

## A designed $\beta$ -hairpin peptide in crystals

(two conformers/octapeptides/ideal pleated sheets/ $\beta$ -turn nucleating segment)

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**ABSTRACT**  $\beta$ -hairpin structures have been crystallographically characterized only in very short acyclic peptides, in contrast to helices. The structure of the designed  $\beta$ -hairpin, *t*-butoxycarbonyl-Leu-Val-Val-D-Pro-Gly-Leu-Val-Val-OMe in crystals is described. The two independent molecules of the octapeptide fold into almost ideal  $\beta$ -hairpin conformations with the central D-Pro-Gly segment adopting a Type II'  $\beta$ -turn conformation. The definitive characterization of a  $\beta$ -hairpin has implications for *de novo* peptide and protein design, particularly for the development of three- and four-stranded  $\beta$ -sheets.

*De novo* peptide and protein design is based on the ability to construct peptide sequences with predictable folding patterns (1–7). Helices and  $\beta$ -sheets have been the focus of considerable synthetic attention in attempts to assemble mimics for helical bundles (8–11) and all  $\beta$ -motifs (12–15). Helix nucleation strategies have been successful in generating both water-soluble helices, using stabilizing side chain interactions (16–19), and incorporating backbone conformational constraints in hydrophobic helices (20–22). The ready crystallizability of peptide helices nucleated with  $\alpha,\alpha$ -dialkylated amino acids has permitted detailed stereochemical characterization by x-ray diffraction (23–26). In contrast, strategies for construction of  $\beta$ -hairpins have been less widely explored (27–32). Furthermore, crystallographic examinations have been made only in two- to five-residue acyclic peptides (33–40). In this report we describe the crystal structure of a designed, synthetic  $\beta$ -hairpin in an acyclic octapeptide.

Analysis of  $\beta$ -hairpin structures in proteins reveal that such features invariably contain Type II' or I'  $\beta$ -turns as the nucleating segment of the chain reversal (41–43). We therefore designed the octapeptide *t*-butoxycarbonyl (Boc)-Leu-Val-Val-D-Pro-Gly-Leu-Val-Val-OMe (11) such that the central D-Pro-Gly segment facilitates a Type II'  $\beta$ -turn conformation. The constraint of pyrrolidine ring formation in D-Pro restricts the value of  $\phi$  to  $+60^\circ \pm 20^\circ$ . The idealized conformational angles for the  $i+1/i+2$  residues of a Type II'  $\beta$ -turn are:  $\phi_{i+1} = +60^\circ$ ,  $\psi_{i+1} = -120^\circ$ ,  $\phi_{i+2} = -80^\circ$ , and  $\psi_{i+2} = 0^\circ$  (44, 45). The valine-rich arms are expected to favor extended strand conformations in view of the established propensity of  $\beta$ -branched residues to occur in  $\beta$ -sheets in proteins (46–48).

### EXPERIMENTAL METHODS

The peptide was synthesized by conventional solution phase procedures and purified by medium pressure liquid chromatography on a C<sub>18</sub> column (40–60 microns), followed by HPLC purification on a C<sub>18</sub> column (10 microns) using methanol-water gradients. The peptide was fully characterized by 400 MHz <sup>1</sup>H NMR (49).

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Colorless crystals in the form of very thin plates were grown by slow evaporation from a CH<sub>3</sub>OH solution, to which a small amount of water was added. Almost all the crystals were composites in the form of a stack of several thin wafers. The crystal selected for data collection had a slightly split profile for some of the reflections. X-ray diffraction data were measured at room temperature, 21°C, with CuK $\alpha$  radiation,  $\lambda = 1.54278 \text{ \AA}$ , on a Siemens P4s four-circle diffractometer (Siemens, Madison, WI) outfitted with an oriented graphite crystal monochromator. The scan mode was  $\theta/2\theta$ , scan speed was a constant  $10^\circ/\text{min}$ , scan range was  $1.3^\circ$  plus the K $\alpha$  separation, and the  $2\theta$  range was  $3\text{--}114^\circ$  (resolution,  $0.93 \text{ \AA}$ ). A background measurement was made at both ends of every scan, each for 50% of the total scan time, and three reflections chosen as standards were remeasured after every 97 measurements. The standards remained constant to within 3%. A total of 8251 reflections were measured, of which 7999 were independent and 5487 were observed with  $F_o > 4\sigma(F)$ . The crystal size was  $0.07 \times 0.27 \times 0.47 \text{ mm}$ . No absorption correction was applied. The cell was triclinic, P1, with  $a = 9.739(2) \text{ \AA}$ ,  $b = 11.579(2) \text{ \AA}$ ,  $c = 26.253(4) \text{ \AA}$ ,  $\alpha = 98.39(1)^\circ$ ,  $\beta = 91.45(2) \text{ \AA}$ ,  $\gamma = 107.80(2)^\circ$ ,  $V = 2766.5(9) \text{ \AA}^3$ , and  $d_{\text{calc}} = 1.113 \text{ g/cm}^3$  for C<sub>45</sub>H<sub>80</sub>N<sub>8</sub>O<sub>11</sub>·H<sub>2</sub>O (formula weight, 927.2) and two independent molecules in the triclinic cell (also two H<sub>2</sub>O molecules per cell). The 130 C, N, and O atom structure was solved with the aid of a vector search and translation procedure contained in the PATSEE program (50). The search model used consisted of 29 C, N, and O atoms containing the  $\beta$ -bend and neighboring backbone atoms in the  $\beta$ -structure of Boc-Cys-Val-Aib-Ala-Leu-Cys-NHMe (32). The correctly placed 29-atom fragment, as determined by PATSEE, was expanded to the complete 130-atom structure by the tangent formula expansion procedure (51). Full-matrix least squares, with 160 H atoms placed in idealized positions and riding with the C or N atom to which each is bonded, was performed in four blocks for each complete cycle on a VAX computer. The final *R* factor was 6.04% for 5487 data ( $>4\sigma$ ) and 1171 variables, where the data to parameter ratio is 4.7:1.0, and  $S = 1.54$ . If structure factors for all 7999 data were calculated, then  $R = 9.0\%$ . The largest difference peak was  $0.27 \text{ e/\AA}^3$  and the largest difference hole was  $0.25 \text{ e/\AA}^3$ . Atomic coordinates for C, N, and O atoms, bond lengths and angles, anisotropic thermal factors, and coordinates for hydrogen atoms have been deposited.

### RESULTS AND DISCUSSION

The conformations of the two independent molecules in the asymmetric unit, molecules 1 and 2, are shown in Fig. 1. Torsional angles are listed in Table 1 and hydrogen bond parameters are shown in Table 2. Molecule 1 possesses an ideal

Abbreviation: Boc, *t*-butoxycarbonyl.

Data deposition: The atomic coordinates have been deposited in the Cambridge Crystallographic Data Base, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K. (reference WABZOF).

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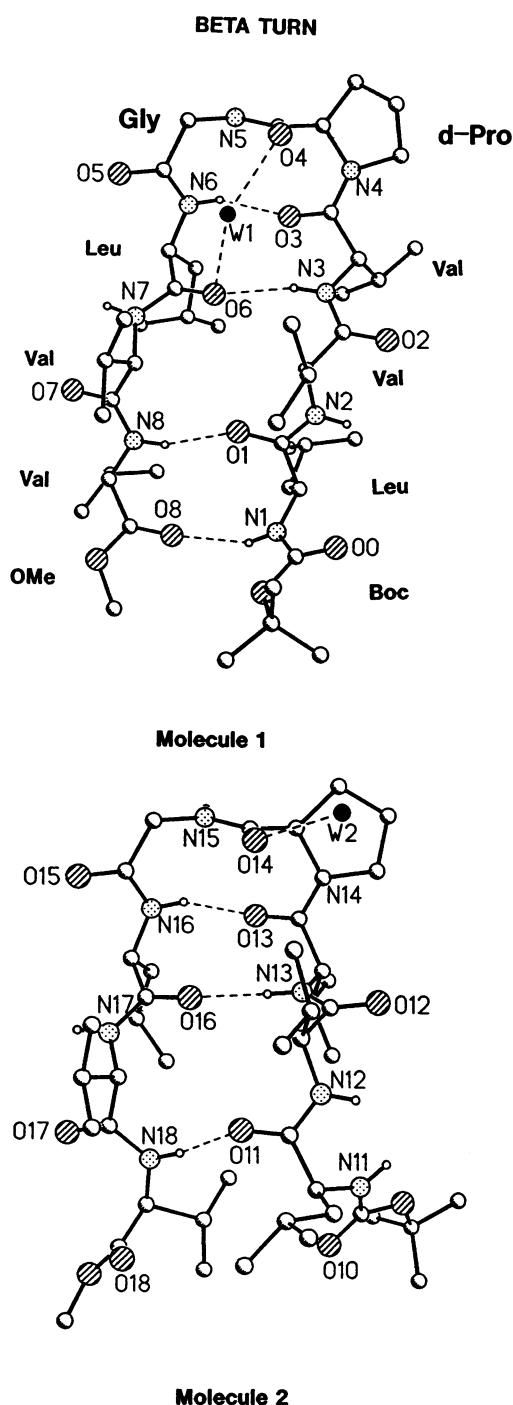


FIG. 1. (Upper) Molecule 1 has an almost ideal  $\beta$ -structure with a Type II'  $\beta$ -bend at D-Pro<sup>4</sup>-Gly<sup>5</sup> and four intramolecular NH $\cdots$ O=C hydrogen bonds. (Lower) The upper 80% of molecule 2 has a conformation similar to molecule 1. The C and N termini of molecule 2 have deviated very significantly from a  $\beta$ -structure. There are only three intramolecular NH $\cdots$ O=C hydrogen bonds in molecule 2. The water molecules W(1) and W(2) will be discussed later.

Type II'  $\beta$ -turn with  $\phi$  and  $\psi$  values of +53° and -132° for D-Pro<sup>4</sup> and -96° and 9° for Gly<sup>5</sup> (Table 1). The extended backbone on either side of the  $\beta$ -turn makes a nearly ideal pleated sheet as seen in the side view of molecule 1 in Fig. 2. The four intramolecular NH $\cdots$ O=C hydrogen bonds, N(1) $\cdots$ O(8), N(8) $\cdots$ O(1), N(3) $\cdots$ O(6), and N(6) $\cdots$ O(3) (Table 2) complete the ideal  $\beta$ -hairpin structure of molecule 1. Molecule 2 is fairly similar to molecule 1 from O(11) to N(18) [compare with O(1) to N(8) in molecule 1]. However, the N and C

Table 1. Torsional angles for the hairpin conformation of Boc-Leu-Val-Val-D-Pro-Gly-Leu-Val-Val-OMe

Residue	Angle	Molecule 1, degrees	Molecule 2,* degrees
Backbone			
Boc(0)	$\psi$	-173	180
	$\omega$	+172	+179
Leu(1)	$\phi$	-86 <sup>†</sup>	-119 <sup>†</sup>
	$\psi$	+119 <sup>†</sup>	-57 <sup>†</sup>
	$\omega$	-177	180
Val(2)	$\phi$	-134	-115
	$\psi$	+118	+112
	$\omega$	-177	-172
Val(3)	$\phi$	-160 <sup>†</sup>	-122 <sup>†</sup>
	$\psi$	+142 <sup>†</sup>	+94 <sup>†</sup>
	$\omega$	-176	-171
D-Pro(4)	$\phi$	+53	+66
	$\psi$	-132	-127
	$\omega$	+175	+174
Gly(5)	$\phi$	-96	-95
	$\psi$	9	7
	$\omega$	-178	178
Leu(6)	$\phi$	-90	-94
	$\psi$	+136	+140
	$\omega$	+165	+176
Val(7)	$\phi$	-128	-121
	$\psi$	+130	+123
	$\omega$	+164	-179
Val(8)	$\phi$	-148 <sup>†</sup>	-82 <sup>†</sup>
	$\psi$	+136	+144
	$\omega$	180	+173
Sidechains			
Leu(1)	$\chi_1$	+174	-178
	$\chi_2$	+63, -175	-171, +67
Val(2)	$\chi_1$	-48, -175	175, -58
Val(3)	$\chi_1$	+58, -175	177, -58
D-Pro(4)	$\chi_1$	-22	+21
	$\chi_2$	+32	-33
	$\chi_3$	-29	+33
	$\chi_4$	+15	-20
	C4 $\beta$ N4C4 $\alpha$ C4 $\beta$	+5	0
Leu(6)	$\chi_1$	+169	180
	$\chi_2$	+66, -174	152, -82
Val(7)	$\chi_1$	-57, +176	-56, +179
Val(8)	$\chi_1$	+56, -176	-62, +174

Torsional angles  $\phi$ ,  $\psi$  and  $\omega$  for the backbone and  $\chi_n$  for the sidechains follow the convention presented in ref. 52. The estimated standard deviations are 1.0–1.5°.

\*Labels for atoms in molecule 2 differ from molecule 1 simply by the addition of the number 10.

<sup>†</sup>Large differences (between 30° and 180°) in torsional angles between molecules 1 and 2.

termini of molecule 2 (see bottom right of Fig. 1 and Fig. 2, Lower) have been twisted away from a  $\beta$ -structure. There is a rotation of +176° about the C(11A)–C(11') bond as compared with the C(1A)–C(1') bond causing the N(11)H moiety to be directed to the exterior of the  $\beta$ -loop rather than to the interior. At the other end of molecule 2, there is a rotation of +66° about the N(18)–C(18A) bond as compared with the N(8)–C(8A) bond in molecule 1, causing the C=O(18) to turn away from the interior of molecule 2. Consequently, in molecule 2, there is no hydrogen bond between N(11) and O(18).

Both molecule 1 and molecule 2 repeat along the crystallographic *a*-axis and each forms a separate, extended  $\beta$ -sheet (Fig. 3) in which each strand is antiparallel to its neighbors. Molecule 1 forms a pair of intermolecular hydrogen bonds, N(2) $\cdots$ O(7) and N(7) $\cdots$ O(2), with molecules on either side. Molecule 2 forms a pair of intermolecular hydrogen bonds

Table 2. Hydrogen bonds

Type	Donor	Acceptor	N...O and O...O, Å	H*...O, Å	Angle C=O...N, degrees
Molecule 1					
Intra	N1	O8	2.977	2.17	161
Inter	N2	O7 <sup>†</sup>	2.936	2.07	163
Intra	N3	O6	3.076	2.21	150
	N4				
Peptide-solvent	N5	W2	2.884	2.04	
Intra	N6	O3	2.869	2.02	151
Inter	N7	O2 <sup>‡</sup>	2.869	1.98	165
Intra	N8	O1	2.946	2.08	160
Molecule 2					
Inter	N11	O17 <sup>‡</sup>	2.860	2.03	152
Inter	N12	O17 <sup>‡</sup>	3.044	2.17	148
Intra	N13	O16	2.888	1.99	154
	N14				
Peptide-solvent	N15	W1	2.807	1.96	
Intra	N16	O13	2.896	2.04	137
Inter	N17	O12 <sup>†</sup>	2.988	2.11	149
Intra	N18	O11	2.882	1.99	150
Solvent					
	W1	O4 <sup>§</sup>	2.754		
	W1	O6 <sup>§</sup>	2.936		
	W2	O14	2.929		
	W2	O15 <sup>‡</sup>	2.987		

\*Hydrogen atoms were placed in idealized positions with N-H = 0.90 Å.

<sup>†</sup>At symmetry equivalent 1+x,y,z. <sup>‡</sup>At symmetry equivalent -1+x,y,z. <sup>§</sup>At symmetry equivalent x,-1+y,z.

similar to those in molecule 1, N(12)...O(7) and N(17)...O(12) and, in addition N(11) (the NH moiety that has distorted the

N terminus of the  $\beta$ -structure of the individual molecule 2), forms a hydrogen bond with O(17).

An edge-on view of the assembly of the  $\beta$ -sheets along the *b*-axis direction, formed by molecule 1, is shown in the middle and the stacking formed by molecule 2 is on the left and repeated on the right in Fig. 4. The individual  $\beta$ -sheets overlap somewhat at the  $\beta$ -turns. A horizontal translation of molecules

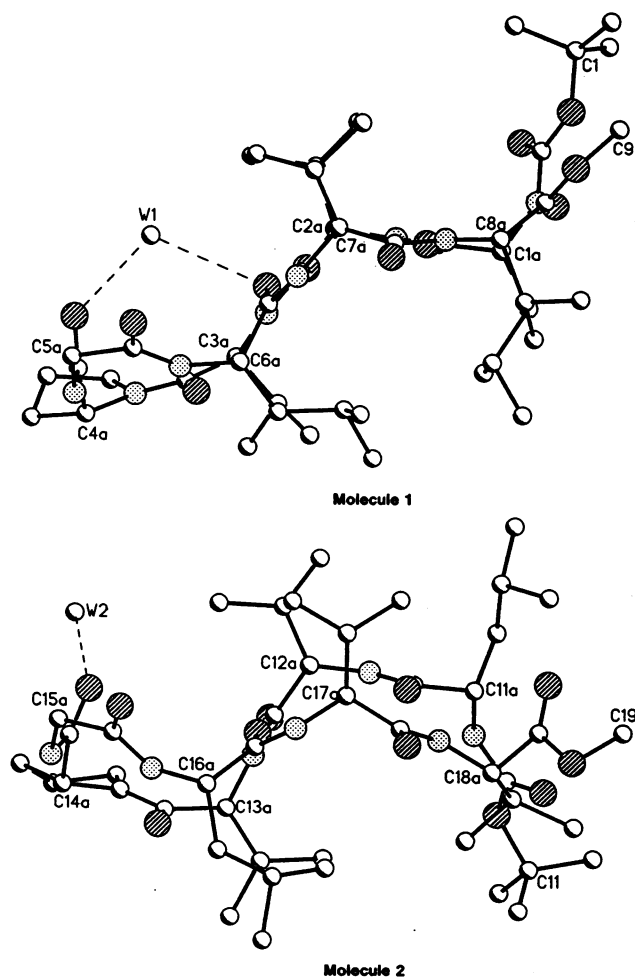


FIG. 2. (Upper) Side view of molecule 1 showing an almost ideal pleated sheet. (Lower) Side view of molecule 2.

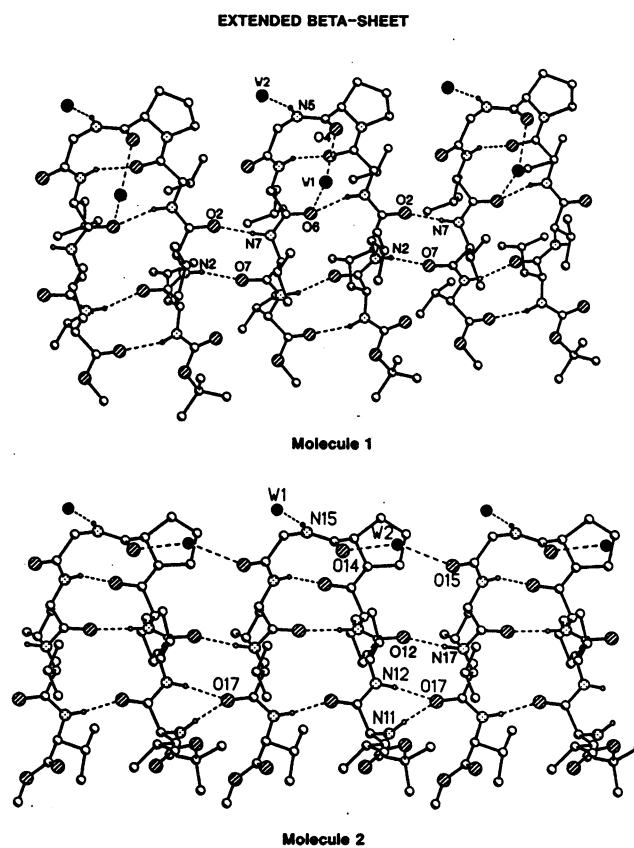


FIG. 3. Individual extended  $\beta$ -sheets separately formed by molecules 1 and 2 by translation of molecules in the *a*-axis direction.

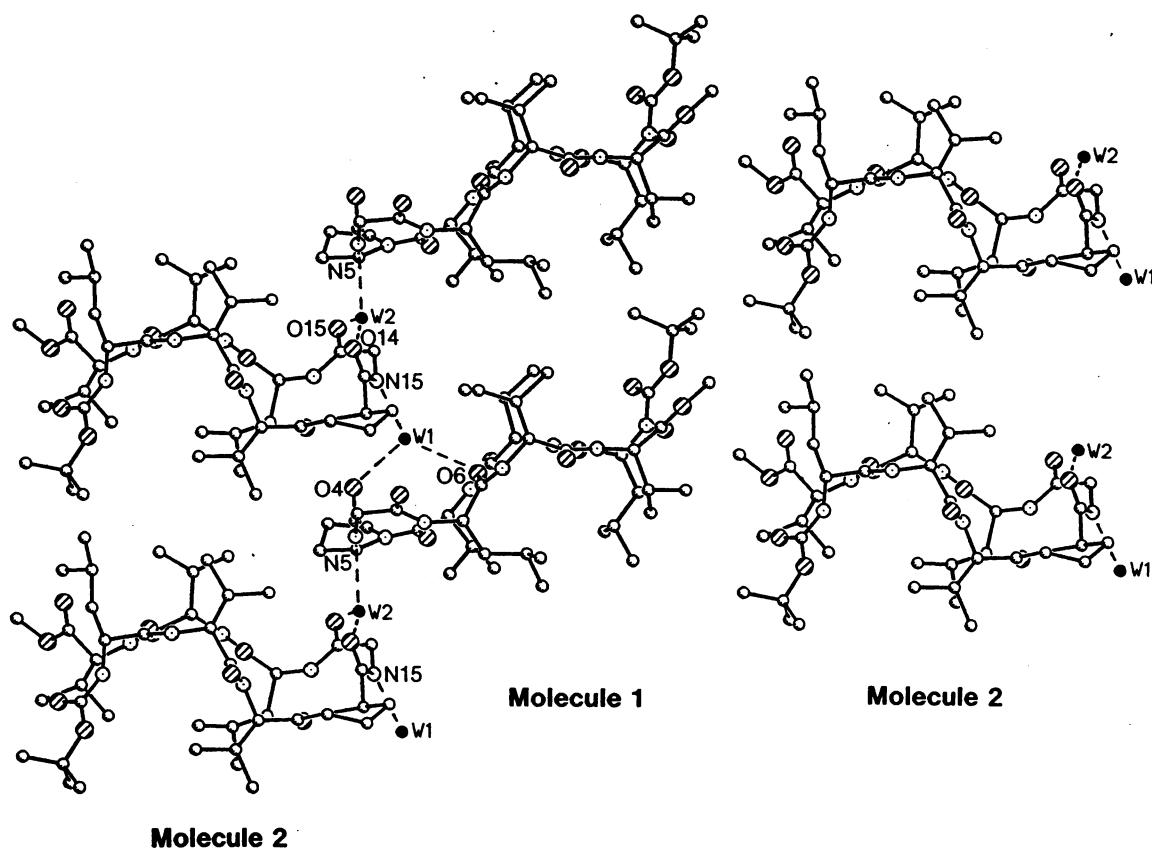


FIG. 4. The supramolecular assembly of individual  $\beta$ -sheets formed by molecules 1 (Middle) and molecules 2 (Left) side and repeated by translation (Right). The  $a$ -axis direction is perpendicular to the paper, and the  $c$ -axis direction is roughly horizontal. The length of the  $c$ -axis corresponds to the combined lengths of the  $\beta$ -loops of one molecule 1 plus one molecule 2. The two molecules W(1) and W(2) connect molecules 1 and 2 by mediating hydrogen bonds between N(15) and O(4) and between N(5) and O(14), atoms in the  $\beta$ -turns. W(1) also forms a hydrogen bond with O(6) and W(2) forms a hydrogen with O(15) in a neighboring molecule directly superimposed on the one shown.

2, by one cell length along the  $c$ -axis, shows the boundary between the N and C termini of the two independent molecules. The only near approaches between the two molecules 1 and 2 at this boundary are between C(9) and O(18) and between C(19) and O(8) at 3.66 Å and 3.51 Å, respectively, and between C(9) and C(11E) at 3.71 Å.

Water molecules W(1) and W(2) shown in Fig. 4, as well as in Figs. 1–3, connect the individual  $\beta$ -sheets of molecules 1 and 2 by hydrogen bond formation involving atoms in the  $\beta$ -turns. W(1) is an acceptor from N(15) (Fig. 4, above) and a donor to O(4) and O(6) (Fig. 4, below; see also Fig. 3). W(2) is an acceptor from N(5) above and a donor to O(14) and O(15) below. The O(14) and O(15) carbonyl oxygens are in neighboring peptide molecules 2 as can be seen in Fig. 3. The water molecules act like a scaffold that connects all the  $\beta$ -sheets into a supramolecular assembly that is two molecules wide, as indicated in Fig. 4.

Although crystal structure determinations of proteins have become commonplace, moderately sized oligopeptides do not crystallize readily, presumably as a result of conformational flexibility in solution. Crystallizability has been successfully enhanced by the introduction of stereochemically constrained noncoded amino acids, particularly  $\alpha$ -aminoisobutyric acid, that stabilize helical folding. Indeed, single crystals and structures up to 16 residues long have been easily obtained in helical  $\alpha$ -aminoisobutyric acid-containing sequences (20–26, 53, 54).  $\beta$ -Sheets have been characterized only in crystals of relatively short peptides, with leucine-enkephalin providing an important example (55). In the octapeptide structure described in the present study, a  $\beta$ -hairpin, which assembles into sheets in crystals, has been definitively characterized. The use of a  $\beta$

turn-nucleating D-Pro-Gly segment undoubtedly stabilizes the observed conformation. Indeed the robustness of the  $\beta$ -hairpin conformation has been demonstrated by the observation of critical interstrand nuclear Overhauser effects in apolar solvents (49). Short hairpins have indeed been a feature of cyclic peptides, but the register of the arms has been maintained by constraints of covalent cyclization, either through peptide bonds or disulfide bridges. Early examples of the former include cyclohexaglycine (56) and ferrichrome A (57), while the hexapeptide disulfide Boc-Cys-Val- $\alpha$ -aminoisobutyric acid-Ala-Leu-Cys-NHMe (32) and the synthetic somatostatin analog octreotide (58) provide examples of the latter. The determination of the  $\beta$ -hairpin conformation in the designed octapeptide suggests that construction of three and four stranded  $\beta$ -sheet structures should be achievable using multiple Type II'-nucleating centers.

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