Highly Sensitive Determination of Amino Acids by LC-MS under Neutral Conditions

Ryota Morimoto, Takumi Matsumoto, Mayuri Minote, Masayuki Yanagisawa, Ryotaro Yamada, Takefumi Kuranaga,* and Hideaki Kakeya*

Department of System Chemistry and Molecular Sciences, Division of Bioinformatics and Chemical Genomics, Graduate School of Pharmaceutical Sciences, Kyoto University; Yoshida, Sakyo-ku, Kyoto 606–8501, Japan.

Received November 27, 2020; accepted December 14, 2020

Peptide drug leads possess unusual structural features that allow them to exert their unique biological activities and ideal physicochemical properties. In particular, these peptides often have D-amino acids, and therefore the absolute configurations of the component amino acids have to be elucidated during the structural determination of newly isolated peptide drug leads. Recently, we developed the highly sensitive labeling reagents d/L-FDVDA and d/L-FDLDA for the structural determination of the component amino acids in peptides. In an LC-MS-based structural study of peptides, these reagents enabled us to detect infinitesimal amounts of amino acids derived from mild degradative analysis of the samples. Herein, we firstly report the improved LC-MS protocols for the highly sensitive analyses of amino acids. Second, two new labeling reagents were synthesized and their detection sensitivities evaluated. These studies increase our understanding of the structural basis of these highly sensitive labeling reagents, and should provide opportunities for future on-demand structural modifications of the reagents to enhance their hydrophobicity, stability, and affinity for applications to specialized HPLC columns.

Key words natural product; structural determination; peptide; D-amino acid

Introduction

Among naturally occurring bioactive compounds,1) mid-sized peptide natural products have attracted significant attention from pharmaceutical scientists owing to their low toxicity and high specificity to target proteins.2) These peptides possess unusual structural features that allow them to exert their unique biological activities and ideal physicochemical properties as drug leads.3) In particular, mid-sized peptide natural products often have D-amino acids, and therefore, the absolute configurations of the component amino acids have to be elucidated during the structural determination of the newly isolated peptide drug leads such as teixobactin4–6) and lysocine E.7–10) However, the absolute configurations of the component amino acids in peptidic natural products cannot be directly determined by NMR and MS/MS, which are used for the determination of the planar structures.

Among the elucidation strategies available for the stereochemistry determination of the component amino acids, Marfey’s method11) is one of the most widely accepted procedures. First, the peptide is completely hydrolyzed to its component amino acids; second, the amino acids are labeled by the reagent for optical resolution; third, the labeled amino acids are chromatographically compared with the standard samples to determine the absolute configuration. This strategy is a powerful tool, not only for the structural elucidation of newly identified natural products but also for confirmation of a total synthesis.12,13) However, the degraded peptide cannot be recovered after using this method, and problems inevitably arise during the peptide hydrolysis stage. To obtain a higher yield of the analyte, peptide 1 has to be hydrolyzed under harsh conditions, which also increases the yield of the epimer 2, leading to the structural misassignment. In contrast, hydrolysis under milder conditions suppresses epimerization. However, the yield of the target hydrolysates is also decreased (Fig. 1).

Results

Recently, inspired by studies on the scarce natural product yaku’amide B,14) we developed the highly sensitive-advanced Marfey’s method (HS-advanced Marfey method) by using our new labeling reagents d/L-FDVDA and d/L-FDLDA for the determination of amino acids15) (Fig. 2a). The new reagents enabled us to obtain the stereochemical information on the component amino acids resulting from mild degradative analysis under smaller amounts of peptide. As a result of our continuing research into the use of these reagents for peptide structural determination, we have optimized the LC-MS conditions and synthesized analogues of the labeling reagents. Herein, we firstly report a method for highly sensitive amino acid analysis under neutral conditions. Second, two newly synthesized labeling reagents 5 and 6, were also investigated in an effort to further understand the structural basis of our reagents (Fig. 2b).

Generally, acidic mobile phase additives (e.g., trifluoroacetic acid (TFA) and formic acid) enhance the sensitivity of

Fig. 1. Problems Encountered the Structural Elucidation of Peptide
(Color figure can be accessed in the online version.)
the analyte for positive ion electrospray ionization (ESI). 16) We have observed higher sensitivity for LC-MS analyses of labeled amino acids under acidic conditions, and reported the structural determination of thioamicolamides, a group of peptidic natural products, using Marfey’s analysis under acidic conditions 17) just before the development of our reagents, D/L-FDVA and D/L-FDLDA. However, it is also well known that an acidic mobile phase can damage a silica gel-based HPLC column. Furthermore, “LC-MS grade” reagents should be used as the additives for LC-MS analysis, otherwise the reliability of the analysis decreases because of trace impurities. If the labeled amino acids can be detected at high sensitivity under neutral conditions, costly LC-MS-grade acidic additives would not be required.

In the preceding study, the ability of 3 and 4 to aid in the separation of diastereomeric derivatives of 20 proteinogenic amino acids was also tested under acidic conditions as well as with conventionally used labeling reagents 18–20). In this report, we investigated the potential of our reagents to be used without acidic additives because our reagents have a tertiary amine, which is easily ionized even under neutral conditions. 21) Initially, the sensitivities of derivatives 3 and 4 under neutral conditions were examined.

The LC-MS analysis of L-leucine was conducted under acidic and neutral conditions (Fig. 3). Under neutral conditions, analytes 7 and 8, labeled with conventional reagents L-FDAA/L-FDVA, were hardly detected even at the same concentration at which they were detectable under acidic conditions. In contrast, analytes 9 and 10, labeled with our reagents 3/4, were clearly detected even without acidic additives. This result suggested that our reagents were useful to analyze amino acids with high sensitivity in the presence of a neutral mobile phase.

Encouraged by these results, the diastereomeric resolutions with 3 and 4 under neutral conditions were then investigated (Table 1). As was the case for the analysis under acidic conditions, all diastereomeric derivatives were chromatographically separated without modifying the HPLC conditions. Although the analysis was performed with a conventional reversed-phase octadecyl silyl (ODS) HPLC column, excellent resolution was observed for all amino acids. Notably, D/L-Asn, Gln and His, which are hardly separable using previously reported reagents, 19) were more clearly resolved with high sensitivities by labeling with 3 and 4 under neutral conditions (Fig. 4).

For future structural modifications of these labeling reagents, the mechanism of their high sensitivities was studied in detail. To extract the structural basis of the labeling technique, two new analogues, 5 and 6, were designed and synthesized (Chart 1). At the outset, to evaluate the importance of the amino acid side chain in the reagent, compound 5 was synthesized from L-phenylalanine in the same manner as for the synthesis of 3 and 4. Next, compound 6 was synthesized to assess the value of the amide bond. The C-terminus of yaku’amide B (22) 22) was directly attached onto 1,5-difluoro-
2,4-dinitrobenzene (DFDNB) under nucleophilic aromatic substitution ($\text{SN}_\text{Ar}$) reaction conditions to afford 6.

With the newly developed reagents in hand, the sensitivities of 5 and 6 under neutral conditions were then investigated. First, L-Leu was labeled by 5 and 6. Second, the derivatives 7, 10, 23, and 24 were equally quantified by UV absorbance (340 nm) derived from the fluorodinitrobenzene structure. Third, the detection sensitivities of the four derivatives were compared by LC-MS under neutral conditions (Fig. 5).

As expected, the detection sensitivities of the new derivatives 23 and 24 surpassed that of 7, which is almost the same as 10. The substitution of the amino acid side chain and removal of the amide bond did not affect the sensitivity in the design of the reagents. These results indicate that in the future, on-demand structural modifications of the reagents should be possible to enhance their hydrophobicity, stability, and affinity for specialized HPLC columns other than ODS.

**Conclusion**

In summary, the highly sensitive determinations of amino acids under the neutral conditions were demonstrated by the originally developed labeling reagents L-FDVDA and L-FDLDA. The amino acids labeled by 3 and 4 were able to be detected in the LC-MS experiments without acidic additives and even under the extremely diluted conditions. Importantly, the diastereomeric separation of the labeled Asn, Gln, and His was drastically improved under the neutral conditions compared with the acidic conditions. Second, the abilities of two newly synthesized labeling reagents, 5 and 6, were also investigated to further understand the importance of the reagent structure to their function. Both reagents also showed high sensitivities similar to 3 and 4 without any acidic additives, confirming the importance of the tertiary amine as a key structural motif of our highly sensitive labeling reagents. These results demonstrate the potential of our reagents for

**Table 1. LC-MS Analysis of 20 Labeled Amino Acids under Neutral Conditions**

| amino acid | elution order | t (L) | t (D) | $\Delta t$ | elution order | t (L) | t (D) | $\Delta t$ |
|------------|---------------|------|------|----------|---------------|------|------|----------|
| Ala        | L $\rightarrow$ D | 12.6 | 22.6 | 10.0     | L $\rightarrow$ D | 20.5 | 26.8 | 6.3      |
| Val        | L $\rightarrow$ D | 17.5 | 28.7 | 11.2     | L $\rightarrow$ D | 23.2 | 32.4 | 9.2      |
| Leu        | L $\rightarrow$ D | 23.6 | 31.9 | 8.3      | L $\rightarrow$ D | 28.8 | 35.7 | 6.9      |
| Ile        | L $\rightarrow$ D | 21.2 | 31.5 | 10.3     | L $\rightarrow$ D | 26.6 | 35.1 | 8.5      |
| Phe        | L $\rightarrow$ D | 23.9 | 31.4 | 7.5      | L $\rightarrow$ D | 28.0 | 34.8 | 6.8      |
| Thr        | L $\rightarrow$ D | 8.7  | 21.5 | 12.8     | L $\rightarrow$ D | 16.8 | 25.4 | 8.6      |
| Ser        | L $\rightarrow$ D | 8.2  | 17.7 | 9.5      | L $\rightarrow$ D | 16.8 | 22.4 | 5.6      |
| His        | L $\rightarrow$ D | 20.6 | 24.2 | 3.6      | L $\rightarrow$ D | 25.1 | 27.4 | 2.3      |
| Lys (12)   | L $\rightarrow$ D | 42.4 | 49.4 | 7.0      | L $\rightarrow$ D | 48.6 | 56.8 | 8.2      |
| Trp        | L $\rightarrow$ D | 23.5 | 30.5 | 7.0      | L $\rightarrow$ D | 27.3 | 33.7 | 6.4      |
| Asp        | L $\rightarrow$ D | 3.8  | 6.3  | 2.5      | L $\rightarrow$ D | 7.3  | 13.0 | 5.7      |
| Asn        | L $\rightarrow$ D | 7.3  | 15.7 | 8.4      | L $\rightarrow$ D | 14.7 | 21.0 | 6.3      |
| Glu        | L $\rightarrow$ D | 5.1  | 13.6 | 8.5      | L $\rightarrow$ D | 10.9 | 20.3 | 9.4      |
| Gln        | L $\rightarrow$ D | 8.1  | 16.4 | 8.3      | L $\rightarrow$ D | 17.4 | 21.6 | 4.2      |
| Arg        | L $\rightarrow$ D | 27.2 | 32.7 | 5.5      | L $\rightarrow$ D | 31.7 | 36.6 | 4.9      |
| Met        | L $\rightarrow$ D | 21.2 | 28.0 | 6.8      | L $\rightarrow$ D | 24.9 | 31.5 | 6.6      |
| Tyr (11)   | L $\rightarrow$ D | 45.0 | 53.2 | 8.2      | L $\rightarrow$ D | 51.9 | 58.1 | 6.2      |
| Pro        | L $\rightarrow$ D | 15.3 | 24.3 | 9.0      | L $\rightarrow$ D | 21.3 | 28.3 | 7.0      |
| Cys (13)   | L $\rightarrow$ D | 40.4 | 47.6 | 7.2      | L $\rightarrow$ D | 45.6 | 54.3 | 8.7      |

| Gly         | 18.5          | 23.2          |
| 3/4         | 42.5          | 46.6          |
| 14/15       | 23.8          | 29.3          |

LC-MS conditions; column: Cosmosil 5C18-AR-II $\phi$ $\times$ 250 mm; eluent: MeCN/H$_2$O = 20/80 (0–10 min) 20/80 to 100/0 (10–70 min) 100/0 (70–80 min); 40°C; detection: ESI-positive, see supplementary materials for details. (Color figure can be accessed in the online version.)
future on-demand structural modifications. Future studies will include the development of other highly sensitive labeling reagents and more advanced applications.

**Experimental**

**General Remarks**

$^1$H- and $^{13}$C-NMR spectra were recorded on a JEOL ECZ600 (600 MHz for $^1$H-NMR and 150 MHz for $^{13}$C-NMR) spectrometer. Chemical shifts are denoted in $\delta$ (ppm) relative to residual solvent peaks as the internal standard (CDCl$_3$, $^1$H $\delta$ 7.26, $^{13}$C $\delta$ 77.0). ESI-MS and LC-MS experiments were recorded on a Shimadzu LCMS-IT-TOF or Shimadzu LC-MS 8040 system. Optical rotations were recorded on a JASCO P-2200 polarimeter. HPLC experiments were performed with a SHIMADZU HPLC system equipped with a LC-20AD intelligent pump. All reactions sensitive to air and/or moisture were conducted under a nitrogen atmosphere using dry, freshly distilled solvents unless otherwise noted. All reagents were used as supplied unless otherwise stated. Analytical TLC was performed using E. Merck Silica gel 60 F254 pre-coated plates. Silica gel column chromatography was performed using 40–50 $\mu$m Silica Gel 60N (Kanto Chemical Co., Inc., Tokyo, Japan).

**Laboratory Instruments**

1. **General:***
   - General Remarks
   - General Remarks

2. **Methods:***
   - General Remarks
   - General Remarks

3. **Results:***
   - General Remarks
   - General Remarks

4. **Discussion:***
   - General Remarks
   - General Remarks

5. **Conclusion:***
   - General Remarks
   - General Remarks

---

**Chart 1. The Synthesis of 5 and 6**

(Color figure can be accessed in the online version.)

**Fig. 4. LC-MS Chromatograms of 16–19**

LC-MS conditions; column: Cosmosil 5C18-AR-II φ2 × 250 mm; eluent for acidic condition: MeCN/H$_2$O/formic acid = 20/80/0.1 (0–10 min) 20/80/0.1 to 100/0/0.1 (10–70 min) 100/0/0.1 (70–80 min); eluent for neutral condition: MeCN/H$_2$O = 20/80 (0–10 min) 20/80 to 100/0 (10–70 min) 100/0 (70–80 min); 0.2 mL/min; 40°C; detection: ESI-positive, see supplementary materials for details. (Color figure can be accessed in the online version.)

**Fig. 5. LC-MS Chromatograms of 7, 10, 23, and 24 under Neutral Conditions**

LC-MS conditions; column: COSMOSIL 5C18-AR-II φ2 × 250 mm; eluent: MeCN/H$_2$O = 40/60 (isocratic), 0.2 mL/min; 40°C; detection: ESI-positive, UV = 340 nm, see supplementary materials for details. (Color figure can be accessed in the online version.)
tral conditions: The samples were prepared in the same manner as the preceding study,\textsuperscript{15} and then analyzed by LC-MS [column: Cosmosil 5C18-AR-II \( \varphi \times \bar{\varphi} \times \Phi \); eluent: MeCN/H\(_2\)O (20/80 (0–10 min) 20/80 to 100/0 (10–70 min) 0/100 (70–80 min); 40°C; detection: ESI-positive).

LC-MS chromatograms of \textbf{16–19}: The samples were prepared in the same manner as the preceding study,\textsuperscript{15} and then analyzed by LC-MS [column: Cosmosil 5C18-AR-II \( \varphi \times \varphi \times \varphi \); eluent for acidic condition: MeCN/H\(_2\)O/formic acid = 20/80/0.1 (0–10 min) 20/80/0.1 to 100/0/0.1 (10–70 min) 100/0/0.1 (70–80 min); eluent for neutral condition: MeCN/H\(_2\)O = 20/80 (0–10 min) 20/80 to 100/0 (10–70 min) 0/100 (70–80 min); 0.2 mL/min; 40°C; detection: ESI-positive).

Amide \textbf{21}: To a solution of tert-butoxycarbonyl (Boc)-L-Phe-OH (204 mg, 1.06 g, 4.00 mmol) in tetrahydrofuran (THF) 20 mL were added N-methylmorpholine (NMM) (0.48 mL, 4.4 mmol) and isobutyl chloroformate (0.58 mL, 4.4 mmol) at 0°C. After the mixture was stirred for 1 h, the reaction mixture was concentrated to give the corresponding amine hydrochloride salt, which was used in the next reaction without further purification.

To a solution of the above amine in acetone (2.0 mL) were added saturated aqueous NaHCO\(_3\) (2.4 mL) and DFDNB (83.1 mg, 0.407 mmol) at room temperature. After being stirred for 1 h, the reaction mixture was concentrated to give the crude amine, which was used in the next reaction without further purification.

To a solution of the above amine in acetone (1.2 mL) were added saturated aqueous NaHCO\(_3\) (0.610 mmol) in CH\(_2\)Cl\(_2\) (16.8 mL) was added TFA (7.2 mL) at 0°C. After being stirred at 0°C for 1 h, saturated aqueous NaHCO\(_3\) was added to the reaction mixture. The resulting solution was extracted with EtOAc three times. The combined organic layer was washed with brine, dried over MgSO\(_4\), and concentrated. The residue was purified by column chromatography (MeOH/CHCl\(_3\) = 5:95) to afford \textbf{6} (108 mg, 93% for 2 steps) as a yellow solid: [\(\alpha\)]\textsubscript{D}\textsuperscript{20} = −4.78 (c = 1.00, acetone); \textsuperscript{1}H-NMR (600 MHz, CDCl\(_3\)) \( \delta \) 9.12 (d, \( J = 7.6 \) Hz, 1H), 8.74 (d, \( J = 7.6 \) Hz, 1H), 6.71 (d, \( J = 13.8 \) Hz, 1H), 3.57 (m, 1H), 2.48 (m, 2H), 2.25 (s, 6H), 2.11 (m, 1H), 1.03 (d, \( J = 6.9 \) Hz, 3H), 0.98 (d, \( J = 6.9 \) Hz, 3H); \textsuperscript{13}C-NMR (150 MHz, CDCl\(_3\)) \( \delta \) 160.8, 159.0, 149.7, 128.2, 127.3, 125.4, 101.5, 60.6, 57.4, 46.0, 30.1, 18.7, 17.4; HRMS (ESI) C\(_{19}\)H\(_{23}\)FN\(_5\)O\(_5\) [M + H\textsuperscript{+}]\textsuperscript{250 mm; eluent for acidic condition: MeCN/H\(_2\)O/formic acid = 20/80/0.1 (0–10 min) 20/80/0.1 to 100/0/0.1 (10–70 min) 100/0/0.1 (70–80 min); eluent for neutral condition: MeCN/H\(_2\)O = 20/80 (0–10 min) 20/80 to 100/0 (10–70 min) 0/100 (70–80 min); 0.2 mL/min; 40°C; detection: ESI-positive).

Acknowledgments We thank Ms. Haruka Ogawa for the preliminary experiments of this study. This work was financially supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan [17H06401 (H.K.), 18K14396 (T.K.), and 19H02840 (H.K.)], and the Platform Project for Supporting Drug Discovery and Life Science Research from the Japan Agency for Medical Research and Development (AMED), Contributions from the SUNBOR GRANT, the Daiichi-Sankyo Award in The Society of Synthetic Organic Chemistry Japan, the Takeda Science Foundation, and The Tokyo Biochemical Research Foundation to T.K. are also greatly appreciated.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References

1) Kakeya H., Nat. Prod. Rep., 33, 648–654 (2016).
2) Fenger-K., Hoffmann T., Drug Discov. Today, 20, 122–128 (2015).
3) Tang D., Sussmann R. D., Acc. Chem. Res., 50, 1566–1576 (2017).
4) Ling L. C., Schneider T., Peoples A. J. et al., Nature (London), 517, 455–459 (2015).
5) Jin K., Sam I. H., Po K. H. L., Lin D., Ghazvini Zadeh E. H., Chen S., Yuan Y., Li X., Nat. Commun., 5, 12394 (2016).
6) Zong Y., Fang F., Meyer K. J., Wang L., Ni Z., Gao H., Lewis K., Zhang J., Rao Y., Nat. Commun., 10, 3268 (2019).
7) Hamamoto H., Urai M., Ishii K., Yasukawa J., Paudel A., Murai M., Kaji T., Kurita N., Tomoda H., Yamada M., Souma M., Kurihara H., Inoue M., Sekimizu K., Nat. Chem. Biol., 11, 127–133 (2015).
8) Murai M., Kaji T., Kuranaga T., Hamamoto H., Sekimizu K., Inoue M., Angew. Chem. Int. Ed., 54, 1556–1560 (2015).
9) Kaji T., Murai M., Itoh H., Yasukawa J., Hamamoto H., Sekimizu K., Inoue M., Chem. Eur. J., 22, 16912–16919 (2016).
10) Itoh H., Tokumoto K., Kaji T., Paudel A., Hamamoto H., Sekimizu K., Inoue M., Nat. Commun., 10, 2992 (2019).
11) Marfey P., Bilo V., Kirchhofer H., Carlsberg Res. Commun., 7746–7752 (2014).
12) Yamashita T., Matoba H., Kuranaga T., Inoue M., Tetrahedron, 70, 7746–7752 (2014).
13) Pan C., Kuranaga T., Kakeya H., J. Nat. Med., (2021), in press. https://doi.org/10.1007/s11418-020-01472-z.
14) Kuranaga T., Mutoh H., Sesoko Y., Goto T., Matsunaga S., Inoue M., *J. Am. Chem. Soc.*, 137, 9443–9451 (2015).
15) Kuranaga T., Minote M., Morimoto R., Pan C., Ogawa H., Kakeya H., *ACS Chem. Biol.*, 15, 2499–2506 (2020).
16) Liigand J., Laaniste A., Kruve A., *J. Am. Soc. Mass Spectrom.*, 28, 461–469 (2017).
17) Pan C., Kuranaga T., Liu C., Lu S., Shinzato N., Kakeya H., *Org. Lett.*, 22, 3014–3017 (2020).
18) Harada K., Fujii K., Hayashi K., Suzuki M., Ikai Y., Oka H., *Tetrahe- 
dron Lett.*, 37, 3001–3004 (1996).
19) Fujii K., Ikai Y., Mayumi T., Oka H., Suzuki M., Harada K., *Anal. Chem.*, 69, 3346–3352 (1997).
20) Fujii K., Ikai Y., Oka H., Suzuki M., Harada K., *Anal. Chem.*, 69, 5146–5151 (1997).
21) Suo R., Watanabe R., Takada K., Suzuki T., Oikawa H., Itoi S., Sugita H., Matsunaga S., *Org. Lett.*, 22, 1254–1258 (2020).
22) Kuranaga T., Sesoko Y., Sakata K., Maeda N., Hayata A., Inoue M., *J. Am. Chem. Soc.*, 135, 5467–5474 (2013).