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MALDI TOF/TOF-Based Approach for the Identification of D- Amino Acids in Biologically Active Peptides and Proteins

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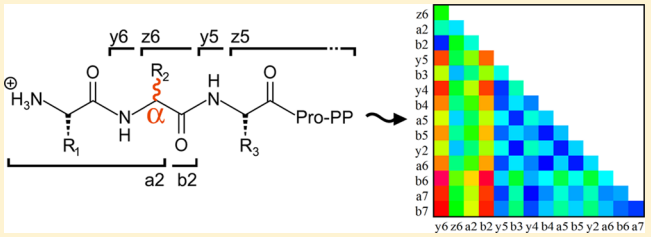
Supporting Information

ABSTRACT: Several biologically active peptides contain a D-amino acid in a well-defined position, which is position 2 in all peptide epimers isolated to date from vertebrates and also some from invertebrates. The detection of such D- residues by standard analytical techniques is challenging. In tandem mass spectrometric (MS) analysis, although fragment masses are the same for all stereoisomers, peak intensities are known to depend on chirality. Here, we observe that the effect of a D-amino acid in the second N-terminal position on the fragmentation pattern in matrix assisted laser desorption time-of-flight spectrometry (MALDI-TOF/TOF MS) strongly depends on the peptide sequence. Stereosensitive fragmentation (SF) is correlated to a neighborhood effect, but the D- residue also exerts an overall effect influencing distant bonds. In a fingerprint analysis, multiple peaks can thus serve to identify the chirality of a sample in short time and potentially high throughput. Problematic variations between individual spots could be successfully suppressed by cospotting deuterated analogues of the epimers. By identifying the [D-Leu2] isomer of the predicted peptide GH-2 (gene derived bombininH) in skin secretions of the toad *Bombina orientalis*, we demonstrated the analytical power of SF-MALDI-TOF/TOF measurements. In conclusion, SF-MALDI-TOF/TOF MS combines high sensitivity, versatility, and the ability to complement other methods.



KEYWORDS: *post-translational modification, D- amino acid containing peptide, chirality, peptidomics, proteomics, isotope label*

INTRODUCTION

Bioactive peptides are involved in numerous biological functions and serve important functions as hormones and neuropeptides for cellular signaling, as secretory peptides for interspecies communication, or as peptide toxins as well as defense peptides against microbes and predatory animals.[1] For the understanding of all of these processes, the knowledge and analysis of their primary structure is essential. In consequence, an array of adequate techniques such as amino acid analysis or tandem MS sequencing is available, which allows for the routine determination of amino acid sequences and the majority of chemical modifications. Some peptides, however, carry a perfidiously unsuspicious post-translational modification that is not readily detected by standard analytical measurements. In these peptides and some proteins, the dogma of ubiquitous protein homochirality is violated by a single D- amino acid substitution. In some cases, a D- residue in peptide linkage can result from age-dependent racemization.[2] However, during peptide biosynthesis, which is completely unrelated to aging, the D- amino acid is generated from the corresponding L-isomer present within the precursor polypeptide by the action of peptidyl-aminoacyl-L/D-isomerases. Interestingly, all L/D-isomerases studied to date from vertebrates act exclusively on the second N-terminal amino acid residue.[3−7] The first peptide, which was found to be processed by such an enzyme, was dermorphin isolated from the skin of a South American tree frog.[8] Further, the discovery of dermorphin as well as the related deltorphins[9] was facilitated by the striking effect of the D- residue on biological activity (i.e., the all-L peptides were inactive). By contrast, more subtle effects were observed in other vertebrate peptides, for which a natural form with a D-amino acid exists as well, for example, the bombinins H from frog skin,[10] and, interestingly, a C-type natriuretic peptide and a β-defensin-like peptide from the venom of male platypus, a primitive mammal.[11,12]

The analysis of D- amino acids within polypeptides poses a challenge,[13] mainly due to two limitations: first, although Edman degradation gives rise to chiral phenylthiohydantoin amino acid derivatives, for the resolution of the enantiomers, chiral media are required;[14] second, stereoisomerisation does not lead to any detectable mass differences. Moreover, fragment masses are identical for all stereoisomers in tandem MS experiments, which nowadays is the commonly used sequenc-

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Table 1. Peptide Epimers Tested by MALDI-TOF/TOF-MS[a]

Sequence	Mass	*R*					
		a2	b2	z6	z5	y6	y5
H-IIGPVLĊ-NH_2	770.5	1.82	1.32	1.27	1.52	1.26	1.28
		(2.60)	(1.77)	(1.66)	(2.15)	(1.86)	(1.96)
H-GIGPVLĊ-NH_2	715.0	-	1.19	1.18	1.18	1.33	1.20
		(2.16)	(1.65)	(1.82)	(1.77)	(2.14)	(1.86)
H-KIGPVLĊ-NH_2	786.1	-	-	-	1.34	*2.22	1.35
					(3.36)	(5.83)	(3.38)
H-EIGPVLĊ-NH_2	787.1	-	-	-	1.39	1.39	1.87
					(2.24)	(2.25)	(3.50)
H-FIGPVLĊ-NH_2	805.1	-	-	-	1.24	1.20	1.21
					(1.53)	(1.59)	(1.59)
H-SIGPVLĊ-NH_2	745.0	-	-	-	1.29	-	1.52
					(2.71)		(3.32)
H-AIGPVLĊ-NH_2	729.0	-	-	-	1.37	1.37	1.54
					(1.85)	(2.18)	(2.60)
H-IKGPVLĊ-NH_2	786.1	1.06	1.11	1.08	1.06	1.06	1.06
		(1.19)	(1.27)	(1.16)	(1.20)	(1.13)	(1.13)
H-IEGPVLĊ-NH_2	787.1	1.47	1.30	1.24	1.38	1.21	1.22
		(1.92)	(1.64)	(1.50)	(1.81)	(1.52)	(1.54)
H-IAGPVLĊ-NH_2	729.0	-	-	1.10	1.15	1.09	1.18
				(1.28)	(1.29)	(1.24)	(1.44)
H-ISGPVLĊ-NH_2	745.0	-	1.49	1.31	1.39	1.25	1.27
			(2.30)	(1.89)	(2.11)	(1.57)	(1.60)
H-IFGPVLĊ-NH_2	805.1	1.79	1.28	1.31	1.49	1.24	1.24
		(2.29)	(1.53)	(1.59)	(1.88)	(1.76)	(1.83)
H-IIFPVLĊ-NH_2	861.2	-	-	1.89	-	4.48	1.73
				(2.87)		(7.25)	(5.27)
H-IIAPVLĊ-NH_2	785.1	-	-	1.76	2.08	4.52	2.16
				(2.78)	(2.94)	(6.90)	(5.29)
H-IIKPVLĊ-NH_2	842.2	-	1.64	1.67	2.19	2.78	1.48
			(2.54)	(3.88)	(5.31)	(4.74)	(2.75)
H-IIDPVLĊ-NH_2	829.1	-	-	1.60	1.74	2.93	1.63
				(3.31)	(3.13)	(5.62)	3.48)
H-IISPVLĊ-NH_2	801.1	-	-	1.54	1.81	3.51	1.66
				(2.70)	(2.67)	(5.47)	(4.25)
H-IITPVLĊ-NH_2	827.2	3.25	5.16	2.49	2.21	5.37	2.16
		(5.78)	(9.47)	(4.12)	(3.41)	(9.86)	(5.92)

[a]Ċ denotes *S*-carbamidomethyl-cysteine. Upper values denote *R* for a given fragment i averaged over all fragments j. Lower values in brackets denote the obtained maximum *R* for a given fragment i. When marked by an asterisk, the data for Y-ions are given instead of y-ions.

ing method. However, recent findings suggest that the stereochemistry of the backbone, modulates fragment ion abundances, which can be expressed by the so-called chiral recognition factor (R_{chiral}, henceforth referred to as *R*).[15] *R* is calculated for a pair of fragment ion peaks i,j according the formulas given in the Methods section. Early successful applications of MS/MS to identify peptide epimers using collision-induced dissociation (CID) comprise peptides with up to four residues,[15,16] and, later, segments of ω-agatoxin IVB and C, and Aβ(17−29) with a focus on serine epimerization after metal complexation.[17] In an alternative approach, electron capture dissociation (ECD) fragmentation was applied to dermorphin and another small protein, the 20-residue Trp-cage.[18] In the case of the Trp-cage, the fragmentation pattern was found to be sensitive also to the tertiary structure. Recently, radical-directed dissociation (RDD) yielded a respectable chiral discrimination when applied to dermorphin and to peptides containing D-Ala, D-Ser, or D-Asp.[19] A recent identification of crustacean hyperglycemia hormones (CHHs) epimers was achieved by using ion mobility spectrometry (IMS) analysis of MS-generated epimeric fragment ions.[20] Most promising results were obtained by application of matrix assisted laser desorption time-of-flight spectrometry (MALDI-MS/MS) metastable decay−CID to dermorphin as well as a heptapeptide derived from the sequence of natural HIV isoforms with a D- residue in varying positions (residue 2, 3, or 5).[21] MALDI-MS/MS yielded good results when validated with several short molluskan peptides (3−5 residues) and could be combined with direct measurement in *Aplysia* neurons.[22,23] However, given several successful examples of D- amino acid identification using MS techniques, stereoselective fragmentation, and the impact of residues adjacent to D- amino acids are still poorly understood.

In this study, we systematically explored the sequence dependence of the stereoselective fragmentation propensity in MALDI-TOF/TOF metastable decay−CID experiments. We focused on model peptides with a single D- amino acid residue in position 2 as peptides bearing an inversion of backbone chirality at this position might be of highest biological significance in vertebrates.[24] We investigated the effect of different D- residues in position 2 as well as the modulating effect of the nearest neighbor residues on the fragmentation pattern. Our data suggest that the stereosensitivity is highly sequence-dependent and can be indeed of practical use for the analysis of a varity of peptide sequences. As a major experimental improvement, the investigation of stereoisomers with unfavorable *R* became feasible by using a deuterated analogue of the suspected epimeric candidate. As a proof-of-

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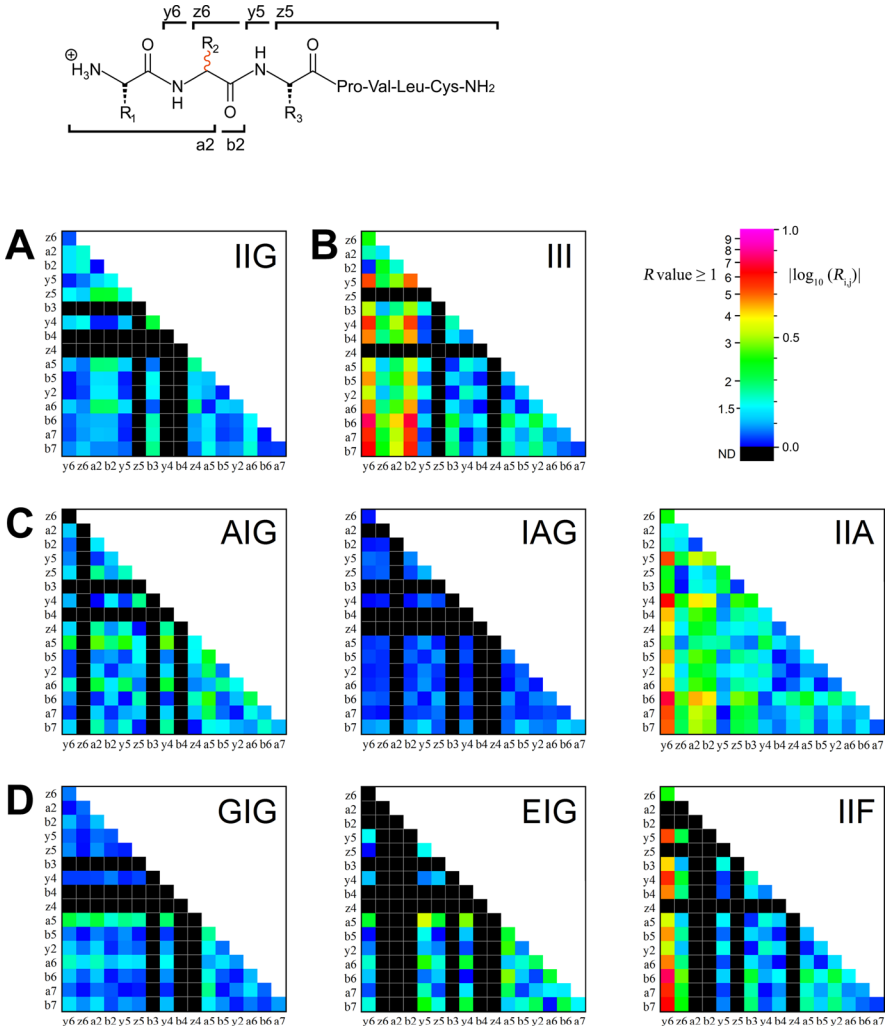

Figure 1. Fragmentation scheme of model heptapeptides templated on the N-terminal heptapeptide of bombininH epimers H2 and H4. Fragments in MALDI-TOF/TOF derived from cleavages in the neighborhood of the epimerized residue are indicated (according to the nomenclature by Roepstorff and Johnson et al.[46,47]). (A) *R* matrices of wild-type bombininH, $R_{1,2}$ = $CH(CH_3)CH_2CH_3$ (Ile), R_3 = H (Gly). (B) III demonstrates the impact of residue 3. (C) *R* matrices of alanine-scanning mutated heptapeptide. (D) *R* matrices for selected peptides: GIG demonstrates non-neighborhood SF. All *R* matrices are shown in Supplementary Figure S-1. Indices i,j are always assigned yielding $R \geq 1$.

concept, we were able to confirm the presence of a D- residue in a predicted peptide from frog skin secretions by means of SF-MALDI-TOF/TOF MS.

EXPERIMENTAL PROCEDURES

Peptide Synthesis

Peptides were synthesized on a continuous-flow synthesizer using standard Fmoc solid-phase chemistry with a Rink Amide AM resin (200−400 mesh, 0.62 meqg^{-1}; Nova-Biochem), PyBOP (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) as condensation reagent, and *N*-methylmorpholine as a base. After cleavage, peptides were derivatized with iodoacetamide and purified by reverse-phase high-performance liquid chromatography (HPLC) over a C-18 column (Vydac) with a linear gradient of acetonitrile (solvent A, 0.1% TFA; solvent B, 80% acetonitrile). N-Fmoc-Val-d8 (*N*-(9-fluorenylmethoxycarbonyl)-L-valine-2,3,4,4,4,5,5,5-d8) was from Aldrich.

MALDI-TOF/TOF Mass Spectrometry

Samples were analyzed on a MALDI-TOF/TOF 4800 analyzer (ABSciex, Framingham, MA) operated in reflector positive mode acquiring 3000 total shots per spectrum with a fixed laser intensity set at 5000. Experiments were carried out using α-cyano hydroxyl cinnamic acid (5 mg/mL in 50% (v/v) acetonitrile) as matrix. Each sample (0.3−0.5 μL) was mixed with 2−3 μL of matrix, and then 0.5 μL of the mixture was spotted on the target plate. Tandem MS experiments were carried out using a 1 kV method with and without collision-induced dissociation using air as collision gas and metastable suppression enabled. Spectra were acquired and processed using the 4800 Analyzer and Data Explorer Software. In most cases, when loss of water peaks were observed, peak areas *a* were combined with their parent ion peaks. Compared to PSD, the application of CID favored multiple fragmentations and, in such a way, complicated data analysis.

R matrices were generated for each compound according to formulas $R_{i,j} = (r_{i,j})_L/(r_{i,j})_D$ and $r_{i,j} = (a_i/a_j)$. Thereby, *a* are the peak areas of two chosen fragment ions i and j in a spectrum of epimer L or D, respectively. $r_{i,j}$ were averaged from three spectra, each of a different spot. For practical purposes, these matrices can be inspected for maximum values without further processing.

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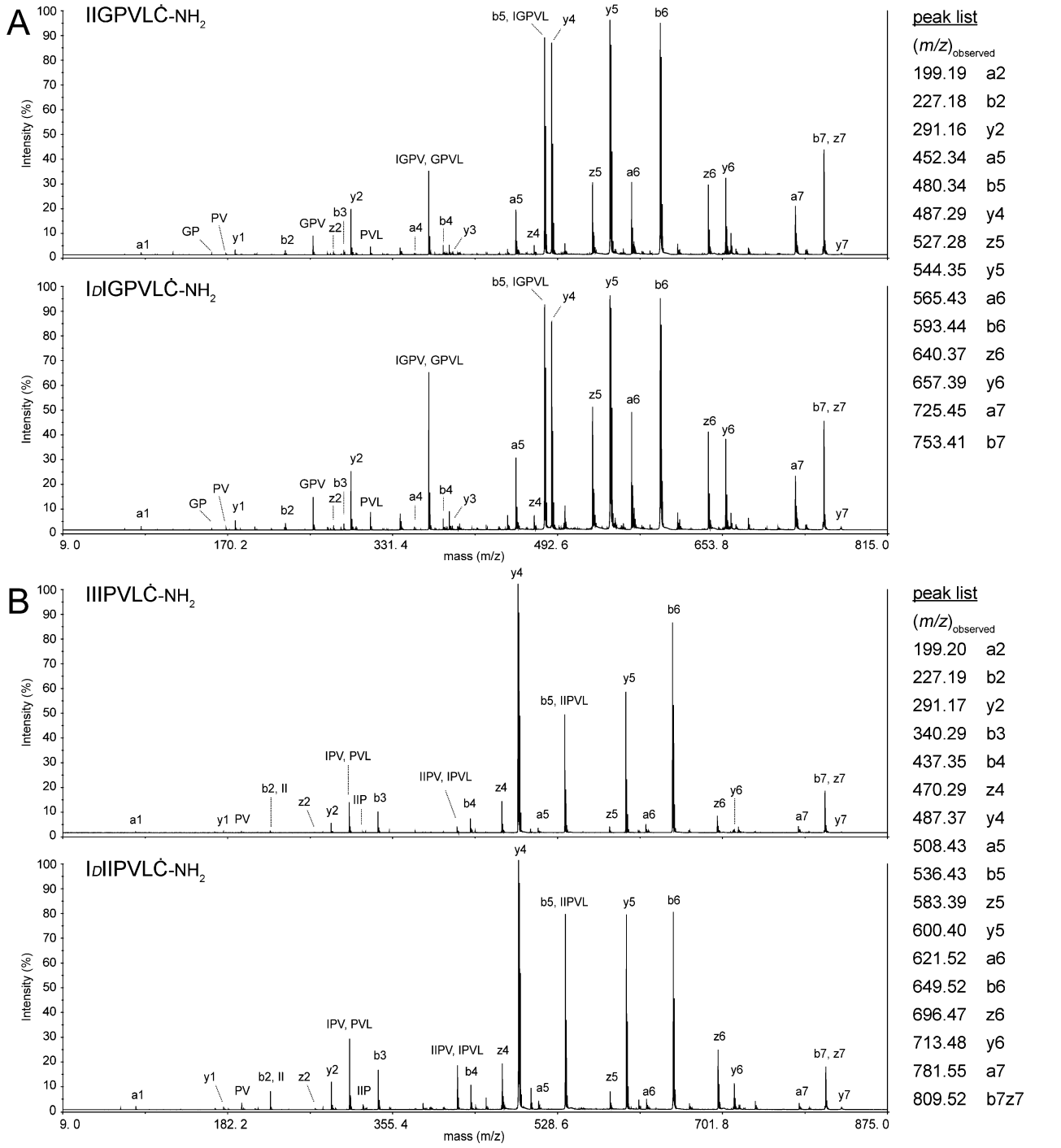

Figure 2. MALDI-MS/MS spectra of peptide epimers IIG (corresponding to wild-type bombininH) and III demonstrate differences in fragmentation patterns. Resulting *R* matrices are shown in Figure 1A and B, Figure S-1-1 and Figure.S-1-18.

Isolation of Peptide GH-2

Skin secretions from *Bombina orientalis* were collected as described[3] and passed through a Centricon filter retaining proteins with a mass of >10 kDa. The filtrate was fractionated over a 218TP C-18 column (Vydac, Hesperia, CA) with a gradient of acetonitrile (solvent A, 0.1% TFA; solvent B, 80% acetonitrile in solvent A). Fractions yielding a mass peak matching the calculated mass of GH-2 peptide were dried in the SpeedVac, subjected to endoproteinase Asp-N (Sequencing grade, Sigma) cleavage in 100 mM NH_4HCO_3 buffer, pH 8.5, and were rechromatographed under the same conditions.

RESULTS

In our experimental approach, we applied MALDI-TOF/TOF metastable decay/CID to a series of epimeric pairs of heptapeptides templated on the N-terminal portion of bombinin H (H-IIGPVLGLVGSALGGLLKKI-NH_2), which contained single amino acid substitutions within the N-terminal tripeptide (Table 1). Previous investigations by means of CD and proton NMR in solution made it unlikely that those peptides adopt any extensive secondary or tertiary structures,[25,26] which might bias the effect of the primary structure. However, in the MALDI-TOF/TOF experiments, more serious

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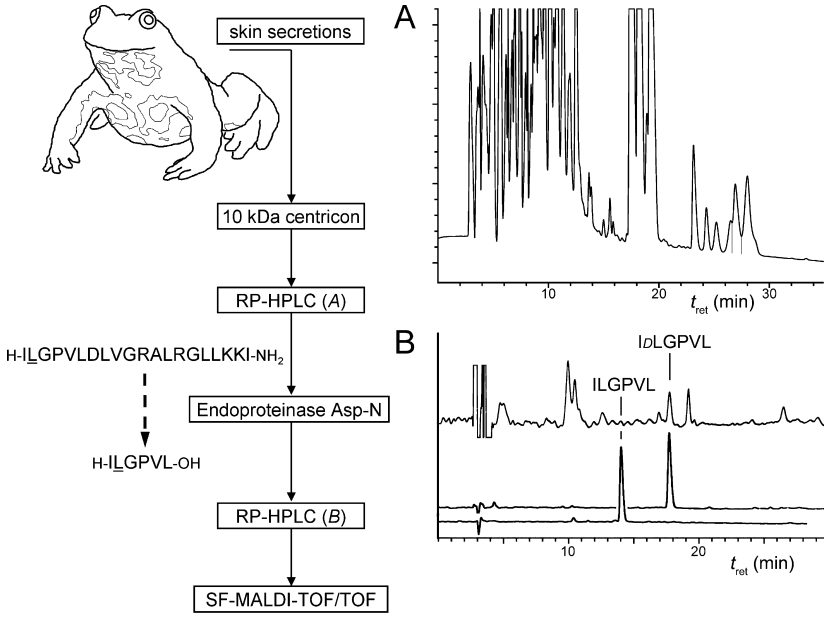

Figure 3. Analysis scheme of natural GH-2 peptide. (A) RP-chromatogram of prefractionized *Bombina* skin secretions. The fraction containing the putative GH-2 epimer (arrow) was subjected to endoproteinase Asp-N cleavage. (B) RP-chromatograms of the enzyme digest (above) and synthetic peptides (below). Arrows denote the positions of the epimers. Peak area of [D-Leu2]-GH-2(1-6) in the sample corresponds to 0.5 μg.

distortions of the results are known to arise from the considerable, method-inherent spot-to-spot variations. Therefore, peak area ratios $r_{ij} = a_i/a_j$ instead of peak areas a_i were averaged after assigning fragment ions to the mass. The r_{ij} values were then found to be satisfactorily reproducible with a mean deviation of typically no more than 10% (within one series of measurements). For the interpretation of fragmentation spectra, all peaks with a relative peak intensity of >5% were considered. From these data, a matrix consisting of all possible chiral recognition factors (i.e., involving all possible pairs of fragments i,j) was generated for each pair of epimers. In the following sections (Table 1, Figure 1, and Figure S-1), i and j were arbitrarily assigned to yield $R_{i,j} \geq 1$ for comparison of the strength of the chiral effect. Please note that $|\log^{10} R_{i,j}| = |\log^{10} R_{j,i}|$ For demonstration of the chiral effect, spectra of two epimers are shown in Figure 2.

Alanine Scan and the Impact of a Chiral Neighborhood

Initially, we applied a partial alanine-scan on the wild-type peptide to delineate contributions from certain residues within the N-terminal tripeptide to the fragmentation propensities and as such to the preference between alternative fragmentation pathways (Figure 1).

The replacement of the Ile or D-allo-Ile in position 2 by an Ala or D-Ala, respectively, lead to decreased *R* values of no more than 1.5 when compared to wild-type (*R* values of up to 2.6) in the MS/MS experiments (Figure 1 and Figure S-1). This observation indicates that the side-chain composition in position 2 is indeed important for chiral discrimination. Remarkably, when Gly-3 was replaced by an Ala, *R* values up to and above 6 ($R_{b6/y6}$) were observed. Conversely, the substitution of Ile-1 by an Ala had only little to no effect on the fragmentation pattern. The contribution of residues 4−7 was not investigated here.

General Substitutions and the Abstraction of Fragmentation Rules

Next, we extended these experiments to more general substitutions within the N-terminal tripeptide (Table 1). The resulting *R* matrices are listed in Figure S-1. Stereoselective fragmentation could often be attributed to contributions by single fragment ions, which were rated by calculation of average *R* based on above matrices.

The residue in position 2 crucially influences chiral recognition, whereby a major chiral recognition is contributed by fragments a2, b2, or z5. The average *R* (Table 1) values for those fragments can be ranked in the order I, *F* > S, E > A > K. Thereby, peptides with amino acid residues I, F, and S gave rise to *R* values above 2 (Figure S-1-1 and Figures S-1-8−12). When a Lys was in this position, stereoselectivity was nearly fully diminished (maximum peak $R_{z5/y4}$ = 1.20). For high *R*, bulky hydrocarbon chains without charged functional group may thus be advantageous.

A replacement of the achiral Gly in position 3 by any other amino acid drastically enhanced the chiral effect. Stereochemistry was then best reported by the y6 fragment ion, which gave rise to *R* values in the order I > F, A > D, S > K ≫ G (Table 1 and Figure S-1-13−18). For example, an Ile in position 3 yielded *R* values up to and above 9 (see Figure 1B and Figure S-1-18). The presence of functional groups in side chains seems to decrease *R* values.

The N-terminal residue had comparably little effect on the stereoselectivity. Nevertheless, polar residues (*S*, *E*) enhanced stereosensitivity in particular of fragment y5, whereas the Lys-peptide gave rise to Y6 and Y7 fragments with high *R* values (and only those), albeit with an extraordinary high error of up to 40% (Table 1 and Figure S-1-2−7).

Please note that, whereas residue effects on fragmentation are well-known, stereoselectivity is an additional, yet naturally not entirely independent effect on fragmentation preferences. In fact, any of the gas-phase reactions involved in fragmentation may be, but not necessarily are, stereospecific.[27,28]

Isolation of GH-2 Epimers from *Bombina* Skin Secretions

For the isolation of GH-2,[29] crude *B. orientalis* skin secretions were prefractionated by ultrafiltration through a 10 kDa centricon filter. The below-10 kDa fraction (filtrate) was

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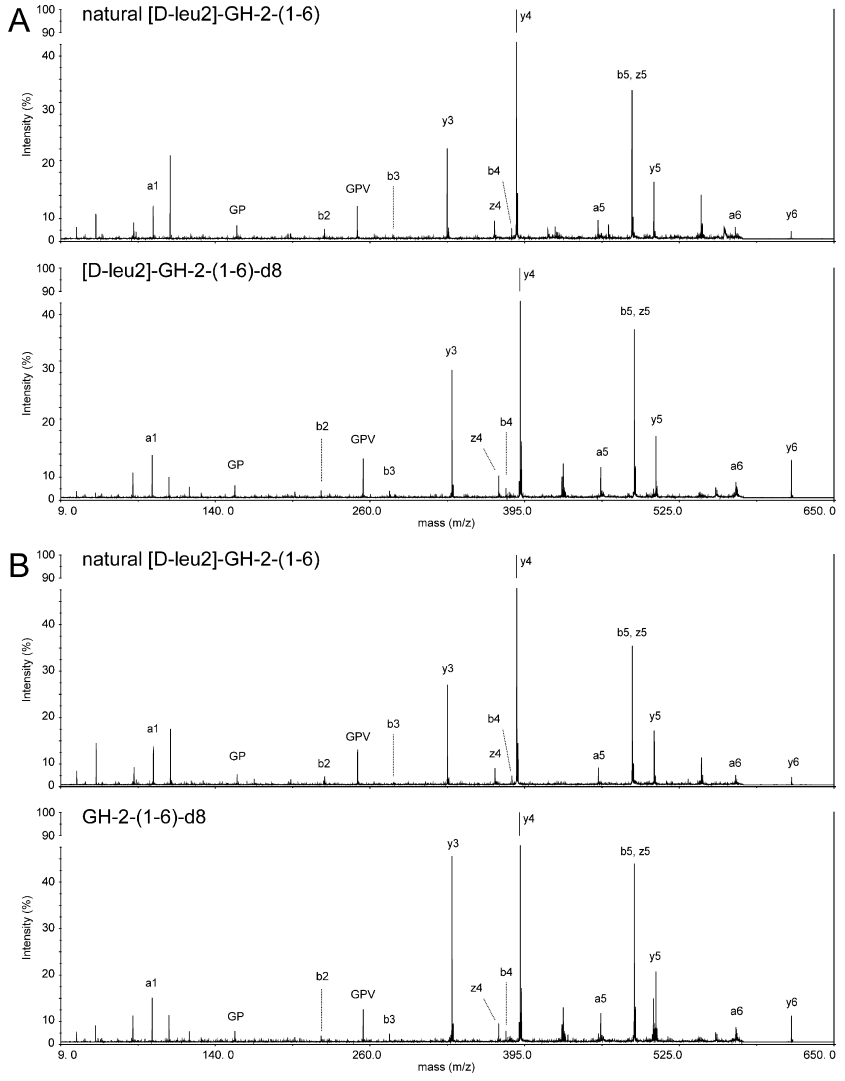

Figure 4. CID-MALDI-TOF/TOF spectra of natural [D-Leu2]-GH-2(1-6) (fraction 17.6 min in chromatogram Figure 2B) and cospotted deuterated synthetic GH-2(1-6)-d8 (panel A) or [D-Leu2]-GH-2(1-6) epimers (panel B). The ratio of sample to standards was 1:1.

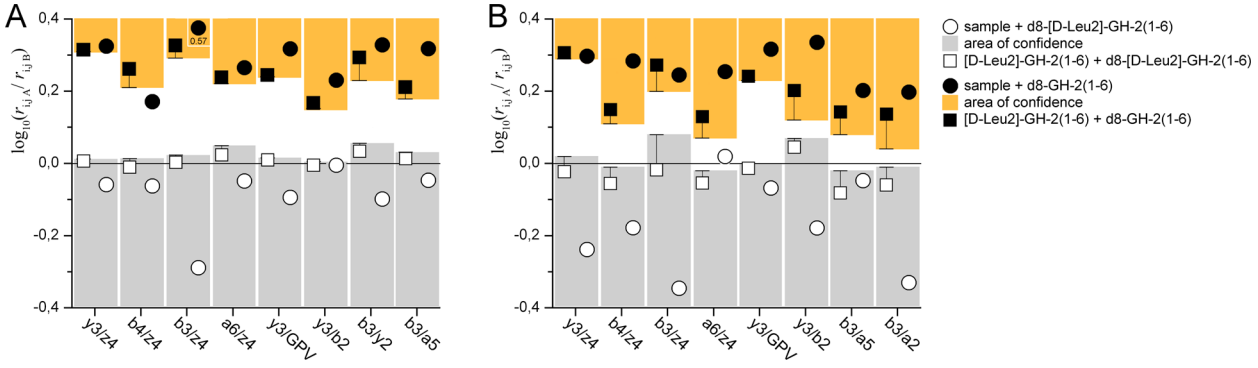

Figure 5. Quantitative evaluation of peak intensities in MALDI-TOF/TOF confirms the natural GH-2(1-6) epimer. Experimental conditions without applying CID (A) and with CID (mass spectra are shown in Figure 4B. Peak ratios for samples cospotted with deuterated d8-[D-Leu2]-GH-2(1-6) ($R = r_{i,j,sample}/r_{i,j,d8\text{-}D}$, open circles) are shown. Gray bars denote areas of high confidence for the positive prediction of an epimeric peptide in this experiment, as determined by cospotting [D-Leu2]-GH-2(1-6) with deuterated d8-[D-Leu2]-GH-2(1-6) (empty squares). Their proximate borders were defined by the mean deviance ($n = 3$) in these experiments (represented by whiskers). In a complementary experiment, peak ratios for sample cospotted with deuterated d8-GH-2(1-6) ($R = r_{i,j,d8\text{-}L}/r_{i,j,sample}$, filled circles) were determined. Orange bars denote areas of high confidence for the positive prediction of an epimeric peptide, as determined by cospotted deuterated d8-GH-2(1-6) and [D-Leu2]-GH-2(1-6) (filled squares). The latter ratios represent the R values. In these data sets, $\log_{10}(R)$ for sample cospotted with deuterated [D-Leu2]-GH-2(1-6) are close to zero, whereas ratios for sample cospotted with deuterated GH-2(1-6) correlate with the respective R values, confirming the presence of [D-Leu2] in the sample.

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fractionated by semipreparative RP-HPLC, which yielded about 40 major peaks (Figure 3A). By MALDI-MS analysis, a mass corresponding to the predicted peptide GH-2 ($[MH]^+_{observed}$ = 2143.5) was found at retention times 25.0 and 27.2 min, respectively. These matched the retention times of synthetic peptide epimers of GH-2. MS/MS produced fragmentation patterns, which were consistent with the sequence of GH-2 (H-ILGPVLDLVGRALRGLLKKI-NH_2) (Figure S-2). Visual inspection did not show differences in fragment ion abundances between the epimers; subtle variations became apparent in a detailed investigation of the spectra, however (data not shown). The fraction containing the putative epimer was subjected to endoproteinase Asp-N cleavage, and the resulting peptides were purified to homogeneity (Figure 3B). A mass corresponding to the N-terminal peptide GH-2(1-6) (H-ILGPVL−OH) was detected at retention time of 17.6 min. This matched the retention time of the synthetic D-peptide epimer, whereas the all-L epimer eluted at 13.9 min.

Confirmation of the D- Form by Application of a Deuterated Analogue

In MALDI MS/MS experiments, GH-2(1-6) epimers yielded only three major peaks and stereoselective fragment pairs with R values no higher than 2 (Figure 4). We therefore aimed to improve the quality of the data by eliminating the experimental spot-to-spot variations. For this purpose, we compared MS/MS results of GH-2(1-6) with cospotted deuterated analogue GH-2(1-6)-d8. We found that the mean deviations of $R_{y3/y4}$ and $R_{z4/y3}$, for example, were reduced by more than 80% when compared to R values obtained from these peptides spotted on different targets. This fact indicates that the spot-to-spot variations indeed contribute significantly to the experimental error of up to 20%.

When applied to natural GH-2(1-6), the sample was cospotted either with GH-2(1-6)-d8 or with [D-Leu2]-GH-2(1-6)-d8 (Figure 4). Peak area ratios from fragment pairs of the sample were directly compared to those of the corresponding peaks of the d8-standard (Figure 5). For example, calculation of ratios between $r_{z4/y3}$ of sample and GH-2(1-6)-d8 yielded 2.1 (taken from MS/MS spectra without CID), which is within the error range of $R_{z4/y3}$ = 2.0 ± 0.1. By contrast, the ratios between sample and [D-Leu-2]-GH-2(1-6)-d8 gave a value close to one, which indicates stereochemical identity and thus confirms the D- residue. The results for selected nonredundant fragment pairs are depicted in Figure 5.

DISCUSSION

In a search for new peptides, extracts from organic tissues are typically crudely fractionated according to size or charge and subsequently subjected to RP-HPLC, which provides both high-resolution separations as well as the removal of excess of undesirable alkali salts, which may obscure the mass spectra of peptides by the formation of adducts. In principle, RP-HPLC can provide separation of epimeric peptides; their resolution, however, depends on the hydrophobicities of the D- residue and its nearest neighbors.[30] In consequence, the observation of a peptide mass appearing in several HPLC peaks may hint toward the presence of epimeric forms albeit other explanations may be likely as well. Moreover, a false positive epimer identification may occur during the highly sensitive MALDI-MS analysis because of detection of traces of the all-L peptide tailing after its actual peak, which often elutes prior to the epimer. At that stage of analysis, additional evidence from other stereosensitive methods is appreciated. Recently, MALDI-MS/MS metastable decay−CID was proposed as an analytical tool, not only for sequence determination but also for the detection of epimeric peptides in biological samples.[21] Whereas earlier studies suggested that the position of the D- residue within the peptide chain is a decisive factor for the obtained chiral recognition, we were particularly interested in the effect exerted by the sequence context in peptides with a D- residue in position two (where, however, stereosensitive fragmentation patterns were recently gained only after N-terminal acetylation[21]). Indeed, as of today, in all vertebrate and several invertebrate peptides the D- amino acid is the second residue of the mature product, whereas it has also been found in other positions in invertebrates.[31−38]

As a first step in dissecting the positional effect from the sequence context, we performed an Ala scan on the N-terminal heptapeptide of bombinin H wild-type, a natural substrate of the *Bombina variegata* L/D-isomerase (Figure 1). Alanine, the most simple but yet chiral amino acid, provides a plain methyl residue for moderate interaction with its nearest neighbors at the site of substitution. At the epimerization site, the substitution of a bulky Ile, (leading to the sequence Ile-Ala or Ile-D-Ala, respectively) is accompanied by a loss of potential steric interactions and results in a considerable drop in stereosensitivity. The same substitution in the neighboring position 1 simply generates the reversed sequence Ala-Ile or Ala-D-allo-Ile but, surprisingly, only leads to an indistinct change in the pattern in the R matrix. At position 3, the other nearest neighbor, the substitution of Gly leads to a remarkable increase in R values. Indeed, glycine is achiral and devoid of a side chain, which could provide any chiral interactions with adjacent residues.

These findings reveal a differential position effect, which necessarily results from placing the racemized amino acid residue between a long C-terminal chain and only one N-terminal amino acid. At position 3, the achiral and highly flexible glycine apparently supports an equal distribution of the kinetic energy independent of the stereochemistry along the polypeptide chain. As such, the native sequence of Bombinin H represents an exceptional setup.

Peptides with amino acid substitutions other than Ala within the N-terminal tripeptide (Table 1) gave rise to results consistent with the general trend yet with subtle variations and revealed a complex sequence dependency. As a rule of thumb, stereochemistry is readily reported by the y6 fragment ions. If there is a glycine in position 3, fragments a2, b2, or y5 (in some cases Y5 or Y6) are reporters. In conclusion, gas-phase disruption of amide bonds between the potential D-residue and its nearest neighbors (Figure 1) is under direct chiral control, and, moreover, cleavages at those bonds are mediated by side-chain interactions.

In many cases, however, we observed that fragment ions derived from breakage sites distant from the D- residue contributed significantly to chiral recognition. This is most evident in the peptide GIG, when the D- residue is embedded in an achiral environment, because both flanking residues are glycines (Figure 1). R values from neighborhood fragmentations were as low as approximately 1.2, which is consistent with the achiral environment in view of the above findings. Nevertheless, an average R of 1.77 was observed for fragment a5 comprising maxima such as $R_{y4/a5}$ = 1.96 ± 0.05 and $R_{y6/a5}$ = 2.14 ± 0.11 (Figure S-1-2). These findings suggest that factors employing chiral interactions other than a neighborhood effect

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influence the stability of bonds elsewhere in the chain and thus affect the fragmentation propensities in the gas phase in dependence on the stereochemistry in position 2. An important factor is possibly the overall conformation, which is known to affect the fragmentation pattern[18])

In consequence of the above findings, neighborhood fragmentations are a good starting point for the determination of chirality when facing an unknown sample. Single *R* values may already suffice in case of peptides with a Phe in position 3 (Figure 1D and Figure.S-1-13), as found in several natural neuropeptide families,[39] for example. However, long-range fragmentations may also serve as feasible reporters. Those probably arise from conformational preferences and are as such difficult to predict by general rules but may be recognized by a classifier program. So, from a more practical point of view, combining *R* values derived from multiple nonredundant fragment ions (i.e., a fingerprint analysis) can serve to increase the confidence in this interpretation. Indeed, this strategy turned out to be effective in the case of the natural epimer [D-Leu2]-GH2 described below (Figure 5).

Conversely, from the experimental point of view, spot-to-spot variations are a major source of error and well-known to complicate or even prevent quantitative investigations.[40] We could achieve a substantial improvement by the use of on-spot reference peptides carrying a deuterium label for the first time in the analysis of peptide epimers. These are fragmented under the very conditions of the particular spot but appear in a different mass window, and the resulting spectra of sample and standard can be compared directly.

Finally, we combined these improvements in data acquisition and interpretation when analyzing a real sample. Starting from *Bombina* skin secretions, we followed the analytical strategy as outlined in Figure 3 to isolate gene-derived bombininH-2 (GH-2).[29] The epimeric form of this peptide has been predicted, yet to our knowledge not been confirmed at the peptide level. In the present paper, evidence for natural [D-Leu-2]-GH-2 was provided by (i) the retention time of full-length [D-Leu-2]-GH-2 in RP chromatograms with the expected mass and fragmentation pattern in MALDI-TOF/TOF, (ii) the retention time of a proteolytically generated fragment [D-Leu-2]-GH-2(1-6) with the expected mass, and (iii) a quantitative fragmentation pattern implicating analysis of multiple $r_{i,j}$ values in MALDI-TOF/TOF consistent with cospotted deuterated [D-Leu-2]-GH-2(1-6) (Figure 5). Beside the advantage of additional verification steps, the incorporation of an endoproteinase step into the processing scheme (Figure 3) opens the field for the chiral analysis of proteins.[41]

CONCLUSIONS

MALDI-TOF/TOF-mass spectrometry is a suitable tool to identify the presence of a D- residue in natural peptides at position 2. In particular, it may effectively be used in the investigation of all peptides, where the existence of epimeric forms has been suggested.[29] A few of these possess a favorable sequence, which can be expected to yield high *R* values in MS/MS experiments, such as some neuropeptides of the RFamide family (which contain Phe in position 2; Figure 1D and Figure.S-1-13). For the majority of peptides, however, this is probably not the case, and their analysis will benefit from the use of a cospotted deuterated reference. This group comprises several ranalexins, temporins 1DYa and PTa, and brevenins 1PTa and 1PTb from diverse Rana species,[42,43] as well as the amyloidogenic dermaseptin-like peptide aDrs from the skin of *Pachymedusa dacnicolor*, a tree frog from southern Mexico,[44] where the D- residue acts as a switch between different superstructure architectures of amyloid.[45] In higher mammals, LAP (lingual antimicrobial peptide), β-defensins 2 and 8 from cattle, the human peptide LL-37, as well as the insulin B chain (from frog, cattle, and man) could be isomerase substrates at least under certain conditions. This compilation of potential natural epimers is certainly not exclusive due to several necessary compromises. For example, predictions relied on the mature N-termini as they are given in the data banks. However, due to alternative processing, the N-termini may vary and need to be experimentally confirmed, which in many cases has not been done. Many more epimeric peptides might exist, even such with a known precursor sequence. We have demonstrated here that stereosensitive fragmentation MALDI-MS/MS has the potential for a future use in high-through-put peptide screening for new epimeric peptides, where the D- residue crucially affects receptor interactions, folding propensities or polypeptide turnover.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b01067.

Figure S-1-1−36: *R* matrices calculated for designated model peptides with or without CID. Figure S-2: MALDI-MS/MS spectra of synthetic and natural [D-Leu2]-GH-2 peptide. (PDF)

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Notes

The authors declare no competing financial interest.

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