Stereoselectivity in the Oligomerization of Racemic Tryptophan *N*-Carboxyanhydride (NCA-Trp) as Determined by Isotope Labeling and Mass Spectrometry

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The polycondensation of tryptophan *N*-carboxyanhydride (NCA-Trp) is investigated in the presence and in the absence of POPC (1-*O*-palmitoyl-2-*O*-oleoyl-*sn*-glycerol-3-*O*-phosphocholine) liposomes with the aim to study and to quantify possible stereoselective effects of the process. A novel technique, based on isotope labeling of one enantiomer, and high performance liquid chromatography mass spectrometry (LC-MS) allow determination of the individual stereoisomer distribution of oligomers up to $n = 10$. For the first time, the preferential homochiral growth and the relative stereoisomer distributions for each oligomer length are directly demonstrated.

Introduction. – The present paper addresses the question, whether *rac*-NCA-amino acid oligomerization leads to a preferential formation of homochiral oligomers (NCA = *N*-carboxyanhydride = cyclic anhydride). The introduction of a novel analytical method allows for the first time the quantitation of the preferential homochiral growth and the determination of the relative stereoisomer distributions for each oligomer length. Results are shown for the case of the NCA-DL-tryptophan (Trp) polycondensation, restricting the analysis up to the decamer ($n = 10$).

Despite the virtual simplicity, the oligomerization of *N*-carboxyanhydrides (NCA) [1] presents a few problems that have not been clarified to date [2]. One of these concerns the polycondensation of NCA-amino-acid racemates. NCA-Amino-acid racemates oligomerize slower than each of the pure NCA enantiomers [2–4]. A few hypotheses have been proposed to explain this difference, and in particular, it has been suggested that the lower rate of condensation of the racemate may correspond to a stereoselective or even stereospecific mechanism leading to homochiral sequences [3–5]. NMR Investigations appear to be consistent with a stereoselective mechanism for some of the amino acids used, and it was reported that the average length of the stereoblocks never exceeded 4 monomer units [6–13]. However, it has not been possible to determine the stereoisomer distribution of oligomers nor to quantitate the amount of homochiral sequence formed. This paper focusses on the quantitative analysis and not on the possible mechanistic reasons that may lead to the observed stereoisomer distributions.

Quantitative analysis has not been performed until now mostly due to the absence of suitable analytical methods. To appreciate the analytical difficulty, consider that the number of the 2^n theoretically possible stereoisomers ($2^n/2$ enantiomer pairs of diastereoisomers) increases with the oligomer length n. The experimental separation

and the determination of the stereosequence of all the 1024 theoretically possible stereoisomers of the different Trp oligomers formed (for $n=10$) seems to be an insurmountable task. When we restrict the analysis only to the determination of the stereoisomers consisting of the same composition of D-amino acid (p) and L-amino acid (q) residues, there are still 65 different stereoisomer subgroups (D_pL_q) of oligomers to be separated.

Isotope labeling of one enantiomeric NCA monomer in combination with liquid chromatography mass spectrometry (LC–MS) analysis leads to stereoisomer subgroups (D_pL_q) of the same length ($n=p+q$), but having different masses. In the present work, L-(D_5)Trp with a five-fold deuterated indole ring was used in the oligomerization of *rac*-NCA-Trp. Each L-(D_5)Trp inserted instead of a D-Trp leads to a 5-Da mass increase in the resulting Trp oligomer. This permits a differentiation of D_pL_q stereoisomer subgroups by their masses, since for $n=p+q$, D_pL_q subgroups of length n have now different masses ($m_{\text{all-D}}+5q$) (where $m_{\text{all-D}}$ is the mass of the homochiral oligo-D-Trp), and masses of different D_pL_q groups are separated by at least 5 Da, maximally 5n Da. If the peaks in the selected-ion-monitoring (SIM) chromatograms of the corresponding masses are integrated and compared to each other, the stereoisomer distribution of D_pL_q subgroups for each oligomer with length n can be determined, provided that the ionization behavior and the mass accuracy of the stereoisomers is similar under the conditions used. An example, including the mass spectra for each stereoisomer subgroup, of the oligo D-Trp/L-(D_5)Trp SIM chromatograms for $n=3$ is shown in *Fig. 1*. That the quantification of different oligo-Trp stereoisomers having the same length by SIM is a good method was shown by positive electrospray ionization (pos. ESI) mass spectrometry (see *Exper. Part*). The integral areas of stereoisomer-subgroup (D_pL_q) peaks in the MS SIM-chromatogram representation, with the mass $m_{\text{all-D}}+5q$ Da (with $q=1, 2, \ldots, n$), are, therefore, proportional to the total relative abundance of diastereoisomers (having the same q value) in that particular subgroup.

Experimental. – *Liposome Preparation*. Liposomes were prepared from POPC (=1-*O*-palmitoyl-2-*O*-oleoyl-*sn*-glycerol-3-*O*-phosphocholine) only. The lipids were first dissolved in $CHCl_3$ in a round-bottom flask. After evaporation of the solvent at 40°, the thin film was dried overnight under high vacuum. The dried lipid film was dispersed in buffer, which led to the formation of mainly multilamellar liposomes of considerable heterogeneity in size [14][15]. The liposome suspension was then frozen 10 times in liq. N_2 (−195°) and thawed in a water bath at 40° (freeze and thaw cycles) to reduce the number of small unilamellar liposomes and increase the content of multivesicular liposomes [16][17]. A significant decrease in size and lamellarity was achieved by successively passing the liposome suspension through two *Nucleopore* polycarbonate membranes ($d=25$ mm) from *Sterico AG* (Dietlikon, Switzerland) with mean pore diameters of 400 nm (10 times), 200 nm (10 times), and 100 nm (10 times), by means of an extruder supplied by *Lipex Biomembranes Inc.*, Vancouver, Canada [16]. Liposomes were characterized by DLS (dynamic light scattering). The extruded liposome suspensions were always freshly prepared and were never stored for longer than two weeks.

CDI-Induced Amino-Acid Condensation. In a first feeding, a freshly prepared soln. of either D-Trp, L-Trp, or L-(D5)Trp in 0.4M 1*H*-imidazole buffer (pH 6.80) was incubated with a 2.5-fold molar excess of CDI (=carbonyldiimidazole=1,1′-carbonylbis[1*H*-imidazole]) at 0° for 2 min. The resulting NCA-Trp soln. was mixed in a 1:3 (*v*/*v*) ratio with a 100-nm-extruded 100 mM POPC liposome suspension in 0.4M 1*H*-imidazole/HCl at pH 6.80, prepared by the extrusion technique with polycarbonate membranes of 100-nm pore diameter for final extrusions [16]. Then it was incubated in a *Vortex-Genie-2* shaker (*Scientific Industries*, USA) for 12 h at r.t. The same volume of NCA-Trp was added in the second feeding and incubated for another 12 h.

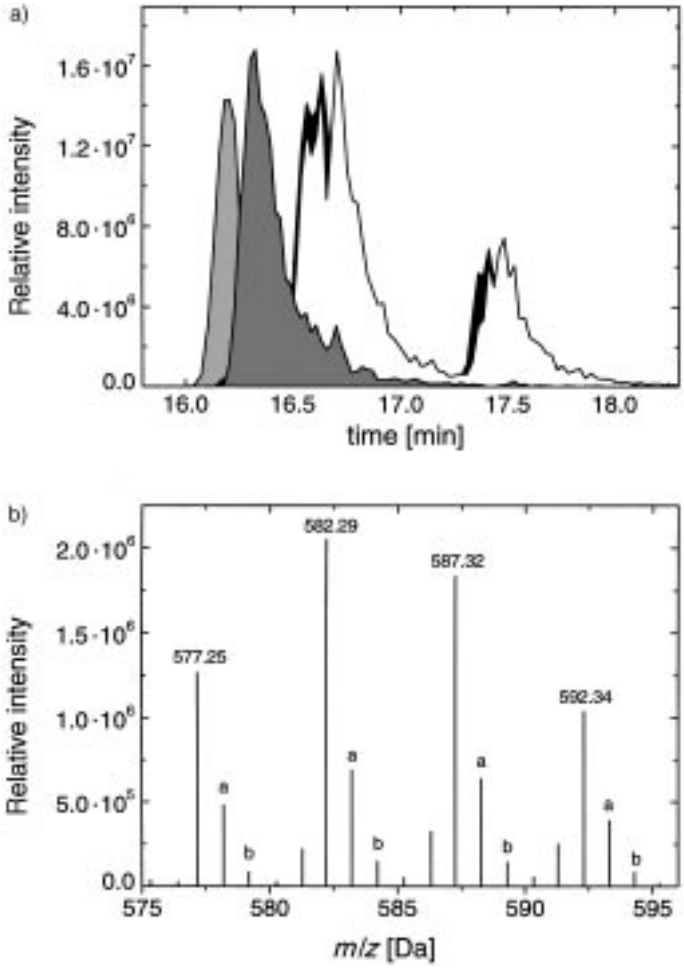

Fig. 1. a) *MS-SIM Chromatograms of the stereoisomer subgroups of oligo-Trp for* n = *3* (trimers) (the stereoisomer subgroups consist of [L-(D_5)Trp]$_3$ (L_3, light gray), [D-Trp]$_3$ (D_3, gray), [D-Trp][L-(D_5)Trp]$_2$ (DL_2, black), and [D-Trp]$_2$[L-(D_5)Trp] (D_2L, white). b) *The corresponding* m/z *values* (pos. ESI, $z = 1$: positively charged N-terminus of each peptide) *and their relative intensities* (L_3, 592.34 Da; D_3, 577.25 Da; DL_2, 587.32 Da, D_2L, 582.29 Da; the letters a and b represent the isotopes of each stereoisomer subgroup: a, $m + 1/z$ Da; b, $m + 2/z$ Da).

The final sample was analyzed by HPLC (*HP1100 LC*; buffer *A*, 0.1% CF_3COOH; buffer *B*, 99.9% MeCN, 0.1% CF_3COOH; starting with 10% *B*, gradient 2.0% *B*/min flow (1 ml/min; *C18* column (*Macherey-Nagel*, *ET 250/4 Nucleosil 100-5*)). The eluate was examined by a diode-array detector (*HP1100 DAD*). Peaks (label: *n*-mer in *Fig. 2*, see below) correspond to Trp oligomers, as verified up to the 10-mer by mass spectrometry (*HP1100 MSD*).

In a typical double-feeding experiment with *rac*-NCA-Trp, a freshly prepared soln. of 60 mM racemic NCA-D-Trp/NCA-L-(D_5)Trp in 0.4M 1*H*-imidazole/HCl (pH 6.80) was mixed in a 1:3 (*v*/*v*) ratio with either buffer only or with a 100-nm-extruded 100 mM POPC liposome suspension in 0.4M 1*H*-imidazole/HCl (pH 6.80) and incubated for at least 12 h at r.t. in a *Vortex-Genie-2* shaker (*Scientific Industries*, USA). The same volume of racemic NCA-D-Trp/NCA-L(D_5)Trp was added again in a second feeding and incubated for another 12 h at

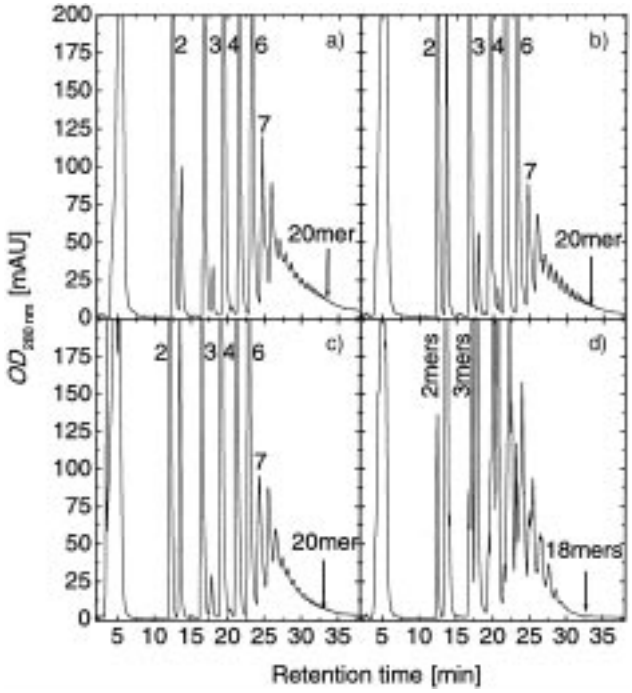

Fig. 2. *HPLC Plots recorded* (at 280 nm) *for the CDI-induced condensation in the presence of POPC liposomes with two feedings of* a) *NCA-*L*-Trp,* b) *NCA-*D*-Trp,* c) *NCA-*L*-(D_5)Trp, or* d) *NCA-*D*-Trp/NCA-*L*-(D_5)Trp racemate*

r.t. After this time, neither NCA-amino acid nor *N*-(1*H*-imidazol-1-yl-carbonyl)amino acid was detected, which indicated that the reaction was complete. The reaction mixtures with liposomes (25 – 50 µl) were injected immediately into the HPLC after removal from the shaker. This was done to ensure a homogeneous distribution of the oligomers formed. It was observed that allowing samples in multiple-feeding experiments with NCA-Trp to stand without shaking for a few hours led to precipitation in the vesicular systems.

Liquid Chromatography/Mass Spectrometry. The condensation products of the reaction samples were separated and analyzed by reversed-phase HPLC (*P4000*, *Finnigan Mat*) connected to a diode-array detector (*UV 6000 LP*, *Finnigan Mat*) and an ion-trap mass-spectrometry detector (*LCQ-Deca*, *Finnigan Mat*). Reversed-phase HPLC: achiral C_{18} column *ET 250/4 Nucleosil 100-5* (*Macherey-Nagel*) at r.t.; buffer solvents *A* (0.1% OF_3COOH) and *B* (99.9% MeCN, 0.1% CF_3COOH), starting with a 2 min isocratic flow of 10% *B* and then driving a typical gradient of 2.0% *B*/min up to 90% *B*; flow 1 ml/min. At high MeCN levels, lipids such as POPC precipitate on the column. Therefore, the column had to be washed with MeOH (*ca.* 100 ml) after every 10 lipid-containing injections to guarantee a good, reproducible separation on the column.

In the case of co-condensation of nondeuterated D- and deuterated L-Trp, the quantification of the different stereoisomer subgroups was achieved by ion-trap mass spectrometry. Typical settings were 350° capillary temp., 80 units sheath gas-flow rate, 20 units auxiliary gas-flow rate, 4.5 kV *I*-spray voltage, 39 V capillary voltage, and – 60 V tube-lense offset. Quantification was carried out by integrating the SIM-chromatogram peaks over time.

To show that oligo-Trp stereoisomers of the same length have similar sensitivities in electrospray-ionization, 60 mM of either NCA-D-Trp, NCA-L-Trp, NCA-L-(D_5)Trp, or racemic NCA-D-Trp/NCA-L-(D_5)Trp were mixed in a ratio of 1:3 (*v*/*v*) with buffer soln. and incubated for 12 h in a shaker at r.t. The samples were then injected into the HPLC and analyzed by LC-MS. Mean values with standard deviations for the *OD* areas at 280 nm divided by the SIM areas and the number of oligomer length (*n*) were calculated with the data of seven injections. For the di-, tri-, and tetramer of oligo-Trp, two subgroups of diastereoisomers could be discerned,

having UV signals sufficiently separated to be integrated independently. In the case of oligomers longer than the tetramer, the UV signals of the stereoisomers could not be related to different groups of diastereoisomers. For oligomers higher than the pentamer, stereoisomers of different length started to overlap in the UV chromatogram. For each Trp-oligomer length, up to the 5-mer, all the ratios obtained for stereoisomer subgroups are within a range of 25% around the mean value of single-enantiomer and racemate condensation. The mean values decrease with increasing length and approach a constant value of *ca.* 20 for oligomers higher than the tetramer. Standard deviations typically were lower for longer oligomers.

Results and Discussion. – We reported earlier that the NCA-Trp condensation in the presence of POPC (1-*O*-palmitoyl-2-*O*-oleoyl-*sn*-glycerol-3-*O*-phosphocholine) liposomes assists the formation of longer Trp oligomers (up to the 29-mer) compared to the aqueous reference system (never longer than the 8-mer) [18]. *Fig. 2* shows that the chiral POPC bilayer has no significant influence on the 1,1′-carbonylbis[^{1}*H*-imidazole](CDI)-induced condensation of either L-Trp, L-(D_5)-Trp, or D-Trp. It can, therefore, be concluded that the only significant effect of the POPC liposomes under the conditions used in the present work, is the formation of longer oligomers in higher yields [18]. As we will see below, the same is true for the *rac*-NCA-Trp condensation. However, when racemate condensation is compared to the condensation of either NCA-L-Trp or NCA-D-Trp alone, the yield of oligomers is lower in the former case, and a decrease in the length of the longest detectable oligomer is observed. This is shown for the liposome system in *Fig. 2*. Such observations have been described before for NCA-Glu condensation in aqueous systems [19].

In the case of racemic NCA-D-Trp/L-(D_5)Trp condensation (*Fig. 2, d*), it has been possible to determine the relative stereoisomer distribution up to the decamer. The stereoisomer distributions as a function of n follow a kinetic pattern that suggests a *Markov* process of second or higher order. Here, since the emphasis is on homochirality, we will consider only the results of $n = 10$ for the liposome system (*Fig. 2, d*) and $n = 7$ for the reference system with no liposomes being present during the condensation experiment. *Fig. 3* represents the experimentally determined stereoisomer distribution and compares them to the one expected for a statistical (*Bernoulli*) distribution (which assumes the same probability for NCA-L-Trp and NCA-D-Trp to be added into a growing chain).

It is apparent that the produced homochiral oligo-Trps (all-L or all-D) are overrepresented compared to the statistically expected distribution (8.3 times in *Fig. 3, a*; 40 times in *Fig. 3, b*). In particular, the comparison of *Fig. 3, a* with *Fig. 3, b*, shows that this effect is also present in bulk H_2O, *i.e.* without liposomes. The presence of liposomes – as suggested earlier [18] – has mostly the function of permitting the synthesis of longer Trp sequences. Being aware of the fact that with the exception of the homochiral sequences (far left and right columns in the histograms), all the stereoisomer groups consist of more than one diasteroisomer, it is clear that the two homochiral oligo-Trps are the most abundant of all stereoisomers in the subgroups shown in *Fig. 3*.

It can be concluded that the *rac*-NCA-Trp condensation is stereoselective. Note that the stereoisomer distributions obtained are more or less symmetrical, which agrees with the above-mentioned observation that the chiral POPC membrane under the conditions used has no significant influence on the condensation. The clear overrepresentation of homochiral sequences evidences a homochiral cooperative effect in the polycondensation: once that (say) a short ·· LL ·· sequence is formed, the

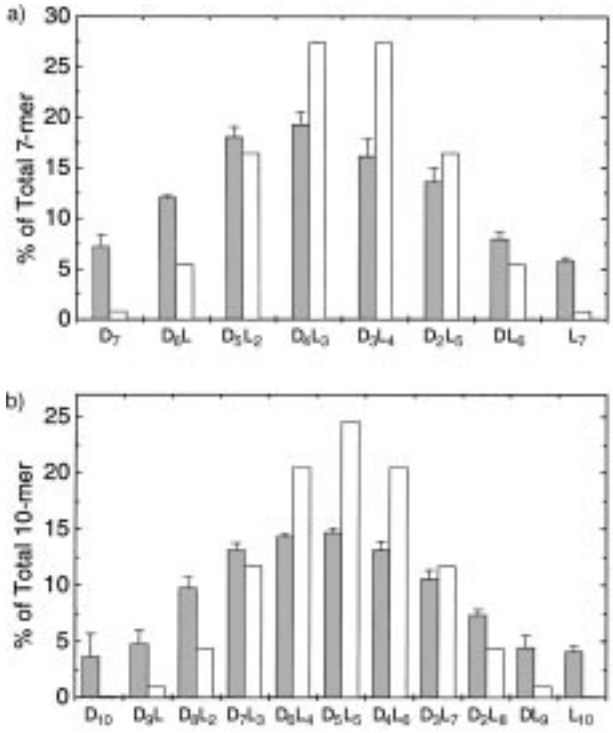

Fig. 3. *Relative abundances for* D_pL_q *-stereoisomer groups of the oligo-Trp* n*-mers* ($n = 7$ and 10, resp.), *obtained after two racemic NCA-Trp feedings* a) *in the absence* ($n = 7$) *and* b) *in the presence of POPC liposomes* ($n = 10$). The relative abundances of the D_pL_q stereoisomer subgroups (light gray columns) are mean values of three measurements. Standard deviations are given as error bars. The white columns correspond to the theoretical distribution, assuming a statistical oligomerization.

incorporation of another L is more favorable than the incorporation of a D unit. The steric factors that determine such a kinetic process are difficult to clarify. Several effects, such as secondary-structure formation, chain aggregation in presence or absence of liposomes, and/or binding at liposomes may play a role.

Aside from this question regarding the mechanism of preferred homochiral chain growth, it will be of great interest to see whether mixtures of different NCA-amino-acid racemates (same amount of D- and L-amino acids, but different amino acid composition) lead also to a preferential formation of homochiral oligopeptides consisting of more than one type of amino acid. Work in this direction is in progress in our laboratory.

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