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Synthesis and Biological Activities of Phenylahistin Derivatives

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Abstract—X-ray crystallographic analysis was performed and several phenylahistin derivatives were synthesized to elucidate the structural components necessary for the anti-microtubule activity of phenylahistin. We primarily focused on the unique isoprenylated dehydrohistidine structure. Our results showed that a uniplanar pseudo-three-ring structure formed by the hydrogen bonding of diketopiperazine and imidazole rings is important for the anti-microtubule activity of phenylahistin.

Introduction

Phenylahistin [**1**], a fungal metabolite from *Aspergillus ustus* NSC-F038, was discovered during screening for new cell cycle inhibitors. It is a member of a new class of colchicine-like microtubule binding agents that exhibit cytotoxic activity against a wide variety of tumor cell lines.[1–3] (−)-Phenylahistin [(−)-**1**], a diketopiperazine derivative, consists of L-phenylalanine and a unique isoprenylated dehydrohistidine residue with a quaternary carbon at the 5-position of the imidazole ring.[2] To develop more potent anti-tumor agents based on this diketopiperazine derivative, it is important to elucidate the structural components important in the anti-microtubule activity of (−)-**1**.

In the present study, we used X-ray crystallography to analyze (+)-**1** and the synthesis of its derivatives, and comprehensively investigate the structural components of phenylahistin that are necessary for anti-microtubule activity. In particular, we focused on the isoprenylated dehydrohistidine structure and found that it forms a rigid uniplanar pseudo-three-ring structure, composed of diketopiperazine and imidazole rings. The rings are attached through a hydrogen bond between N8-H and N3 and an α, β-unsaturated bond (C6–C7). The structure of this component is particularly important for the anti-microtubule activity of phenylahistin.

Key words: Amino acids and derivatives; antitumor compounds; X-ray crystal structure; natural products.

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Chemistry

Simple alkylation and reduction of **1** and modification of cyclo(Gly-Phe) with imidazole derivatives seem to be necessary for the synthesis of derivatives of **1**. However, the complete synthesis and subsequent analogue development of **1** has not yet been accomplished due to the presence of a unique quaternary carbon at the imidazole ring. Derivatives **2**–**5** were synthesized from **1** by alkylation or reduction. As shown in Scheme 1, **2** and **3** were synthesized by hydrogenation of each enantiomer of **1** over 10% palladium on carbon in MeOH under atmospheric hydrogen at room temperature. Two-hour hydrogenation yielded derivative **2**, in which the 1,1-dimethyl-2-propenyl group of **1** was reduced. Further hydrogenation (24 h) with **2** yielded derivative **3**, in which the dehydrohistidine was reduced. The latter reduction resulted in only one diastereomer, with an optical rotation polarity similar to that of **2**. Although we did not determine the absolute configuration at the α-position of the substituted histidine residue of **3**, this diastereoselective hydrogenation may be due to the steric hindrance of the phenyl ring of the Phe residue. This conclusion is based on the results of (+)-**1** X-ray crystallography which indicates that this phenyl ring is positioned above and overlays the diketopiperazine ring (see Results and Discussion).

As shown in Scheme 2, methylation of **1** with MeI and NaH in DMF yielded mono- and tri-methylated compounds. The extent of methylation could be controlled by manipulating the reaction temperature rather than the amount of reagents. Mono-methylated derivative **4** was predominantly obtained in a reaction with 10

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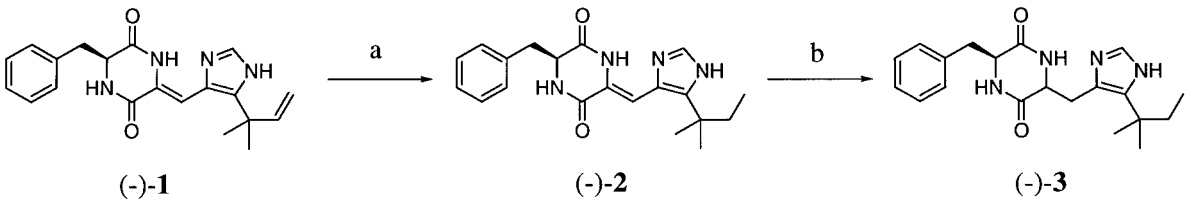
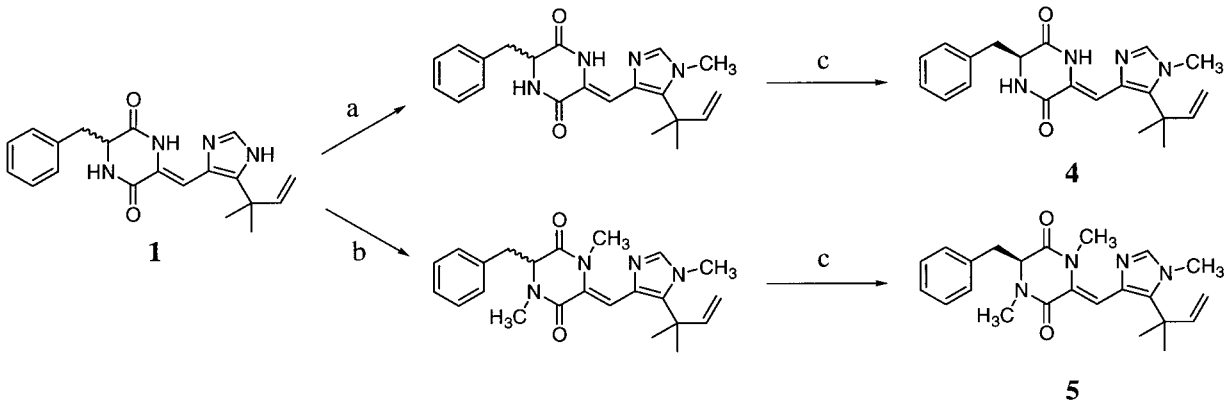
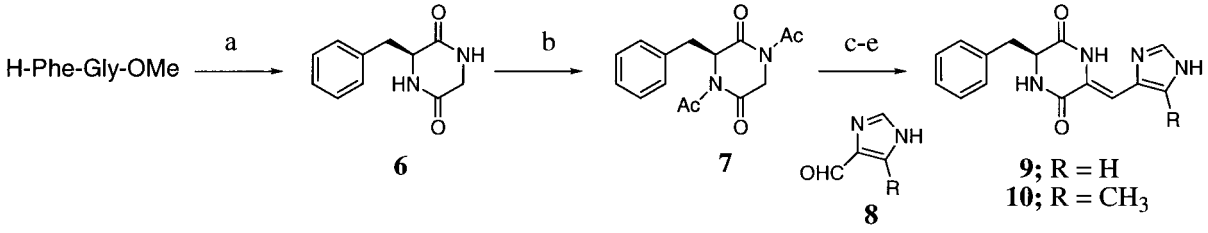


Scheme 1. (a) H_2/10% Pd-C, MeOH, rt, 2 h; (b) H_2/10% Pd-C, MeOH, rt, 24 h.



Scheme 2. (a) MeI, NaH, DMF, −30°C, 2 h; (b) MeI, NaH, DMF, rt, 2 h; (c) chiral HPLC.



Scheme 3. (a) MeOH, reflux, 14 h; (b) Ac_2O, AcONa, 80°C, 14 h; (c) **8** LDA, HMPA, DMF, −60°C, 0.5 h; (d) Tf_2O, pyridine, rt, 10 min; (e) NH_4OH, rt, overnight.

equivalent of the reagents at −30°C. Methylation at the τ nitrogen of the imidazole ring, was detected by NMR analysis.[4] On the other hand, the tri-methylated derivative **5** was obtained in a reaction with 30 equivalent of the reagents at room temperature. Compounds **4** and **5** with an L-phenylalanine residue were separated by HPLC with a chiral column.[5]

Derivatives **9** and **10** were synthesized from cyclo (Gly-Phe) **6**, which was prepared by cyclization of H-Phe-Gly-OMe. After acetylation of the two amide nitrogens on the diketopiperazine ring of **6**, 4(5)-imidazolecarboxaldehyde or 4-methyl-5-imidazolecarboxaldehyde (**8**) was introduced in the presence of lithium diisopropylamide (LDA) and hexamethylphosphoramide (HMPA).[6] Subsequent dehydration with triflic anhydride-pyridine and deacetylation with aqueous NH_4OH gave compounds **9** and **10**, although less than 30% racemization was observed in these steps. Compounds **9** and **10** with an L-phenylalanine residue were purified by chiral column HPLC.

Results and Discussion

X-ray crystallographic analysis

X-ray analysis of (+)-**1** is shown in Figure 1 and Table 2. The stereochemistry of the C6–C7 double-bond was confirmed to be *Z*, and the presence of a hydrogen bond between N8-H and N3 was noted. These findings were in agreement with those established in NMR studies (low field-shift of N8-H d 12.08 ppm).[2,7] The results

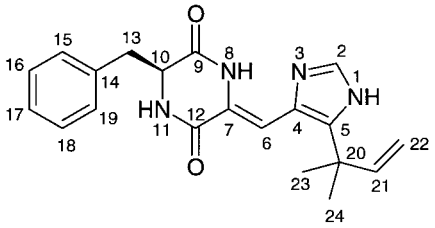
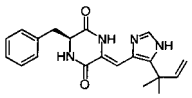
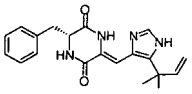
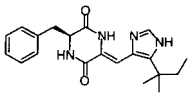
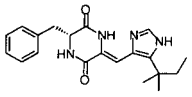
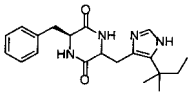
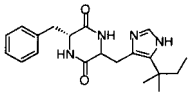
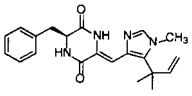
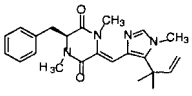
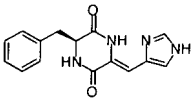
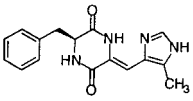
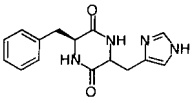


Figure 1. Structure of (−)- phenylahistin [(−)-**1**].

Table 1. Biological activity of phenylahistin and its derivatives

Compound	Structure	IC_{50} (μM)	
		Microtubule protein[a] polymerization	P388 proliferation
(−)-**1**		25	0.21 ± 0.02[b]
(+)-**1**		> 200 [15%][c]	10 ± 1.5
(−)-**2**		30	0.23 ± 0.05
(+)-**2**		> 200 [12%]	19 ± 4.2
(−)-**3**		> 200	> 200
(+)-**3**		> 200	> 200
4		100	0.95 ± 0.03
5		> 200	160 ± 5.5
9		> 200 [14%]	> 200
10		> 200 [28%]	7.5 ± 0.5
11[d]		> 200	> 200
	colchicine[d]	16	0.031 ± 0.01

[a]Microtubule protein was prepared from bovine brain by two cycles of the assembly and disassembly method.[9] The concentration of microtubule protein used for the assay was 1.5 mg/mL.
[b]Values are mean ± SEM of three experiments.
[c]The inhibition percentage at 200 μM is indicated within the square brackets when the inhibition was observed, since diketopiperazine derivatives are insoluble at concentrations more than 200 μM.
[d]Cyclo(His-Phe) **11** and colchicine were purchased from Sigma (St. Louis, MO).

Table 2. Results of X-ray crystallographic analysis of (+)-**1**

Crystal parameters	
Empirical formula	$C_{20}H_{22}N_4O_2$
Formula weight	350.42
Crystal system	tetragonal
Lattice parameters:	a = 15.3509(7) Å
	c = 8.309(2) Å
	V = 1958.0(2) Å^3
	$Z = 4$
Space group	$P4_2$ (#77)
Dcalc.	1.189 g/cm^3
μ (CuK_α)	6.37 cm^{-1}
Refinement parameters	
No. of reflections measured	2229
Nonzero reflections (I > 1.50σ)	1724
R-index Residuals: *R*[a]	0.057
Residuals: *RW*[b]	0.067
Goodness of fit indicator[c]	4.31

[a]$\Sigma \parallel Fo| - |Fc \parallel / \Sigma |Fo|$.
[b]$[\Sigma w((|Fo| - |Fc|)^2 / \Sigma w Fo^2)]^{1/2}$.
[c]$[\Sigma w(|Fo| - |Fc|)^2 / (No - Nv)]^{1/2}$.

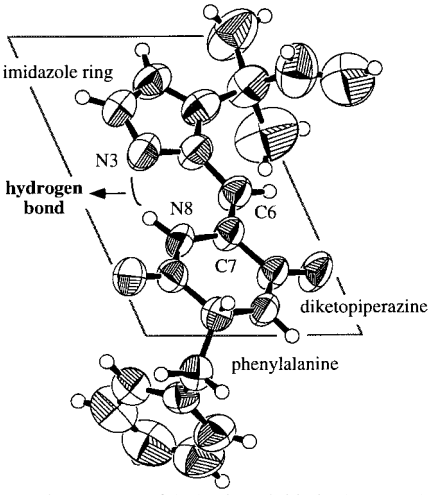


Figure 2. Crystal structure of (+)-phenylahistin (ORTEP).

suggested that the two heterocycles, i.e., the diketopiperazine and imidazole rings, were fixed in the same plane by forming a pseudo-three-ring structure. The benzyl group of the Phe residue was located out of this plane and over the diketopiperazine ring, in a conformation which is reported as the most energetically favorable for a diketopiperazine with an aromatic amino acid residue.[8] The movement of the 1,1-dimethyl-2-propenyl group at the imidazole ring was restricted by the steric hindrance of a hydrogen atom at the β-position (C6) of the α,β-unsaturated His derivative. These findings indicate that the conformation of (+)-phenylahistin is highly restricted. Since the (−)-form of **1**, the biologically active enantiomer, only varies from the (+)-form in conformation at the α-position of the Phe residue, the rigid conformation of phenylahistin may be important for binding to the microtubule protein.

Biological activities of synthetic derivatives

To understand the relationship between the postulated rigid plane structure of (−)-**1** and its biological activity, we synthesized derivatives of (−)-**1** and examined its effects on the polymerization of microtubule protein prepared from the bovine brain[9,10] and the proliferation of P388 cells. We have recently demonstrated that (−)-phenylahistin [(−)-**1**] exhibited colchicine-like inhibition of microtubule polymerization ($IC_{50} = 25\ \mu M$).[1] This inhibitory activity of (−)-**1** was similar to that of colchicine ($IC_{50} = 16\ \mu M$), although the anti-proliferative activity of (−)-**1** ($IC_{50} = 0.21\ \mu M$) was about seven times less than that of colchicine ($IC_{50} = 0.031\ \mu M$). The differences in activity may be due to other biological activities of colchicine, or lower cellular permeability of (−)-**1** in anti-proliferative assays, since (−)-**1** has a more hydrophilic structure than colchicine. We also demonstrated that (−)-**1** with the L-Phe residue was 50 times more active than its (+)-form in anti-proliferative assays. (+)-**1** also exhibited a weak inhibitory activity against microtubule polymerization (15% inhibition at 200 μM) compared with that of (−)-1. These results indicate that the spatial arrangement of the benzyl group of the phenylalanine residue is important for the potent biological activity.

In the present study, compound **2**, in which the 1,1-dimethyl-2-propenyl group of **1** was reduced (Table 1), showed the same activity as **1**, indicating that this double bond is not important for the anti-microtubule activity. However, compound (−)-**3**, in which the dehydrohistidine of (−)-**2** was reduced, completely lost the inhibitory activity. Modification of the pseudo-three-ring structure in the phenylahistin structure alters activity, suggesting that inhibitory activity is dependent upon a planar arrangement of the diketopiperazine and imidazole rings.

Compound **4** was derived through methylation of the nitrogen atoms of **1**. When the τ nitrogen of the imidazole ring of (−)-**1** was methylated, the compound was approximately five times less active than (−)-**1**. This finding suggests that the imidazole nitrogen participates in binding with the microtubule protein or that this methylation affords structural hindrance to the conformation of the 1,1-dimethyl-2-propenyl group on the imidazole ring. The tri-methylated compound **5** had a severe reduction in inhibitory activity. This result also suggests that the rigid plane conformation of (−)-**1** is important for anti-microtubule activity, since methylation of the diketopiperazine nitrogen (N8) disrupts the formation of the hydrogen bond necessary for the rigid pseudo-three-ring conformation.

To determine the importance of the alkyl group at the 5-position on the imidazole ring, the 1,1-dimethyl-2-propenyl group of (−)-**1** was replaced with a hydrogen atom or a methyl group, in compounds **9** and **10** respectively. Compound **9** containing a hydrogen atom at position 5 lost most inhibitory activity. Compound **10** with a methyl group at the 5-position also exhibited decreased activity in the anti-microtubule assay, although a weak inhibitory effect on P388 cell proliferation was observed. The marked decrease in biological activity of **9** and **10** suggests that an alkyl group of the proper length or a quaternary carbon at the 5-position

of the imidazole ring is very important for the anti-microtubule activity. Further SAR studies are necessary to establish the importance of this component. Total synthesis of phenylahistin, including the formation of a chemical bond containing a quaternary carbon on the imidazole ring, should provide this answer.

Conclusion

In the present study, we analyzed the X-ray crystallography of (+)-phenylahistin and evaluated the biological activity of phenylahistin derivatives. We determined that the component of (−)-phenylahistin necessary for anti-microtubule activity is the rigid uniplanar pseudo-three-ring structure formed by a hydrogen bond. Our results should be useful for the development of more potent anti-microtubule agents based on the diketopiperazine structure.

Experimental

Microtubule protein polymerization assay

Microtubule protein was prepared from bovine brain tissue by two cycles of assembly and disassembly.[9] Polymerization of microtubule protein was monitored by an increase in turbidity at 37°C in the microtubule assembly buffer[10] containing 100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP. Polymerization of microtubule protein was initiated by a temperature shift from 0°C to 37°C. Turbidity was measured with a thermo-controlled spectrophotometer (Beckman DU-20, Fullerton, CA) at 360 nm. Compounds for all experiments were dissolved in DMSO at a final concentration of 2% (v/v).

Alamar Blue™ assay

P388 cells in exponential growth phase were seeded into 96-well tissue culture plates (5×10^3 cells/100 µl/well) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum for 16 h. The compounds (DMSO solution) were then added to each well at various concentrations, and the cells were cultured for an additional 48 h. Live cells were counted using Alamar Blue™ (BioSource International, Camarillo, CA).[11]

Single-crystal X-ray diffraction analysis

A pale yellow crystal of (+)-**1** with approximate dimensions of $0.4\times0.4\times0.3$ mm, which was crystallized from EtOAc, was used for single-crystal X-ray diffraction analysis. All X-ray measurements were performed on a Rigaku AFC7R diffractometer with graphite monochromated CuK_α radiation and a rotating anode generator. The crystal data and refinement parameters are summarized in Table 2. Of the 2229 collected reflections, 2064 were unique (Rint = 0.019). The data were corrected for Lorentz and polarization effects, and a correction for secondary extinction was applied. The structure was determined by direct methods[12] and expanded using Fourier techniques.[13] All calculations were performed using the teXscan crystallographic software package from Molecular Structure Corporation.

Chemistry

Melting points were determined on a Mettler FP62 apparatus and are uncorrected. ^{1}H NMR and ^{13}C-NMR spectra were recorded on JEOL GSX270J spectrometer. The spectra were recorded with tetramethylsilane (δ = 0.0 for 1H); DMSO-d_6 (δ = 39.5 for ^{13}C); $CDCl_3$ (δ = 77.0 for ^{13}C) as internal reference. Mass spectra (electrospray ionization, methanol as the mobile phase) were analyzed with a Finnigan SSQ 7000 spectrometer. High-resolution fast atom bombardment mass spectra were analyzed with a JEOL JMS-DX303 spectrometer. Infrared spectra were recorded on a JEOL JIR-5500 infrared spectrophotometer in KBr pellets. Silica-gel column chromatography was performed using Merck 70–230 mesh silica gel 60. Optical rotations were measured on a Horiba SEPA-200 polarimeter, and are expressed in 10^{-1} deg cm^2 g^{-1}. Elemental analyses were performed with a Fisons EA 1108 elemental analyzer.

(−)-Cyclo-[5-(1,1-dimethylpropyl)dehydrohistidinyl-L-phenylalanine] (−)-2. 10 mg of 10% palladium on carbon was added to a solution of (−)-**1** (30 mg, 0.086 mmol) in MeOH (10 mL) and the mixture was stirred at room temperature for 2 h under hydrogen at atmospheric pressure. After the catalyst was filtered off and the filtrate was concentrated under reduced pressure, the residue was purified by column chromatography on silica (5 g) using $CHCl_3$:MeOH (50:1) as an eluant. The desired fractions were collected and the solvent was evaporated. The residual white powder was recrystallized with EtOH–hexane to yield 12 mg (39%) of (−)-**2** as a colorless solid; mp 224–226 °C; $[\alpha]_D^{25}$ −295 (c = 0.15, MeOH); UV (MeOH) nm 322 (ε 23300), 231 (ε 8830), 203 (ε 16800); IR (KBr) cm^{-1} 3310, 3220, 2970, 1670, 1440; ^{1}H NMR (270 MHz, $CDCl_3$) δ 12.09 (br s, 1H), 8.98 (br s, 1H), 7.56 (s, 1H), 7.29–7.38 (m, 5H), 6.87 (s, 1H), 5.67 (s, 1H), 4.34 (ddd, J = 2, 3, 10 Hz, 1H), 3.51 (dd, J = 3, 14 Hz, 1H), 2.29 (dd, J = 10, 14 Hz, 1H), 1.74 (q, J = 7 Hz, 2H), 1.40 (s, 6H), 0.74 (t, J = 7 Hz, 3H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 164.6, 159.9, 138.1, 135.5, 132.2, 132.1, 129.5 (2C), 129.1 (2C), 127.4, 123.6, 105.4, 57.2, 41.3, 36.2, 35.4, 27.9 (2C), 9.2; HRMS m/z 352.1926 (M^+) (calcd for $C_{20}H_{24}N_4O_2$: 352.1899). Anal. calcd for $C_{20}H_{24}N_4O_2$: C, 68.16; H, 6.86; N, 15.90, Found: C, 68.34, 6.78, 15.82.

(+)-Cyclo-[5-(1,1-dimethylpropyl)dehydrohistidinyl-D-phenylalanine] (+)-2. This compound was prepared using the same procedure applied for the preparation of (−)-**2**. 44% yield from (+)-**1**; colorless solid; mp 222–223 °C; $[\alpha]_D^{25}$ +284 (c = 0.10, MeOH); UV (MeOH) nm 322 (ε 22800), 231 (ε 8720), 203 (ε 16300); IR (KBr) cm^{-1} 3310, 3220, 2970, 1670, 1440; ^{1}H NMR (270 MHz, $CDCl_3$) δ 12.09 (br s, 1H), 8.98 (br s, 1H), 7.56 (s, 1H), 7.29–7.38 (m, 5H), 6.87 (s, 1H), 5.67 (s, 1H), 4.34 (ddd, J = 2, 3, 10 Hz, 1H), 3.51 (dd, J = 3, 14 Hz, 1H), 2.29 (dd, J = 10, 14 Hz, 1H), 1.74 (q, J = 7 Hz, 2H), 1.40 (s,

6H), 0.74 (t, $J=7$ Hz, 3H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 164.6, 159.9, 138.1, 135.5, 132.2, 132.1, 129.5 (2C), 129.1 (2C), 127.4, 123.6, 105.4, 57.2, 41.3, 36.2, 35.4, 27.9 (2C), 9.2; HRMS *m/z* 352.1932 (M^+) (calcd for $C_{20}H_{24}N_4O_2$: 352.1899). Anal. calcd for $C_{20}H_{24}N_4O_2$: C, 68.16; H, 6.86; N, 15.90. Found: C, 68.09, 6.87, 15.84.

(−)-Cyclo-[5-(1,1-dimethylpropyl)histidinyl-L-phenylalanine] (−)-3. This compound was prepared from (−)-**2**, using the same procedure used for the preparation of (−)-**2**, but with a 24 h reaction time. 60% yield from (−)-**2**; white powder; mp 224–225 °C; $[\alpha]_D^{25}$ −96 (*c* = 0.16, MeOH); UV (MeOH) nm 257 (ε 194), 205 (ε 18100); IR (KBr) cm^{-1} 3380, 3200, 2970, 1670, 1440; ^{1}H NMR (270 MHz, DMSO-d_6) δ 11.55 (br s, 1H), 8.25 (br s, 1H), 7.86 (br s, 1H), 7.41 (s, 1H), 7.29–7.14 (m, 5H), 4.24 (br s, 1H), 3.94 (br d, $J=11$ Hz, 1H), 3.11 (dd, $J=14$, 4 Hz, 1H), 2.88 (dd, $J=14$, 5 Hz, 1H), 2.82 (dd, $J=15$, 2 Hz, 1H), 1.45 (q, $J=7$ Hz, 2H), 1.32 (dd, $J=15$, 11 Hz, 1H), 1.13 (s, 3H), 1.12 (s, 3H), 0.60 (t, $J=7$ Hz, 3H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 166.9, 165.4, 135.9, 132.5, 131.6, 131.2, 130.3 (2C), 127.9 (2C), 126.6, 55.7, 54.3, 38.2, 35.0, 34.3, 31.9, 27.6, 27.6, 9.1; HRMS *m/z* 354.2098 (M^+) (calcd for $C_{20}H_{26}N_4O_2$: 354.2055). Anal. calcd for $C_{20}H_{26}N_4O_2\cdot 1/3H_2O$: C, 66.64; H, 7.46; N, 15.54, Found: C, 66.75, 7.41, 15.52.

(+)-Cyclo-[5-(1,1-dimethylpropyl)histidinyl-D-phenylalanine] (+)-3. This compound was prepared from (+)-**2** according to the procedure described for the preparation of (−)-**3**. 46% yield from (+)-**2**; white powder; mp 226–227 °C; $[\alpha]_D^{25}$ +99 (*c* = 0.10, MeOH); UV (MeOH) nm 257 (ε 174), 205 (ε 18200); IR (KBr) cm^{j1} 3380, 3200, 2970, 1670, 1440; ^{1}H NMR (270 MHz, DMSO-d_6) δ 11.55 (br s, 1H), 8.25 (br s, 1H), 7.86 (br s, 1H), 7.41 (s, 1H), 7.29–7.14 (m, 5H), 4.24 (br s, 1H), 3.94 (br d, $J=11$ Hz, 1H), 3.11 (dd, $J=14$, 4 Hz, 1H), 2.88 (dd, $J=14$, 5 Hz, 1H), 2.82 (dd, $J=15$, 2 Hz, 1H), 1.45 (q, $J=7$ Hz, 2H), 1.32 (dd, $J=15$, 11 Hz, 1H), 1.13 (s, 3H), 1.12 (s, 3H), 0.60 (t, $J=7$ Hz, 3H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 166.9, 165.4, 135.9, 132.5, 131.6, 131.2, 130.3 (2C), 127.9 (2C), 126.6, 55.7, 54.3, 38.2, 35.0, 34.3, 31.9, 27.6, 27.6, 9.1; HRMS *m/z* 354.2110 (M^+) (calcd for $C_{20}H_{26}N_4O_2$: 354.2055). Anal. calcd for $C_{20}H_{26}N_4O_2\cdot 1/2H_2O$: C, 66.09; H, 7.49; N, 15.41, Found: C, 65.80, 7.50, 15.30.

(−)-Cyclo-[(N^{im}-methyl-5-(1,1-dimethyl-2-propenyl))dehydrohistidinyl-L-phenylalanine] (−)-4. 45 mg (1.88 mmol) of sodium hydride (NaH) (60% in mineral oil) was added in portions to a solution of **1** (200 mg, 0.57 mmol) in DMF (15 mL) and the mixture was stirred at −30 °C for 10 min. To this mixture, 1.0 mL (17.1 mmol) of MeI was added dropwise and stirred at −30 °C for 2 h. 20 mL of saturated aqueous NH_4Cl was added to the reaction mixture and the mixture was extracted three times with 50 mL of EtOAc. The combined organic layers were washed with saturated NaCl, dried over Na_2SO_4 and concentrated in vacuo. Chromotography of the residual white powder (150 mg) over 20 g of silica gel with a $CHCl_3$:MeOH eluant (50:1) yielded 130 mg (63%) of **4**: The (−)-form of **4** was isolated by HPLC using a chiral column (CHIRALCEL OD, 10×250 mm) eluted with a mixture of hexane and ethanol (3:1) at a flow rate of 6.0 mL/min on a Waters system (600E series). 19% yield from **1**; white powder; mp 214–215 °C; $[\alpha]_D^{25}$ −285 (*c* = 0.30, MeOH); UV (MeOH) nm 317 (ε 25400), 232 (sh, ε 8640), 204 (ε 16800); IR (KBr) cm^{-1} 3200, 2980, 1680, 1440; ^{1}H NMR (270 MHz, $CDCl_3$) δ 12.20 (br s, 1H), 7.39 (s, 1H), 7.38–7.24 (m, 5H), 7.09 (s, 1H), 6.01 (dd, $J=18$, 9 Hz, 1H), 5.68 (br s, 1H), 5.14 (d, $J=9$ Hz, 1H), 5.00 (d, $J=18$ Hz, 1H), 4.33 (m, 1H), 3.66 (s, 3H), 3.50 (dd, $J=14$, 3 Hz, 1H), 2.92 (dd, $J=14$, 10 Hz, 1H), 1.59 (s, 6H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 164.6, 160.1, 145.6, 138.5, 136.5, 135.6, 134.6, 129.5 (2C), 129.1 (2C), 127.4, 124.0, 112.7, 106.6, 57.1, 41.3, 39.2, 34.9, 28.9, 28.8; HRMS *m/z* 364.1909 (M^+) (calcd for $C_{21}H_{24}N_4O_2$: 364.1899). Anal. calcd for $C_{21}H_{24}N_4O_2\cdot 1/4H_2O$: C, 68.36; H, 6.69; N, 15.19, Found: C, 68.44, H, 6.67, N, 15.01.

(−)-Cyclo-*N*,*N*′-dimethyl-[(N^{im}-methyl-5-(1,1-dimethyl-2-propenyl))dehydrohistidinyl-L-phenylalanine] (−)-5. This compound was prepared from **1** with 10 equivalents of NaH and 30 equivalents of MeI at room temperature using a procedure similar to that used for the preparation of (−)-**4**. For the purification of (−)-**5**, a mixture of hexane and ethanol (4:1) was used as an eluant in the previously mentioned chiral column HPLC. 8% yield from **1**; white powder; mp 95–98 °C; $[\alpha]_D^{25}$ −632 (*c* = 0.50, MeOH); UV (MeOH) nm 287 (ε 11100), 203 (ε 15900); IR (KBr) cm^{-1} 2900, 1690, 1640, 1380; ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.76 (br s, 1H), 7.33–7.21 (m, 5H), 7.08 (s, 1H), 6.00 (dd, $J=18$, 11 Hz, 1H), 5.28 (d, $J=11$ Hz, 1H), 5.08 (d, $J=18$ Hz, 1H), 4.19 (dd, $J=10$, 4 Hz, 1H), 3.86 (s, 3H), 3.38 (dd, $J=14$, 4 Hz, 1H), 3.13 (dd, $J=14$, 10 Hz, 1H), 2.92 (s, 3H), 2.55 (s, 3H), 1.60 (s, 3H), 1.57 (s, 3H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 166.8, 160.2, 143.5, 137.8, 136.5, 134.8, 129.4 (2C), 128.8 (2C), 127.2, 124.6, 114.4, 105.6, 65.9, 40.6, 38.8, 36.6, 34.9, 33.1, 28.4, 28.1; HRMS *m/z* 392.2223 (M^+) (calcd for $C_{23}H_{28}N_4O_2$: 392.2212). Anal. calcd for $C_{23}H_{28}N_4O_2$: C, 70.38; H, 7.19; N, 14.27, Found: C, 70.26, H, 7.30, N, 14.27.

Cyclo-[glycinyl-L-phenylalanine] 6. A solution of H-Phe-Gly-OMe, prepared from Boc-Phe-Gly-OMe with 4 N HCl-dioxane (20 g, 59 mmol), in MeOH (100 mL), was refluxed for 16 h. The white precipitate that appeared during reflux was washed three times with 10 mL of MeOH and collected to yield 7.5 g (62%) of **6** as a white powder, mp 262–263 °C (decomp.); $[\alpha]_D^{25}$ +60 (*c* = 0.15, DMSO); UV (MeOH) nm 257 (ε 101), 206 (ε 5770); IR (KBr) cm^{-1} 3340, 3200, 3060, 1680, 1470, 1340; ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.16 (br s, 1H), 7.90 (br s, 1H), 7.32–7.15 (m, 5H), 4.07 (br dd, $J=7$, 4 Hz, 1H), 3.35 (dd, $J=18$, 3 Hz, 1H), 3.10 (dd, $J=14$, 4 Hz, 1H), 2.88 (dd, $J=14$, 5 Hz, 1H), 2.75 (d, $J=18$ Hz, 1H); ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 167.1, 165.7, 136.0, 130.1 (2C) 128.1 (2C), 126.8, 55.5, 43.7, 38.8; MS (ESI) *m/z* 205 $(M+H)^+$; Anal. calcd for $C_{11}H_{12}N_2O_2\cdot 1/5H_2O$: C, 63.57; H, 6.01; N, 13.48, Found: C, 63.85, H, 5.86, N, 13.40.

Cyclo-*N*,*N*′-diacetyl-[glycinyl-L-phenylalanine] 7. The mixture of **6** (0.5 g, 2.45 mmol) and fused sodium acetate

(201 mg, 2.45 mmol) in acetic anhydride (10 mL) was heated for 16 h at 100 °C under nitrogen. After removal of the acetic anhydride in vacuo at 45 °C, the residue was dissolved in EtOAc and the resulting organic layer was washed with saturated NaCl, dried over Na_2SO_4. **7** was crystallized from EtOAc. 88% yield (0.62 g); colorless solid; mp 84–85 °C; $[\alpha]_D^{25}$ +7.8 ($c = 0.52$, MeOH); UV (MeOH) nm 209 (ε 20400); IR (KBr) cm^{-1} 1720, 1400, 1380, 1240; ^1H NMR (270 MHz, $CDCl_3$) δ 7.33–7.26 (m, 3H), 7.08–7.05 (m, 2H), 5.44 (t, $J = 5$ Hz, 1H), 4.49 (d, $J = 19$ Hz, 1H), 3.35 (dd, $J = 14$, 5 Hz, 1H), 3.20 (dd, $J = 14$, 5 Hz, 1H), 2.58 (s, 3H), 2.55 (s, 3H), 2.48 (d, $J = 19$ Hz, 1H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 171.2, 171.0, 167.9, 166.0, 134.3, 129.7 (2C), 129.1 (2C), 128.2, 59.0, 46.0, 38.7, 27.1, 26.8; MS (ESI) m/z 311 $(M + Na)^+$; Anal. calcd for $C_{15}H_{16}N_2O_4$: C, 62.49; H, 5.59; N, 9.72, Found: C, 62.50, H, 5.50, N, 9.67.

Cyclo-[dehydrohistidinyl-L-phenylalanine] 9. To a solution of **7** (311 mg, 0.93 mmol) in dimethoxyethanol (DME) (10 mL) was added 0.6 mL of 2 M lithium diisopropylamide (LDA, 1.2 mmol) and the mixture was stirred at −70 °C for 10 min. To this solution was then added a solution of 4(5)-formylimidazole (120 mg, 1.25 mmol, Maybridge Chemical Co., Cornwall, UK) in HMPA (4 mL)-DME (6 mL) at −70 °C. The solution was allowed to warm to −30 °C. After stirring for 30 min at this temperature, triflic anhydride (370 μL, 2.2 mmol) and pyridine (180 μL, 2.2 mmol) were added and the solution was allowed to warm to room temperature. After an additional hour of stirring, aqueous ammonia (3 mL) was added and stirred for 14 h at room temperature. The reaction mixture was extracted with $CHCl_3$ three times, dried over Na_2SO_4 and concentrated in vacuo. The resultant residue was purified by HPLC with a reverse phase column (Waters, mBondasphere 19×150 mm, 10 μm, C-18) employing a gradient from 60 to 80% CH_3CN in 0.1% TFA at a flow rate of 17 mL/min on a Waters system (600E series). However, since purified **9** contained 28% of a racemized compound with D-phenylalanine (44% *ee*), further purification by HPLC with a chiral column was performed using the same procedure described for the purification of (−)-**4**. 8% yield from **7**; white powder; mp 208–209 °C; $[\alpha]_D^{25}$ −257 ($c = 0.21$, DMSO); UV (MeOH) nm 307 (ε 16700); IR (KBr) cm^{-1} 3400, 3120, 1680, 1440; ^1H NMR (270 MHz, DMSO-d_6) δ 12.88 (br s, 1H), 11.09 (br s, 1H), 8.44 (s, 1H), 8.13 (br s, 1H), 7.48 (s, 1H), 7.24–7.11 (m, 5H), 6.25 (s, 1H), 4.48 (m, 1H), 3.19 (dd, $J = 14$, 4 Hz, 1H), 2.95 (dd, $J = 14$, 5 Hz, 1H); ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 164.8, 158.6, 135.8, 135.5, 132.3, 130.0 (2C), 128.0 (2C), 126.7, 125.6, 118.3, 101.8, 55.9; 38.9 (overlapping DMSO-d_6); HRMS m/z 282.1084 (M^+) (calcd for $C_{15}H_{14}N_4O_2$: 282.1117). Anal. calcd for $C_{15}H_{14}N_4O_2 \cdot CF_3COOH$: C, 51.52; H, 3.81; N, 14.14, Found: C, 51.15, H, 3.62, N, 13.84.

Cyclo-[(5-methyl)dehydrohistidinyl-L-phenylalanine] 10. This compound was prepared from **7** with 4-methyl-5-imidazolecarboxaldehyde using the same procedure described for the preparation of **9**. Since HPLC purified **10** contained 21% of a racemized compound with D-phenylalanine (58% *ee*), further purification by HPLC with a chiral column was performed using the same procedure described for the purification of (−)-**4**. 3% yield from **7**; colorless solid; mp 285–286 °C (decomp); $[\alpha]_D^{25}$ −267 ($c = 0.21$, DMSO); UV (MeOH) nm 319 (ε 22800); IR (KBr) cm^{-1} 3400, 3180, 1680, 1450; ^1H NMR (270 MHz, DMSO-d_6) δ 11.50 (br s, 1H), 8.35 (br s, 1H), 7.74 (s, 1H), 7.24–7.14 (m, 5H), 6.20 (s, 1H), 4.48 (m, 1H), 3.33 (br s, 1H), 3.20 (dd, $J = 14$, 4 Hz, 1H), 2.93 (dd, $J = 14$, 5 Hz, 1H), 2.19 (s, 1H); ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 164.2, 158.7, 135.6, 134.6, 132.3, 130.0 (2C), 128.0 (2C), 127.5, 126.6, 123.4, 101.7, 55.9, 38.7, 8.9; High-resolution MS m/z 296.1261 (M^+) (calcd for $C_{16}H_{16}N_4O_2$: 296.1273). Anal. calcd for $C_{16}H_{16}N_4O_2 \cdot 1/5H_2O$: C, 64.07; H, 5.51; N, 18.68. Found: C, 64.39, H, 5.65, N, 18.29.

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5. Compounds **4** and **5** with the L-phenylalanine residue were determined by the chiral HPLC detection (see ref. 2) for L-Phe in the hydrolysate (6 N HCl at 110 °C for 24 h) of each peak in the chiral HPLC separation.
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