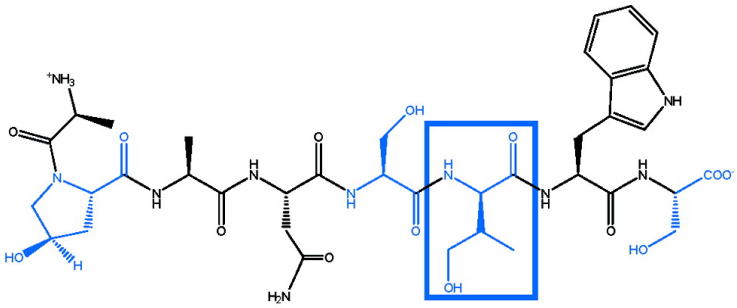
Article

Polypeptide Chains Containing d-β-Hydroxyvaline

Katarzyna Pisarewicz, David Mora, Fred C. Pflueger, Gregg B. Fields, and Frank Mar

J. Am. Chem. Soc., **2005**, 127 (17), 6207-6215• DOI: 10.1021/ja050088m • Publication Date (Web): 08 April 2005



γ-Hydroxyconophan gld-V*: Ala-Hyp-Ala-Asn-Ser-D-Hyv-Trp-Ser

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 5 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 04/08/2005

Polypeptide Chains Containing D-γ-Hydroxyvaline

Katarzyna Pisarewicz, David Mora, Fred C. Pflueger, Gregg B. Fields, and Frank Marí*

Contribution from the Department of Chemistry & Biochemistry and Center of Excellence in Biomedical & Marine Biotechnology, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431

Received January 6, 2005; E-mail: mari@fau.edu

Abstract: Life has an unexplained and distinct L-homochirality. Proteins typically incorporate only L-amino acids into their sequences. In the present study, D-Val and D-γ-hydroxyvaline (D-Hyv; V*) have been found within ribosomally expressed polypeptide chains. Four conopeptides were initially isolated, gld-V*/gld-V*′ from the venom of *Conus gladiator* and mus-V*/mus-V*′ from the venom of *Conus mus*. Their complete sequences (gld-V*/gld-V*′ = Ala-Hyp-Ala-Asn-Ser-D-Hyv-Trp-Ser and mus-V*/mus-V*′ = Ser-Hyp-Ala-Asn-Ser-D-Hyv-Trp-Ser) were determined by a combination of nano/pico-NMR and MS/MS methods. The amino acid triad that contains the γ-hydroxylated residue, Ser-D-Hyv-Trp, is a novel structural motif that is stabilized by specific interactions between the D-amino acid and its neighboring L-counterparts. These interactions inhibit lactonization, a peptide backbone scission process that would normally be initiated by γ-hydroxylated residues. Conopeptides possessing the Ser-D-Hyv-Trp motif have been termed γ-hydroxyconophans. We have also isolated analogous conopeptides (gld-V and mus-V) containing D-Val instead of D-Hyv; these are termed conophans. γ-Hydroxyconophans and conophans are particularly atypical because (i) they are not constrained as most conopeptides, (ii) they are extremely short in length, (iii) they have a high content of hydroxylated residues, and (iv) their sequences have no close match with other peptides in sequence databases. Their modifications appear to be part of a novel hyperhydroxylation mechanism found within the venom of cone snails that enhances neuronal targeting. The finding of D-Val and D-Hyv within this family of peptides suggests the existence of a corresponding D-stereospecific enzyme capable of D-Val oxidation.

Introduction

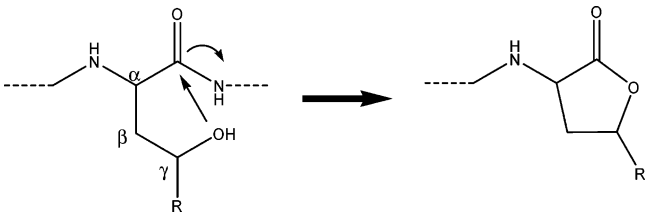
Modification of L-amino acids within polypeptide chains often delineates protein function and is a ubiquitous biochemical process.[1] Peptides found in the venom of predatory marine mollusks belonging to the genus *Conus* (cone snails) possess diverse modifications that modulate their activities. The *Conus* venom is a complex mixture of peptides (conopeptides) that elicit a wide range of neurophysiological responses.[2−5] Several conopeptides have been shown to be valuable therapeutic agents for the treatment of a variety of neurologically related conditions.[6−9] The venom apparatus of cone snails consists of a muscular bulb used to propel the venom and is coupled to a long duct (where the venom is produced), which is ultimately connected to a specialized radular tooth that serves as both a harpoon and disposable hypodermic needle. The polypeptide components of the venom are produced by epithelial cells[10] in the venom duct and are expressed in the ribosome as protein precursors that subsequently undergo posttranslational modifications and proteolytic cleavage to form the mature conopeptide.[11] Conopeptides contain multiple combinations of modified amino acids, such as cystines, hydroxyproline, γ-carboxyglutamate, Br-Trp, D-Trp, D-Leu, pyro-Glu, glycosylated Ser/Thr, and sulfated Tyr.[12−14] These modifications provide stability and exquisite specificity toward neuronal targets,[12,15,16] aiding these marine snails in prey capture.

Modification of polypeptide chains by epimerization of standard L-amino acids to produce their D-counterparts is rarely observed.[17] D-Amino acids were believed to be produced only

(1) Krishna, R. G.; Wold, F. In *Proteins: Design and Analysis*; Angeletti, R. H., Ed.; Academic Press: San Diego, CA, 1998; pp 121−206.
(2) Terlau, H.; Olivera, B. M. *Physiol. Rev.* **2004**, *84*, 41−68.
(3) Mari, F.; Fields, G. B. *Chim. Oggi* **2003**, *21*, 43−48.
(4) Jones, R. M.; Bulaj, G. *Curr. Pharm. Des.* **2000**, *6*, 1249−1285.
(5) Newcomb, R.; Miljanich, G. *Handbook of Neurotoxicology*; Massaro, E. J., Ed.; Humana Press: Totowa, NJ, 2002; Vol. 1, pp 617−651.
(6) Heading, C. E. *Curr. Opin. Invest. Drugs (PharmaPress Ltd.)* **2002**, *3*, 915−920.
(7) Adams, D. J.; Alewood, P. F.; Craik, D. J.; Drinkwater, R. D.; Lewis, R. J. *Drug Dev. Res.* **1999**, *46*, 219−234.
(8) McIntosh, J. M.; Jones, R. M. *Toxicon* **2001**, *39*, 1447−1451.
(9) Shen, G. S.; Layer, R. T.; McCabe, R. T. *Drug Discovery Today* **2000**, *5*, 98−106.
(10) Marshall, J.; Kelley, W. P.; Rubakhin, S. S.; Bingham, J. P.; Sweedler, J. V.; Gilly, W. F. *Biol. Bull.* **2002**, *203*, 27−41.
(11) Milne, T. J.; Abbenante, G.; Tyndall, J. D.; Halliday, J.; Lewis, R. J. *J. Biol. Chem.* **2003**, *278*, 31105−31110.
(12) Craig, A. G.; Bandyopadhyay, P.; Olivera, B. M. *Eur. J. Biochem.* **1999**, *264*, 271−275.
(13) Craig, A. G.; et al. *J. Biol. Chem.* **1999**, *274*, 13752−13759.
(14) Craig, A. G.; Park, M.; Fischer, W. H.; Kang, J.; Compain, P.; Piller, F. *Toxicon* **2001**, *39*, 809−815.
(15) McIntosh, J. M.; Olivera, B. M.; Cruz, L. J. *Methods Enzymol.* **1999**, *294*, 605−624.
(16) Myers, R. A.; Cruz, L. J.; Rivier, J. E.; Olivera, B. M. *Chem. Rev.* **1993**, *93*, 1923−1936.
(17) Mitchell, J. B. O.; Smith, J. *Proteins* **2003**, *50*, 563−571.

10.1021/ja050088m CCC: $30.25

Scheme 1



by prokaryotes, with most D-amino acid containing polypeptide chains being of nonribosomal origin and assembled by enzymatic processes within unicellular organisms. A few examples of D-amino acids occurring within bioactive peptides and proteins of multicellular organisms have been reported, thus challenging our understanding of the homochirality of life.[18] D-Amino acids are found in position 2 of frog skin opionoid peptides such as dermorfine,[19] in neuropeptides of land mollusks such as achatin,[19] and even in mammalians, as revealed in the 39-residue C-type natriuretic peptide from platypus venom.[20] D-Phe is found in position 3 of the 72-residue crustacean hyperglycemic hormone.[21] D-Trp (and sometimes D-Leu) is found as the second residue after the first Cys in contryphans.[22−24] The modifications of L- to D-amino acids are determinants of stability and potency. For example, D-Ser46 in the 48-residue funnel-web spider venom ω-agatoxin IVB provides more resistance to the major venom protease and is a more potent blocker of the P-type voltage-sensitive calcium channels than its L-Ser46 (ω-agatoxin IVC) counterpart.[25] More recently, a 46-residue conotoxin belonging to the I-gene superfamily has been found to possess D-Phe in position 44, which has been determined to be essential for the neuroexcitatory properties of the conopeptide.[26] In general, D-amino acids within polypeptide chains can provide unique structural determinants that allow the stabilization of turns[27] or resistance to enzymatic breakdown.[25]

γ-Hydroxylation of non-Pro amino acids is an even rarer process than epimerization, since a hydroxyl group in the γ-position of any amino acid (except Pro) could undergo nucleophilic attack at the contiguous peptide bond to form a stable five-membered ring lactone (Scheme 1).

Nonetheless, γ-hydroxyarginine has been found as part of the sequence of polyphenolic proteins that form the adhesive plaques of marine mussel species.[28] The presence of γ-hydroxyarginine provides trypsin resistance to mussel glue proteins. γ-Hydroxylysine (γ-Hyk) has been reported within the sequence of crytonomad algae biliproteins;[29] however, the role of γ-Hyk in protein function has not been defined. The oxidation of Leu to produce hydroxyleucine (presumably in either the δ- or γ-position) has been described as an unusual post-translational modification present in unstable forms of hemoglobin associated with patients afflicted with hemolytic anemia.[30,31] The significance of this modification has not been established. γ-Hydroxyproline (Hyp) is commonly found in collagen and is vital for collagen structural stability.[32,33] The unique cyclic nature of Pro impedes lactonization. Nonproteinogenous γ-hydroxylated amino acids have been found within enzymatically produced cyclic peptides.[34−37]

The present study has identified D-Val and D-γ-hydroxyvaline (D-Hyv; V*) within ribosomally expressed polypeptide chains. D-Hyv was found within the sequences of four conopeptides from the venom of *Conus gladiator* (gld-V* and gld-V*′) and *Conus mus* (mus-V* and mus-V*′). We have also isolated analogous peptides that contain D-Val (gld-V and mus-V). Hyv was first described as a novel amino acid isolated from plants.[38] Hyv is an unexpected modified residue in proteins and peptides, as its hydroxyl group could readily cleave a peptide bond by intraresidue cyclization to form a lactone (Scheme 1). The stability of Hyv within conopeptides has been explained by the D-configuration at the α-carbon in conjunction with specific interactions with its surrounding L-amino acids. The doubly modified D-Hyv along with its neighboring residues defines a novel structural motif that characterizes a new family of conopeptides termed γ-hydroxyconophans.

Results and Discussion

We initially isolated three unusual conopeptides, gld-V*, gld-V*′, and gld-V (Figure 1A), from the venom of *Conus gladiator* (species code gld), a cone snail species that inhabits the tropical Eastern Pacific region and preys upon worms. Concurrent with isolation of these conopeptides, related conopeptides mus-V*, mus-V*′, and mus-V were isolated from *Conus mus* (species code mus), a cone snail species related to *C. gladiator* that inhabits the Western Atlantic region (Figure 1B). The gld conopeptides were isolated in nanomolar quantities, whereas the mus conopeptides were isolated in picomolar quantities. Nano/pico-NMR techniques[39,40] allowed the acquisition of their spectra and revealed almost identical compositions for these octapeptides, including an unusual amino acid for gld-V* and gld-V*′ (Figure 2), whereas gld-V showed Val in its place (Figure 4). The mass spectra of gld-V*/gld-V*′ and gld-V gave molecular ions of 863.3 and 847.3 Da, respectively. gld-V* and gld-V*′ had the same covalent structures. The mus

(18) Yang, H. B.; Zheng, G.; Peng, X. Z.; Qiang, B. Q.; Yuan, J. G. *FEBS Lett.* **2003**, *552*, 95−98.
(19) Fujii, N. *Origins Life Evol. Biosphere* **2002**, *32*, 103−127.
(20) Torres, A. M.; Menz, I.; Alewood, P. F.; Bansal, P.; Lahnstein, J.; Gallagher, C. H.; Kuchel, P. W. *FEBS Lett.* **2002**, *524*, 172−176.
(21) Soyez, D.; Toullec, J. Y.; Ollivaux, C.; Geraud, G. *J. Biol. Chem.* **2000**, *275*, 37870−37875.
(22) Jimenez, E. C.; Olivera, B. M.; Gray, W. R.; Cruz, L. J. *J. Biol. Chem.* **1996**, *271*, 28002−28005.
(23) Jimenez, E. C.; Watkins, M.; Juszczak, L. J.; Cruz, L. J.; Olivera, B. M. *Toxicon* **2001**, *39*, 803−808.
(24) Jacobsen, R.; Jimenez, E. C.; Grilley, M.; Watkins, M.; Hillyard, D.; Cruz, L. J.; Olivera, B. M. *J. Pept. Res.* **1998**, *51*, 173−179.
(25) Heck, S. D.; et al. *Science* **1994**, *266*, 1464−1464.
(26) Buczek, O.; Yoshikami, D.; Bulaj, G.; Jimenez, E. C.; Olivera, B. M. *J. Biol. Chem.* **2005**, *280*, 4247−4253.
(27) Imperiali, B.; Moats, R. A.; Fisher, S. L.; Prins, T. J. *J. Am. Chem. Soc.* **1992**, *114*, 3182−3188.
(28) Papov, V. V.; Diamond, T. V.; Biemann, K.; Waite, J. H. *J. Biol. Chem.* **1995**, *270*, 20183−20192.
(29) Sidler, W.; Kumpf, B.; Suter, F.; Morisset, W.; Wehrmeyer, W.; Zuber, H. *Biol. Chem. Hoppe-Seyler* **1983**, *366*, 233−244.
(30) Brennan, S. O.; Shaw, J.; Allen, J.; George, P. M. *Br. J. Haematol.* **1992**, *81*, 99−103.
(31) Brennan, S. O.; Shaw, J. G.; George, P. M.; Huisman, T. H. J. *Hemoglobin* **1993**, *17*, 1−7.
(32) Haading, J. J.; Crabbe, M. J. C. *Post-translational Modifications of Proteins*; CRC Press: Boca Raton, FL, 1992.
(33) Perret, S.; Merle, C.; Bernocco, S.; Berland, P.; Garrone, R.; Hulmes, D. J. S.; Theisen, M.; Ruggiero, F. *J. Biol. Chem.* **2001**, *276*, 43693−43698.
(34) Terui, Y.; Nishikawa, J.; Hinoo, H.; Kato, T.; Shoji, J. *J. Antibiot.* **1990**, *43*, 788−795.
(35) Shoji, J.; Hinoo, H. *J. Antibiot.* **1975**, *28*, 60−63.
(36) Morita, H.; Kayashita, T.; Uchida, A.; Takeya, K.; Itokawa, H. *J. Nat. Prod.* **1997**, *60*, 212−215.
(37) McGahren, W. J.; Morton, G. O.; Kunstmann, M. P.; Ellestad, G. A. *J. Org. Chem.* **1977**, *42*, 1282−1286.
(38) Pollard, J. K.; Sondheimer, E.; Steward, F. C. *Nature* **1958**, *182*, 1356−1358.
(39) Barbara, T. M. *J. Magn. Reson. Ser. A* **1994**, *109*, 265−269.
(40) Barbara, T. M.; Bronnimann, C. E. *J. Magn. Reson.* **1999**, *140*, 285−288.

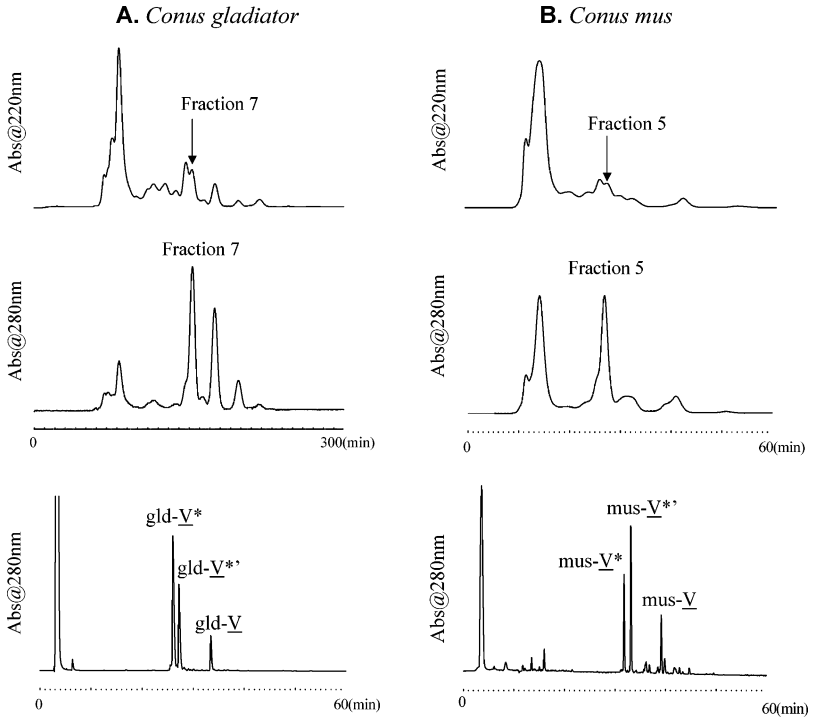


Figure 1. Conopeptide isolation from venom of (A) *C. gladiator* and (B) *C. mus*. The *Conus* venom was fractionated using (top and middle) SE-HPLC (Superdex-30, buffer = 0.1 M NH_4HCO_3). The elution SE-HPLC profiles are shown at λ = 220 (top) and 280 nm (middle), respectively. The arrows indicate the selected fractions of Trp-containing gld and mus peptides. The Trp-containing fractions were further separated using (bottom) RP-HPLC (Vydac C18, H_2O/60% CH_3CN linear gradient over 100 min with 0.1% TFA).

octapeptides revealed sequence information identical to their gld counterparts, except that their molecular weights were shifted by 16 Da. Combined Edman degradation sequencing, MS/MS (Figure 3), and NMR analyses (Figure 2) revealed the structures (Chart 1) of these octa-conopeptides [hydroxylated amino acids are shown in blue, including the modified amino acids (O and V*)].

NMR analysis, along with MS/MS spectra of gld-V*, gld-V*′, mus-V*, and mus-V*′, revealed the presence of Hyv in these conopeptides, representing the first examples of this amino acid found within polypeptide chains. A shielded doublet (~0.5 ppm) that corresponds to a methyl group appears in all these spectra. The 2D-TOCSY spectra of the gld-V*/gld-V*′ pair indicates that this shielded doublet is part of a spin system that corresponds to Hyv. The presence of resonances at δ = 0.52, 1.89, 3.15, 4.45, and 7.99 ppm defines a unique spin system that matches the reported values of Hyv (Figure 2). This assignment is supported by the fragmentation pattern in the MS/MS spectra, where the mass difference between the b_6 and the b_5 fragment also corresponds to Hyv (Figure 3). Other internal fragments, such as OANSV* and NSV*, provide additional support for the presence of Hyv in these peptides.

The configuration of the α-carbon in Hyv was determined by detailed analysis of the proton chemical shifts and the splitting patterns caused by proton−proton coupling in the ^{1}H NMR spectra. The chemical shifts and splitting patterns of the βH protons within the Ser-Val-Trp triad are quite sensitive to the different chiralities of their respective α-carbons (Figure 4). Direct comparison of the NMR splitting patterns and chemical shifts between the gld-V*, gld-V*′, and gld-V indicates that the stereochemistry of the α-carbon at residue six (Hyv for gld-V* and gld-V*′ and Val for gld-V) has been preserved upon hydroxylation. This is particularly noticeable for the βH signal of Trp-7 in gld-V*, gld-V*′, and gld-V (Figures 2 and 4), since their splitting patterns are most sensitive to the different stereochemistries of the different analogues of gld-V. Splitting patterns for the synthetic analogues of gld-V with L-configurations in residue six are completely unrelated to the one observed in the native conopeptides. By way of contrast, these patterns are identical to the D-Val synthetic analogue and are the same in all native conopeptides (gld-V*, gld-V*′, and gld-V). The NMR findings are consistent with the MS/MS spectra of gld-V and its synthetic analogues (Figure 5), as the fragmentation of the native conopeptide corresponds only to the fragmentation observed in the synthetic analogue with D-Val. Since the synthetic analogues of gld-V clearly established the D-configuration of residue six in gld-V, the absolute configuration of Hyv in gld-V* and gld-V*′ also corresponds to the D-amino acid.

The gld-V*/gld-V*′ and mus-V*/mus-V*′ pairs have the same covalent structures, respectively. However, their chromatographic behavior revealed differences in hydrophobicity in a temperature-independent fashion. In principle, these differences might be attributed to *cis*/*trans* isomerism of the peptide bond involving Hyp in residue 2, as suggested by NMR evidence in other related conopeptides.[42] Ultraviolet resonance Raman spectroscopy suggested that two conformational states within conopeptides could be attributed to the differences of the χ dihedral angles of the Trp within their sequence.[23] However, in these cases temperature dependency of the distribution of the conformers has been observed.[24] Closer analysis of the MS/MS data of the gld and mus γ-hydroxyconophans reveals

(41) Siethoff, C.; Lohaus, C.; Meyer, H. E. In *Microcharacterization of Proteins*; Kellner, R., Lottspeisch, F., Meyer, H. E., Eds.; Wiley-VCH: Weinheim, 1999.
(42) Pallaghy, P. K.; He, W.; Jimenez, E. C.; Olivera, B. M.; Norton, R. S. *Biochemistry* **2000**, *39*, 12845−12852.

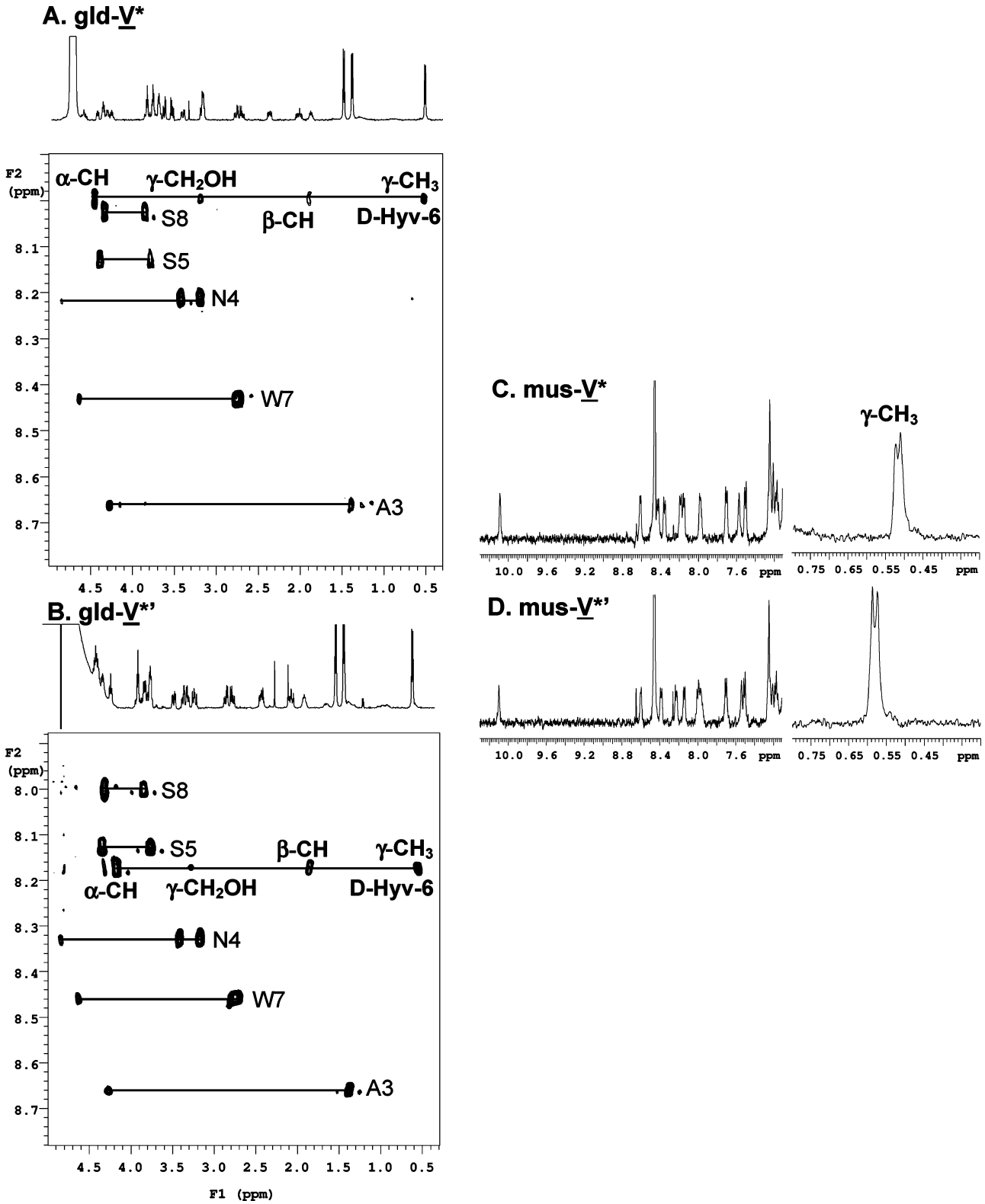


Figure 2. NMR spectra of the γ-hydroxyconophans from *C. gladiator* (A gld-V* and B gld-V*′) and *C. mus* (C mus-V* and D mus-V*′). A and B show the 1D proton spectra along with its corresponding 2D-TOCSY spectrum of 35 nmol of gld-V* and 22 nmol of gld-V*′, respectively. Part A was recorded at 25 °C using a gHX HR-MAS probe. The NMR assignments of the γ-hydroxyvaline (i.e., for gld-V*: HN: d 7.99, 8 Hz; αH: 4.45, m; γCH_2 m 3.15; βCH m 1.89, 6.9 Hz; γCH_3: d 0.52, 7.1 Hz) correlated well with the reported values of the synthetic amino acid.[43] C and D show the 1D proton spectra of picomolar quantities of mus-V* and mus-V*′, respectively. Spectra in B, C, and D were recorded using 3 mm tubes (see Experimental Section).

that the fragmentation patterns within the pairs differ in the intensity of the b_6 fragment (Figure 3), which suggests structural differences within the Hyv residue. This difference is more significant when utilizing an ESI-ion trap instrument to record the MS/MS spectra (data not shown, see Experimental Section). Furthermore, the largest chemical shift differences between the gld-V* and gld-V*′ are within the resonances of α-CH and γ-CH2 proton of Hyv (Figure 2). Hyv bares a chiral center at

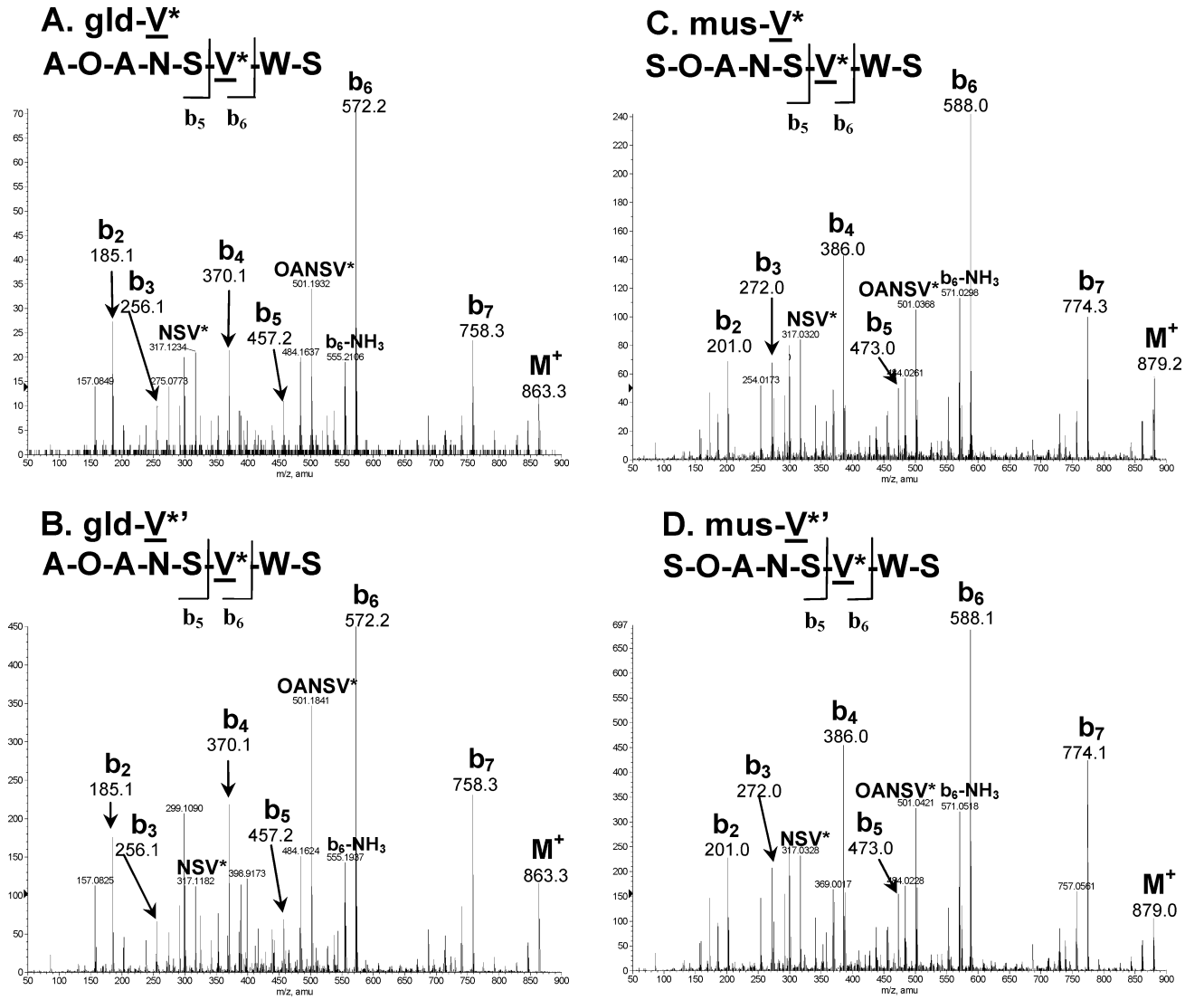


Figure 3. MALDI-MS/MS of the γ-hydroxyconophans from *C. gladiator*: (A) gld-V* and (B) gld-V*′; and *C. mus*: (C) mus-V* and (D) mus-V*′. These spectra were recorded using the AB Q-TOF instrument (see Experimental Section). Assignments of the b fragments and others were carried out using standard procedures.[41]

the β-carbon; therefore, gld-V* and gld-V*′ are likely to be diastereomers, epimeric at the β-carbon of Hyv, as reflected in the differences in the NMR chemical shifts of the groups attached to the β-position of Hyv. There are slight differences reported for the chemical shifts of the 2*S*,3*S* and 2*S*,3*R* diastereomers of free Hyv;[43] however, these differences cannot be used to determine the absolute configuration of Hyv within these diastereomeric conopeptides.

The γ-hydroxylation of any amino acid (except for γ-Hyp) would be unexpected, as it introduces susceptibility to lactonization. This susceptibility would explain why the Edman degradation analysis of gld-V* yielded its sequence only up to the residue preceding Hyv (data not shown). The basic conditions (pH ≥ 9 at 40−55 °C) required for the coupling reaction of phenylisothiocyanate to the free *N*-terminal amino group of a polypeptide chain, or the strongly acidic conditions necessary to produce the PTH-amino acid,[44] might catalyze the intraresidue cyclization process leading to early termination of the complete peptide sequence. Intraresidue cyclization is possible in proteins/peptides containing γ-hydroxyarginine[28] and γ-hydroxylysine.[29] Both amino acids have been reported as part of the sequences of proteins. However, these are positively charged amino acids and their hydroxyl groups are secondary alcohols. These two factors diminish the nucleophilicity of the hydroxyl group and thus deter lactonization. Furthermore, in the case of γ-hydroxyarginine in the mussel adhesive protein Mefp-3, it has been suggested that γ-hydroxyarginine is capable of hydrogen bonding with Dopa, the next residue in the protein sequence. The interresidue interactions of this dyad of contiguously modified amino acids represent a stable structural motif that confers Mefp-3 with resistance to proteases and appears to be involved in the molecular interactions necessary for adhesion.[28]

What is the basis for the stability of Hyv within gld and mus conopeptides? The distinctive allowed Ramachandran space for D-amino acids could place contiguous residues in close proximity.[17] This is confirmed by X-ray and NMR analyses of

(43) Easton, C. J.; Merrett, M. C. *Tetrahedron* **1997**, *53*, 1151−1156.

(44) Lottspeisch, F.; Houthaeve, T.; Kellner, R. In *Microcharacterization of Proteins*; Kellner, R., Lottspeisch, F., Meyer, H. E., Eds.; Wiley-VCH: Weinheim, 1999.

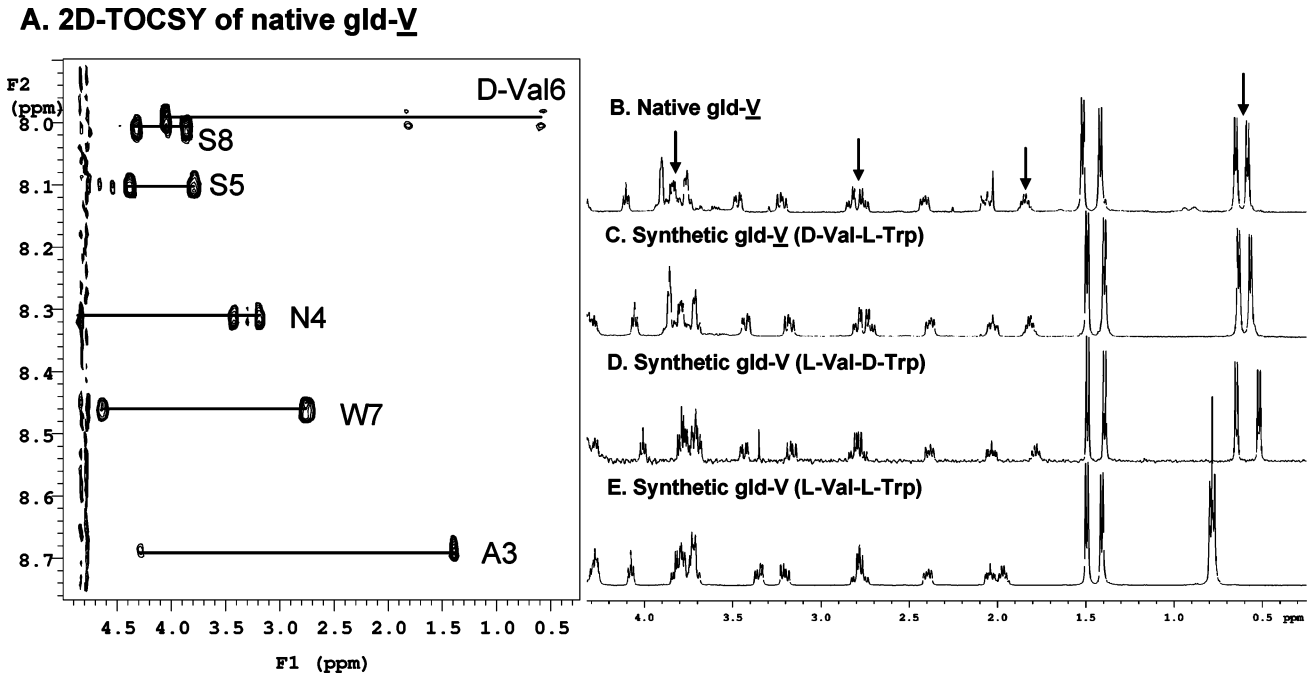
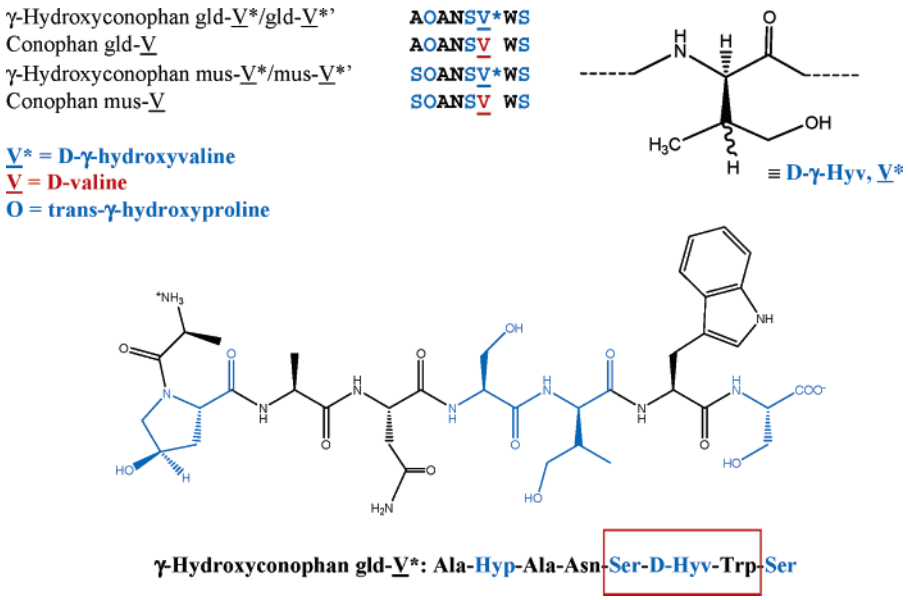


Figure 4. NMR spectra of the conophan from *C. gladiator* (A and B gld-V) and its corresponding synthetic peptides containing L-Val-L-Trp (C), L-Val-D-Trp (D), and D-Val-L-Trp (E). Part A shows the ^{1}H NMR 2D-TOCSY spectrum of 21 nmol of native gld-V isolated from the venom of *C. gladiator*. B, C, D, and E show the 0.2−4.5 ppm region (αH and side-chains) of the 1D proton spectra of native gld-V (B), synthetic gld-V incorporating D-Val-L-Trp (C), synthetic gld-V incorporating L-Val-D-Trp (D), and synthetic gld-V using L-Val-L-Trp (E). A and B were recorded at 25 °C using a gHX HR-MAS probe. C, D, and E were recorded using 3 mm tubes in the gHCX probe. The arrows in B indicate resonances of interest that substantiate (by comparison with the synthetic analogue) the chirality assignment of Val-6 in gld-V.

Chart 1



polypeptide chains that contain D-amino acids, such as dermophins and achatins, where all side-chains of the Xaa_{i-1}-D-Xaa_i-Xaa_{i+1} motif are on the same face of the polypeptide chain because of the central D-amino acid configuration.[45−47] In the case of the γ-hydroxyconophans gld-V*/gld-V*′ and mus-V*/mus-V*′, structural stability can be explained by specific interactions of the D-Hyv with it neighboring L-amino acids. Trp provides (i) steric hindrance and (ii) a stabilizing van der Waals interaction between the D-Hyv methyl group and the Trp aromatic ring that impede cyclization. Evidence of the van der Waals interaction is observed in the chemical shift of the Hyv methyl group, which is shielded (δ = 0.52 ppm) in the same manner as methyl groups are shifted by aromatic residues within folded proteins.[48] X-ray data on the Tyr-D-Ala-Phe triad found in dermophin indicates the presence of a C−H···π interaction between the D-Ala methyl group and the Tyr aromatic ring; this is a defining feature of the Tyr-D-Ala-Phe structural topology.

(45) Slabicki, M. M.; Potrzebowski, M. J.; Bujacz, G.; Olejniczak, S.; Olczak, J. *J. Phys. Chem. B* **2004**, *108*, 4535−4545.
(46) Ishida, T.; In, Y.; Inoue, M.; Yasuda-Kamatani, Y.; Minakata, H.; Iwashita, T.; Nomoto, K. *FEBS Lett.* **1992**, *307*, 253−256.
(47) Kamatani, Y.; Minakata, H.; Iwashita, T.; Nomoto, K.; In, Y.; Doi, M.; Ishida, T. *FEBS Lett.* **1990**, *276*, 95−97.
(48) Storch, E. M.; Grinstead, J. S.; Campbell, A. P.; Daggett, V.; Atkins, W. M. *Biochemistry* **1999**, *38*, 5065−5075.

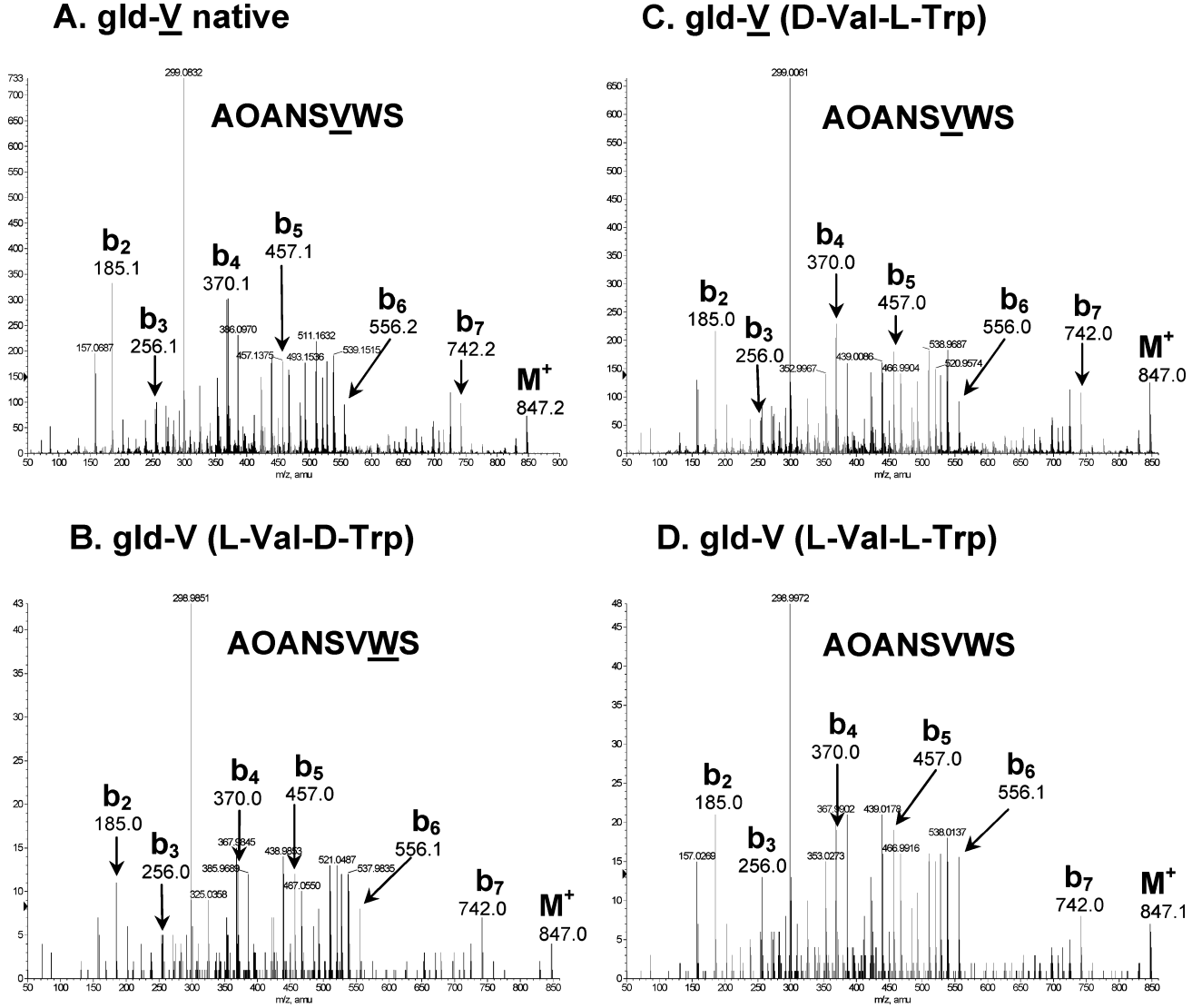


Figure 5. MS/MS of the conophan from *C. gladiator*, gld-V: (A) native gld-V; (B) synthetic gld-V incorporating L-Val-D-Trp; (C) synthetic gld-V incorporating D-Val-L-Trp; and (D) synthetic gld-V incorporating L-Val-L-Trp. These spectra were recorded using the AB Q-Star XL Q-TOF instrument (see Experimental Section). The stereochemistry of the Val residue was determined by comparing the NMR (Figure 4) and MS/MS spectra shown here of the native gld-V with its synthetic peptide analogues. The influence of the chirality of Val-6 on the MS/MS fragmentation patterns of these peptides supports the NMR evidence shown in Figure 4.

The same interaction appears to be present in the gld-V*/gld-V*′ and mus-V*/mus-V*′ Ser-D-Hyv-Trp triad, as indicated by the NMR spectra described above and rationalized by molecular modeling (Figure 6). Stabilizing interactions within the Ser-D-Hyv-Trp triad can also be provided by hydrogen bonding between Ser and D-Hyv side-chains due to their proximity. Overall, the D-Hyv is held in a "locked" conformation by the C−H···π interaction with Trp on one side and an H-bond with Ser on the other side, explaining the inability of Hyv to undergo lactonization.

The amino acid triad that contains the γ-hydroxylated amino acid, Ser-D-γHyv-Trp, is a novel structural motif that defines a new class of conopeptides termed γ-hydroxyconophans. The corresponding analogous peptides that contain just D-Val, such as gld-V, are termed conophans. The initial finding of γ-hydroxyconophans within the venom of *Conus gladiator* led to their isolation from *C. mus*. *C. gladiator* and *C. mus* are closely related cone snail species.[49] These species evolved separately from a common ancestor as the Isthmus of Panama separated the Atlantic and Pacific Oceans 3−3.5 million years ago. It is remarkable that these two *Conus* species, which nowadays inhabit different oceans, bear the same biochemical imprint within their venom through a novel set of posttranslational modifications. While this is an indication of their common ancestral origins, γ-hydroxyconophans, conophans, and peptides that contain related structural motifs are likely to be found in other *Conus* species. Here, the difference between the gld conophans and their mus counterparts is Ser in residue 1 in *C. mus* as opposed to Ala in *C. gladiator*. This appears to be part of the hyperhydroxylation strategy used by cone snails to optimize their venom efficacy.

It is noteworthy that the sequences of these conopeptides have an extremely high number of hydroxylated residues. Perhaps, just as in the case of collagen, hydroxylation is the preferred strategy used by the cone snails to increase hydrogen-bonding capabilities. However, in the *Conus* case, hydrogen bonding is

(49) Duda, T. F.; Palumbi, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10272−10277.

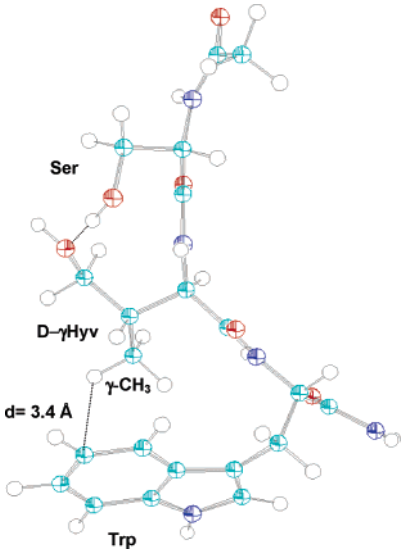


Figure 6. Molecular model of γ-hydroxyconophan structural motif H_3-CC(O)-Ser-D-Hyv-Trp-NH_2. This model was constructed on the basis of the X-ray structure of the dermorphin Tyr-D-Ala-Phe "message sequence" and illustrates the proximity of the γ-methyl of D-Hyv to the Trp side-chain and the hydroxyl group of Hyv to the Ser side-chain, which are shown as being hydrogen bonded. This proximity enables specific interactions between the side-chains of all these residues (see text). Evidence of the D-Hyv-Trp side-chain interaction is provided by the strongly shielded chemical shift of the D-Hyv γ-methyl group (see Figures 2 and 4). Trp also provides steric impediment to the lactonization process and aids the stability of polypeptides chains that include this structural motif.

directed toward increasing binding strength and selectivity toward their neuronal targets. Polyhydroxylation is a recurrent feature in natural products of marine and land origins.[50−53] Polyhydroxylated compounds, ranging from Taxol to Dermostatin, rely on the hydrogen-bonding capabilities of their hydroxyl groups to interact with their molecular targets. In the case of most polyhydroxylated natural products, their complex molecular scaffolds are the product of an intricate multienzymatic biosynthetic pathway that incorporates diverse metabolites in manners unique to the organisms that produce the compounds. On the other hand, cone snails have relied on a universal mechanism for protein synthesis and modification for the production of small molecule-like structural scaffolds that fulfill the specialized task of targeting specific neuronal receptors. These versatile peptide engineers have developed a wide range of protein processing schemes that allow them to efficiently carry out multiple posttranslational modifications on polypeptide chains across the many conopeptide families. While this process is reminiscent of the biosynthetic production of complex natural products, the cone snails are using a reduced set of enzymes capable of modifying a wide range of ribosomally expressed polypeptide chains. This strategy is aimed at enhancing the molecular complexity and diversity of the venom. The need for the development of such biochemically diverse venom is likely to be an evolutionary adaptation designed to compensate for the lack of mobility of cone snails when compared to other marine predators. Within this scheme, the epimerization and subsequent hydroxylation of Val provides further diversity to the venom by adding a new protein scaffold that is so far unique to *Conus*. However, just as other posttranslational modifications found in *Conus* venom were previously described in other organisms,[3,54] it will not be surprising to find the γ-hydroxyconophan scaffold in other organisms. In addition to the unprecedented presence of D-Hyv in their sequence, these γ-hydroxyconophans are unusual because (i) they are linear conopeptides and not constrained like the conotoxin and contryphan families, (ii) they are extremely short in length, (iii) they have a high content of hydroxylated residues, and (iv) their primary structure has no close match in the sequence databases.

The epimerization of Val by cone snails has produced the first example of D-Val within a ribosomally expressed polypeptide chain. Most epimerizations found in small linear peptides occur near the N-terminal and preferentially at the second position. The D-Val in these conophans is at the third amino acid from the *C*-terminal, the same relative position as in the larger disulfide-constrained ω-agatoxin[25] and the r11a I-superfamiliy conotoxin,[26] which have 48 and 46 residues, respectively. Apparently, the epimerization has a strong preference at this position near the C-terminal regardless of the nature of the amino acid (D-Ser in ω-agatoxin, D-Phe in the r11a conotoxin, or D-Val in conophans) or size and nature of the expressed protein. In fact, it is likely that the two-base enzymatic mechanism proposed for the epimerization of D-Ser in ω-agatoxin is in effect in all these cases, as the epimerase in the funnel-web spider is also know to epimerize other amino acids, such as Ala, Cys, and *O*-methylserine.[55] However, the substrate for this epimerase has a recognition site Leu-Xaa-Phe-Ala, observed neither in the r11 conotoxin nor in the conophans. Furthermore, the spider epimerase is capable of converting Xaa in small peptides at several positions within the polypeptide chain.[55] Therefore, it is likely that different epimerases with distinct specificities are operating in each of these cases.

The presence of D-Hyv in gld-V*/gld-V*′, as opposed to D-Val in gld-V, suggests the existence of an enzyme capable of D-Val oxidation. This putative enzyme could be using gld-V, or its precursor protein, as a substrate to modify D-Val and generate the D-Hyv form of the toxin. This process would be analogous to Glu γ-carboxylation of certain conopeptides, which require the action of a specific carboxylase on the precursor form of the peptide[56−59] to produce conantokins and related Gla-containing conopeptides. The isolation and identification of a hydroxylase with D-amino acid specificity is under investigation.

Experimental Section

Peptide Isolation. Specimens of *Conus gladiator* (species code gld) were collected from several locations off the Pacific coast of Costa Rica. The venom was dissected from the venom ducts, pooled, lyophilized (~50 mg from 47 snails), and initially fractionated using

(50) Cui, J.; Wang, Y.; Lin, C.; Zeng, L. *Tianran Chanwu Yanjiu Yu Kaifa* **2000**, *12*, 94−100.
(51) Watson, A. A.; Fleet, G. W. J.; Asano, N.; Molyneux, R. J.; Nash, R. J. *Phytochemistry* **2001**, *56*, 265−295.
(52) Zeng, L.; Su, J.; Zhong, Y.; Fu, X.; Peng, T.; Zhu, Y.; Meng, Y.; Cen, Y.; Xu, X.; Zheng, Y.; Wang, G. *Pure Appl. Chem.* **1999**, *71*, 1147−1151.
(53) Danieli, B.; Riva, S. *Pure Appl. Chem.* **1994**, *66*, 2215−2218.
(54) Olivera, B. M. *Annu. Rev. Ecol. Syst.* **2002**, *33*, 25−47.
(55) Heck, S. D.; Faraci, W. S.; Kelbaugh, P. R.; Saccomano, N. A.; Thadeio, P. F.; Volkmann, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4036−4039.
(56) Bandyopadhyay, P. K.; Colledge, C. J.; Walker, C. S.; Zhou, L.-M.; Hillyard, D. R.; Olivera, B. M. *J. Biol. Chem.* **1998**, *273*, 5447−5450.
(57) Bandyopadhyay, P. K.; Garrett, J. E.; Shetty, R. P.; Keate, T.; Walker, C. S.; Olivera, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1264−1269.
(58) Czerwiec, E.; Begley, G. S.; Bronstein, M.; Stenflo, J.; Taylor, K.; Furie, B. C.; Furie, B. *Eur. J. Biochem.* **2002**, *269*, 6162−6172.
(59) Walker, C. S.; Shetty, R. P.; Clark, K.; Kazuko, S. G.; Letsou, A.; Olivera, B. M.; Bandyopadhyay, P. K. *J. Biol. Chem.* **2001**, *276*, 7769−7774.

size exclusion-HPLC on a Pharmacia Superdex-30 column (2.5 × 100 cm) with elution by 0.1 M NH_4HCO_3 buffer at a flow rate of 1.5 mL/min (Figure 1A). The column eluent was monitored on a PDA detector (TSP SM-5100) at $\lambda = 220$ and 280 nm. The material in the major peak (gld_07) in the $\lambda = 280$-detected chromatogram was further separated using an RP-HPLC Vydac C18 column (10 × 250 mm, 5 μm, 300 Å) eluted with a linear gradient of H_2O/60% CH_3CN over 100 min (Figure 1B). 0.1% TFA was used as ion-pairing reagent. Three peptide fractions were separated (gld-V*, gld-V*′, and gld-V) and subsequently analyzed by MS and NMR. Similarly, specimens of *Conus mus* were collected off the Florida Keys (Plantation, Monroe County); 12 mg of crude venom was extracted and processed as described for *C. gladiator*.

Peptide Sequencing and Synthesis. Sequencing was carried out by Edman degradation chemistry on an Applied Biosystems (AB) Procise cLC and Procise 491A instruments. Peptide synthesis was performed on an AB 433A peptide synthesizer using Fmoc chemistry.[60] Peptide-resin cleavage utilized appropriate scavengers[61,62] to avoid Trp modification. Cleaved peptides were purified by RP-HPLC as described above.

Mass Spectrometry. The MS/MS spectra of all conopeptides were obtained either on an AB Q-Star XL Q-TOF spectrometer equipped with an oMALDI-2 or on a Micromass Q-TOF micro instrument equipped with a nanospray source. Additional MS experiments were carried out using a Finningan LCQ-Deca instrument. Samples (∼1 pmol) analyzed using the Q-TOF instrument were desalted using a C18 ZipTip and introduced with a nanospray ion source.[63,64] Glu-fibrinogen, $m/z = 785.85$ doubly charged, was used as an internal standard. Approximately 10 pmol of sample was applied for analyses using the LCQ instrument. Samples were analyzed by flow injection using 30%ACN/0.1% acetic acid as a carrier.

NMR Spectroscopy. NMR spectra were acquired on a Varian Inova 500 MHz spectrometer equipped with three rf channels, pulse field gradients, and waveform generators. Initially, 1D- and 2D-TOCSY spectra were recorded using a gHX HR-MAS probe[39,40] for 1 nmol of gld-V* in 35 μL. Larger sample quantities (20−35 nmol of the gld peptides) were analyzed using 3 mm sample tubes in 130 μL of NMR solution in a 5 mm gHCX triple resonance probe. 1D spectra were acquired using 512 scans, whereas 2D spectra were acquired using 96 increments in t1 with 256 scans per increment in a phase-sensitive mode. 2D spectra were processed using linear predictions in t1 to 1024 points and transformed to final size of 2k × 2k. The 1D spectra of picomolar amounts of the mus conopeptides were acquired overnight using 3 mm sample tubes in 130 μL of NMR solution in a 5 mm gHCX triple resonance probe. All spectra were recorded at 25 and 0 °C in an NMR solution that consisted of 90% H_2O/10% D_2O using TSP as an internal standard. The pH for this solution was adjusted to 3.6 using 0.01 M solutions of HCl and NaOH and a Phoenix micro-pH probe. Water suppression was achieved using Watergate[65] and Excitation Sculpting[66] for the 2D experiments and WET[67] and presaturation for the 1D ^1H spectra. The resonance assignments were carried out using standard biomolecular NMR procedures.[68]

Molecular Modeling. Molecular models were built based on the X-ray structure of the dermophin "message sequence" Tyr-D-Ala-Phe.[45] The extended conformation consistent with the NMR data was used for the initial model and optimized to self-consistency by the MMX force field as previously described.[69] The structural motif that characterizes the gld/mus γ-hydroxyconophan family of conopeptides [Ser-D-Hyv-Trp] was capped using an acetyl group at the *N*-terminus and an amide group at the *C*-terminus to simulate a protein-like environment.

Acknowledgment. We thank J. Lauer-Fields, Mare Cudic, and Jose Rivera-Ortiz for synthesis of peptides; A. Franco for collection of specimens; M. Crawford for peptide sequencing; and C. Byrdwell and K. Stone for mass spectrometry analysis. We thank the government of the Republic of Costa Rica for providing the permits to collect the necessary specimens. This work was supported by the Florida Sea Grant College Program (R/LR-MB-18) and the NIH (GM 066004 to F.M., CA 77402 to G.B.F.). This work is contribution P200503 from the Center of Excellence in Biomedical and Marine Biotechnology.

Supporting Information Available: Complete refs 13 and 25. This material is available free of charge via the Internet at http://pubs.acs.org.

JA050088M

(60) Fields, G. B.; Lauer-Fields, J. L.; Liu, R.-q.; Barany, G. *Synthetic Peptides: A User's Guide*, 2nd ed.; ed.; W. H. Freeman & Co.: New York, 2001.
(61) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Peptide Protein Res.* **1990**, *36*, 255−266.
(62) Fields, C. G.; Fields, G. B. *Tetrahedrom Lett.* **1993**, *34*, 6661−6664.
(63) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. *Nature* **1996**, *379*, 466−469.
(64) Wilm, M.; Mann, M. *Anal. Chem.* **1996**, *68*, 1−8.
(65) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661−665.
(66) Callihan, D.; West, J.; Kumar, S.; Schweitzer, B. I.; Logan, T. M. *J. Magn. Reson. Ser. B* **1996**, *112*, 82−85.
(67) Smallcombe, S. H.; Patt, S. L.; Keifer, P. A. *J. Magn. Reson. Ser. A* **1995**, *117*, 295−303.
(68) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley & Sons: New York, 1986.
(69) Mari, F.; Lahti, P. M.; McEwen, W. E. *J. Am. Chem. Soc.* **1992**, *114*, 813−821.