41. (Cholestanyloxycarbonyl)benzyl Esters as Peptide Substituents: Conformational Properties of Fully Protected Oligo-L-Lysines with C-Terminal (Cholestanyloxycarbonyl)benzyl and Benzyl Ester Moieties

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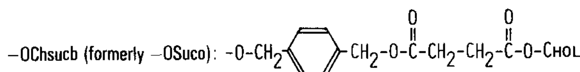
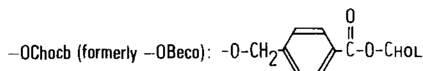
This study involves L-lysine oligo peptides, protected at the N-terminus by the Nps and at the ε-amino functions by Boc groups. Two series were prepared from dimer to octamer, one containing the *p*-[(cholestan-3β-yloxy)carbonyl]benzyl, the other one the benzyl ester group at the C-terminus. Conformational analyses were performed by IR absorption. The occurrence of the intermolecular β-structure in the solid state and in CH_2Cl_2 solution was demonstrated for the highest oligomers. The relative stabilities of the self-associated species were determined by adding a variety of polar solvents to the CH_2Cl_2 solutions. The cholestanyl-containing peptides have a lower propensity to self-aggregate than the benzyl-ester analogues. Self-aggregation and decreasing solubility run in parallel. It was also directly shown that soluble urea derivatives may disrupt intermolecular H-bonds in CH_2Cl_2, a point of practical interest, particularly in solid-phase peptide synthesis.

Introduction. – C-Terminal protecting groups containing the cholestanol moiety are promising tools in the synthesis and conformational analysis of peptides, since they enhance peptide solubilities in organic solvents of low polarity [1–5]. In previous work, *p*-{[4-(cholestan-3β-yloxy)succinyloxy]methyl}benzyl (= Chsucb (formerly Suco)) peptide esters were mainly used, but their preparation is rather cumbersome. Therefore, other carboxyl substituents containing cholestanol were proposed [6], *inter alia* the *p*-[(cholestan-3β-yloxy)carbonyl]benzyl (= Chocb (formerly Beco)) ester group. In the present communication, we describe two complete series of $[Lys(Boc)]_n$ peptides with $n = 2$ to 8, both carrying at the N-terminus the Nps protecting group. At the C-terminus,





one series (series **A**) carries the OChocb group, the other one (series **B**) the benzyl ester group (OBzl). These peptides were subjected to solid-state and solution conformational analysis, performed by IR spectroscopy, and the conformational preferences and relative stabilities were compared between the two series.

Preparation and Characterization of Peptides. – Peptides *(cf. Table)* were prepared in solution according to established procedures by means of the two-phase-purification method [7] [8]. The series **A** carrying the OChocb group at the C-terminal carboxyl group (**IA** to **VIIIA**) starts with Nps-Lys(Boc)-OChocb which was obtained by esterification of Nps-Lys(Boc)-OH with HOChocb. Similarly, the series **B (IB** to **VIIIB)** was initiated with Nps-Lys(Boc)-OBzl. Stepwise elongation, after removal of Nps, was carried out with Nps-Lys(Boc)-OH using carbodiimide derivatives in the presence of 1,2,3-benzotriazol-1-ol. The peptides carrying an unprotected N-terminus were not kept, but immediately elongated to the next higher homologue. The fully protected peptides appeared generally homogeneous on TLC, however special purification steps were required at some stages. The Nps group has not been frequently used in peptide synthesis recently, since the formation of a number of side-products during deblocking may give raise to difficulties. Such problems can be minimized although not fully eliminated by using ammonium thiocyanate in the presence of suitable indole derivatives for deprotection and by working in the dark below room temperature.

Table. *Characterization of Protected* L-*Lysine Oligopeptides by TLC*

Peptides[a])		R_f Values[b])					
		Series **A**			Series **B**		
		1	*2*	*3*	*1*	*2*	*3*
I	Nps-Lys(Boc)-OR	0.85	0.67	0.81	0.83	0.77	0.77
II	Nps-Lys(Boc)-Lys(Boc)-OR	0.87	0.41	0.61	0.77	0.65	0.64
III	Nps-Lys(Boc)-Lys(Boc)-Lys(Boc)-OR	0.80	0.33	0.36	0.69	0.45	0.50
IV	Nps-Lys(Boc)-[Lys(Boc)]$_2$-Lys(Boc)-OR	0.77	0.31	0.22	0.61	0.33	0.35
V	Nps-Lys(Boc)-[Lys(Boc)]$_3$-Lys(Boc)-OR	0.73	0.28	0.16	0.52	0.26	0.25
VI	Nps-Lys(Boc)-[Lys(Boc)]$_4$-Lys(Boc)-OR	0.71	0.23	0.14	0.50	0.23	0.21
VII	Nps-Lys(Boc)-[Lys(Boc)]$_5$-Lys(Boc)-OR	0.68	0.18	0.13	0.48	0.20	0.18
VIII	Nps-Lys(Boc)-[Lys(Boc)]$_6$-Lys(Boc)-OR	0.66	0.17	0.12	0.47	0.18	0.15

a) In series **A**, OR = OChocb; in series **B**, OR = OBzl.
b) Chromatography on 5 × 10 cm silica-gel TLC plates *60F 254, Merck*; system *1*: $CHCl_3$/MeOH 9:1; system *2*: $CHCl_3$/MeOH 95:5; system *3*: CH_2Cl_2/EtOH 14:1.

Solid-State Conformational Analysis. – The solid-state conformational properties of the Nps-Lys(Boc) peptide series were examined by IR absorption. Significant examples are shown in *Fig. 1*. The amide-A (3450–3250 cm^{-1}) and amide-I (1720–1620 cm^{-1}) bands appear to be the most informative for interpreting conformational changes as a function of increasing number of residues in the peptide chain [5] [9] [10]. The maxima of the amide-II (1600–1500 cm^{-1}) and amide-V (750–600 cm^{-1}) bands proved not to be reliably diagnostic for conformational analysis, inasmuch as multiple contributions due to the aromatic rings are present in the same spectral region [10]. The small band (shoulder) at about 1740 cm^{-1} in the lowest oligomers, which disappears in the highest oligomers, is assigned to the carbonyl stretching mode of the ester functions of the C-terminal substituents [5] [10].

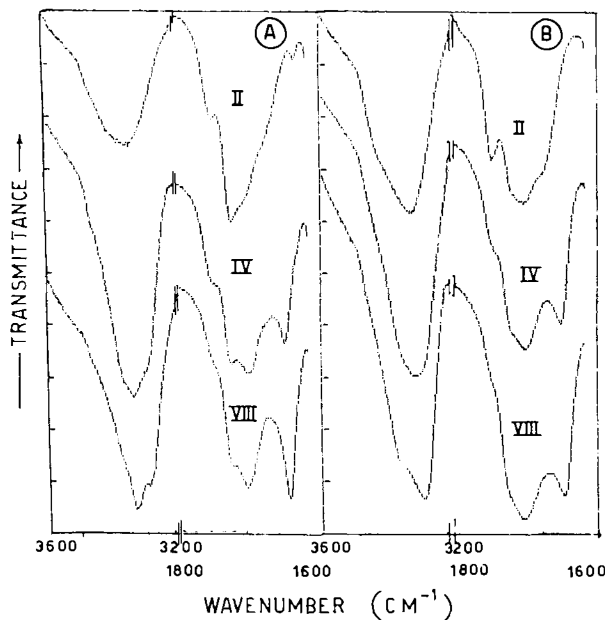


Fig. 1. *Solid-state IR spectra in the 3600–3200 cm⁻¹ and 1800–1600 cm⁻¹ regions of the di-, tetra-, and octapeptides* **II**, **IV**, *and* **VIII**, *respectively with the OChocb* (**A**) *and the OBzl* (**B**) *substituent at the C-terminus*

In the highest oligomers of both series **A** and **B**, characteristic bands are seen at 3300–3284 cm^{-1} (N–H stretching vibration of strongly H-bonded groups) and at 1631–1628 cm^{-1} (amide C=O stretching vibration of strongly H-bonded groups), both indicative of the occurrence of the β-structure [5] [9–12]. These absorptions are first visible as a small band or shoulder at the tetrapeptide stage. It proved to be impossible to discriminate between the parallel and antiparallel types of β-structure adopted by these peptides using the diagnostic 1690 cm^{-1} band of the antiparallel β-structure [11] [12], since the absorption in that region of the carbonyl groups of the side-chain urethane moieties is very intense [5] [9]. In none of the highest oligomers of the two series a band near 1655 cm^{-1} is found, indicating the onset of the α-helical structure [5] [9] [12]. This is not surprising in view of the known observation that the critical chain length for α-helix formation in the solid state occurs at or above the decapeptide stage [5] [9] [13].

The influence of the cholestanyl moiety appears to be slight. A tentative assignment of the factor responsible for the small differences in the relative intensities of the bands in the 3330–3285, 1720–1690 and 1630 cm^{-1} regions of the two series may be a less regular interaction of the ε-Boc side chains in the Chocb peptide esters.

Solution Conformational Analysis. – In the low-polarity solvent CH_2Cl_2, at 10^{-2}M concentration, the tendency to give strongly self-associated species to a relevant degree is clearly visible in the IR spectra of the hexa- and heptapeptides of series **A** *(Fig. 2)*. The β-structure diagnostic bands are found at 3280 cm^{-1} and 1625 cm^{-1} [5] [9–12]. However, in contrast to the solid state *(Fig. 1)*, under these conditions there are still some NH groups that are not H-bonded (3445 cm^{-1}) [5] [10]. The onset of the β-structure is first seen at the hexapeptide stage *(Fig. 2)*. By a 10-fold dilution of the CH_2Cl_2 solution to 10^{-3}M, the

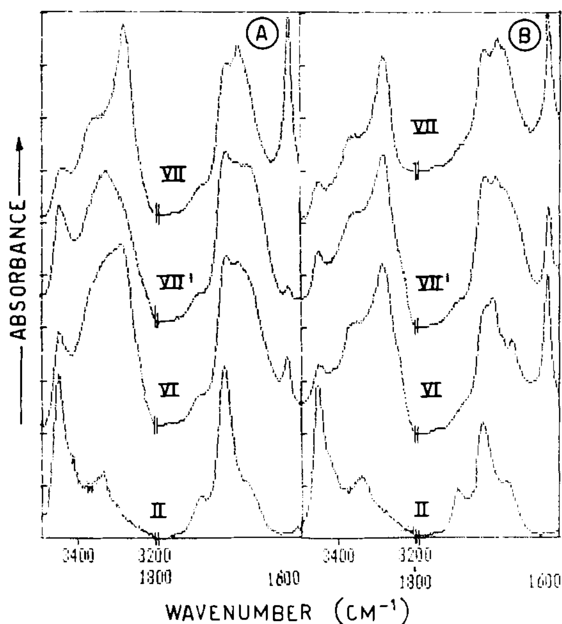


Fig. 2. *IR spectra (CH_2Cl_2) in the 3500–3200 cm^{-1} and 1800–1610 cm^{-1} regions of the di-, hexa-, and heptapeptides* **II**, **VI**, *and* **VII**, *respectively with the OChocb* (**A**) *and the OBzl* (**B**) *substituent at the C-terminus.* Concentration 10^{-2}M. The spectra of the two heptamers at 10^{-3}M, **VII'A** and **VII'B**, are also shown.

extent of β-structure formation of the highest oligomers is drastically reduced, showing that this structure is of the intermolecular type [5]. In *Fig. 2* the spectra of significant oligomers of series **B** are also exhibited. A comparison of the relative intensities of the informative bands in the spectra of corresponding peptides in the two series supports the conclusion that the OBzl peptides tend to aggregate much more strongly than the Chocb analogs.

To get a better insight into the relative stabilities of the β-structures in the highest oligomers of both peptide series, we took advantage of the IR-titration method recently introduced by some of us [14–19]. Self association in CH_2Cl_2 was disrupted by adding a variety of polar organic solvents including DMSO (dimethylsulfoxide), HMPTA (hexamethylphosphortriamide), trimethyl phosphate, and sulfolane (= tetrahydrothiophene-1,1-dioxide) in increasing amounts. This process was monitored by following the disappearance of the band near 1630 cm^{-1}.

The solvent-titration curves in CH_2Cl_2/DMSO mixtures are shown in *Fig. 3*. For each peptide, the relative intensity was calculated from the area of the 1630 cm^{-1} band, taking the value observed in CH_2Cl_2 as relative intensity 1.0 [14–19]. The effect produced by increasing main-chain length is clearly apparent in both series. The increasing stability of the self-associated species of these short peptides with an increase of main-chain length is not unexpected [14–19] and should be related to the decreasing influence of the end portions of the molecules which are more easily solvated. From a comparison of the titration curves of corresponding peptides in the two series **A** and **B**, additional evidence is obtained for the higher stability of the self-associated species formed by the OBzl peptides.

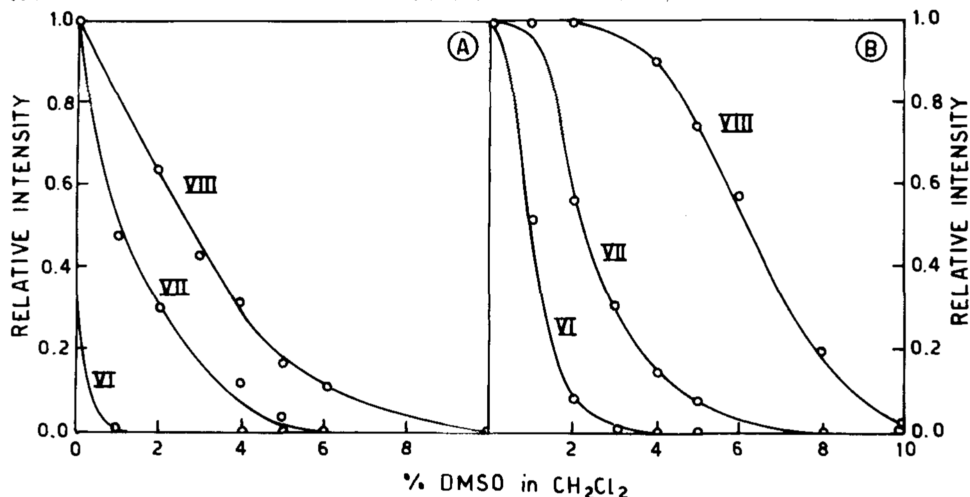


Fig. 3. *Relative intensity of the amide-I C=O stretching band related to strongly self-associated molecules in the IR spectra of the hexa-, hepta-, and octapeptides* **VI**, **VII**, *and* **VIII**, *respectively, of series* **A** (OChocb peptides) *and series* **B** (OBzl peptides) *in CH_2Cl_2/DMSO as a function of increasing percentages of DMSO.* Peptide concentration is 10^{-2}M.

Fig. 4 illustrates the solvent effect in the case of the OChocb octapeptide **VIIIA**. It is clear that DMSO and HMPTA are almost equally effective as structure-disrupting solvents [19]. However, there is a significant difference between the capabilities of a) the two S=O containing solvents DMSO and sulfolane and b) the two P=O containing solvents HMPTA and trimethyl phosphate, DMSO and HMPTA being the more effec-

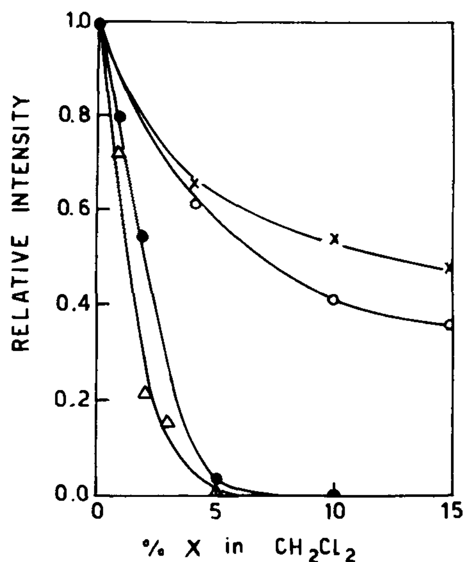


Fig. 4. *Relative intensity of the amide-I C=O stretching band related to strongly self-associated molecules in the IR spectra of octapeptides* **VIIIA** *in CH_2Cl_2/X mixtures as a function of increasing percentages of X.* X: HMPTA Δ, DMSO ●, trimethyl phosphate ○, and sulfolane X. Peptide concentration is 10^{-3}M.

tive in the two groups of solvents [19]. From a comparison of the curve in CH_2Cl_2/DMSO mixtures (concentration 10^{-3}M) with the corresponding one in *Fig. 3* (concentration 10^{-2}M) it turns out again, that the H-bonded structures formed are of the intermolecular type [17–19].

One of us has recently shown the advantages of performing peptide synthesis using *N,N'*-disubstituted carbodiimides which form CH_2Cl_2-soluble ureas [20]. In this connection it seems important, particularly for solid-phase peptide synthesis, to determine whether soluble ureas interact with the peptide chain. If so, this phenomenon would permit to overcome the unfavorable consequence of self-aggregation of peptide chains attached to insoluble polymeric matrices [21–24]. *Fig. 5* clearly shows that this interaction

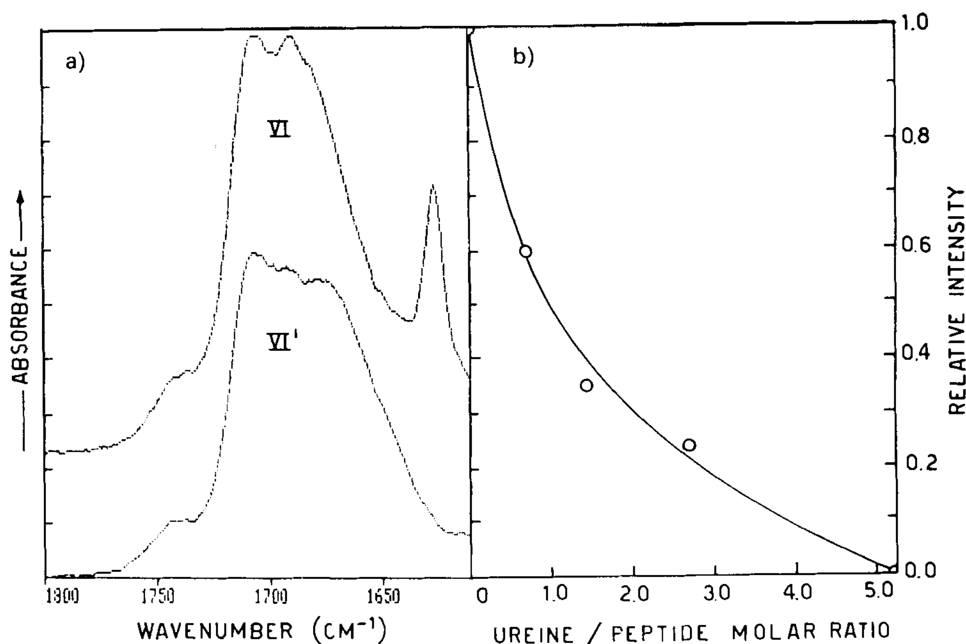


Fig. 5. a) *IR spectra in the 1800–1610-cm⁻¹ region of hexapeptide* **VIA** (1.5 × 10^{-2}M) *in* CH_2Cl_2 *in the absence* (**VI**) *and presence* (**VI'**) *of added* N,N'*-diisopropylurea at a molar ratio peptide/*N,N'*-diisopropylurea of 1:5.5.* For **VI'**, the reference cuvette contained an appropriate solution of *N,N'*-diisopropylurea in CH_2Cl_2. b) *Relative intensity of the amide-I C=O stretching band related to the strongly self-associated molecules of the hexapeptide* **VIA** *as a function of increasing* N,N'*-diisopropylurea/peptide molar ratio.*

does indeed take place and that the reasonably soluble *N,N'*-diisopropylurea, at an *N,N'*-diisopropylurea/peptide molar ratio of 5.5:1, is able to disrupt completely the self-associated species formed in CH_2Cl_2 by 1.5 × 10^{-2}M Nps-Lys(Boc)-[Lys(Boc)]$_4$-Lys-(Boc)-OChocb (**VIA**). We have also been able to demonstrate that the two unsymmetrical derivatives *N*-methyl-*N'*-(*tert*-butyl)urea and *N*-ethyl-*N'*-cyclohexylurea are also effective in disrupting (peptide)C=O···H–N(peptide) intermolecular H-bonds (data not shown).

Discussion. – In this work, we have been able to detect the critical chain length for intermolecular β-structure formation in the solid state and in CH_2Cl_2 for the $[Lys(Boc)]_n$-peptide series in the form of either OChocb or OBzl esters. In the solid state, this ordered secondary structure is first seen at $n = 4$ in both series. This result compares well with those previously reported for related oligopeptide series under the same experimental conditions [5] [9]. In particular, the onset of the β-structure has been reported to occur at the pentapeptide stage for the H-Lys(Boc)-$[Lys(Boc)]_n$-Lys(Boc)-OChocb series [5] and at the tetrapeptide stage for the Boc-Lys(Z)-$[Lys(Z)]_n$-Lys(Z)-Gly-OCH_2-1,4-(2-$NO_2C_6H_3$)-CONH-polyethyleneglycol series [9].

It is now well established that the onset and increase of the proportion of peptide self-associated species are paralleled by a marked decrease in solubility [25]. These phenomena are of practical significance, since they can have an adverse effect on rates of aminolysis in peptide synthesis and on the ease of purification of the intermediates and final products. Therefore, the knowledge of the relationship between structure and solubility is of paramount importance in planning a correct strategy of synthesis. Since the OChocb group has been used to increase the solubility of medium-sized peptides in halohydrocarbons [6], it was of interest to ascertain whether this moiety is solubility-increasing solely on the basis of its lipophilic properties or whether it may also impede the formation of the poorly soluble β-structure in these solvents.

The results described here allow the conclusion that the low solubility in CH_2Cl_2 of Nps-Lys(Boc)-$[Lys(Boc)]_n$-Lys(Boc)-OChocb, $n = 4$–6, is related to the onset of an intermolecularly H-bonded β-structure, as already shown for other medium-sized peptides [5] [9] [10] [14–19] [25] [26]. The addition of more polar solvents, particularly DMSO and HMPTA, to the CH_2Cl_2 solutions of the hexa- to octapeptides is able to convert the β-structure into a solvated, statistically coiled conformation, by forming effective (peptide)N–H···O=X(solvent) H-bonds, where X is either S or P, thereby disrupting (peptide)N–H···O=C(peptide) intermolecular H-bonds [5] [14–19]. Interestingly, such additions are commonly used to enhance the solubility of peptides during coupling reactions.

In addition, the conformational analysis in CH_2Cl_2 solution strongly supports the view that the OChocb peptide esters have a lower propensity to self-aggregate than their OBzl analogues. This difference might be due to steric effects operative in the (cholestanyl oxycarbonyl)benzyl esters. The reason why it is not seen in the solid state should probably be found in the crystal forces present under those conditions which tend to level off all slight different contributions. Interestingly, in CH_2Cl_2 solution, the β-structure is first seen at the heptapeptide stage in the H-Lys(Boc)-$[Lys(Boc)]_n$-Lys(Boc)-OChsucb series [5].

It is gratifying to note that the quantitative scale of increasing tendency to self-associate of the peptides examined parallels the observed qualitative scale of decreasing solubility in CH_2Cl_2. An additional lipophilic C-terminal blocking group, the stearyl substituent, has been proposed by *Hollosi et al.* [27] in their recent conformational study of β-turn forming model peptides. However, no detailed investigation of the role played by this aliphatic moiety in the solubilization of oligopeptides has been performed thus far.

Finally, we have been able to present the first direct evidence that (peptide)N–H···O=C(peptide) intermolecular H-bonds in CH_2Cl_2 can be disrupted by adding an appropriate molar ratio of a soluble urea. We argue that the main factor responsible for this phenomenon is a competition of (urea)N–H···O=C(peptide) H-bonds with

the intermolecular (peptide)N–H···O=C(peptide) H-bonds characteristic of the β-structure [28] [29]. This finding is of interest, since it represents an additional point in favour of the use of carbodiimides which form CH_2Cl_2-soluble ureas in solid-phase peptide synthesis.

Experimental Part

General. Nps-Lys(Boc)-OH was obtained from *Fluka AG*, Buchs. HOChocb was prepared according to [6]. All products were subjected to TLC on silica gel *60, F254, Merck*, and detected by adequate reagents and by using a *Camag* TLC scanner with a *Hewlett Packard 3390 A* integrator. In particular, peptide spots in the **A** series were detected with H_2SO_4 (cholestanol) and in the **A** and **B** series with ninhydrin after heating. See also [30] for chromatographic methods.

Peptides. Esterification of Nps-Lys(Boc)-OH with HOChocb was carried out with *N,N'*-dicyclohexylcarbodiimide in the presence of 4-(dimethylamino)pyridine [31]. Elongations of the peptide chains with Nps-Lys(Boc)-OH were essentially according to *König* and *Geiger* [32] with *N,N'*-dicyclohexylcarbodiimide in series **A** and *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide hydrochloride in series **B**. Solvents for the couplings were THF for **IA** to **VIA**; THF/DMF/CH_2Cl_2 15:10:5 for **VIIA**; DMF for **VIIIA** and **IB** to **VIIIB**. For the standard extractions in the spray-column extractor according to the two-phase-purification method [2], all reaction solutions were diluted 50–100 fold with CH_2Cl_2. Removal of Nps groups was performed with $Na_2S_2O_3$ according to [33] or with NH_4SCN/(2-methyl-1-indolyl)acetic acid according to [34], mainly in series **A**. Purification by column chromatography on silica gel *60* was necessary for **IIA**, **IIIA**, **IIB** and **VIIIB**.

Spectroscopy. IR spectra were recorded using a *Perkin-Elmer* model *580 B* spectrophotometer, equipped with a *Perkin-Elmer* model *3600* data station and a model *660* printer. The solution IR spectra, reported in absorbance, have been obtained from the recorded transmittance spectra by using the TAAT function of the CDS II software of the data station. For the solution measurements, cells with path lengths of 1.0 and 0.1 mm were used. CH_2Cl_2, HMPTA and sulfolane were obtained from *Fluka*; DMSO and trimethyl phosphate were *Merck* products. For the solid-state measurements, the KBr disk technique was employed. The band positions are accurate to ± 1 cm^{-1}.

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