is presented in Table 1. The following results were obtained:

1. Nine of the peptides were constructed using a prelabeled Lys(biotin), yielding from 0% to 88% correct product by HPLC. In three of these peptides, the Lys(biotin) was not efficiently coupled.
2. Eleven laboratories used Fmoc-Lys(Dde)-OH (as did the PSRG). Three of these submitted samples contained no correct product. One contained the Lys(biotin) but was missing the Arg. The other two samples contained two biotins. The attachment of the extra biotin was the result of the fact that Boc-Ala-OH was not employed as the N-terminal residue. These last two laboratories also used modified biotins.
3. Nine laboratories used Fmoc-Lys(Mtt)-OH. Of these samples, one had no correct product and was missing the biotin, and another contained only low levels of the requested peptide. For this synthesis, the Mtt group must be selectively

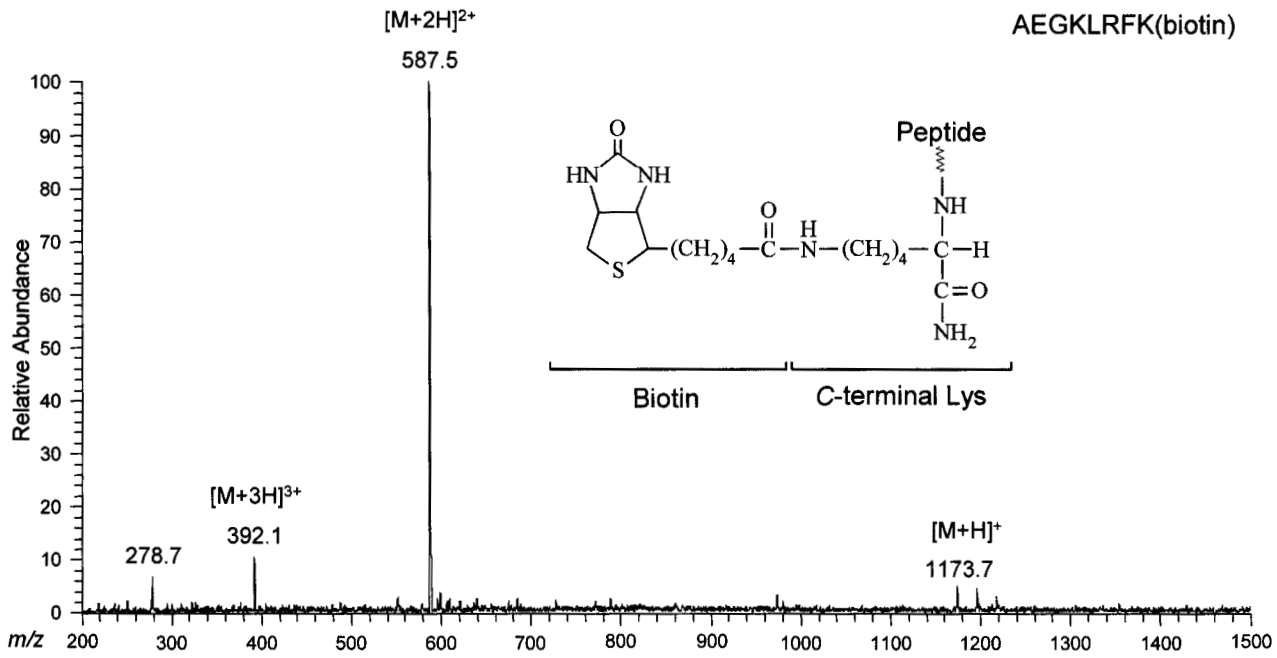

FIGURE 2

Electrospray ionization mass spectrum of the test peptide, AEGKLRFK(biotin). Three charge states of the peptide are readily detected, verifying a molecular mass of 1173 daltons.

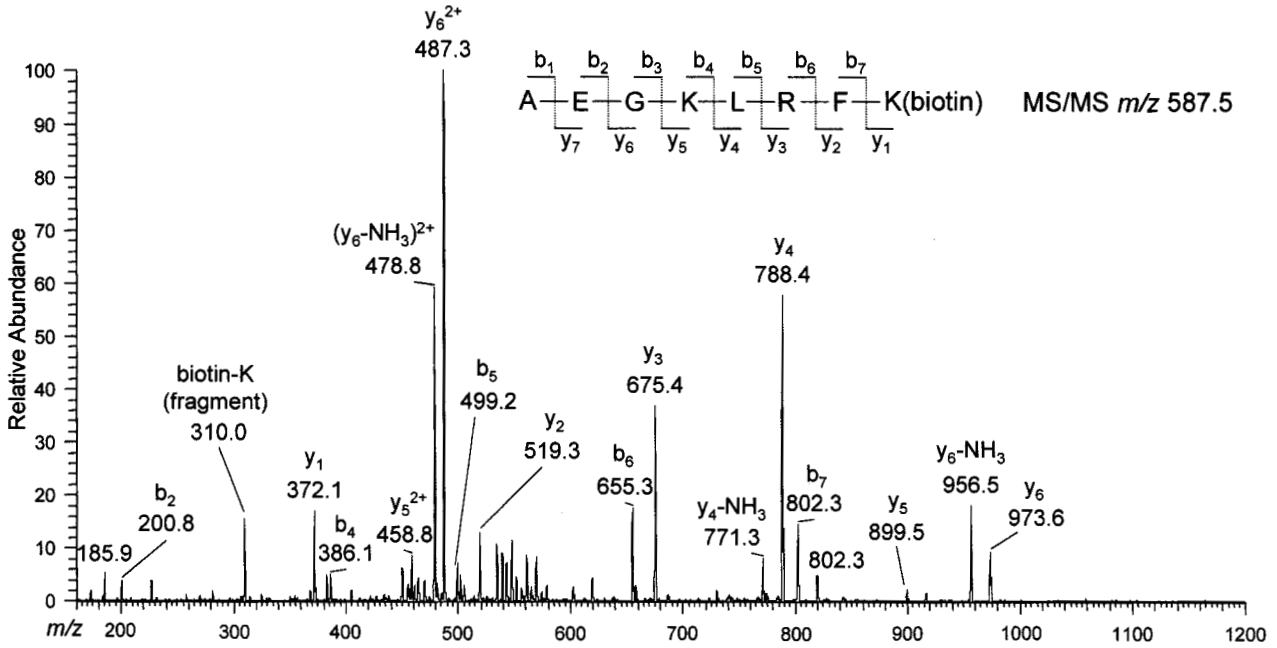

FIGURE 3

Tandem mass spectrum obtained by collision-induced dissociation of $[M+2H]^{2+}$ (m/z 587.5) for the test peptide. The fragmentation pattern confirms the expected sequence.

TABLE 1

Summary of Results from ABRF Peptide Synthesis Research Group Study

	Percentage Correct Product		Mass Spectrometry Data							
			Major Product		Other	Lys				
Sample[a]	RP-HPLC	CE	Correct Mass	Identity	Compo-nents	Protec-tion	Biotin	Chemistry	Instrument	Cleavage Reagent
1340A	88.9	85.9	Yes			Aloc	D-Biotin	Fmoc/HBTU	Rainin Symphony	Modified K
4761	12.5	31	Yes		Multiple	Aloc	EZ-link NHS 9050 Biotin	Fmoc	Milligen	R
0715	86.4	88	Yes		− Lys (biotin)	Biotin		Fmoc/PyBOP/ HOBt	Shimadzu Pssm-8	R
1231	82.2	81.7	Yes		− Glu, − Ala, − Lys (biotin)	Biotin		Fmoc/HBTU/ HOBt	Rainin Symphony	Modified B
1354	54.9	76.8	Yes		− Lys (biotin)	Biotin		Fmoc/HBTU/ HOBt	ABI 431	Modified B
4612	28.5	58.9	Yes		peptide +16, − Lys (biotin)	Biotin		Fmoc/HBTU/ HOBt	ABI 431A	B
8105	0	0	No	− Lys (biotin) and Glu-to-Gln	Glu-to-Gln	Biotin		Fmoc/HBTU/ HOBt	ABI 433A	Modified K
8398A	73.8	80.1	Yes			Biotin		Fmoc/HATU	Rainin Symphony	B
8583	11.7	12.7	No	− Lys (biotin)		Biotin		Fmoc/HBTU/ HOBt	ABI 433A	K
MPCF	76.6	83.8	Yes		+ Pmc	Biotin		Fmoc/HBTU	Rainin Symphony	Modified B
8398B	59.3	81.7	Yes	(Biotin caproate)	+ Extra Lys(biotin caproate)	Biotin caproate resin		Fmoc/HATU	Rainin Symphony	B
2958	50.4	47	Yes		+ 91 Amu, possible bzl on Glu	Boc-Lys (2ClZ)	D-Biotin	Boc/HBTU/ HOBt	AB 430	5 mL HF/ 500 μL Anisole
0690	61.2	84	Yes	(Biotin caproate)	+ Pmc	Dde	(±)Biotin	Fmoc/HBTU/ HOBt	AB 433A	Reagent-K
2311	0	0	No	− Arg		Dde	Biotin	Fmoc/HBTU/ HOBt	AB 430	Reagent-K
2913	52.9	71.1	Yes		+ Pbf	Dde	D-Biotin	Fmoc/HBTU/ HOBt	AB 431A	Modified Reagent-B
4005	0	0	No	2 Biotin caproates[b]	1902 daltons[c]	Dde	Biotin-- amido-caproate NHS ester	Fmoc/PyBOP	AMS 422	Modified Reagent-B
4209D	61.7	65.6	Yes			Dde	(±)Biotin	Fmoc/HBTU	ACT 396MBS	Modified Reagent-B

(continued)

TABLE 1

Summary of Results from ABRF Peptide Synthesis Research Group Study *(continued)*

Sample[a]	Percentage Correct Product		Mass Spectrometry Data			Lys Protection	Biotin	Chemistry	Instrument	Cleavage Reagent
			Major Product		Other Components					
	RP-HPLC	CE	Correct Mass	Identity						
IRBJ	0	0	No	2 Biotins[b]		Dde	Biotin-N-hydroxy-succinimide	Fmoc/HBTU	ACT MPS 396	Modified Reagent-B
1068	84.3	87.2	Yes		+ Pbf	Dde	NHS-LC-Biotin	Fmoc/PyBOP	Perseptive Pioneer	Reagent-K
1374	82.8	83.2	Yes		− Ala	Dde	Succin-imidyl D-Biotin	Fmoc/HBTU/HOBt	AB 431	Reagent-R
5130	72.9	82.9	Yes			Dde	D-Biotin	Fmoc/HOBt/DCC	AB 431A	Reagent-K
5557	96.1	90	Yes			Dde	D-Biotin	Fmoc/HBTU/HOBt	AB 433	Reagent-K
9818	92.1	92.5	Yes			Dde	D-Biotin	Fmoc/HBTU/HOBt	AB 432	Modified Reagent-B
9453	81.6	81.5	Yes		Peptide + 90	Fmoc	(±)Biotin	Boc	AB 430	90:10 HF:Anisole
1340T	96	90	Yes			MeOtrt	D-Biotin	Fmoc/HBTU	Rainin Symphony	Modified Reagent-K
0775	85.1	84	Yes		+ Biotin[b]	Mtt	(±)Biotin	Fmoc/TBTU	Rainin Symphony	Modified Reagent-B
1524	33.7	63.4	Yes	(Biotin caproate)	− Biotin caproate	Mtt	Sulfo-NHS-LC-Biotin	Fmoc/TBTU/HOBt	Milligen 9050	Reagent-K
1609	74.1	66.1	Yes		+ Biotin[b]	Mtt	D-Biotin	Fmoc/HBTU/HOBt	AB 432	Reagent-R
1633	60	55.8	Yes		− Biotin	Mtt	EZ-link NHS Biotin	Fmoc/HOBt/DCC	AB 431A	Reagent-K
3845	0	0	No	− Biotin		Mtt	NHS-LC-Biotin	Fmoc Opfp/HOBt	Milligen 9050 Plus	Modified Reagent-B
4209M	7	6.8	No	− Biotin		Mtt	(±)Biotin	Fmoc/HBTU	ACT 396MBS	Modified Reagent-B
7298	81.2	70.8	Yes		+ Biotin[b]	Mtt	D-Biotin	Fmoc/HBTU/HOBt	AB 433	Reagent-K
7299	89.9	80.8	Yes			Mtt	D-Biotin	Fmoc/HBTU/HOBt	AB 433	Reagent-K
7347	78	83.5	Yes		+ Biotin[b]	Mtt	(±)Biotin	Fmoc/HBTU	Perseptive Pioneer	Reagent-B

[a]The samples are grouped according to Lys protection and then listed in numerical order.
[b]Did not use Boc-Ala-OH.
[c]The extra mass is on the Lys(biotin) by tandem mass spectrometry.
±, with and without; CE, capillary electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography.

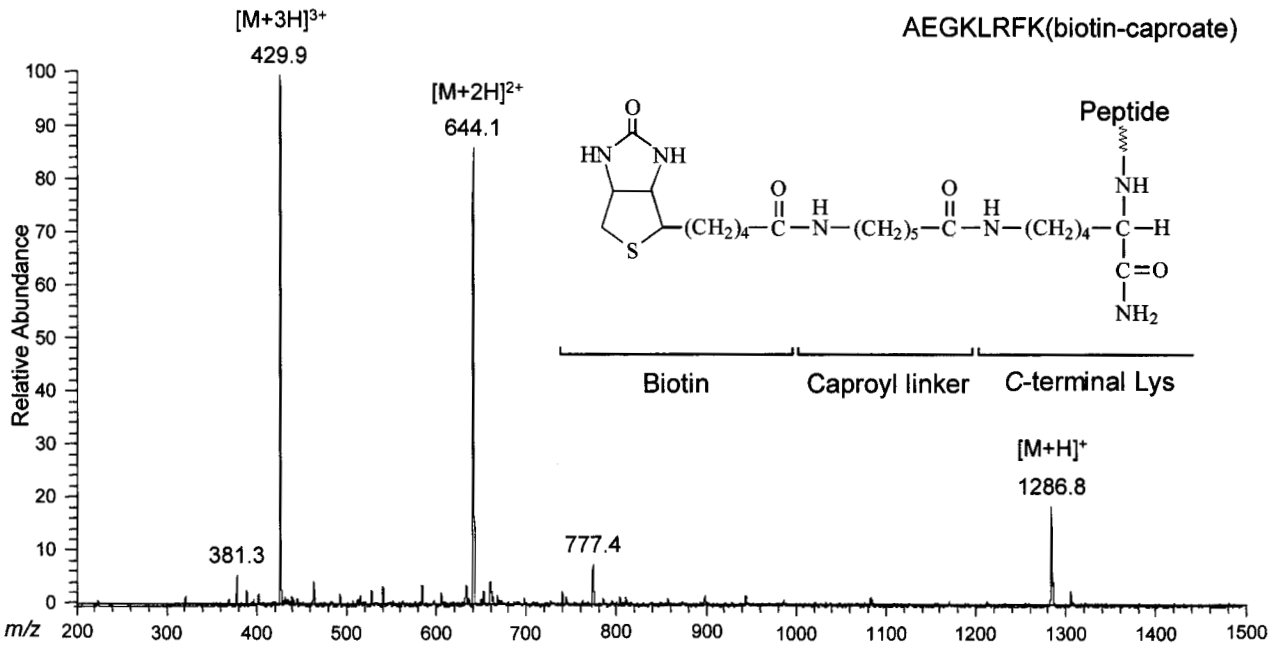

FIGURE 4

Electrospray ionization mass spectrum of a submitted sample of AEGKLRFK(biotin) in which there was a caproyl linker between biotin and the C-terminal Lys. The three charge states detected for this peptide indicated a molecular mass of 1286 daltons.

removed using dilute TFA or acetic acid/trifluoroethanol/methylene chloride (AcOH/TFE/DCM).[4]

4. Two laboratories used Fmoc-Lys(Aloc)-OH. One of these samples contained multiple components, with the product of interest at only 12.5%. For this synthesis, the Aloc group must be selectively removed using Pd(0),Bu_3SnH,[5] or Pd(0), HOAc, *N*-methylmorpholine in chloroform.[6]
5. Two laboratories constructed their peptides using *t*-Boc chemistry. In both of these samples, the product of interest was the major component by HPLC.

Overall, nine of the samples did not have the product of interest as the major component by HPLC.

Two types of biotin were used by the participating laboratories: biotin and biotin caproate (b-c). The caproate arm increased the mass of the correct peptide to 1286 daltons (Fig. 4) and caused an extra peak in the amino acid analysis corresponding to the

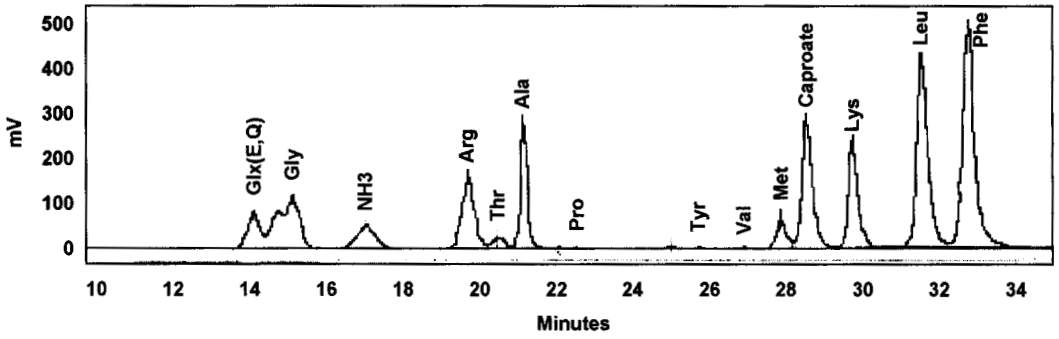

FIGURE 5

Amino acid analysis of a peptide constructed with a caproyl linker to biotin. An extra peak was observed at 28.6 minutes between Met and Lys.

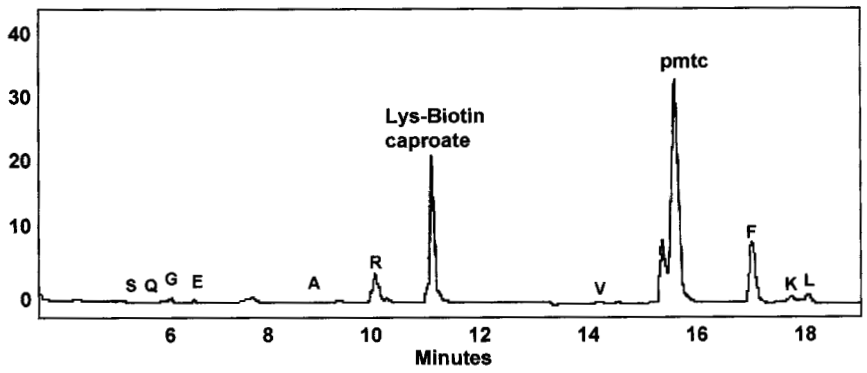

FIGURE 6

Elution position of PTH-Lys biotin caproate(Lys(BC)) by Edman sequence analysis. Lys(BC) was not readily resolved from PTH-Tyr using the gradient conditions described in Methods.

derivatized caproate. (Fig. 5) The retention time of PTH-Lys(b-c) during Edman degradation analysis was within 0.1 minute of that of PTH-Tyr and therefore was not well resolved (Fig. 6).

ESI/MS was a valuable tool in the analysis of these crude products. In peptide 8105, ESI/MS and subsequent ESI/MS/MS analysis rapidly identified a Glu-to-Gln substitution in the peptide in addition to a significant quantity of a truncated product (Figs. 7 and 8). Analysis of this peptide illustrates the fact that tandem mass spectrometry can readily identify an amino acid substitution that would be undetected by amino acid analysis.

Peptide 8398B was synthesized using a prelabeled resin. ESI/MS analysis indicated that, in addition to the expected biotin caproate peptide, there was an additional product which presumably corresponds to the peptide plus an extra Lys(b-c) (Fig. 9). This was verified by subsequent MS/MS analysis (Fig. 10). The mono-Lys(b-c) peptide constituted 17.5% of the sample by RP-HPLC.

It is interesting to note that a variety of different cleavage cocktails were developed in the 1990s. These reagents contain different scavengers in TFA to reduce side reactions of the reactive cations generated from cleaved protecting groups. Reagent-K

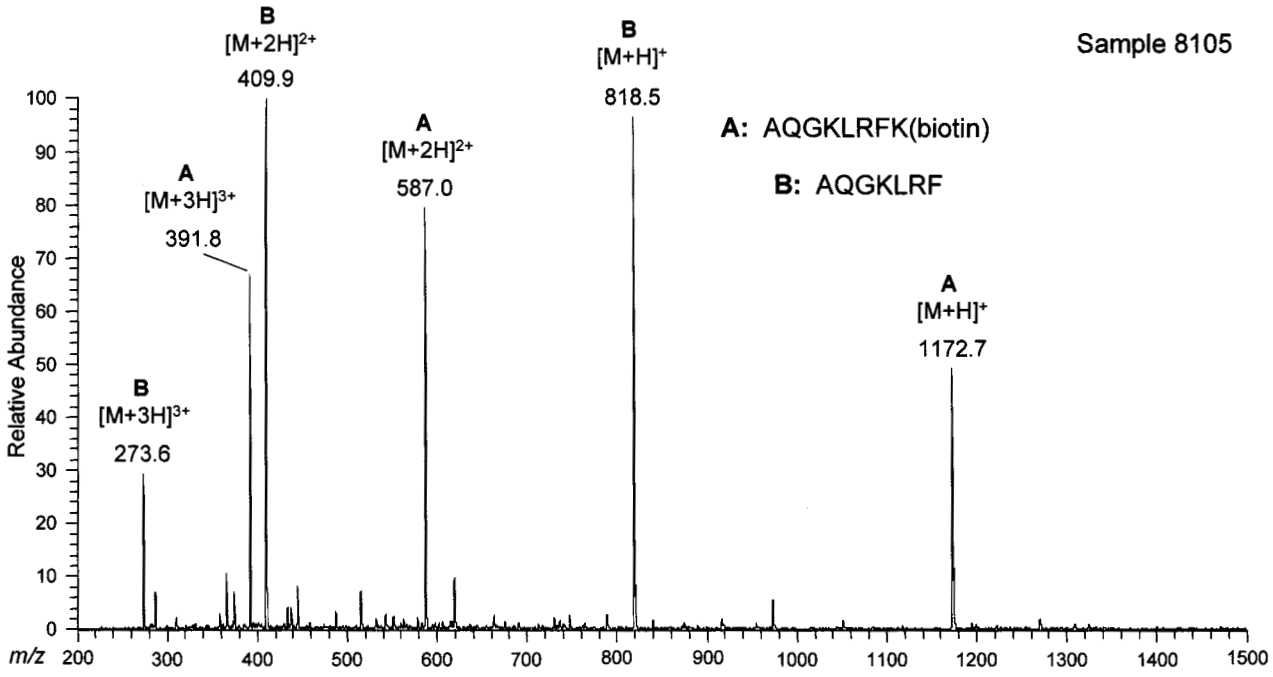

FIGURE 7

Electrospray ionization mass spectrum of sample 8105 containing a Glu-to-Gln substitution in the requested biotin-labeled peptide **(A)** in addition to a substantial quantity of a truncated product **(B)**.

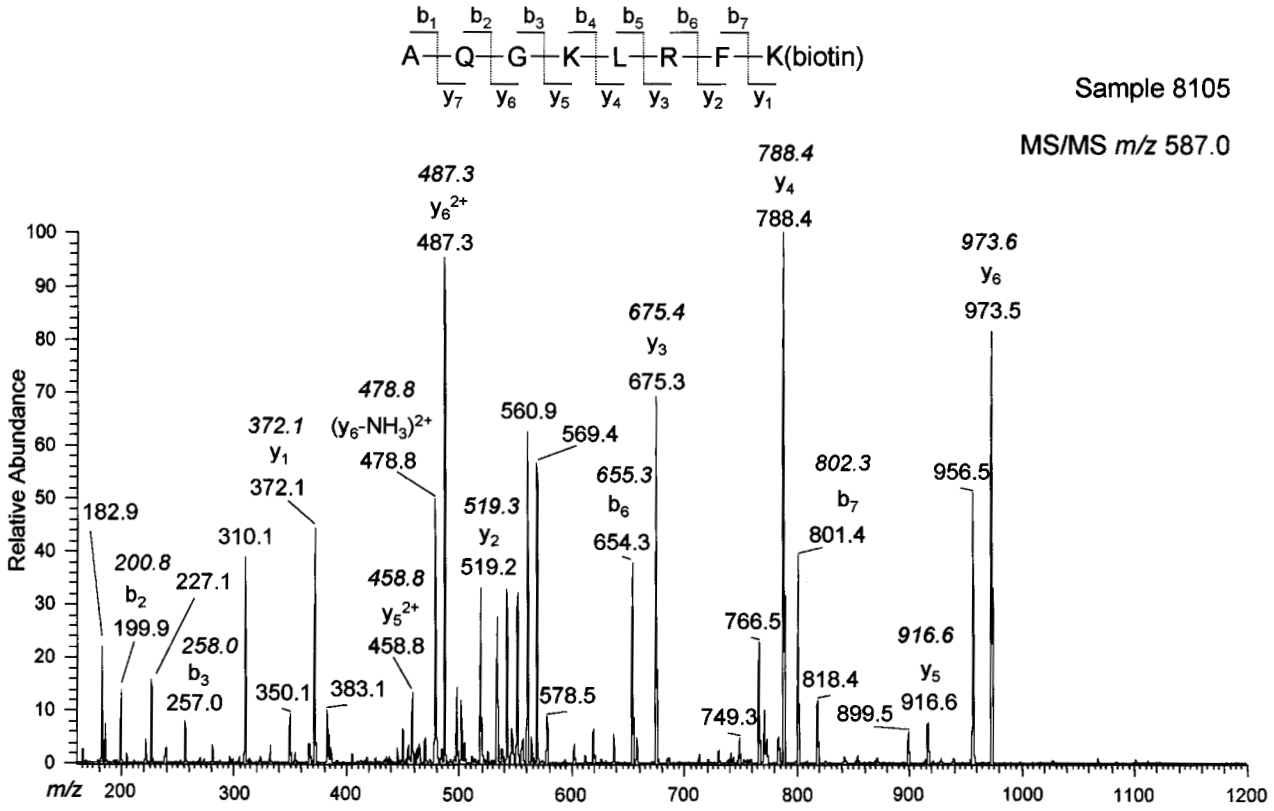

FIGURE 8

Tandem mass spectrum obtained by collision-induced dissociation of $[M+2H]^{2+}$ (m/z 587.0) for the biotin-containing peptide in sample 8105 indicating the Glu-to-Gln substitution.

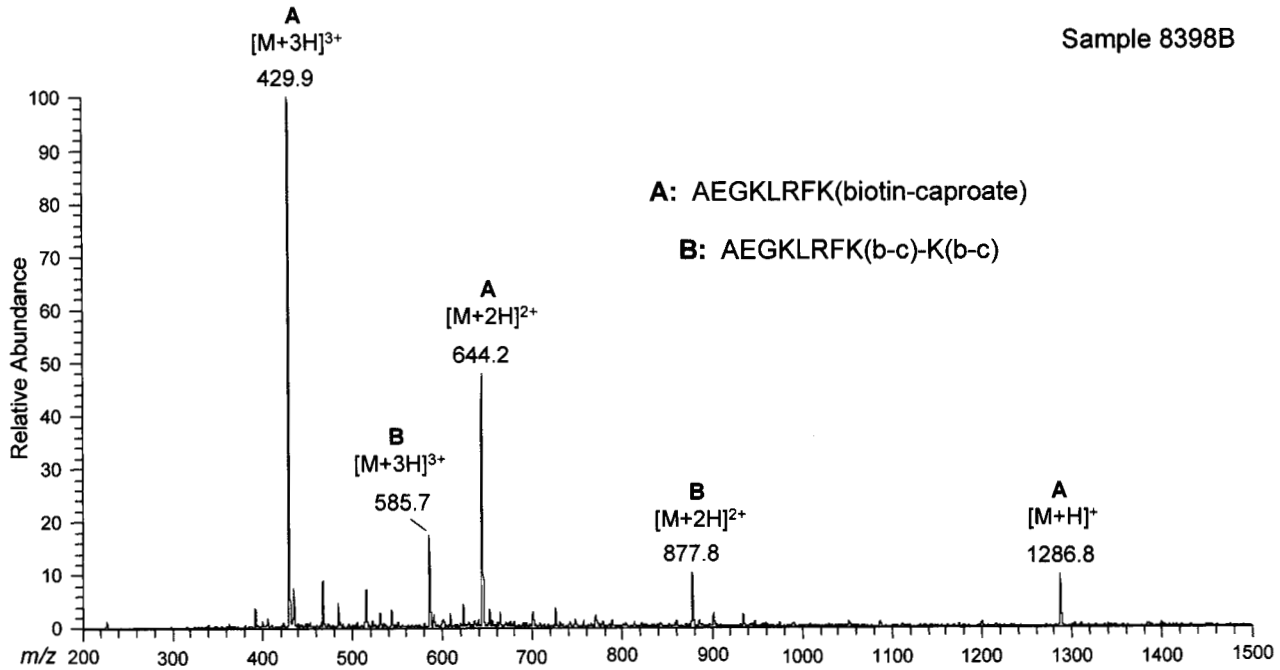

FIGURE 9

Electrospray ionization mass spectrum of sample 8398B indicating the presence of peptides containing one **(A)** and two **(B)** residues of biotin caproate.

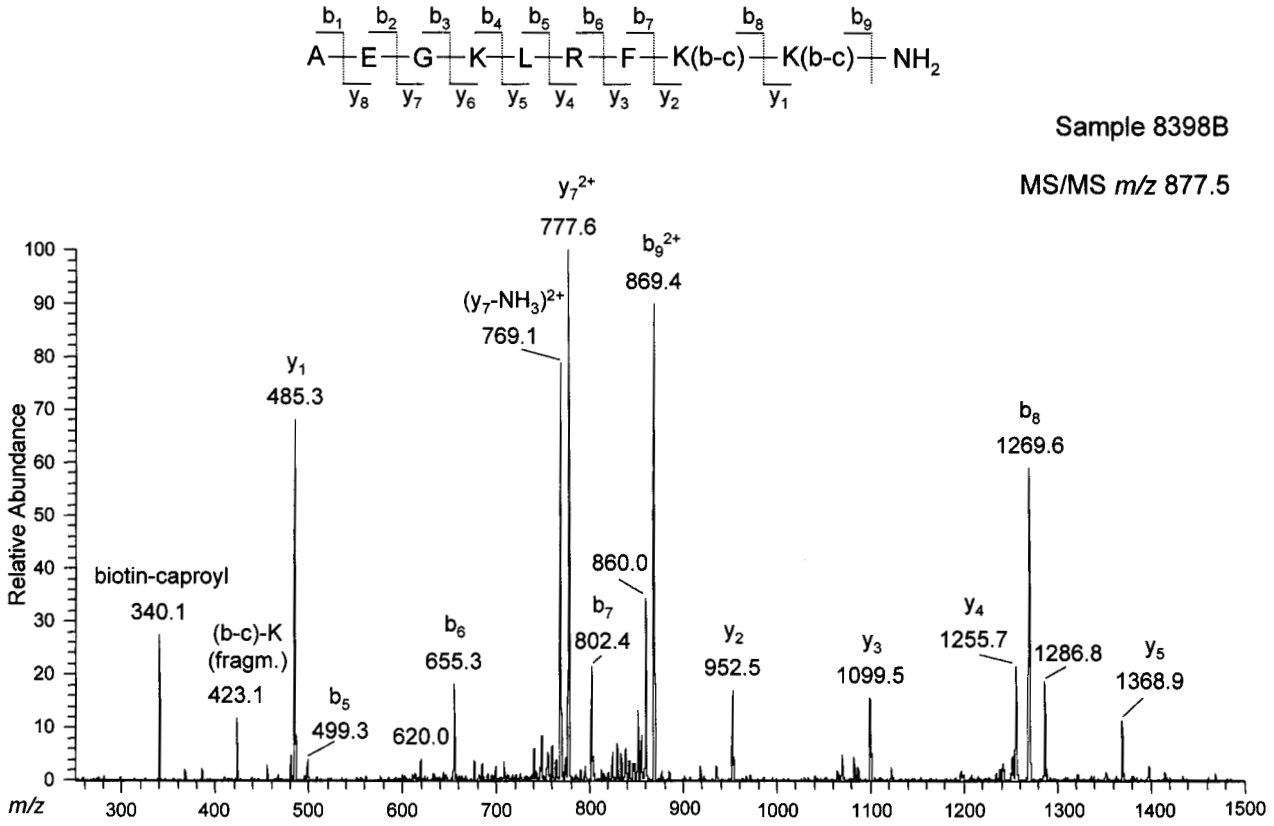

FIGURE 10

Tandem mass spectrum obtained by collision-induced dissociation of $[M+2H]^{2+}$ (m/z 877.5) for the (biotin caproate)$_2$-containing peptide in sample 8398B. The fragmentation pattern verifies the presence of two adjacent biotin caproates.

(TFA-phenol-H_2O-thioanisole-1,2-ethandithiol 82.5:5:5:5:2.5),[7] Reagent-B (TFA-phenol-H_2O-triisopropylsilane 88:5:5:2),[8] and Reagent-R (TFA thioanisole-1,2-ethanedithiol-anisole (90:5:3:2)[9] are the most commonly used. Reagent-B is becoming more popular because it provides clean, highly efficient cleavage and does not contain any malodorous thiol components. Forty-one percent of the respondents used cleavage Reagent-B for this study.

The results of this study showed that use of either a prelabeled amino acid or resin did not guarantee a good product; 37% of the peptides constructed using prelabeled starting materials were failures. It is also clear that when an Fmoc-Lys(Dde)-OH derivative is employed to introduce a label into a peptide, a Boc amino acid must be utilized for the *N*-terminus because the 2% hydrazine[3] employed for selective deprotection of the Dde group will also remove all Fmoc groups on the peptide. Finally, for characterization of synthetic peptides, MALDI-TOF/MS and ESI/MS were extremely valuable for qualitative, but not quantitative, analysis.

Considerable potential exists for the use of the Rink amide MBHA resin coupled with the Fmoc-Lys(Dde)-OH approach for the incorporation of various labels into peptides. One new and exciting application would be in the synthesis of peptides for FRET. FRET is based on the ability of molecules called quenchers to block fluorescence when in close proximity to fluorescent dyes. FRET has been used for the identification of novel proteases.[10] Incorporating a fluorophore and a quencher during peptide synthesis would avoid labeling and purification problems in peptides used for future FRET studies. It is hoped that core facilities will be able to make use of this strategy.

ACKNOWLEDGMENTS

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