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Synthesis and Solid-Phase Application of Suitably Protected γ-Hydroxyvaline Building Blocks

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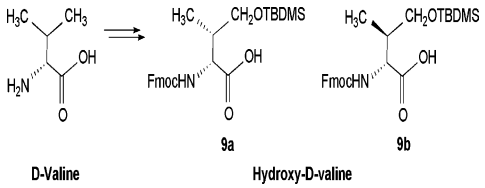

Recently, an unexpected modified residue, γ-hydroxy-D-valine (D-Hyv), was identified within ribosomally expressed polypeptide chains of four conopeptides from the venoms of *Conus gladiator* and *Conus mus*. To assemble Hyv-containing peptides, we have explored several routes for the synthesis of appropriately functionalized Hyv building blocks. D-Hyv was produced from D-Val by using a variation of the previously published $K_2PtCl_4/CuCl_2$ oxidative method. Direct synthesis of Boc- or Cbz-D-Hyv lactone proceeded in low yield; additionally, the lactones are too unreative for solid-phase applications. 9-Borabicyclononane or copper-complexed D-Hyv was prepared and treated with *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf) to produce D-Hyv(*O*-TBDMS). The most efficient complex disruption was achieved by Chelex 110 resin (Na^+ form) treatment of copper-complexed D-Hyv(*O*-TBDMS). Reaction of D-Hyv(*O*-TBDMS) with Fmoc-OSu produced Fmoc-D-Hyv(*O*-TBDMS) in 26% yield from D-Val. The Fmoc-D-Hyv(*O*-TBDMS) diastereomers were separated by preparative RP-HPLC in 13% yield from D-Val. Fmoc-D-Hyv(*O*-TBDMS) was used for the synthesis of the conopeptide gld-*V** from *Conus gladiator*. The isolated synthetic and natural products had coincidental mass and NMR spectra. The methodology presented herein will greatly facilitate biological studies of Hyv-containing sequences, such as receptor responses to hydroxylated versus nonhydroxylated conopeptides and the relative susceptibility of proteins to modification by oxidative stress.

Introduction

Hydroxylated amino acids constitute an important modification in proteins. The best characterized of the post-translationally hydroxylated amino acids are γ-hydroxy-Pro (Hyp) and δ-hydroxy-Lys (Hyl), which are commonly found in collagen. γ-Hydroxy-L-Pro offers enhanced stabilization of the collagen triple-helix compared with L-Pro, whereas δ-hydroxy-L-Lys is the primary site of collagen glycosylation and participates in intermolecular collagen cross-links.[1] γ-Hydroxy-Arg has been found as part of the sequence of polyphenolic proteins that form the adhesive plaques of marine mussel species.[2] The hydroxylation of Arg provides trypsin resistance to these mussel glue proteins. Recently, an unexpected modified residue, γ-hydroxy-D-valine (D-Hyv), was identified within ribosomally expressed polypeptide chains of four conopeptides from the venoms of *Conus gladiator* and *Conus mus*.[3] These conopeptides were the first known examples of a naturally occurring polypeptide chain containing Hyv. In general, γ-hydroxyamino acids are not that common in nature (except γ-hydroxy-Pro) since a hydroxyl group in the γ-position can undergo intramolecular cyclization to form a lactone, cleaving the peptide bond. For example, *N*-acetyl-γ-L-hydroxyvaline lactone had been isolated from streptomycete obtained from marine sediments.[4] The stability of Hyv within conopeptides has been explained by the D-

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(1) Kivirikko, K. I.; Myllylä, R.; Pihlajaniemi, T. In *Post-translational Modifications of Proteins*; Harding, J. J., Crabbe, M. J. C., Eds.; CRC Press: Boca Raton, FL 1992; pp 1−51.

(2) Papov, V. V.; Diamond, T. V.; Biemann, K.; Waite, J. H. *J. Biol. Chem.* **1995**, *270*, 20183−20192.

(3) Pisarewicz, K.; Mora, D.; Pflueger, F. C.; Fields, G. B.; Marí, F. *J. Am. Chem. Soc.* **2005**, *127*, 6207−6215.

(4) Hernandez, I. L. C.; Godinho, M. J. L.; Magalhaes, A.; Schefer, A. B.; Ferreira, A. G.; Berlinck, R. G. S. *J. Nat. Prod.* **2000**, *63*, 664−665.

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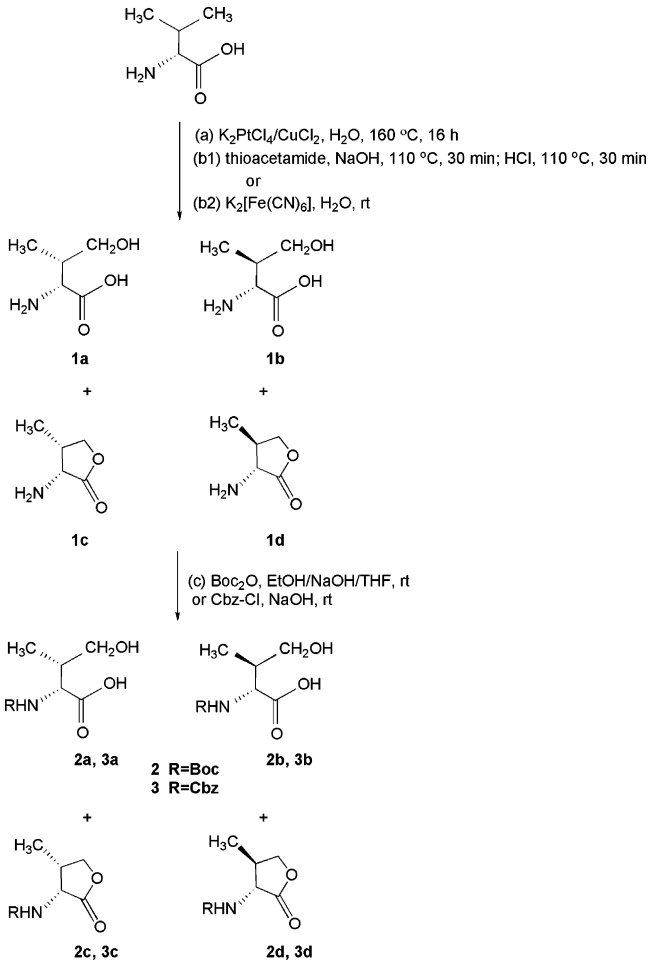
configuration at the α-carbon in conjunction with specific interactions with the surrounding L-amino acids.[3]

Conopeptides elicit a wide range of strong neurophysiological responses by targeting specific ion channels and receptors with very high affinities and selectivities.[5] As such they are promising therapeutic agents. Given this role, there is much interest in developing efficient methods for conopeptide synthesis.[6] Additionally, Hyv may be a useful marker for studying protein oxidation in biological systems under oxidative stress.[7] We have presently considered the synthesis of Hyv derivative suitably protected for Fmoc solid-phase chemistry and its subsequent incorporation into a conopeptide sequence. The synthesis of Hyv derivatives is complicated by the fact that Hyv contains two asymmetric centers and therefore exists as four stereoisomers: L-iso [(*S*,*S*)], D-iso [(*R*,*R*)], L-allo [(*S*,*R*)], and D-allo [(*R*,*S*)]. Hyv has been prepared previously as a racemic mixture by a modified Erlenmeyer synthesis.[8,9] The first stereocontrolled synthesis involved radical chlorination of L-Val, followed by hydrolysis of the product chlorides to give a mixture of Hyv diastereomers in very low yields.[10] In a second approach selectivity was achieved via asymmetric radical hydrogen bromide addition.[11] Most recently, a catalytic system based on platinum in combination with copper(II) as oxidant was utilized for C−H bond functionalization.[12] Yields of up to 67% were reported when L-Val was oxidized in the presence of K_2PtCl_4 as a catalyst and $CuCl_2$ as an oxidant. Good regio- and stereoselectivity [3:1 ratio of (3*S*,4*S*) to (3*S*,4*R*)] and limited racemization (<5%) was observed. Isolated L-Hyv lactones were subsequently converted to Boc-Hyv lactones. However, the Boc-Hyv lactone represented somewhat of a dead end, as the lactone form is too unreactive to use for Hyv incorporation into peptide chains. With the need for the open form of Hyv for peptide synthesis, side chain hydroxyl protection is also required to prevent lactonization during activation of the carboxyl group.[13,14]

To assemble Hyv-containing conopeptides, we have developed an efficient method for the synthesis of an appropriately functionalized Fmoc-Hyv building block, Fmoc-D-Hyv(*O*-TBDMS). We utilized a variation of the procedure of Sames et al.[12] for the efficient production of D-Hyv from D-Val. Copper-complexed D-Hyv was prepared and treated with TBDMSOTf to produce D-Hyv(*O*-TBDMS). Reaction of D-Hyv(*O*-TBDMS) with Fmoc-OSu gave the desired Fmoc-D-Hyv(*O*-TBDMS). Fmoc-D-Hyv(*O*-TBDMS) was characterized by NMR spectroscopy and mass spectrometry, and used in the synthesis of the conopeptide gld-*V** from *Conus gladiator*. The isolated synthetic and natural products had coincidental mass and NMR spectra.

SCHEME 1. Synthesis of Boc- or Cbz-Hyv



Results and Discussion

Initially, optimized conditions for the catalytic hydroxylation of Val were developed (Scheme 1). D-Val was treated with catalytic amounts of K_2PtCl_4 (10 mol %) in the presence of 10 equiv of $CuCl_2$ in H_2O at 160 °C for 16 h. The reaction was performed in a glass pressure vessel. Dangel et al.[12] applied gaseous H_2S for removal of excess $CuCl_2$, which is often used for the precipitation of the copper as cupric sulfide. A serious drawback to this method is the H_2S toxicity. Moreover, the reaction with gaseous H_2S often requires prolonged heating in highly acidic conditions to produce manageable precipitates. Even under those conditions the CuS sometimes stays colloidal and eludes filtration.[15] Thioacetamide had been successfully used for the decomposition of amino acid copper complexes.[15,16] The alkaline hydrolysis of thioacetamide results in a more tractable precipitate of CuS that remains readily filterable even upon subsequent acidification.[15] The reaction mixture described above was treated with thioacetamide under alkaline conditions (pH 8) for 30 min at 110 °C, and then followed by acidic conditions (pH 2) for an additional 10 min at 110 °C (Scheme 1, route b1). The insoluble CuS was filtered off upon cooling. We also explored the use of potassium ferrocyanide for copper precipitation (Scheme 1, route b2). An aqueous solution of potassium ferrocyanide was added to the reaction mixture and a red-brown

(5) Sharpe, I. A.; Gehrmann, J.; Loughnan, M. L.; Thomas, L.; Adams, D. A.; Atkins, A.; Palant, E.; Craik, D. J.; Adams, D. J.; Alewood, P. F.; Lewis, R. J. *Nat. Neurosci.* **2001**, *4*, 902−907.
(6) Marí, F.; Fields, G. B. *Chim. Oggi* **2003**, *21* (6), 43−48.
(7) Fu, S.; Hick, L. A.; Sheil, M. M.; Dean, R. T. *Free Radical Biol. Med.* **1995**, *19*, 281−292.
(8) Galantay, E.; Szabo, A.; Fried, J. *J. Org. Chem.* **1963**, *28*, 98−102.
(9) Englisch-Peters, S. *Tetrahedron* **1989**, *45*, 6127−6134.
(10) Faulstich, H.; Döling, J.; Michl, K.; Wieland, T. *Liebigs Ann. Chem.* **1973**, 560−565.
(11) Easton, C. J.; Merrett, M. C. *Tetrahedron* **1997**, *53*, 1151−1156.
(12) Dangel, B. D.; Johnson, J. A.; Sames, D. *J. Am. Chem. Soc.* **2001**, *123*, 8149−8150.
(13) Remmer, H. A.; Fields, G. B. In *Peptides*: *Frontiers of Peptide Science*; Proceedings of the Fifteenth American Peptide Symposium, June 14−19, 1997, Nashville, TN; Tam, J. P., Kaumaya, P. T. P., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; pp 287−288.
(14) Cudic, M.; Lauer-Fields, J. L.; Fields, G. B. *J. Peptide Res.* **2005**, *65*, 272−283.
(15) Taylor, U. F.; Dyckes, D. F.; Cox, J. R., Jr. *Int. J. Peptide Protein Res.* **1982**, *19*, 158−161.
(16) Crivici, A.; Lajoie, G. *Can. Synth. Commun.* **1993**, *23*, 49−53.

precipitate formed. The $Cu[Fe(CN)_6]$ precipitate was removed by centrifugation. In either case (route b1 or b2), evaporation of the filtrate gave D-Hyv as a mixture of open form isomers **1a** and **1b** and lactone isomers **1c** and **1d** (Scheme 1).

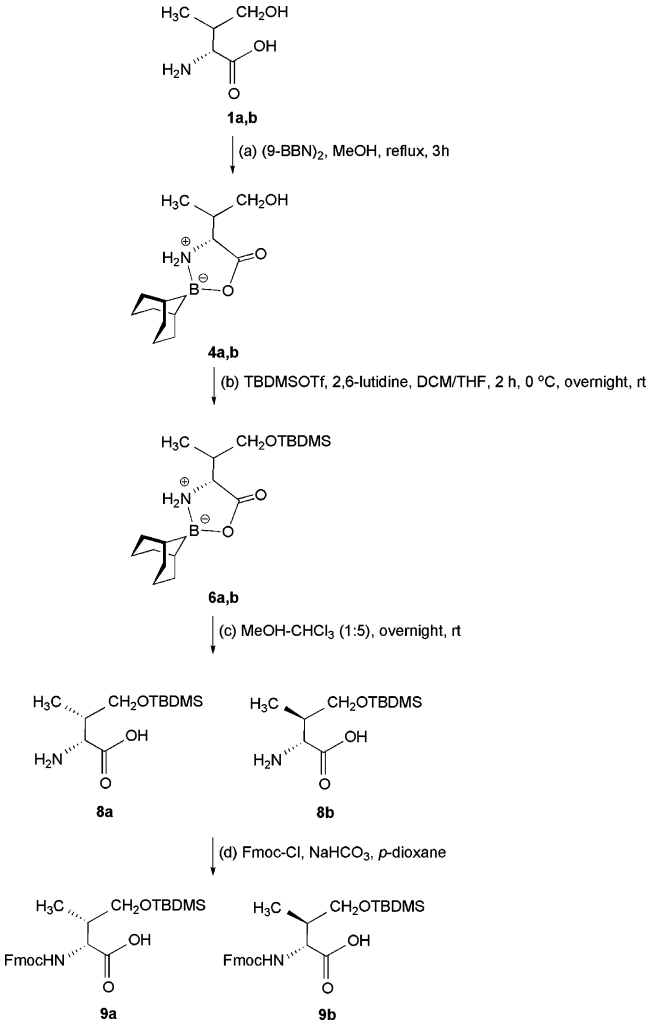
A diastereoselective ratio of 3:1 (3*S*,4*S*) to (3*S*,4*R*) for the crude reaction material was observed by ^{1}H NMR spectroscopy in D_2O (Supporting Information, Figure 1). The value was based on the ratio of integration units for the CH_3 protons of the product and unreacted D-Val. The result is in accordance with that previously published.[12] The level of L-Hyv in the product was not evaluated, as it had been shown previously that racemization was negligible during the synthesis of L-Hyv from L-Val.[12]

Initial attempts to protect the α-amino group of D-Hyv with the *tert*-butyl dicarbonate (Boc_2O) or benzyl chloroformate (Cbz-Cl) group (Scheme 1) resulted in low yields. The low yields could be explained by competing formation of the corresponding N^{α}-protected Hyv in cyclic (lactone) form and open (linear) form. It has been shown that the synthesis of *N*-substituted homoserines required longer reaction times than *N*-substitutions of most amino acids, most likely due to the intramolecular hydrogen bonding between the amino and the γ-hydroxyl group of homoserine.[17] The purified major isomer of N^{α}-Boc protected Hyv lactone (**2c**) and both major and minor isomers of N^{α}-Cbz protected Hyv lactone (**3c** and **3d**) were isolated and characterized by ^{1}H NMR spectroscopy (Supporting Information, Figures 2−4).

An alternative α-amino group protection scheme that included prior hydroxyl protection was developed to prevent lactone formation. Previous studies have indicated that 9-borabicyclononane (9-BBN) protected amino acids are very tolerant to a wide range of reaction conditions, and the solubility of the corresponding borane complexes is significantly improved in various organic solvents.[18−20] The crude reaction mixture of Val and Hyv was treated with 9-BBN while refluxing in methanol to give a mixture of soluble borane complexes of Val and Hyv (Scheme 2) that were successfully separated by column chromatography. The hydroxyl group of Hyv was protected by treatment with *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf) in the presence of 2,6-lutidine (Scheme 2). The major isomer **6a** was separated from the minor isomer **6b** by column chromatography. Starting from **4a** and **4b**, **6a** and **6b** were isolated in a combined 88% yield.

Subsequent cleavage of the borane complex proceeded with more difficulties than expected. Borane complexes of amino acids are typically cleaved with either aqueous HCl or by exchange with ethylenediamine in methanol. When 9-BBN was used for regioselective protection of the α-amino and α-carboxyl groups of (5*R*)-δ-hydroxy-L-Lys, complete decomplexation was reported via treatment with a mixture of chloroform and methanol over 12 h at room temperature.[20] Decomplexation of the borane complex of Hyv **6a** was slow in the presence of ethylenediamine or a chloroform−methanol mixture and was observed only after heating the reaction mixture for several hours at 50 °C. The product **8a** was reacted with Fmoc-Cl and the resulting residue was purified by flash chromatography to provide Fmoc-D-Hyv(*O*-TBDMS) **9a** in 12% yield starting from **6a**. NMR and mass spectra and RP-HPLC analysis confirmed the purity and composition of Fmoc-D-Hyv(*O*-TBDMS) (Supporting Information, Figures 5 and 6). The overall yield of **9a** utilizing the 9-BBN complex approach was 2% starting from D-Val.

SCHEME 2. Synthesis of Fmoc-Hyv(*O*-TBDMS) with 9-BBN Complexation



Due to the difficulties with 9-BBN complex decomposition, a copper complex approach was examined (Scheme 3). Copper-complexed Hyv **5a** and **5b** was prepared as previously described.[14] The hydroxyl group of Hyv was protected by treatment with TBDMSOTf in the presence of DMAP in pyridine. The desired copper-complexed D-Hyv(*O*-TBDMS) **7a** and **7b** was separated from copper-complexed Val by extraction with EtOAc−H_2O. Disruption of the copper complex was achieved by using Na^+ Chelex 100 resin in a pyridine−H_2O mixture.[21] The product **8a** and **8b** was reacted with Fmoc-OSu and the resulting residue purified by flash chromatography to provide Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** in 56% yield starting from **8a** and **8b**. NMR and mass spectra confirmed the purity and composition of Fmoc-D-Hyv(*O*-TBDMS) (Supporting Information, Figures 7 and 8). The overall yield of **9a** and **9b** utilizing the copper complex approach was 26% starting from

(17) Ozinskas, A. J.; Rosenthal, G. A. *J. Org. Chem.* **1986**, *51*, 5047−5050.

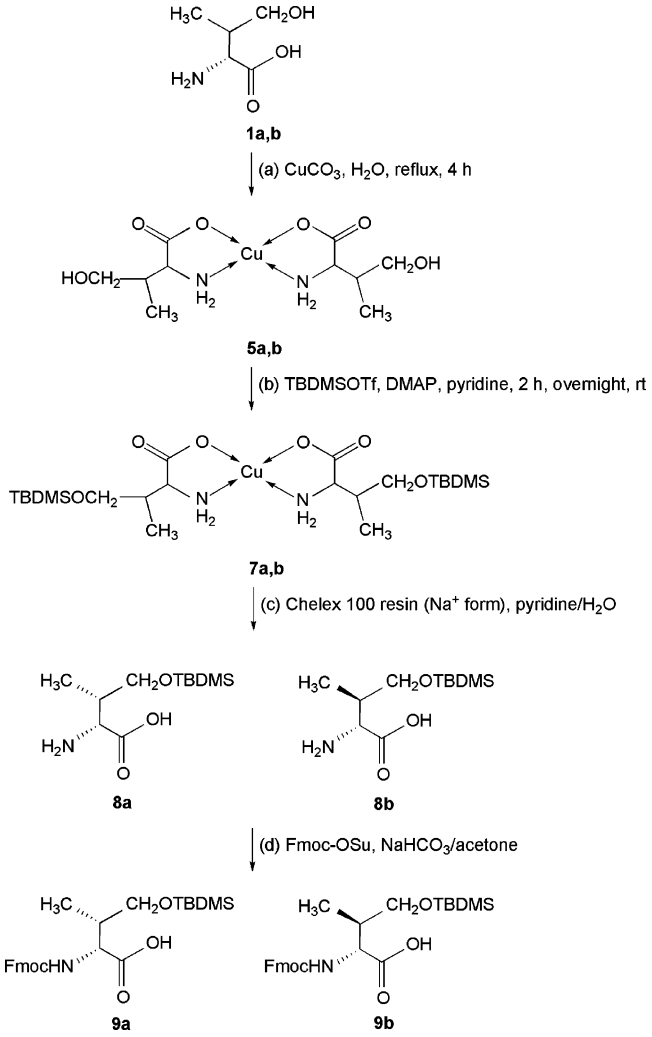
(18) Dent, W. H., III; Randal Erickson, W.; Fields, S. C.; Parker, M. H.; Tromiczak, E. G. *Org. Lett.* **2002**, *4*, 1249−1251.

(19) Walker, W. H., IV; Rokita, S. E. *J. Org. Chem.* **2003**, *68*, 1563−1566.

(20) Syed, B. M.; Gustafsson, T.; Kihlberg, J. *Tetrahedron* **2004**, *60*, 5571−5575.

(21) Barral, I.; Savrda, J. *Synthesis* **1973**, 795−796.

SCHEME 3. Synthesis of Fmoc-Hyv(*O*-TBDMS) with Copper Complexation



D-Val. RP-HPLC analysis (Supporting Information, Figure 9) of Fmoc-D-Hyv(*O*-TBDMS) revealed the presence of two peaks (at 17.7 and 17.9 min) whose areas corresponded well to the observed diastereoselective ratio of 3:1 (3*S*,4*S*) to (3*S*,4*R*) for the D-Hyv mixture of **1a** and **1b**. By comparison, Fmoc-D-Hyv(*O*-TBDMS) **9a** isolated from the 9-BBN complex approach showed the presence of only one peak by RP-HPLC at 17.7 min (Supporting Information, Figure 6). Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** from the copper complexation approach were then conveniently separated by preparative RP-HPLC (Supporting Information, Figures 10 and 11) with an overall yield of 13% starting from D-Val.

The mixture of the major and minor isomers of Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** was used for the solid-phase synthesis of the D-Hyv containing conopeptide gld-*V** (Ala-Hyp-Ala-Asn-Ser-D-Hyv-Trp-Ser-NH_2). The peptide was assembled by automated Fmoc chemistry. Fmoc-D-Hyv(*O*-TBDMS) was coupled by using a 2-fold molar excesses of Fmoc-amino acid and 1-hydroxybenzotriazole (HOBt), a 1.8-fold molar excess of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and a 4-fold molar excess of *N*,*N*-diisopropylethylamine. Peptide-resin cleavage and side chain deprotection proceeded with H_2O−thioanisole−TFA (1:1:18) for 2 h. The peptide was purified by preparative RP-HPLC. The product was homogeneous by analytical RP-HPLC (Supporting Information, Figure 13), and MALDI-TOF-MS analysis gave the desired mass (Supporting Information, Figure 12).

The synthetic gld-*V** conopeptide was compared with material isolated from *Conus gladiator*. Due to the Hyv chiral center at the β-carbon, *Conus gladiator* gld-*V** exists as diastereomers, which are referred to as gld-*V** and gld-*V**′.[3] Prior analysis indicated that gld-*V** corresponds to the Hyv (3*S*,4*S*) configuration, whereas gld-*V**′ corresponds to the Hyv (3*S*,4*R*) configuration.[3] Analysis of the synthetic peptide product by using RP-HPLC conditions designed to separate out gld-*V** and gld-*V**′[3] showed more than one component of the same mass (data not shown). NMR spectroscopic analysis found that the most prominent isolated product corresponded to naturally occurring gld-*V** (Supporting Information, Figure 14). Analysis of the Fmoc-Hyv(*O*-TBDMS) derivative used for peptide synthesis indicated 75% of the (3*S*,4*S*) configuration present (see earlier discussion), and thus it is logical that the most abundant synthetic peptide diastereomer is the one containing the Hyv (3*S*,4*S*) configuration (gld-*V**).

Overall, an efficient 5 step synthesis of an appropriately functionalized Hyv building block, Fmoc-D-Hyv(*O*-TBDMS), has been developed (Scheme 3). Fmoc-D-Hyv(*O*-TBDMS) was subsequently utilized for the solid-phase synthesis of a Hyv-containing conopeptide. The (3*S*,4*S*) and (3*S*,4*R*) configurations of Hyv could be separated out following synthesis of either complexed D-Hyv(*O*-TBDMS), Fmoc-D-Hyv(*O*-TBDMS), or the desired peptide. The methodology presented herein will greatly facilitate biological studies of Hyv-containing sequences, such as receptor responses to hydroxylated versus nonhydroxylated conopeptides[3] and the relative susceptibility of proteins to modification by oxidative stress.[7]

Experimental Section

Synthesis of 4-Hydroxy-D-valine (γ-Hydroxy-D-Val) (1). Synthesis of γ-hydroxy-D-Val was performed as described.[12] Briefly, D-Val (5 mmol, 585 mg) was treated with a catalytic amount of K_2PtCl_4 (10 equiv, 10 mol %, 0.5 mmol, 208 mg) in the presence of $CuCl_2 \times 2H_2O$ (10 equiv, 50 mmol, 8.5 g) and heated to 160 °C for 16 h in a pressure vessel (Chemglass). Copper(II) sulfide was removed by treatment with thioacetamide or K_2PtCl_4 (see below).

(1) Method A: Thioacetamide Removal of Copper(II) Sulfide. Thioacetamide (1.1 equiv, 55 mmol, 4.25 g) was added to the reaction mixture followed by NaOH (4 M) until the pH of the suspension was brought to 8. The suspension was heated at 110 °C for 30 min. The pH of the suspension was brought to 2 with 2 M HCl, followed by heating for 10 min at 110 °C. The reaction mixture was cooled down and filtered through a bed of Celite. After filtration, the pH of the solution was brought to 8 and the solution left in the hood overnight and than evaporated. D-Hyv was obtained as a mixture of open chain isomers **1a** and **1b** and lactone isomers **1c** and **1d**. The product to starting material ratio ($\times$100) as well as the isomer ratio was determined by ^{1}H NMR spectroscopy (Supporting Information, Figure 1) as described.[12]

(2) Method B: Potassium Ferrocyanide Removal of Copper(II) Sulfide. The reaction mixture was cooled down to room temperature. Precipitated CuCl was filtered off and the precipitate washed well with cold H_2O. Potassium ferrocyanide (5 mmol, 2.1 g) was dissolved in H_2O (20 mL) and added to the solution. The red-brown precipitate formed was removed by centrifugation and then washed two times with H_2O. Filtrates were collected and evaporated to dryness. The residue was dissolved in small amount of ethanol (10 mL). Nondissolved material (salt) was filtered and evaporation of filtrate gave D-Hyv as a mixture of open chain isomers **1a** and **1b** and lactone isomers **1c** and **1d**. The product to

starting material ratio (×100) as well as the isomer ratio was determined by ^{1}H NMR spectroscopy as described.[12]

Synthesis of *N*-*tert*-Butyloxycarbonyl-4-hydroxy-D-valine (2). Boc-protected γ-hydroxy-D-Val lactone **2c** and **2d** was prepared as described.[12] Briefly, the crude reaction mixture of D-Hyv was dissolved in ethanol (10 mL) and NaOH (2 M) was added until the pH 10. Boc_2O (1.4 equiv, 7 mmol, 1.5 g) was dissolved in THF (5 mL) and added to the reaction mixture. The reaction was stirred at room temperature and monitored by TLC (EtOAc−toluene−HOAc, 5:5:1). Organic solvents were removed by rotary evaporation before acidification. The reaction was diluted with H_2O, cooled to 0 °C, and adjusted to pH 3 with HCl (2 M). The aqueous mixture was extracted with EtOAc. The organic phase was dried with anhydrous Na_2SO_4, filtered, and concentrated to an oil. The product was a mixture of Boc-Val and Boc-Hyv in lactone and open forms. This material was purified by flash chromatography (EtOAc−toluene−HOAc, 5:5:1). The total yield of Boc-Hyv lactone **2c** and **2d** was 120 mg (10% starting from D-Val). Isolated **2c** lactone was characterized by ^{1}H NMR spectroscopy (Supporting Information, Figure 2) as described.[12]

Synthesis of *N*-Benzyloxycarbonyl-4-hydroxy-D-valine (3). Cbz-protected γ-hydroxy-D-Val lactone **3c** and **3d** was prepared starting from the crude reaction mixture of D-Hyv in H_2O. NaOH (2 M) was added until pH 8 and then Cbz-Cl was added (1.5 equiv, 7.5 mmol, 1080 μL). The reaction was stirred at room temperature and monitored by TLC (EtOAc−toluene−HOAc, 1:10:1). The reaction was cooled to 0 °C and adjusted to pH 3 with HCl (2 M). The aqueous mixture was extracted with EtOAc. The organic phase was dried with anhydrous Na_2SO_4, filtered, and concentrated to an oil. The product was a mixture of Cbz-Val and Cbz-Hyv in lactone and open forms. This material was purified by flash chromatography (EtOAc−toluene−HOAc, 1:10:1). The total yield of Cbz-Hyv lactone **3c** and **3d** was 120 mg (9% starting from D-Val). Compounds **3c** and **3d** were characterized by ^{1}H NMR spectroscopy (Supporting Information, Figures 3 and 4).

Synthesis of 4-Hydroxy-D-valinatobicyclononylboron 4. The crude reaction mixture of Val and Hyv (starting from 5 mmol) was dissolved in methanol (20 mL) and 9-BBN dimer (6 mmol, 1.464 g, 1.2 equiv) was added. The reaction mixture was refluxed for 3 h. Methanol was evaporated and the crude residue was purified by flash chromatography (EtOAc−toluene, 10:1). Val borane complex was successfully separated from Hyv borane complex **4a** and **4b**. The total yield of Hyv borane complex **4** was 178 mg (14% starting from **1**).

Synthesis of 4-Hydroxy-D-valine Copper Complex 5. The copper complex of D-Hyv **5a** and **5b** was prepared as described.[14] Two equivalents of $CuCO_3$ (8−10 mmol) was used and the reaction was refluxed for 4 h. The reaction mixture was evaporated and dried well in a desiccator, then was used without further purification.

Synthesis of 4-*O*-*tert*-Butyldimethylsilyl-D-valine. (1) Method A: Starting from 4-Hydroxy-D-valinatobicyclononylboron 4a and 4b. 9-BBN-complexed Hyv **4a** and **4b** (180 mg, 0.7 mmol) were dissolved in a mixture of DCM and THF (2:1, 20 mL), whcih was cooled to 0 °C, then TBDMSOTf (242 μL, 1 mmol) was introduced in the presence of 2,6-lutidine (165 μL, 1.38 mmol) as catalyst.[20] The reaction mixture was stirred overnight. The solution was evaporated and the residue was purified by flash chromatography (EtOAc−toluene, 1:1), which allowed for separation of the major isomer **6a** from the minor isomer **6b**. The fractions were collected and after evaporation 230 mg of **6a** and **6b** combined was obtained, which corresponded to 88% yield starting from **4a** and **4b**. The major isomer (**6a**) was dissolved in a mixture of methanol and chloroform (1:5, 10 mL) and the solution stirred overnight at room temperature. There was no noticeable decomposition of the complex; therefore, the reaction mixture was stirred for an additional 16 h at 50 °C. The solution containing decomplexed D-Hyv(*O*-TBDMS) **8a** was evaporated to dryness under reduced pressure and the residue was used in the next step without further purification.

(2) Method B: Starting from 4-Hydroxy-D-valine Copper Complex 5a and 5b. A copper-complexed mixture of Val and Hyv **5a** and **5b** (starting from 5 mmol) was dissolved in pyridine (20 mL) and TBDMSOTf (10 mmol, 2.2 mL) was introduced in the presence of DMAP (5 mmol, 604 mg) as catalyst as previously described for copper-complexed Hyl(Boc).[14] After overnight stirring pyridine was evaporated. The residue was partitioned between EtOAc and H_2O. TLC analysis (*n*-BuOH−HOAc−H_2O, 12:3:5) of the EtOAc and H_2O layers revealed that the desired copper-complexed D-Hyv(*O*-TBDMS) **7a** and **7b** was present in the EtOAc layer and copper-complexed Val in the H_2O layer. EtOAc was evaporated and the residue was dissolved in a mixture of pyridine and H_2O (1:1, 30 mL). Na^+ Chelex 100 resin (10 g) was added to the stirred solution. After overnight stirring, the resin was filtered and the colorless solution was evaporated to dryness under reduced pressure to provide the sodium salt of D-Hyv(*O*-TBDMS) **8a** and **8b** (570 mg, 42% yield from **5a** and **5b**). This material was used in the next step without further purification.

Synthesis of *N*-(Fluoren-9-ylmethoxycarbonyl)-4-*O*-*tert*-butyldimethylsilyl-D-valine. D-Hyv(*O*-TBDMS) **8a** and **8b** (570 mg, 2.1 mmol) and $NaHCO_3$ (350 mg, 4.2 mmol) were dissolved in H_2O (10 mL), and a solution of Fmoc-OSu (1.06 g, 3.15 mmol) in acetone (10 mL) was added. Additional acetone (10 mL) was added, and the reaction proceeded overnight at room temperature. Acetone was removed under reduced pressure and the remaining H_2O solution was acidified to pH ∼2 with 1 M HCl. The aqueous suspension was extracted three times with 20 mL of $CHCl_3$. The $CHCl_3$ layer was washed three times with 20 mL of H_2O, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc−toluene−HOAc, 5:10:1, v/v) to obtain Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** (580 mg, 56% yield from **8a** and **8b**). Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** were characterized by ^{1}H NMR spectroscopy (Supporting Information, Figure 7) and MALDI-TOF-MS (*m*/*z* expected 492.62 $[M + Na]^+$; observed 492.52 $[M + Na]^+$). A total of 27 mg of Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** was then purified by preparative RP-HPLC, yielding 11 mg of **9a** and 2 mg of **9b** (Supporting Information, Figures 10 and 11). This represented an overall yield of **9a** and **9b** of 13% starting from D-Val.

D-Hyv(*O*-TBDMS) **8a** (0.6 mmol), obtained from 9-BBN-complexed D-Hyv(*O*-TBDMS) **6a**, was reacted with Fmoc-Cl (1.05 mmol) with use of similar conditions as described above. The major isomer **9a** was isolated in 14% yield starting from **6a** and 2% overall yield starting from D-Val, and characterized by ^{1}H NMR spectroscopy (Supporting Information, Figure 5).

Peptide Synthesis. Peptide-resin assembly was performed on an automated peptide synthesizer by using Rink amide MBHA resin with an initial load of 0.72 mmol/g. Standard Fmoc chemistry was used throughout with a 4-fold molar excess of the acylating amino acids, and HBTU and HOBt as coupling reagents.[22] Fmoc-D-Hyv-(*O*-TBDMS) **9a** and **9b** were coupled manually in 2-fold molar excess to reduce consumption of this amino acid. The peptide was cleaved from the resin with H_2O−thioanisole−TFA (1:1:18) for 2 h. The peptide (Ala-Hyp-Ala-Asn-Ser-D-Hyv-Trp-Ser-NH_2) was purified by preparative RP-HPLC. The product was homogeneous by analytical RP-HPLC and MALDI-TOF-MS analysis gave the desired mass: *m*/*z* expected 862.89 $[M + H]^+$; observed 863.49 $[M + H]^+$ and 885.48 $[M + Na]^+$. Diastereomeric peptides were separated by using C_{18} RP-HPLC.[3] Initially, peptides were separated with an elution gradient of 0−100% B over 100 min where A was 0.1% TFA in H_2O and B was 0.1% TFA in CH_3CN−H_2O (3:2). Isolated fractions were then further purified via isocratic elution at 20.3% B. One-dimensional ^{1}H NMR spectra of the most prominent diastereromeric peptide and gld-*V** isolated from *Conus gladiator* were recorded at 25 °C with use of a 1.7 mm tube in a 3 mm gHCN probe. The NMR assignments of the γ-hydroxy-D-Val (HN: d 7.99,

(22) Fields, C. G.; Lloyd, D. H.; Macdonald, R. L.; Otteson, K. M.; Noble, R. L. *Peptide Res.* **1991**, *4*, 95−101.

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) were in accordance with the reported values of the synthetic amino acid.[11]

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Supporting Information Available: General experimental methods; one-dimensional ^{1}H NMR spectra of D-Hyv **1**, Boc-D-Hyv lactone **2c**, Cbz-D-Hyv lactone **3c** and **3d**, Fmoc-D-Hyv(*O*-TBDMS) **9a**, Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b**, and isolated and synthesized gld-*V**; RP-HPLC analysis of Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b**, Fmoc-D-Hyv(*O*-TBDMS) **9a**, Fmoc-D-Hyv(*O*-TBDMS) **9b**, and synthetic gld-*V**; and MALDI-TOF mass spectra of Fmoc-D-Hyv(*O*-TBDMS) **9a** and **9b** and synthetic gld-*V**. This material is available free of charge via the Internet at http://pubs.acs.org.

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