was heated at reflux for 2.5 h. The mixture was cooled, and the solid was collected to give 3.10 g (74%) of **29**: mp 242-244 °C; IR (Nujol) 1700 (C=O), 1605 (C=N) cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.87 (s, 2, triazole CH), 8.46 (s, 1, C2-H), 8.46 (d, 1, C5-H), 8.04-7.45 (m, 3, remaining aromatic). Anal. ($C_{10}H_7N_5O$) C, H, N.

Biological Test Procedures. The PCA test used in these studies is similar to that described by Goose and Blair.[23] The backs of anesthetized (sodium pentobarbital) male Sprague-Dawley rats were shaven prior to receiving 100-μL injections of two dilutions of an homologous antiserum rich in IgE antiovalbumin antibodies. The two dilutions were prepared to yield average reaction diameters of 7 and 14 mm in control animals. Forty-eight to seventy-two hours later, the rats received test compound by intraperitoneal (ip) injection (60 mg/kg, prepared in 50:50 polyglycol E-200-water, v:v) or oral (po) gavage (100 mg/kg, prepared in 20:80 ethanol-water, v:v). The rats were challenged intravenously with 0.1 mg of ovalbumin and 2.5 mg of Evan's Blue dye contained in 0.5 mL of saline 5 min after ip or 30 min after po compound administration. Thirty minutes after challenge, the rats were sacrificed, the dorsal skin was reflected, and the mean reaction diameters were determined from measurements of two perpendicular axes. The sum of the two mean diameters determined the score for each animal. A minimum of four animals was used for both treatment and vehicle control groups, but results from repeated trails were pooled, causing both treatment and control n values to range from 4 to 12. Percent inhibition was calculated on the basis of difference in scores between control and treated animals and reported as mean percent inhibition plus or minus the standard error.

The PPA test used in these studies is a modificaton of a test described by Spicer et al.[24] Rats were passively sensitized with 2 mL of a dilution of mouse ascites fluid rich in IgE-anti-DNP_3OV antibodies. (A 100-μL intradermal injection of the dilution would produce a PCA reaction approximately 14 mm in diameter.) Two hours later the animals were given an ip injection of either saline or test drug. Thirty seconds later the animals were challenged by an ip injection of 2 mg of ovalbumin in 5 mL of Burn's modified Tyrode's solution containing 25 units of heparin. Five minutes after challenge the animals were sacrificed and the peritoneal shock fluid was collected. Cells were removed by centrifugation (4000g/min). Protein was removed from each sample by precipitation with an equal volume of 0.8 N $HClO_4$ and recentrifugation (434000g/min). Histamine analysis was performed by use of a continuous-flow adaptation of the double extraction procedure described by von Redlich and Glick.[25]

Means of data from the PCA experiments were compared by use of the two-tailed Student's t test, with $p < 0.05$ chosen as the level of statistical significance.

Analytical Procedures. The HPLC conditions employed for the percent determinations of **9** and **10** were as follows: column, 10 μm Alltech C18, 250 mm × 6.4 mm o.d.× 4.6 mm i.d.; mobile phase, 0.005 M tetraethylammonium perchlorate in a mixture of 15:85 methanol-0.02 M McIlvaine[26] buffer; flow rate, 1.3 mL/min; injection volume, 20 μL; detection, UV absorbance at 254 nm.

Registry No. **4**, 610-14-0; **5**, 87693-28-5; **6**, 87693-21-8; **7**, 118-48-9; **8**, 87693-08-1; **9**, 87693-14-9; **10**, 95923-25-4; **11a**, 87693-22-9; **11a** (acid), 13506-76-8; **11a** (acid chloride), 66232-57-3; **11b**, 87693-23-0; **11b** (acid chloride), 38818-49-4; **11c**, 87693-24-1; **11c** (acid chloride), 50424-93-6; **11d**, 87693-25-2; **11d** (acid chloride), 4194-44-9; **11e**, 87693-26-3; **11e** (acid chloride), 15865-57-3; **11f**, 87693-27-4; **11f** (acid chloride), 50425-29-1; **12a**, 87693-15-0; **12b**, 87693-16-1; **12c**, 87693-17-2; **12d**, 87693-18-3; **12e**, 87693-19-4; **12f**, 87693-20-7; **13a**, 87693-02-5; **13b**, 87693-03-6; **13c**, 87693-04-7; **13d**, 87693-05-8; **13e**, 87693-06-9; **13f**, 87693-07-0; **14a**, 87693-09-2; **14b**, 87693-10-5; **14c**, 87693-11-6; **15**, 63598-72-1; **16**, 104035-16-7; **19**, 104035-17-8; **20**, 104035-18-9; **21**, 104035-19-0; **22**, 104035-20-3; **23**, 103060-67-9; **24**, 104035-22-5; **25**, 104035-23-6; **26**, 584-13-4; **27**, 104035-24-7; **28**, 104035-25-8; **29**, 104035-26-9; $H_3CC(OEt)_3$, 78-39-7; $EtC(OEt)_3$, 115-80-0; $C_6H_5C(OEt)_3$, 1663-61-2; 6-nitro-1,3-benzodioxole-5-carboxaldehyde, 712-97-0; 5-amino-tetrazole, 4418-61-5.

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Structure and Solution Conformation of the Cytostatic Cyclic Tetrapeptide WF-3161, *cyclo*[L-Leucyl-L-pipecolyl-L-(2-amino-8-oxo-9,10-epoxydecanoyl)-D-phenylalanyl]

Megumi Kawai, Richard S. Pottorf, and Daniel H. Rich*

School of Pharmacy, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received March 24, 1986*

The sequence and configuration of amino acids in the cytostatic cyclic tetrapeptide WF-3161 are established as *cyclo*(L-Leu-L-Pip-L-Aoe-D-Phe) where Pip = pipecolic acid and Aoe = 2-amino-8-oxo-9,10-epoxydecanoic acid. In chloroform, WF-3161 adopts a conformation with a possible γ-turn between Leu NH and Aoe C=O and a cis amide bond between Leu and Pip. The torsion angles for this conformation are L-Aoe, ϕ, -95°, ψ, +85°, ω, -155°; D-Phe, ϕ, +120°, ψ, -80°, ω, -175°; L-Leu, ϕ, -145°, ψ, +35°, ω, -10°; L-Pip, ϕ, +20°, ψ, -135°, ω, -170°. The cis,trans,trans,trans amide bond sequence is related to the dimethyl sulfoxide conformation of chlamydocin, another cytostatic cyclic tetrapeptide.

WF-3161, a cyclic tetrapeptide isolated from culture filtrates of *Petriella guttulata*, is an inhibitor of cell growth in mouse P-388 leukemia cells (LD_{50} = 200 mg/kg).[2] Umehara et al. showed that WF-3161 contains the unusual amino acid 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe)[3] within the cyclic tetrapeptide *cyclo*(Leu-Pip-Aoe-Phe),[2] where Pip = pipecolic acid. However, the amino acid configurations were not assigned beyond the observation that L-Phe-L-Leu is present in partially hydrolyzed samples of WF-3161.

While characterizing the solution conformation of WF-3161 by nuclear magnetic resonance (NMR) spec-

(1) Abbreviations used herein: Pip, pipecolic acid (2-carboxypiperidine); Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid.
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(3) Hirota, A.; Suzuki, A.; Aizawa, K.; Tamura, S. *Agric. Biol. Chem.* **1973**, *37*, 955-956.

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troscopy, we obtained evidence that the proposed amino acid sequence *cyclo*(Leu-Pip-Aoe-Phe) must contain either a D-phenylalanine or a D-leucine residue. We report here the results of amino acid oxidase studies that establish the configuration of the amino acids and NMR data that confirm the amino acid sequence and establish the ring-system conformation of WF-3161 in chloroform.

Results

The chemical-shift assignments obtained by standard double resonance and 2D-COSY experiments at 200 MHz for WF-3161 were for the most part consistent with those reported previously.[2] The small temperature coefficient ($\Delta\delta/\Delta T = -1.50 \times 10^{-3}$ ppm/°C) for the leucine amide proton is consistent with this proton being shielded from solvent,[4] while the Aoe (-5×10^{-3} ppm/°C) and Phe (-2.67×10^{-3} ppm/°C) are protons exposed to the solvent. The ^{13}C NMR data for WF-3161 were compared with those of chlamydocin, a closely related cyclic tetrapeptide,[5] and with other related cyclic tetrapeptides, to assign all the chemical shifts of WF-3161 with the exception of the Pip β-, γ-, and δ-carbons, which are ambiguous. Important to the conformational argument is the upfield leucine α-carbon resonance at 47.18 ppm, in contrast to the resonance of normal Leu α-carbons near 50–54 ppm.[10] In addition, the epoxy C-9 and C-10 carbons are reassigned to peaks at 46.04 and 53.41 ppm.[2]

Nuclear Overhauser effect (NOE) experiments were performed at 21 °C. Upon irradiation of the Leu α-CH (4.91 ppm), a large enhancement was observed at 4.78 ppm (13.3%, Pip α-CH), establishing that these two protons are close in space and therefore part of a Leu-Pip sequence. Irradiation of the Aoe α-proton (at 4.48 ppm) led to the enhancement of the Phe NH (at 6.17 ppm, 7.3%), establishing their adjacency. Enhancements also were observed between the Phe α-proton (4.68 ppm) and the Leu NH proton (5.87 ppm, 7.2%) and between the Aoe NH proton (5.90 ppm) and the Pip ϵ-proton (2.67 ppm, 1.8%). These data established the sequence of WF-3161 as *cyclo*(Pip-Aoe-Phe-Leu).

The NOE effects also establish that all primary amide bonds have the trans configuration. The large enhancement observed between the Leu α-CH and the Pip α-CH requires that this tertiary amide bond has the "cis" configuration. This conclusion is consistent with the substantial downfield-shifted (at ~4.63 ppm) resonance of one of the Pip ϵ-protons. In a cis amide conformation, one of the Pip ϵ-protons lies in the deshielding region of the Leu carbonyl group. The ^{13}C NMR data are consistent with a cis Leu-Pip unit because the Leu α-carbon resonates 5 ppm upfield (47.18 ppm) from normal due to the steric compression (α-effect) between the Leu and Pip α-carbons.[11]

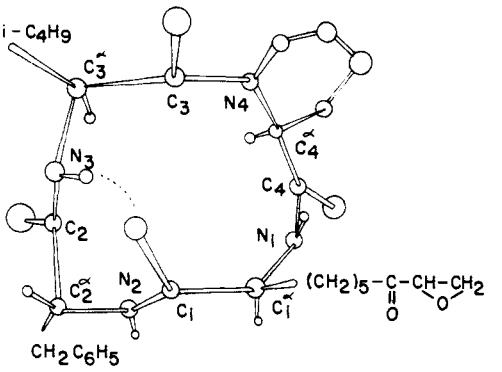

Figure 1. Schematic drawing of sequence and conformation of WF-3161 in chloroform-*d*. Dashed line indicates a possible γ-turn hydrogen bond. Torsional angles (±20°) are the following: L-Aoe, ϕ, -95°, ψ, +85°, ω, -155°; D-Phe, ϕ, +120°, ψ, -80°, ω, -175°; L-Leu, ϕ, -145°, ψ, +35°, ω, -10°; L-Pip, ϕ, +20°, ψ, -135°, ω, -170°.

A D configuration for phenylalanine or leucine was suggested by the following coupling constant and NOE data. Enhancement of the Phe α-CH proton when the Leu NH proton was irradiated established that this amide bond has the trans configuration. In addition, the large coupling constants for Phe ($^3J_{\alpha,NH} \geq 10.0$ Hz) require that this dipeptide unit must have either the L-Phe-D-Leu or D-Phe-L-Leu sequence and not the L-Phe-L-Leu sequence suggested.[2]

Treatment of the amino acids obtained from WF-3161 separately with L- and D-amino acid oxidase established that phenylalanine has the D configuration and that leucine has the L configuration. The configurations of L-pipecolic acid and L-Aoe were not obtained from the amino acid oxidase data. The configurations of these two amino acids were assigned from the NMR data.

Conformation of WF-3161. The conformation of WF-3161, *cyclo*(L-Leu-L-Pip-L-Aoe-D-Phe), contains three transoid amide bonds and one cis amide bond between L-Leu and L-Pip. An intramolecular hydrogen bond, suggested by the small temperature coefficient for the Leu NH ($\Delta\delta/\Delta T = -1.50 \times 10^{-3}$ ppm/°C), is possible but probably distorted due to geometrical constraints. The moderate temperature dependence of the Phe NH proton probably is due to the hydrophobic environment of the two bulky side chains (Phe and Aoe). The L-Aoe NH proton is clearly exposed to the solvent. These data are consistent with an approximately mono γ-turn structure -L-Aoe-D-Phe-L-Leu- plus the cis Leu–Pip bond (Figure 1). The conformation shown in Figure 1 incorporates nonplanar transoid amide bonds (torsion angle twisted ±25° from planarity), which molecular models indicated were needed to attain the 12-membered ring system.

Theoretical calculations[18] indicate that nonplanar transoid amide bonds are likely in cyclic tetrapeptides that contain more than two trans amide bonds.[5,6] In addition,

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a negative ellipticity at 290 nm ([θ] = 800) requires a 9*S* configuration for the epoxy ketone functionality in Aoe.[12]

Discussion

The results here lead to the assignment of the configurations of all amino acids in WF-3161. The revision in the configuration of phenylalanine was prompted by the difficulty in finding a cyclic tetrapeptide conformation that would simultaneously accommodate the trans Phe–Leu amide bond and the large vicinal coupling constant for phenylalanine, which restricted the α-CH and NH vicinal angle to near 180°. The D-Phe configuration was confirmed by L- and D-amino acid oxidase reaction of the amino acid mixture obtained from acid hydrolysis of WF-3161.

The solution conformation of WF-3161 contains a cis amide bond between L-Leu and L-Pip. The configuration of this amide bond was determined by three converging NMR observations. The 5-ppm upfield shift in the leucine α-carbon ^{13}C NMR resonance is characteristic of upfield shifts due to steric interactions in cyclic tetrapeptides[13] and other systems.[11] The strong NOE between the Leu α-CH and Pip α-CH protons requires these two protons to be within 3 Å of each other, which is possible only in a cis amide bond sequence. The deshielding of one ϵ-proton in Pip by the Aoe carbonyl is possible only in the proposed conformation. The remaining amide bonds have trans configurations, and the large coupling constants ($^3J_{\alpha,NH}$) for the three amide protons suggest the dihedral angles ($\theta\alpha$,NH) near 180° may be for transoid amide bonds.[14] The dihedral angles approach an inverse γ-turn structure at D-Phe as shown in Figure 1.

The conformation of WF-3161 in chloroform closely resembles the conformation of the cytostatic cyclic tetrapeptide chlamydocin in dimethyl sulfoxide[5,15] and the conformation of Cyl-2.[16] All of these cyclic tetrapeptides are active at concentrations between 2 and 300 nM in a murine antimitogenesis assay,[17] suggesting that they bind to a common receptor molecule. However, the orientations of the respective L-pipecolic and D-prolyl side chains are different because of the opposite configurations of the amino acids, but the remaining ring-system torsion angles are similar. It is notable that in chloroform WF-3161 does not exist in the bis γ-turn conformation found for chlamydocin[5] or HC-toxin[13] in this solvent. Thus, WF-3161 has a more "constrained" cyclic tetrapeptide ring system conformation, a property that will prove useful for determining the "bioactive" conformation of these structurally and biologically related peptides.

Experimental Section

The sample of WF-3161 used for these experiments was obtained from Fujisawa Pharmaceutical Co.[2] Proton NMR spectra were recorded on a Nicolet 200-MHz spectrometer operated in the FT mode. Detailed descriptions of experimental methods utilized for these studies have been reported previously.[5,6] The pulse sequence 90°–t_1–90°–(acquisition t_2) was employed for the two-dimensional ^1H-shift correlated (COSY) NMR experiment. The peptide concentration was 4.0 mg in 0.5 mL of chloroform-*d* for all NMR experiments unless otherwise stated. Chloroform-*d* [100% atom % D] was purchased from Aldrich Chemical Co. CD spectra were obtained on a JASCO J-40A spectropolarimeter in 0.5 mm–1 cm cells and are reported as mean residue molar ellipticities.

200-MHz ^1H NMR data for WF-3161 at 22 °C: δ ($CDCl_3$) 0.81 (d, 3 H, J = 6.2 Hz, Leu δ-CH_3), 0.82 (d, 3 H, J = 6.2 Hz, Leu δ'-CH_3), 1.13–1.88 (m, 17 H, Leu β, γ, Pip β, γ, δ, Aoe β, γ, δ, ϵ), 2.18–2.52 (m, 2 H, Aoe ω), 2.67 (t, 1 H, J = 13.1 Hz, Pip CH-ϵ), 2.85 (dd, 1 H, J = 2.3, 5.6 Hz, β-keto epoxy), 2.87 (dd, 1 H, J = 6.8, 13.7 Hz, Phe β_1), 2.99 (dd, 1 H, J = 4.6, 5.5 Hz, β'-keto epoxy), 3.25 (dd, 1 H, J = 8.7, 13.7 Hz, Phe β_2), 3.42 (dd, 1 H, J = 2.5, 4.6 Hz, α-keto epoxy), 4.48 (m, 1 H, Aoe α), 4.61–4.71 (m, 2 H, Phe α, Pip ϵ), 4.78 (d, 1 H, J = 4.6 Hz, Pip α), 4.91 (ddd, 1 H, J = 2.7, 8.2, 10.2 Hz, Leu α), 5.87 (d, 1 H, J = 10.0 Hz, Leu NH), 5.90 (d, 1 H, J = 10.5 Hz, Aoe NH), 6.17 (d, 1 H, J = 10.9 Hz, Phe NH).

Carbon-13 chemical shifts of WF-3161 in chloroform-*d* (2.4 mg in 100 μL of $CDCl_3$, 81 600 scans): Phe α, 55.69; β, 34.99; aromatic 1′, 136.84; 2′,6′, 129.21; 3′,5′, 128.45; 4′, 126.71; Aoe α, 54.22; B, 28.71; γ, 25.51; δ, 27.73; ϵ, 22.86; ω, 36.30; epoxy α, 53.41; β, 46.04; Leu α, 47.18; β, 40.63; γ, 24.86; β, δ', 22.15, 20.80; Pip α, 57.26; β, 24.21; γ, 23.07; δ, 26.86; ϵ, 41.33; C=O, 207.27 (Aoe epoxy); 174.56, 174.17; 171.30, 170.05.

The amino acid configurations of Leu and Phe were determined by hydrolyzing WF-3161 in 6 N HCl at 110 °C for 22 h. The residues were reacted separately with L- and D-amino acid oxidases. For L-amino acid oxidase, the procedure of Sieber et al.[7] was used except that a 0.2 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 7.2 was used to avoid contamination with buffer in the amino acid analyses. The obtained residues were analyzed for the component amino acids on a Beckman 119CL amino acid analyzer: Leu, 0.05; Phe, 1.00.

For D-amino acid oxidase, 0.2 M sodium pyrophosphate (pH 8.1) was used. The buffer contained 10 μM NaCN to inhibit any residual catalase present in the enzyme preparation.[8,9] Amino acid analysis gave the following: Leu, 1.00; Phe, 0.00.

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