28. On Intrahelical Hydrogen Bonding and Stability of β-Helices: the Behavior of Some D,L-Alternating Oligoleucines with an *N*-Methylated Residue

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An *N*-methylated residue at the $n-3$ position of the chain was used to reduce the maximum number of H-bonds realizable by some D,L-alternating oligopeptides in $\beta^{4.4}$-, $\uparrow\uparrow\beta^{5.6}$- and $\uparrow\downarrow\beta^{5.6}$-helices and thus increase for the oligopeptides, the relative stability of larger β-helices. With D,L-alternating oligoleucines of the series Boc-Leu$_n$-OMe, however, this approach did not produce the helices expected. Although $\uparrow\downarrow\beta^{7.2}$-helices with only one free NH per strand would theoretically be possible, the *N*-methylated oligoleucines formed instead flawed $\beta^{4.4}$-helices having three free NH's in $CHCl_3$ as well as in other solvents of low polarity. These observations confirm that the stability of β-helices does not depend only on the number of intra- or interstrand H-bonds, and corroborate the idea that β-helices with large cavities are inherently unstable.

Introduction. – As shown by several studies [1–6], $\beta^{4.4}$- and $\uparrow\uparrow\beta^{5.6}$- or $\uparrow\downarrow\beta^{5.6}$-helices[2]) are among the most stable conformations for D,L-alternating peptides. Single- and double-stranded β-helices with a higher number of residues per turn appear to be much less stable. In fact, such β-helices seem to exist only in systems where special interactions with solvent molecules [7], ions [8], or a lipid/H_2O environment [9] [10] would enhance their stability. In connection with our interest [11] in the controversial question of the true β-helical structure of gramicidin A in ion-conducting channels and also with our work [12] on porous *Langmuir-Blodgett* films of β-helical oligopeptides, we were interested in $\uparrow\downarrow\beta^{7.2}$-helices that would be stable in homogeneous, salt-free solutions. Considering the importance of intrahelical H-bonding for the stability of β-helices [1] [2], we thought that stable $\uparrow\downarrow\beta^{7.2}$-helices might be obtained for oligomers for which these helices would represent conformations of maximal intrahelical H-bonding. Therefore, we prepared such oligomers by synthesizing D,L-alternating oligoleucines of the series Boc-Leu$_n$-OMe with an *N*-methylated residue at the $n-3$ (fourth last) position (see *Scheme*) and we report here the results obtained.

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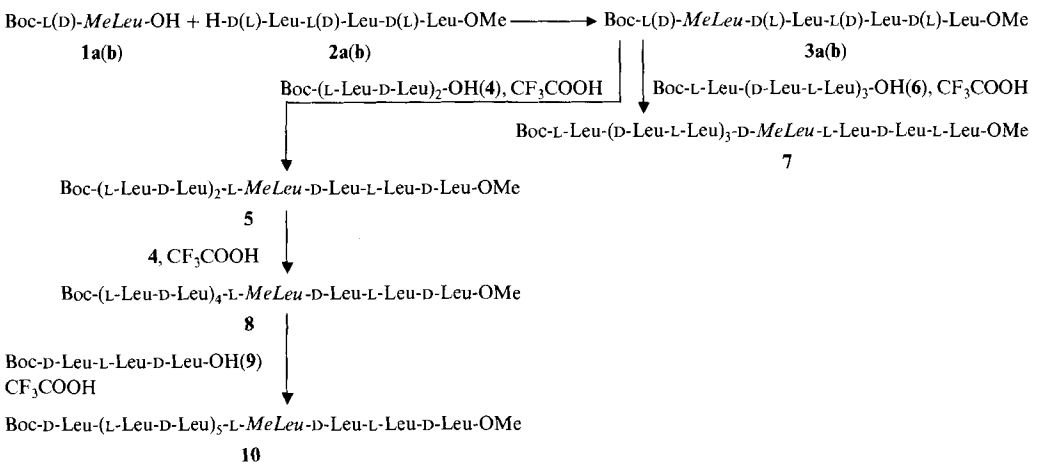
[2]) The superscript indicates the approximate number of residues per turn, ↑↑ and ↑↓ mean parallel and antiparallel helices.

Scheme. *Synthesis of* N-*Methylated Oligoleucines*



Experimental Part – 1. *General.* ^{1}H-NMR Spectra: *Bruker AMX 400* or *AMX 500* operating at 400.13 and 500.13 MHz, resp. ^{13}C-NMR Spectra: *Bruker AMX 500* at 125.27 MHz, with full ^{1}H-decoupling using the WALTZ16 sequence. TOCSY and NOESY Spectra [13] of **8** and **10**: mixing times for the TOCSY, 0.1451 s (AMX 400) or 0.151 s (AMX 500), and for the NOESY, 0.8 s; these spectra were used for the individual assignment of the NH signals; ambiguities in the assignment of **10** (overlap of N*H* and *H*–C(α) resonances, respectively) were resolved by recording an ω_1-selective (CO) heteronuclear (^{13}C,^{1}H) long-range correlation spectrum on the *AMX 500*.

2. *Syntheses* (see *Scheme*). *Boc*-L-MeLeu-*OH* (**1a**) *and Boc*-D-MeLeu-*OH* (**1b**) were obtained from the corresponding Boc-amino acids as oils following *Olsen*'s procedure [14]. ^{1}H-NMR ($CDCl_3$, 16 mg/ml, 25°): 4.83, 4.63 (2*m*, 1 H, H–C(α)); 2.82, 2.80 (2*s*, 3 H, MeN); 1.7 (*m*, 2 H, $CH_2(\beta)$); 1.6 (*m*, 1 H, H–C(γ)); 1.47, 1.46 (2*s*, 9 H, Boc); 0.96, 0.94 (2*d*, 6 H, Me(δ)).

H-D-*Leu*-L-*Leu*-D-*Leu-OMe* (**2a**) *and its Enantiomer* **2b** were obtained from the corresponding Boc- and MeO-protected oligoleucines [15].

Boc-L-MeLeu-D-*Leu*-L-*Leu*-D-*Leu-OMe* (**3a**) *and its Enantiomer* **3b** were prepared from **1a** and **2a** and **1b** and **2b**, resp. M.p. 64–65° (from AcOEt/hexane). ^{1}H-NMR ($CDCl_3$, 21 mg/ml, 25°): 6.98, 6.62, 6.55 (3*d*, 3 H, NH); 4.59–4.45, 4.21 (4*m*, 4 H, H–C(α)); 3.71 (1*s*, MeO); 2.75 (1*s*, MeN); 2.0–1.6 (2*m*, 12 H, $CH_2(\beta)$, H–C(γ)); 1.48 (1*s*, 9 H, Boc); 0.94 (1*m*, 24 H, Me(δ)).

Boc-(L-*Leu*-D-*Leu*)$_2$-L-MeLeu-D-*Leu*-L-*Leu*-D-*Leu-OMe* (**5**) was obtained from **3a** and Boc-(L-Leu-D-Leu)$_2$-OH (**4**; from Me ester [15].

Boc-L-*Leu*-(D-*Leu*-L-*Leu*)$_3$-D-MeLeu-L-*Leu*-D-*Leu*-L-*Leu-OMe* (**7**) was synthesized from **3b** and Boc-L-Leu-(D-Leu-L-Leu)$_3$-OH (**6**; from Me ester [15]).

Boc-(L-*Leu*-D-*Leu*)$_4$-L-MeLeu-D-*Leu*-L-*Leu*-D-*Leu-OMe* (**8**) was obtained from **4** and **5**.

Boc-D-*Leu*-(L-*Leu*-D-*Leu*)$_5$-L-MeLeu-D-*Leu*-L-*Leu*-D-*Leu-OMe* (**10**) was synthesized from **8** and Boc-D-Leu-L-Leu-D-Leu-OH (**9**; from Me ester [15]).

Removal of Boc Group. After 10 h treatment with $CF_3COOH/CHCl_3$ 1:5 at r.t., the soln. was evaporated. Acetone was added to the residue and the soln. obtained evaporated again. This operation was repeated twice. Excess CF_3COOH still remaining in the trifluoroacetate salts thus obtained was neutralized with 4-methylmorpholine (NMM).

Ester Hydrolysis. After 10 h treatment with 2 equiv. of base in 1N NaOH/MeOH 1:5 at r.t., the soln. was neutralized with 0.5N HCl and the precipitate filtered, washed, and recrystallized from AcOMe/Hexane.

Peptide Coupling. The peptide acids in $CHCl_3$ were first treated with isobutyl chloroformate (ClCOO(i-Bu) 1 equiv.); and NMM (1 equiv.) at –15° to give the mixed anhydride, and then with the trifluoroacetate salt (1 equiv.) and NMM (1 equiv.). After stirring *ca.* 15 h at r.t., the soln. was washed 3 times with H_2O, 1N HCl, 1N $NaHCO_3$,

and H_2O, dried ($MgSO_4$), and evaporated. The products were purified by recystallization from $CHCl_3$/hexane. Octapeptide **5** was further purified by column chromatography (silica gel *60* (*Merck*), toluene/AcOH 3:1). All final products were pure by TLC and characterized by NMR.

Results. – ^{1}H-NMR spectra ($CDCl_3$, 25°) of solutions of **5** are characterized by bands of uncertain conformational origin, while those of solutions of **7**, **8** and **10** in the same solvent are well resolved and rather directly attributable to β-helices. In particular, several of their N*H* signals are located at a very low-field (see *Fig. 1*), indicating extensive H-bonding, and the coupling constants ^3J(N*H*,*H*–C(α)) (*Table 1*) are generally large, pointing to an extended conformation. In view of the length of the peptide chains, the presence in the ^{1}H-NMR spectra of essentially only *n* N*H* signals implies [1] long-lived β-helices of a unique type. According to their chemical shift, there can be [1], in each case,

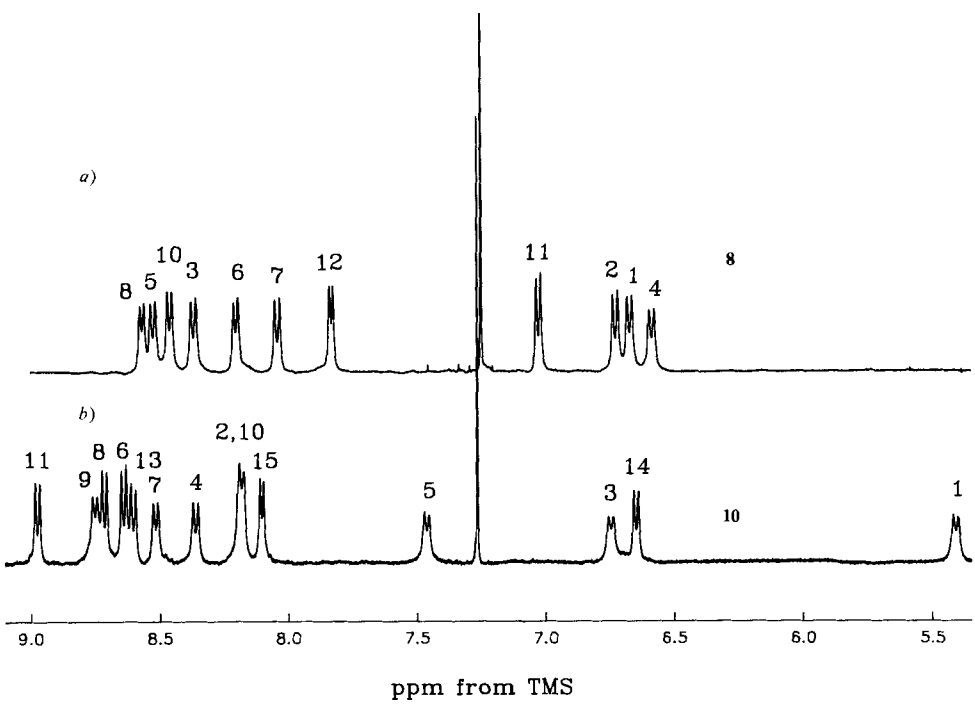


Fig. 1. *NH Region of ^{1}H-NMR spectra* ($CDCl_3$, 25 °C) *of solutions of* a) **8** (36 mg/ml) *and* b) **10** (20 mg/ml). The numbers denote the amino-acid residues to which the N*H* signals are assigned.

only 3 N*H* groups not involved in H-bonding, these being 2 amide-N*H*'s and the urethane-N*H* in the cases of **7** and **10**, and 3 amide-N*H*'s in the case of **8**. These data are *per se* sufficient for identifying the β-helices as right- (**7**) or left-handed (**8** and **10**), $\beta^{4,4}$-helices. In such helices, the N*H*'s of the residues 1, 3, and $n-1$ (**7** and **10**), or 2, 4, and $n-1$ (**8**), should not be involved in H-bonding, and this is in accord with the corresponding δ(N*H*)'s (*Fig. 1*).

Table 1. *^{1}H-NMR Chemical Shifts* (in ppm rel. to TMS) *of the Terminal, MeN, and NH Groups and Coupling Constants* $^3J(NH,H-C(\alpha))$ (in Hz) *for* **7**, **8**, *and* **10** *in* $CDCl_3$ *at 25°*[a])

	7	**8**	**10**
Boc	1.48	1.52	1.48
MeN	3.02	3.02	3.01
MeO	3.70	3.70	3.71
NH[b])	5.3 (c), residue 1)	6.67 (J = 9.5, residue 1)	5.43 (J = 9.0, residue 1)
	6.78 (J = 10.0, residue 10)[d])	6.59 (J = 9.7, residue 4)	6.58 (J = 8.4, residue 14)
	6.84 (J = 8.0, residue 3)[d])	6.73 (J = 9.7, residue 2)	6.71 (J = 9.4, residue 3)
	7.58 (J = 9.2)	7.03 (J = 8.4, residue 11)	7.45 (J = 9.2, residue 5)
	7.77 (J = 6.7, residue 11)[d])	7.84 (J = 6.7, residue 12)	8.11 (J = 6.8, residue 15)
	7.94 (J = 8.5)	8.05 (J = 9.0, residue 7)	8.17 (J = 8.8, residue 2)
	8.09 (J = 9.7)	8.23 (J = 8.2, residue 6)	8.18 (J = 8.8, residue 10)
	8.26 (J = 8.2)	8.38 (J = 9.3, residue 3)	8.35 (J = 9.4, residue 4)
	8.41 (J = 9.4)	8.47 (J = 8.7, residue 10)	8.51 (J = 8.7, residue 7)
	8.63 (J = 6.7)	8.54 (J = 9.2, residue 5)	8.60 (J = 9.2, residue 13)
		8.59 (J = 7.7, residue 8)	8.64 (J = 9.1, residue 6)
			8.72 (J = 9.0, residue 8)
			8.75 (J = 9.1, residue 9)
			8.98 (J = 8.7, residue 11)

[a]) **7**, 6.3 mg/ml; **8**, 36.0 mg/ml; **10**, 20.0 mg/ml.
[b]) The residues are numbered consecutively starting from the N-terminus.
[c]) Because of the overlap with the signal of a $H-C(\alpha)$, no coupling constant can be given.
[d]) The assignment is not established.

The sequence-specific assignment of the NH's and $H-C(\alpha)$'s was achieved for **8** and **10** by TOCSY and NOESY measurements. *Fig. 2* gives a section of the NOESY spectrum of **10** and shows *a*) the assignment of the relevant protons in the chain *via* $NH(i)\rightarrow H-C(\alpha.i)\rightarrow NH(i+1)$, and $H-C(\alpha.11)\rightarrow$MeN(12) contacts, and *b*) important inter-residue contacts of the types $H-C(\alpha.i)/NH(i+4)$ (D-residues) and $NH(i)/H-C(\alpha.i+4)$ (L-residues). The latter establish unambiguously that the helices are of the type $\beta^{4,4}$.

Even solutions with concentrations up to 36 mg/ml gave no indication of the occurrence of double-stranded helices. Similar NMR results were obtained for **7**, **8**, and **10** using benzene or other solvents, including pentachloroethane. In no case, spectral changes were observed by repeating the NMR measurements after the solutions had been left standing at room temperature for several days.

Stereoviews of the $\beta^{4,4}$-helices of **8** and **10** are presented in *Figs. 3* and *4*, respectively. Note that the C-terminal residue, for which lower values of $J(NH,H-C(\alpha))$ were found (*Table 1*), is in both cases tilted slightly away from the helical axis, as expected in view of steric interactions with the N-Me group.

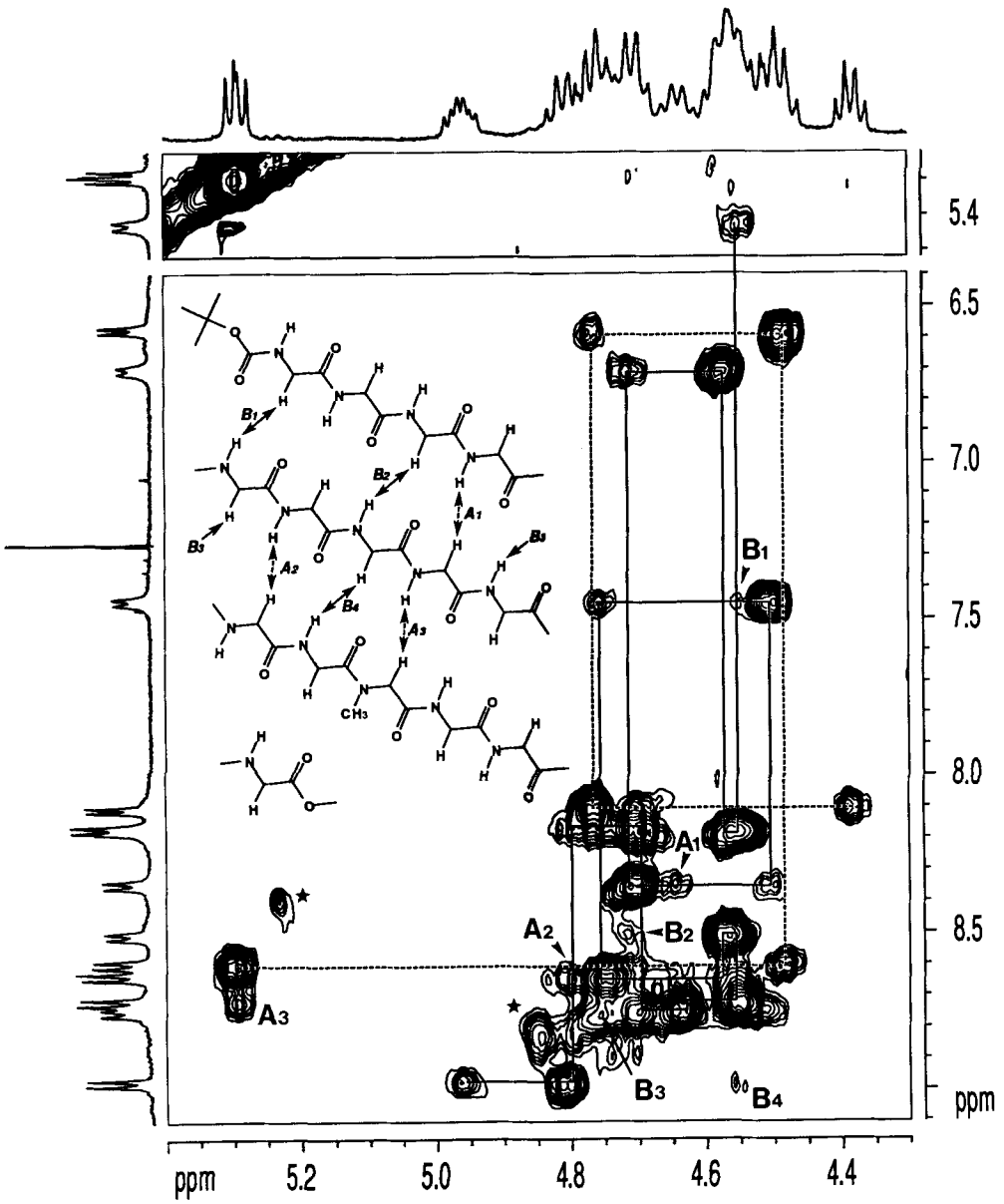


Fig. 2. *Section of the 500.13-MHz NOESY spectrum of* **10** *showing details of the sequence-specific assignment of* NH *and* H–C(α) *resonances.* The solid line pertains to the sequence (N*H*(1)/*H*–C(α.11), and the dashed line to the sequence *H*–C(α.12)/*H*–C(α.15). The identity of the $\beta^{4,4}$-helix is demonstrated by the NOE cross-peaks due to contacts of the *H*–C($\alpha.i$)/N*H*($i+4$) (B1–B4) and N*H*(i)/*H*–C($\alpha.i+4$) (A1–A3) between protons of D- and L-residues, on contiguous turns, respectively. The two cross-peaks denoted by an asterisk derive from an impurity

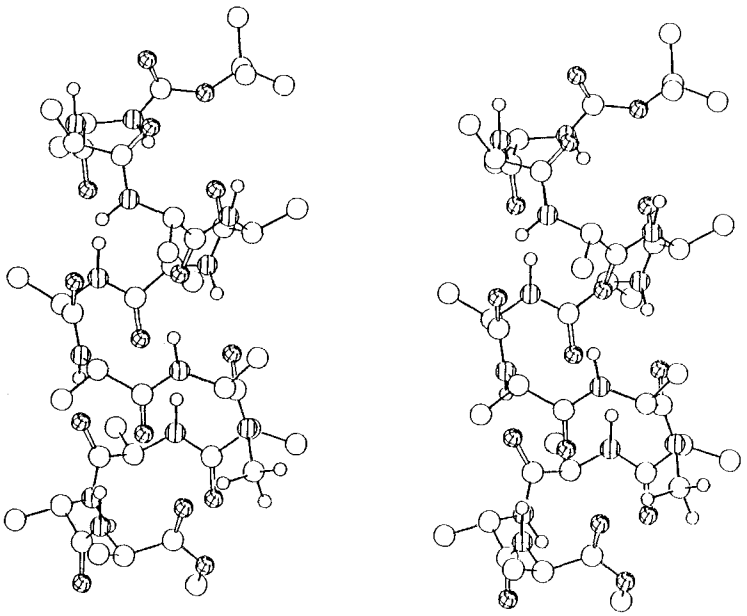


Fig. 3. *Stereoview of the $\beta^{4,4}$-helix of* **8**. For sake of clarity, the side chains are reduced to the C(β)-atom; only the protons N*H* and *Me*N are shown (smallest circles); N- and O-atoms are circles with parallel and spherical-grid lines, respectively. Represented is a minimum-energy conformation as obtained from the program INSIGHT II/DISCOVER (*Biosym Technologies Inc.*, 10065 Barnes Canyon Road, San Diego, CA 92121, USA).

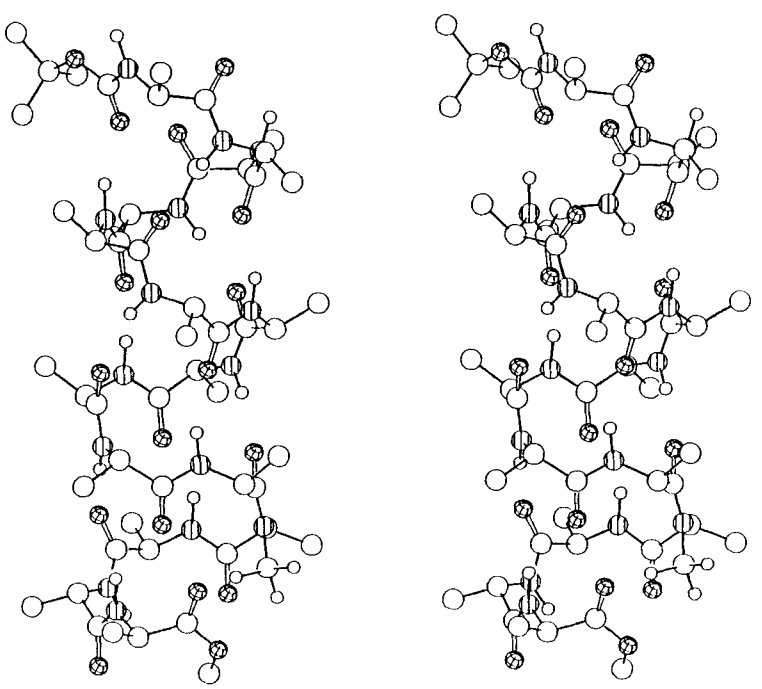


Fig. 4. *Stereoview of the $\beta^{4,4}$-helix of* **10**. See *Fig. 3* for explanations.

Discussion. – For unsubstituted D,L-alternating oligo(-α-amino acids) of formula Boc-X_n-OMe, the β-helices with the highest possible number ($n-1$) of intrahelical H-bonds are $\uparrow\uparrow\beta^{5.6}$- and $\uparrow\downarrow\beta^{5.6}$-helices (*Table 2*). For analogs such as those studied here, with an *N*-methylated residue at the $n-3$ position, it is the $\uparrow\downarrow\beta^{5.6}$- and $\uparrow\downarrow\beta^{7.2}$-helices which can have the highest possible number ($n-2$) of H-bonds (*Table 2*). Therefore, as regards intrahelical H-bonding, for the methylated analogs investigated in this work, $\uparrow\downarrow\beta^{7.2}$-helices would appear to represent conformations as likely as $\uparrow\downarrow\beta^{5.6}$-helices. For

Table 2. *Maximum Number of H-Bonds per Chain (N_{max}) Realizable in Different Types of β-Helices by* D,L-*Alternating Peptides of General Formula Boc-X_n-OMe* (X = α-amino-acid residue) *and by Analogs Having an* N-*Methylated Residue at the Position* n – 3

Type of β-helix[2])	N_{max}[a])		
	Boc-X_n-OMe	*N*-Methylated analogs	
		Even *n*	Odd *n*
$\uparrow\uparrow\beta^{5.6}$	$n-1$	–[b])	–[b])
$\uparrow\downarrow\beta^{5.6}$	$n-1$	$n-2$	$n-2$[c])
$\uparrow\uparrow\beta^{7.2}$	$n-2$	–[b])	–[b])
$\uparrow\downarrow\beta^{7.2}$	$n-2$	$n-2$	$n-2$
$\beta^{4.4}$	$n-3$	$n-4$[c])	$n-4$[c])

[a]) For the peptides with a D-residue at the position *n*, the helices with N_{max} are left-handed, for those with a L-residue at this position, they are right-handed.

[b]) Only helices exhibiting a string of two or more non-H-bonded residues can be constructed.

[c]) Earlier classified as not possible [17]. In this helix, the MeN group prevents a carbonyl O-atom on an adjacent turn from forming an intrahelical H-bond and sterically influences its position.

analogs with odd *n*, the $\uparrow\downarrow\beta^{7.2}$-helices would even appear more likely, since these helices are not sterically disturbed by the MeN group (*Table 2*). The results presented above, however, show that **7**, **8**, and **10**, (**5** is probably too short to form stable β-helices in solution) do not form these, but rather, and with very strong preference, $\beta^{4.4}$-helices, even if this results in two H-bonds less per chain (*Table 2*) and some steric distortion at the C-terminal residue (*Fig. 3* and *4*). These observations underscore the difficulty, already foreseen on theoretical grounds [16], of obtaining β-helices with large cavities and indicate that other factors, beside the number of intrahelical H-bonds contribute to the stability of β-helices. Results [17] obtained for some methylated oligonorleucines confirm that one of these factors is the nature of the side chains. It is possible that the use of oligonorleucines or of oligoleucines having the *N*-methylated residue at the $n-4$ (fifth last) position of the chain [17] may represent a better approach for obtaining $\uparrow\downarrow\beta^{7.2}$- or other wide β-helices than that used in this work.

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