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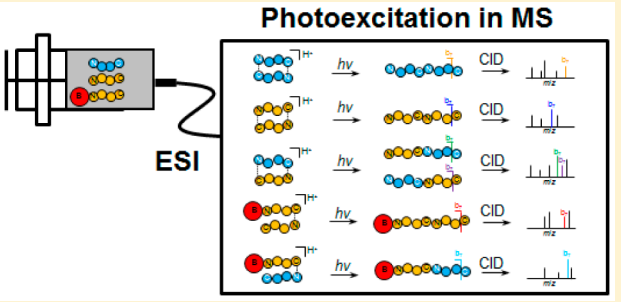
# Photosynthesis of a Combinatorial Peptide Library in the Gas Phase

Sunyoung Lee,[†] Matthew S. Glover, James P. Reilly, and David E. Clemmer*

Department of Chemistry, Indiana University, Bloomington, Indiana 47405, United States

Supporting Information

**ABSTRACT:** A strategy for generating large numbers of peptides from a relatively small number of precursors based on photosynthetic combination in the gas phase is presented. In this approach, electrospray ionization is used to create a combination of proton-bound dimers from a specified set of peptides present in solution. The dimers are then accumulated and isolated in an ion trap mass spectrometer. Photoexcitation (at 157 nm) leads to water elimination and the formation of larger peptide sequences that are characterized by subsequent isolation and collision-induced dissociation. The method is illustrated by using a set of four enkephalin-related and acetylated peptides to generate 12 larger peptide sequences. The ability to synthesize, isolate, and characterize many amino acid sequences from only a few precursors provides a fast and efficient means of characterizing properties of such species (e.g., dissociation patterns and reactivities).






We recently reported that a covalent peptide bond can be formed between two peptide monomers upon 157 nm ultraviolet (UV) photoexcitation of noncovalent, proton-bound peptide dimer ions stored in an ion trap.[1,2] The result is the formation of a new, longer amino acid chain. Despite minuscule yields of the newly synthesized peptide ions, the method generates sufficient products for mass spectrometric characterization experiments (e.g., fragmentation studies). Recently, peptide bond formation in a mass spectrometer was demonstrated through ion/ion reactions with efficiencies up to 31%.[3] Furthermore, the coupling of components within a complex to form large biomolecules may have relevance to the origins of such species.[4,5] In this paper, we demonstrate the combinatorial nature of this approach by using a relatively small set of precursor sequences to generate a library of new peptides.

The solid-phase methods pioneered by Merrifield in the 1960s[6] revolutionized peptide synthesis and was foundational in the establishment of combinatorial chemistry. This chemistry makes it possible to simultaneously synthesize vast numbers of amino acid sequences, including those that contain unnatural amino acids.[7−11] The product of these syntheses is libraries of related molecules that can be screened to identify species of interest.[12−18] The method that we present below is unlikely to yield quantities that are sufficient for such traditional screening strategies; however, the ability to produce, isolate, and characterize new peptides is of interest. For example, in high-throughput studies it would be valuable to be able to produce a range of related sequences and directly generate fragmentation spectra from known standards in order to confirm assignments that are made by database searching methods.[19,20]

## EXPERIMENTAL SECTION

**Sample Preparation.** Methionine enkephalin (YGGFM), leucine enkephalin (YGGFL), [des-Tyr[1]]-leucine enkephalin (GGFL), acetylated Ala-Ala-Ala tripeptide (acAAA) were purchased from Sigma-Aldrich (St. Louis, MO) and used for the combinatorial gas-phase peptide synthesis without further purification. Singly protonated peptide monomer and dimer ions were produced by electrospraying a mixture of four peptides containing 100 $\mu$M of each peptide in a 49.5:49.5:1.0 (v/v/v) water/methanol/acetic acid solution. The solutions were infused at a flow rate of 3.0 $\mu$L min$^{-1}$ using a syringe pump. A potential of 3.0 kV was applied between an emitter and capillary ion entrance. All spectra presented in this study were recorded for a total of ∼10 min (an average of 600 microscans, which are each ∼1 s in duration).

**Mass Spectrometry.** Experiments were performed on a modified LTQ Velos mass spectrometer (Thermo Electron, San Jose, CA). A detailed description of the ion trap instrument used for this study can be found elsewhere.[21−24] Briefly, a fluorine ($F_2$) laser (EX100HF-60, GAM Laser, Orlando, FL) was aligned to the back of the ion trap through a vacuum line to introduce 157 nm UV laser light. For photoexcitation experiments, electrosprayed dimer ions were accumulated in the ion trap for 1 s. Then, the complex ions were mass-selected with ±2 Th width in the ion trap. The stored ions were activated by 157 nm irradiation. A single pulse of laser light was introduced into the ion trap at the beginning of a 10 ms activation step with 0% normalized collision energy and an activation $q$ of 0.1. Product ions obtained from photoexcitation

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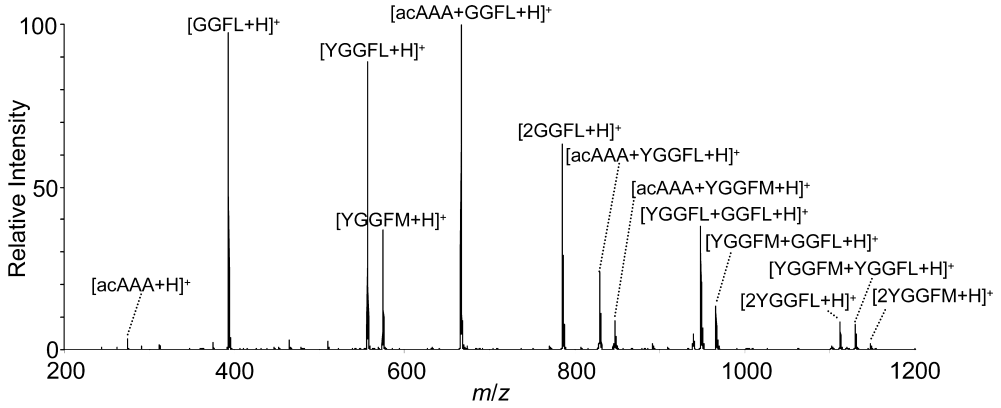


**Figure 1.** Mass spectrum showing a mixture of proton-bound dimer ions formed upon electrospraying the mixture of YGGFM, YGGFL, GGFL, and acAAA peptides. All ions were produced by electrospraying a mixture of four peptides (100 $\mu$M of each compound).

were isolated (±1 Th) and subjected to collision-induced dissociation (CID) analysis by applying a resonant rf excitation waveform for 10 ms with activation $q$ of 0.25 and normalized collision energy of 30%.

## RESULTS AND DISCUSSION

**Formation of Noncovalent Dimer Ions.** Electrospray ionization (ESI)[25] of a solution containing a mixture of four enkephalin-related and acetylated peptides generates singly protonated peptide monomer ions observed at $m/z$ = 274.3, 393.3, 556.3, and 574.3 (corresponding to $[acAAA + H]^+$, $[GGFL + H]^+$, $[YGGFL + H]^+$, and $[YGGFM + H]^+$ ions, respectively, in Figure 1). In addition to the monomer ions, it is straightforward to obtain a range of noncovalent, proton-bound dimer ions by electrospraying a solution with relatively high concentration.[26,27] Upon ESI, a mixture of the four starting peptides gives a total of nine dimer complexes, including three homo- and six heterodimer complexes (Figure 1). Theoretically, four homodimer complexes can be made from four different peptides. It turns out that three homodimer $[2GGFL + H]^+$, $[2YGGFL + H]^+$, and $[2YGGFM + H]^+$ ions appeared at $m/z$ = 785.5, 1111.5, and 1147.5, respectively. There is no peak at $m/z$ = 547.5, corresponding to the homodimer $[2acAAA + H]^+$ ions. N-terminal acetylation of the peptide obstructs a head-to-tail arrangement between peptides that maximizes electrostatic interactions of the amino-terminus of each peptide with the carboxylic acid end of the other.

Six heterodimer complexes can mathematically be formed from four starting peptides. As shown in Figure 1, the peaks assigned to the heterodimer $[acAAA + GGFL + H]^+$, $[acAAA + YGGFL + H]^+$, $[acAAA + YGGFM + H]^+$, $[YGGFL + GGFL + H]^+$, $[YGGFM + GGFL + H]^+$, and $[YGGFM + YGGFL + H]^+$ ions are observed at $m/z$ = 666.4, 829.4, 847.4, 948.5, 966.4, and 1129.5, respectively. Individual complexes can be isolated in the ion trap and collisionally activated to confirm their noncovalent bonding between two peptide units. Alternatively, the isolated complex ions can be activated with UV laser irradiation, yielding "new" peptide ions by linearly coupling two peptide units.

**Combinatorial Synthesis of Gas-Phase Peptide Ions.** Electrospraying a mixture of several peptides leads to combinatorial formation of dimer complex ions. Different $m/z$ values of the complex ions allow isolation of individual dimer ions and consequently photoexcitation of the isolated complex ions generates differently sequenced peptide ions. As previously demonstrated,[1] a peptide bond can be formed between the C-terminus of one peptide and the N-terminus of the other peptide upon photoexcitation of a peptide dimer complex. Peptide bond formation occurs through a Norrish Type I-like mechanism in which 157 nm light induces homolytic cleavage of the bond between the carbonyl carbon and hydroxyl group of the C-terminus.[28] Subsequently, radical rearrangement prompts water loss and formation of a covalent peptide bond as previously described in detail.[2] Such a radical-induced fragmentation mechanism is unlikely to occur via a slow-heating method such as CID for singly protonated peptide multimers. We note that this mechanism requires a free N- and C-terminus between the pairs for peptide bond formation. However, we have also demonstrated cross-linking of amino acid side chains that have amine and carboxyl groups such as Lys and Asp residues, respectively.[2] If the covalent bond is formed from homodimer complex ions, the coupling of either N-/C-terminal side of the complex results in same peptide sequence (Figure 2a). This means one homodimer complex produces a single peptide sequence. In this sense, three peptides (peptide no. 1–3 listed in Table 1) can be synthesized from the three observed homodimer complexes.

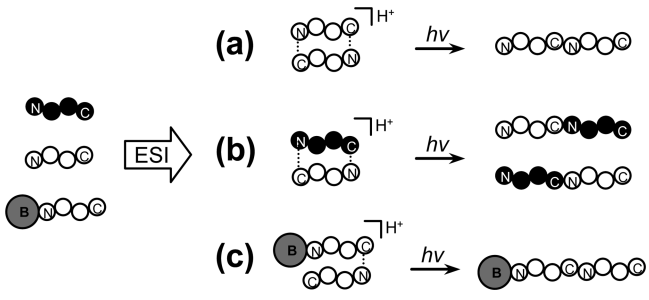


**Figure 2.** Combinatorial synthesis of gas-phase peptide ions in a mass spectrometer. Circled N and C indicate N- and C-terminal amino acids, respectively. Circled B denotes blocking group.

A heterodimer complex leads to two peptide sequences depending on which N-/C-terminal side of the complex forms a covalent bond (Figure 2b). Because photoexcitation of the heterodimer ions nonspecifically links the N-/C-terminal side of the complex, two different peptide ions can be obtained at the same time. However, N- or C-terminal modification of one peptide prevents water elimination from the blocking side of the complex. This controls the formation of specific peptide




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**Table 1. Combinatorial Library Synthesized from the YGGFM, YGGFL, GGFL, and acAAA Peptides**

| peptide no. | precursor dimer[a] | $m/z$[b] | synthesized peptide[c] | $m/z$[d] |
|---|---|---|---|---|
| 1 | 2YGGFM | 1147.5 | YGGFMYGGFM | 1129.6 |
| 2 | 2YGGFL | 1111.5 | YGGFLYGGFL | 1093.4 |
| 3 | 2GGFL | 785.5 | GGFLGGFL | 767.4 |
| 4 | YGGFM + YGGFL | 1129.5 | YGGFMYGGFL | 1111.5 |
| 5 | YGGFM + YGGFL | 1129.5 | YGGFLYGGFM | 1111.5 |
| 6 | YGGFM + GGFL | 966.4 | YGGFMGGFL | 948.4 |
| 7 | YGGFM + GGFL | 966.4 | GGFLYGGFM | 948.4 |
| 8 | YGGFL + GGFL | 948.5 | YGGFLGGFL | 930.5 |
| 9 | YGGFL + GGFL | 948.5 | GGFLYGGFL | 930.5 |
| 10 | acAAA + YGGFM | 847.4 | acAAAYGGFM | 829.5 |
| 11 | acAAA + YGGFL | 829.4 | acAAAYGGFL | 811.4 |
| 12 | acAAA + GGFL | 666.4 | acAAAGGFL | 648.5 |

[a]All dimer ions are singly protonated by electrospray ionization of a mixture solution of four enkephalin-related and acetylated peptides. [b]Monoisotopic $m/z$ values of singly protonated dimer ions that are observed in the ESI spectrum. [c]Singly protonated synthesized peptide ions are obtained from photoexcitation of the precursor dimer ions. [d]Monoisotopic $m/z$ values of singly protonated, synthesized peptide ions that are observed in the photoexcitation spectra.

sequences (Figure 2c). In this study, there are two types of heterodimer complexes; one contains free N-/C-terminal peptides and the other contains a modified N-terminal peptide and a free N-/C-terminal peptide. Each of three observed heterodimer [YGGFL + GGFL + H]$^+$, [YGGFM + GGFL + H]$^+$, and [YGGFM + YGGFL + H]$^+$ ions without any modified termini can result in two peptide sequences (peptide no. 4−9 listed in Table 1). One specific peptide ion (peptide no. 10−12 listed in Table 1) can be produced from the other three observed heterodimer [acAAA + GGFL + H]$^+$, [acAAA + YGGFL + H]$^+$, and [acAAA + YGGFM + H]$^+$ ions containing the modified peptide. Therefore, it should be possible to obtain a total of 12 new peptides from four starting peptides, YGGFM, YGGFL, GGFL, and acAAA. Combinatorial peptide libraries that can be synthesized in a mass spectrometer with the starting peptides are shown in Table 1.

**Formation of Peptide Ions from Homodimer Complexes.** As mentioned above, a new peptide can be obtained by selecting a specific homodimer. Figure 3 shows CID spectra of the newly synthesized peptides from homodimer [2YGGFM + H]$^+$, [2YGGFL + H]$^+$, and [2GGFL + H]$^+$ ions. Photoexcitation of the [2YGGFM + H]$^+$ complex generates water-loss product ions at $m/z$ = 1129.6. This is followed by collisional activation to confirm the identity of the product ions at $m/z$ = 1129.6, corresponding to [YGGFMYGGFM + H]$^+$ ions (Figure 3a). In separate experiments, the assignment of CID fragments of the water-loss product ions ($m/z$ = 1093.4) obtained from photoexcitation of the [2YGGFL + H]$^+$ complex verifies that two YGGFL peptide monomers are coupled to form [YGGFLYGGFL + H]$^+$ ions (Figure 3b). For the last homodimer [2GGFL + H]$^+$ ions, a peak corresponding to the water-loss product ions at $m/z$ = 767.4 is detected by isolating and photoactivating the complex ions. Similar to CID analyses of water-loss product ions obtained from the other complexes, collisional activation of the water-loss product ions generates several unique fragment ions that indicate the formation of [GGFLGGFL + H]$^+$ ions (Figure 3c).

CID spectra of the water-loss product ions from homodimer complexes show a series of b- and y-type ions that show nearly

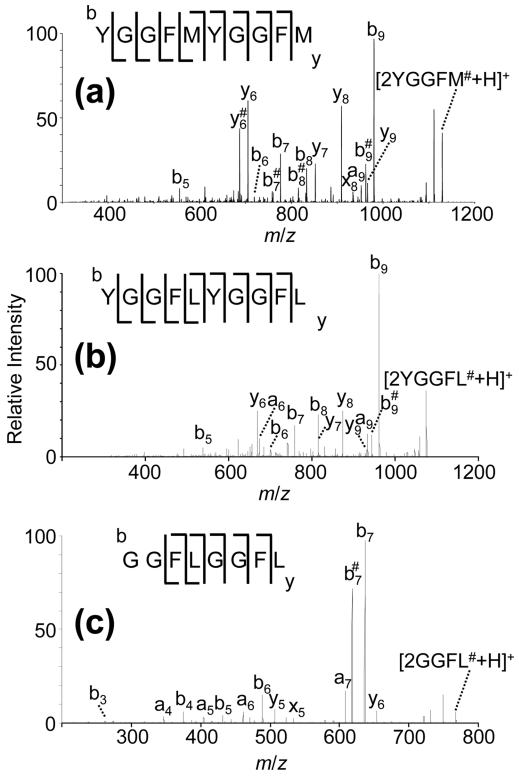


**Figure 3.** CID spectra of the newly synthesized peptide ions, (a) [YGGFMYGGFM + H]$^+$, (b) [YGGFLYGGFL + H]$^+$, and (c) [GGFLGGFL + H]$^+$, obtained from photoexcitation of [2YGGFM + H]$^+$, [2YGGFL + H]$^+$, and [2GGFL + H]$^+$ dimer ions, respectively. # indicates water loss. Peaks are assigned according to the peptide sequence in each spectrum.

complete sequence coverage of the newly synthesized peptides. Furthermore, each CID spectrum is indistinguishable from that obtained upon fragmentation of individual [YGGFMYGGFM + H]$^+$, [YGGFLYGGFL + H]$^+$, and [GGFLGGFL + H]$^+$ ions, where the precursor peptides (here, used as standards) were produced by solid-phase synthesis (see the Supporting Information). These results indicate that three peptide ions from three homodimer complexes are truly synthesized with the direct UV excitation approach.

**Formation of Mixed Peptide Ions from Heterodimer Complexes.** Photoexcitation of a heterodimer complex produces two peptide isomers because each N-/C-terminal side of the complex can be linked. Both peptide YGGFLYGGFM and YGGFMYGGFL sequences have identical $m/z$ values of 1111.5 for singly protonated species and are simultaneously obtained from the heterodimer [YGGFM + YGGFL + H]$^+$ ions upon UV laser irradiation. The CID analysis confirms the formation of the newly synthesized peptides via the coupling reaction of the complex by UV excitation. The b- and y-type ions from the two peptides are shown in one fragmentation spectrum (Figure 4a). Therefore, the mixed fragmentation pattern makes identification of the newly synthesized peptide ions complicated. Several y-type ions ($y_6$, $y_7$, $y_8$, and $y_9$ ions) of both peptides appear at the same $m/z$ values of 687.3, 834.4, 891.4, and 948.4, respectively, because the only difference in their sequences is the reversed location between Leu and Met amino acid residues. However, there are many unique peaks observed in the CID spectrum that can be used to distinguish the two peptide sequences. The peaks at $m/$




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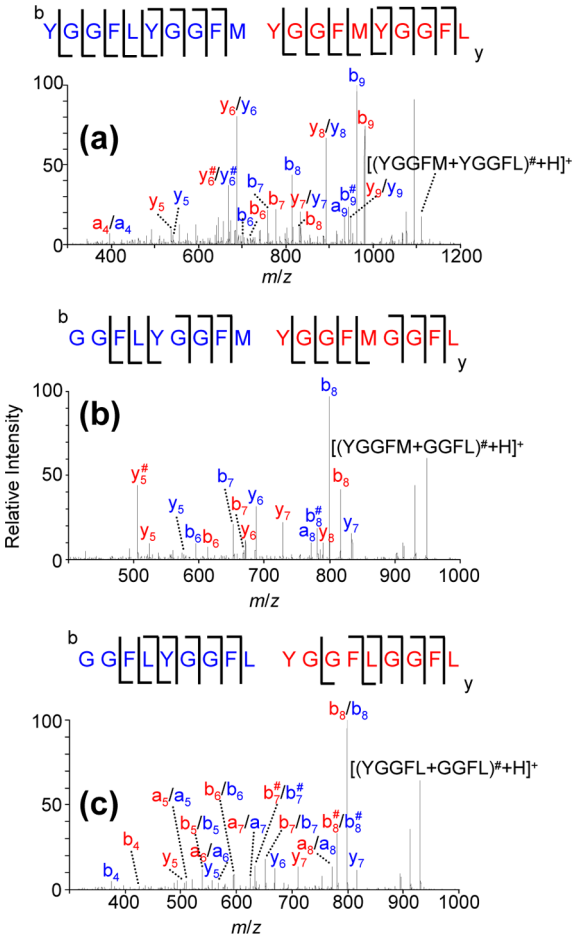


**Figure 4.** CID spectra of the newly synthesized peptides ions, (a) $[YGGFMYGGFL + H]^+$ and $[YGGFMYGGFL + H]^+$, (b) $[GGFLYGGFM + H]^+$ and $[YGGFMGGFL + H]^+$, and (c) $[GGFLYGGFL + H]^+$ and $[YGGFLGGFL + H]^+$, obtained from photoexcitation of $[YGGFM + YGGFL + H]^+$, $[YGGFM + GGFL + H]^+$, and $[YGGFL + GGFL + H]^+$ dimer ions, respectively. # indicates water loss. Peaks are assigned according to peptide sequences in each spectrum. Fragments in color are generated from the peptide sequence of the same color.

$z$ = 701.3, 758.3, 815.4, and 962.4 can be specifically assigned to $b_6$, $b_7$, $b_8$, and $b_9$ ions, respectively, of the YGGFLYGGFM peptide. The fragments observed at $m/z$ = 719.3, 776.3, 833.3, and 980.4 correspond to $b_6$, $b_7$, $b_8$, and $b_9$ ions, respectively, of the YGGFMYGGFL peptide. The observation of several distinctive b-type ions allows identification of each peptide ion.

Photoexcitation of the $[YGGFM + GGFL + H]^+$ complex results in the formation of GGFLYGGFM and YGGFMGGFL peptide ions having the same $m/z$ value of 948.4. Two newly synthesized peptides have different first and last amino acid residues in their sequences. As a result, $m/z$ values of b- and y-type fragments are all distinguishable. In this case, although the fragmentation patterns are overlapped, the identification of the peptide sequences is less problematic due to their unique fragments. As shown in Figure 4b, collisional activation of the water-loss products obtained from UV excitation of the $[YGGFM + GGFL + H]^+$ complex gives rise to unique b- and y-type ions for GGFLYGGFM and YGGFMGGFL peptides. Similar to the CID of the two peptides synthesized from the $[YGGFM + YGGFL + H]^+$ complex, several b-type ions with identical $m/z$ values are generated by collisional activation of two product ions ($[GGFLYGGFL + H]^+$ and $[YGGFLGGFL + H]^+$ at $m/z$ = 930.5) obtained from the $[YGGFL + GGFL + H]^+$ complex. However, distinctive y-type ions ($y_5$, $y_6$, and $y_7$ ions for GGFLYGGFL; $y_5$ and $y_7$ ions for YGGFLGGFL) are useful for identifying individual peptides (Figure 4c).

**Formation of Specific Peptide Ions from Heterodimer Complexes.** To avoid the simultaneous syntheses of two peptides from a heterodimer complex, specific modification can be incorporated into the starting peptide sequence. As listed in Table 1, three heterodimer complexes containing acetylated tripeptide (acAAA) lead to three specific peptide sequences. The N-terminal modification of AAA peptide prevents the UV-induced coupling reaction between the modified N-terminus of the acAAA peptide and C-terminus of other peptide. Therefore, free C-terminus of acAAA peptide links to the N-terminus of the other peptide, which results in specific peptide ions. It is important to note that the N- or C-terminal modification can be employed in a mixture of starting peptides to produce specific peptide sequences.

Acetylated peptide $[acAAAYGGFM + H]^+$ ions are obtained by selecting and photoactivating the heterodimer $[acAAA + YGGFM + H]^+$ ions. As shown in Figure 5a, several fragments

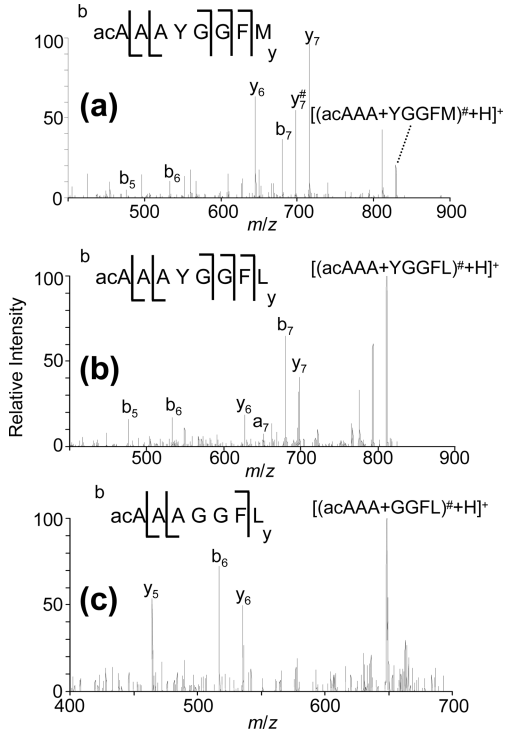


**Figure 5.** CID spectra of the newly synthesized peptides ions, (a) $[acAAAYGGFM + H]^+$, (b) $[acAAAYGGFL + H]^+$, and (c) $[acAAAGGFL + H]^+$, obtained from photoexcitation of $[acAAA + YGGFM + H]^+$, $[acAAA + YGGFL + H]^+$, and $[acAAA + GGFL + H]^+$ dimer ions, respectively. # indicates water loss. Peaks are assigned according to the peptide sequences in each spectrum.

at $m/z$ = 476.3, 533.2, 645.3, 680.3, and 716.3 corresponding to $b_5$, $b_6$, $y_6$, $b_7$, and $y_7$ ions, respectively, are observed from CID analysis of the gas-phase synthesized acAAAYGGFM peptide. Incorporating a modified peptide in the dimer complex makes fragmentation spectrum much simpler. Similarly, the other heterodimers $[acAAA + YGGFL + H]^+$ and $[acAAA + GGFL + H]^+$ ions result in specific peptide acAAAYGGFL and




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acAAAGGFL sequences, respectively, by coupling the C-terminus of the acAAA peptide with the N-terminus of the other peptide (Figure 5b,c). On the basis of their sequences, observed CID fragments of the newly synthesized peptides are fully assigned.

## CONCLUSIONS

We have described a simple strategy for the gas-phase synthesis of combinatorial libraries. Upon electrospraying a mixture of four starting peptides, a total of nine noncovalently bound homo- and heterodimer ions are generated. Each dimer complex can be isolated and activated with UV irradiation, leading to a covalent bond formation between N- and C-termini of two starting peptides. Because the UV-induced coupling reaction occurs in a mass spectrometer, subsequent CID analysis of the newly synthesized peptides makes it possible to identify individual peptide sequences. It is noteworthy that the proof-of-principle demonstration here could be utilized as a rapid means of synthesizing large numbers of sequences for MS/MS characterization. This type of approach would allow MS/MS data to be recorded for many types of sequences without the need to synthesize material in bulk.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02179.

Fragmentation spectra of $[GGFLGGLF + H]^+$ ions (PDF)

## AUTHOR INFORMATION


### Corresponding Author

*E-mail: clemmer@indiana.edu.

### Present Address

†S.L.: Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea.


### Notes

The authors declare no competing financial interest.

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