Cyclic Polypeptides by Solvent-Induced Polymerizations of α-Amino Acid *N*-Carboxyanhydrides

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ABSTRACT: Solutions of DL-phenylalanine-NCA in eight different solvents were thermostated at 20 °C for 4 days (and in two cases at 60 °C) without addition of initiators. Spontaneous polymerizations were observed in dimethylformamide (DMF), in *N*-methylpyrrolidone (NMP), and in dimethyl sulfoxide (DMSO). In dimethyl sulfoxide also sarcosine-NCA, L-alanine-NCA, D,L-leucine-NCA, and DL-valine-NCA underwent spontaneous polymerizations. However, in *N*-methylpyrrolidone, only sarcosine-NCA and L-alanine-NCA polymerized. The products of all these solvent-induced polymerizations were identified by MALDI−TOF mass spectrometry as cyclic oligo- and polypeptides. This fact and the finding that only the most nucleophilic solvents induce spontaneous polymerizations indicate a zwitterionic polymerization mechanism.

Introduction

Synthetic polypeptides played an important role as models of natural polypeptides and proteins over the past 50 years. Most synthetic polypeptides were prepared by ring-opening polymerization of α-amino acid-NCAs.[1−7] This synthetic approach allows for the preparation of homopolypeptides (poly(amino acid)s), copolypeptides with nearly random sequences, or two-block and three-block copolymers. However, copolypeptides having alternating sequences of two or three different amino acids require a different synthetic strategy.

The initiators and the polymerization mechanisms of α-amino acid−NCAs were the object of numerous studies.[3−7] End groups, such as NH_2−R or HO_2C−R, were determined by acid−base titrations. Other end groups were identified and quantified by ^{1}H NMR spectroscopy. However, the formation (or absence) of cyclic oligo- and polypeptide was not studied because the analytical methods suited for such study were not available in previous decades. This situation has turned better with the appearance of MALDI−TOF mass spectroscopy. In recent studies[8,9] of primary amine-initiated polymerizations of several α-amino acid NCAs, it was found that the polypeptides isolated from polymerizations of DL-Phe-NCA or L-Phe-NCA in dimethylformamide (DMF) or *N*-methylpyrrolidone (NMP) contained small amounts of cyclic oligopeptides. This finding was surprising because it is clear that amino end groups cannot cleave the peptide bonds of the polymer backbone at room temperature, so that a "backbiting equilibration" of cycles and linear chains (which are known from polyesters and polyamides above 200 °C) does not exist. Therefore, it is obvious that a special polymerization mechanism must exist, favoring the formation of cyclopeptides at room temperature. In this context, the present work was undertaken to elucidate how the structure of the NCA and the nature of the solvent influence the cyclization tendency and to formulate a pertinent polymerization mechanism.

Experimental Section

Materials. Methylchloroformate, phosphorus tribromide, sarcosine (Sar), L-alanine (L-Ala), DL-phenylalanine (DL-Phe), DL-leucine (DL-Leu), and DL-valine (DL-Val) were purchased from Aldrich Co. (Milwaukee, WI) and used as received. Ultradry DMSO was also purchased from Aldrich Co. Sulfolane (Aldrich Co.) and tetramethylurea (TMU) (Aldrich Co.) were azeotropically dried with toluene and distilled in vacuo. Dichloromethane (Bayer AG, Germany) was distilled over phosphorus pentoxide. Dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP) were distilled over phosphorus pentoxide in vacuo. Dioxane (Aldrich Co.) was refluxed and distilled over sodium.

Syntheses of NCAs. Five NCAs were used in this work, namely Sar-NCA, L-Ala-NCA, DL-Phe-NCA, DL-Leu-NCA, and DL-Val-NCA. All these NCAs were described in numerous publications.[5,6,8,9] In this work all five NCAs were prepared in the same way, namely, by cyclization of *N*-methoxycarbonyl-amino acid trimethylsilyl esters with PBr_3. This synthetic method was also described in several previous publications.[8−10] The absence of bromide ions was tested in such a way that the NCAs were refluxed in 2 N nitric acid for 5 min followed by addition of two drops of a $AqNO_3$ solution in 2 N nitric acid.

Polymerizations. *DL-Phe-NCA (Table 1).* DL-Phe-NCA (10 mmol) was dissolved in dry NMP (10 mL) by shaking the mixture in a 50 mL Erlenmeyer flask having silanized glass walls. The reaction vessel was closed with either a freshly prepared calcium chloride drying tube or by a glass stopper and a highly elastic steel spring (allowing for the escape of CO_2 pressure). After thermostatization for 4 days at 20 °C (or 60 °C) the reaction mixture was poured into diethyl ether (250 mL), and the precipitated polypeptide was isolated by filtration.

Analogous experiments were conducted in other solvents (see Table 1).

Various NCAs (Table 2*).* Sar-NCA (10 mmol) was dissolved in dry DMSO (20 mL) and polymerized at 20 °C as described above. Analogous experiments were conducted in NMP and with L-Ala-NCA, DL-Leu-NCA, and DL-Val-NCA. All reaction mixtures were prepared under an atmosphere of dry argon.

Co-Initiation with Water. DL-Phe-NCA (10 mmol) was dissolved in dry NMP (10 mL), and 5 min after the addition of the NCA, a 2 M solution of water in NMP (0.1 mL) was injected. The reaction vessel was closed with a glass stopper and steel spring. After 4 days at 20 °C, the reaction mixture was poured into diethyl ether and the precipitated polypeptide isolated by filtration. Yield 99%; $\eta_{inh} = 0.15$ dL/g in dichloroacetic acid. According to the MALDI−TOF mass spectrum, the cyclopeptides were the main reaction products.

Acetylation of Polypeptides. Benzylamine-initiated poly(L-Ala), poly(DL-Leu), or poly(DL-Phe) (0.5 g, average DP = 40)

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Table 1. Solvent-Induced Polymerizations of DL-Phe-NCA in Various Solvents

expt no.	solvent	DC[c]	μ[d] (D)	temp (°C)	yield (%)	η_{inh}[e] (dL/g)
1[a]	dichloromethane	8.9	1.5	20	0	
2[a]	dioxane	2.2	0.4	20	0	
3[b]	dioxane	2.2	0.4	20	0	
4[b]	dioxane	2.2	0.4	60	0	
5[a]	sulfolane	44.0	4.8	20	0	
6[a]	nitrobenzene	34.8	4.0	20	0	
7[a]	DMF	36.7	3.8	20	14	0.11
8[a]	DMF	36.7	3.8	60	23	0.13
9[a]	NMP	33.0	4.1	20	99	0.16
10[a]	TMU	23.1	3.4	20	0	
11[a]	TMU	23.1	3.4	60	37	0.13
12[b]	TMU	23.1	3.4	60	0	
13[a]	DMSO	48.9	3.9	20	95	0.20

[a] The reaction vessel was closed with a calcium chloride drying tube. [b] The reaction vessel was closed with a glass stopper. [c] Dielectricity constant at 25 °C. [d] Dipole moment. [e] Measured at 20 °C with $c = 2$ g/L in dichloroacetic acid.

Table 2. Solvent-Induced Polymerizations of Various α-Amino Acid NCAs (Reaction Time 4 days)

expt[a] no.	solvent	temp (°C)	NCA of	yield (%)	η_{inh}[c] (dL/g)
1	DMSO	20	sarcosine	99	0.17
2	DMSO	20	L-alanine	68	0.38
3[b]	DMSO	60	DL-leucine	16	0.22
4	DMSO	60	DL-leucine	13	0.17
5	DMSO	60	DL-valine	40	0.14
6	NMP	20	sarcosine	92	0.23
7	NMP	20	L-alanine	23	0.22
8	NMP	60	DL-leucine	0	
9	NMP	60	DL-valine	0	

[a] The reaction vessel was closed with a glass stopper. [b] The reaction vessel was closed with a drying tube. [c] Measured at 20 °C with $c = 2$ g/L in dichloroacetic acid.

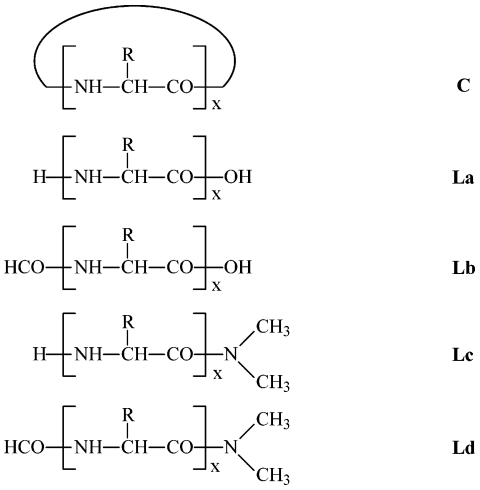
Table 3. Calculated Masses (Da) of Cyclic Oligo- and Polypeptides

DP of cycles	sarcosine and alanine	phenylalanine	leucine	valine
10	710.79	1471.8	1131.6	991.3
15	1066.19	2207.7	1697.4	1486.95
20	1421.58	2943.6	2263.2	1982.6
25	1776.98	3679.5	2829.0	2478.25
30	2132.37	4415.4	3394.8	2973.9

were suspended in dry DMF (9 mL), and acetic anhydride (1 mL) was added. This mixture was stirred at 60 °C for 24 h and poured into cold water. The preciptated polypeptides were isolated by filtration and dried at 60 °C in vacuo over P_4O_{10}. Analogous acetylation experiments were performed with cyclic poly(L-Ala), no. 2, Table 2, poly(DL-Phe), no. 9, Table 1, and poly(DL-Leu), no. 3, Table 2.

Measurements. The inherent viscosities were measured in dichloroacetic acid with an automated Ubbelohde viscometer thermostated at 20 °C. The 100.4 MHz ^{13}C NMR spectra were recorded with a Bruker Avance 400 spectrometer in 10 mm o.d. sample tubes using deuterated trifluoroacetic acid as solvent and internal TMS as shift reference. The MALDI−TOF mass spectra (MS) were recorded on a Bruker Biflex III equipped with a nitrogen laser ($\lambda = 337$ nm). All MS were equipped in the reflection mode with an acceleration voltage of 20 kV. The irradiation targets were prepared from solutions of polypeptides in trifluoroacetic acid with dithranol as matrix and K^+-trifluoroacetate as dopant. In addition to K^+-doped masses, peaks of H^+- and Na^--doped polypeptides were also detectable in the MS. The masses of H^+- and K^--doped cyclopeptides are listed in Table 3. The ^{13}C NMR CP/MAS measurement of poly(L-Ala) was performed on a Bruker Avance 500 NMR spectrometer at a spinning rate of 8000 Hz with a contact time of 1 ms and a repetition time of 3 s.

Scheme 1. Structures of Potential Reaction Products



Results and Discussion

Polymerizations in Various Solvents. Since the formation of cyclopeptides was at first observed for polymerization of DL-Phe-NCA,[9] this monomer was used for the first series of experiments. Eight different solvents were compared at 20 °C (see Table 1). Although DL-Phe-NCA was not completely soluble in dichloromethane, this solvent was included in this work because it was widely used for other NCAs as reported in the literature[4] and in our previous publications.[8,9] The first series of experiments was conducted in such a way that the reaction vessels were closed with freshly prepared calcium chloride drying tubes, allowing for the escape of the liberated carbon dioxide (a standard procedure in the field of NCA polymerization). With this procedure polymerization was observed in dioxane at 20 °C (no. 2, Table 1). The MALDI−TOF MS of the isolated product proved that all poly(DL-Phe) chains were initiated by water (**La** chains in Scheme 1). Therefore, the experiment was repeated in such a way that the reaction vessel was closed with a glass stopper and steel spring, allowing for the escape of CO_2 when pressure was built up. No polymerization occurred under these conditions, even when the temperature was raised to 60 °C (nos. 3 and 4, Table 1). Analogous results were obtained from experiments conducted with TMU. The product obtained at 60 °C contained a significant fraction (possibly more than 50%) of **La** chains in addition to cyclopeptides, when the calcium chloride drying tube was used. Yet, when this experiment was repeated so that the reaction vessel was closed with a glass stopper, no polymerization occurred. These results suggest that water vapor penetrating the drying tube was responsible for the initiation of the **La** chains.

The so-called spontaneous or solvent-induced polymerizations were characterized by the formation of cyclic oligo- and polypeptides as evidenced by MALDI−TOF mass spectrometry. The most rapid polymerization was observed in NMP, and the isolated poly(DL-Phe) exclusively consisted of cycles (labeled **C** in Scheme 1) as evidenced by the MS presented in Figure 1. A somewhat slower polymerization occurred in DMSO, and the reaction product contained a trace of **La** chains in addition to the cyclopeptides. The spontaneous polymerization in DMF was significantly slower, and

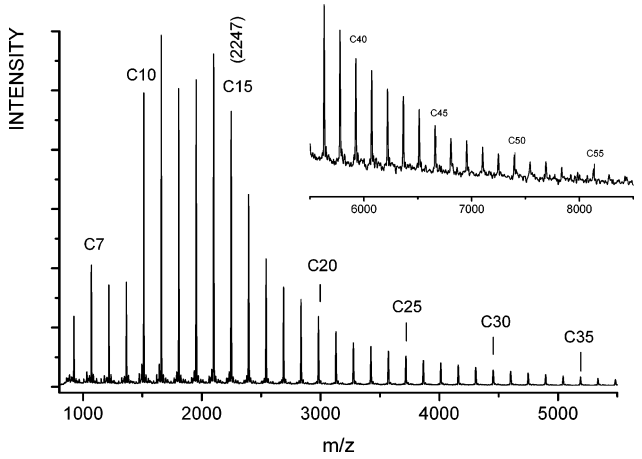


Figure 1. MALDI−TOF mass spectrum of poly(DL-Phe) obtained in NMP at 20 °C (no. 9, Table 1).

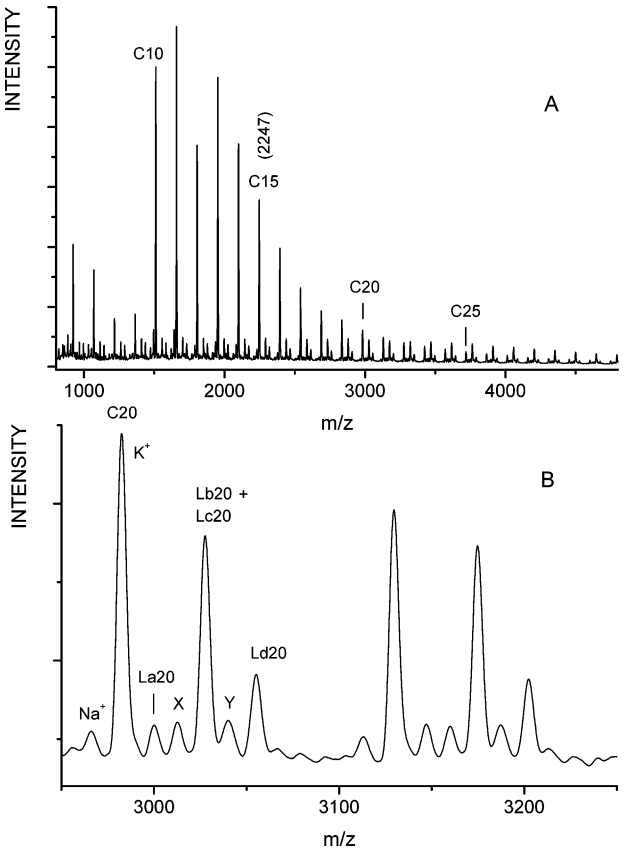


Figure 2. MALDI−TOF mass spectrum of poly(DL-Phe) obtained in DMF at 60 °C (no. 8, Table 1).

initiation by water (invading the reaction vessel via the drying tube) successfully competed with the spontaneous polymerization. As illustrated by the MS of Figure 2, **La**, **Lb** (and/or **Lc**), and **Ld** chains were identified in addition to the prevailing cyclopeptides. As already reported previously[8,9] for primary amine-initiated polymerizations of several NCAs, the amino end groups of growing peptide chains can react with DMF at 60 °C, so that formamide end groups are formed (**Lb** or **Ld** chains, eq 1). The liberated dimethylamine, which is far more nucleophilic than water, acts in turn as initiator (eq 2). Therefore, initiation with a trace amount of moisture initiates a sequence of "side reactions" in DMF at 60 °C. A comparison of the dielectric constants (Table 1) demonstrates that the polarity of the solvents does not play a key role for their reactivity as catalyst. Obviously, their nucleophilicity and donicity (i.e., capacity as electron donor and stabilizer of a positive charge) are the decisive properties. The rates of the spontaneous polymerizations of DL-Phe-NCA listed in Table 1 were so different that even without detailed measurements the reactivity as catalyst can be grouped in the following order: NMP > DMSO > DMF > TMU.

The previously reported polymerizations of DL-Phe NCA in DMF or NMP initiated with primary amines gave also the following information. When the NCA was dissolved in the neat solvent for a few minutes and the primary aliphatic amine added after complete dissolution, the reaction product contained a small amount of cyclic poly(DL-Phe). A longer delay prior to the addition of the amine resulted in a higher content of cyclopeptides. When aniline, which is less nucleophilic than an aliphatic amine, was used as initiator, the content of cycles was higher than that obtained with *n*-hexylamine under identical conditions, but the amide-terminated **Le** chains (Scheme 1) were still the main products. However, when water, an even poorer nucleophile than aniline, was added, the cyclopeptides became the main products and the **La** chains were the minority (see Experimental Section). These observations suggest that the formation of zwitterions, i.e., the initiation, is the rate-determining step, and both propagation and cyclization are relatively rapid. The inverse kinetic pattern would have the consequence that all slowly growing zwitterionic chains will react with the amines so that all polypeptides have amide end groups (**Le** chains). The assumption of a relatively slow initiation is in perfect agreement with the well-known catalytic effect of pyridine and DMF in acylation reactions of dicarboxylic anhydrides, acid chlorides, and thionyl chloride. This catalytic effect is ascribed to the charge separation. As soon as a pair of ions including acylium ions is formed, the following acylation reaction is quite rapid.

Polymerizations of Various NCAs. The polymerizations of DL-Phe-NCA have shown that NMP and DMSO are the most active catalysts of solvent-induced polymerizations. Therefore, these solvents were used for all further polymerizations (Table 2). Whereas in the case of DL-Phe-NCA NMP proved to be the most active catalyst, the experiments with Sar-NCA, L-Ala-NCA, DL-Leu-NCA, and DL-Val-NCA evidenced the opposite trend. All four monomers polymerized in DMSO, whereas DL-Leu-NCA and DL-Val-NCA did not polymerize in NMP, even when the temperature was raised to 60 °C. Eight out of nine experiments were conducted in reaction vessels closed by a glass stopper, and the polypeptides isolated from such experiments exclusively consisted of cyclic polypeptides. The MS of poly(Sar) and poly(L-Ala) presented in Figures 3 and 4 exemplarily illustrate this result. Since DL-Leu-NCA proved to be the least reactive monomer in this study, two procedures were compared in DMSO at 60 °C (nos. 3 and 4, Table 2). The reaction vessel was closed either with a glass stopper or with a drying tube (no. 3). In the latter case the isolated poly(DL-Leu) contained **La** chains resulting from an initiation by water, as illustrated in Figure 4. Obviously, the slow initiation of the solvent-induced polymerization allowed for a successful competition of moisture to penetrate the drying tube and to initiate a polymerization via amino end groups. In contrast, the product isolated from the reaction vessel closed with a glass stopper exclusively consisted of cycles.

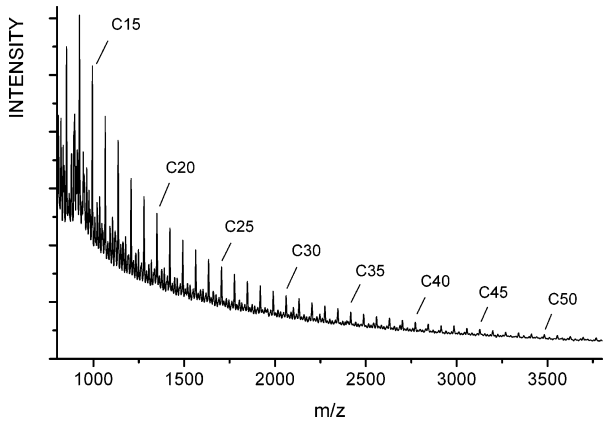


Figure 3. MALDI−TOF mass spectrum of poly(Sar) obtained in NMP at 20 °C. The strong peaks result from H^+ doping and weaker peaks from Na^+ and K^+ doping (no. 5, Table 2).

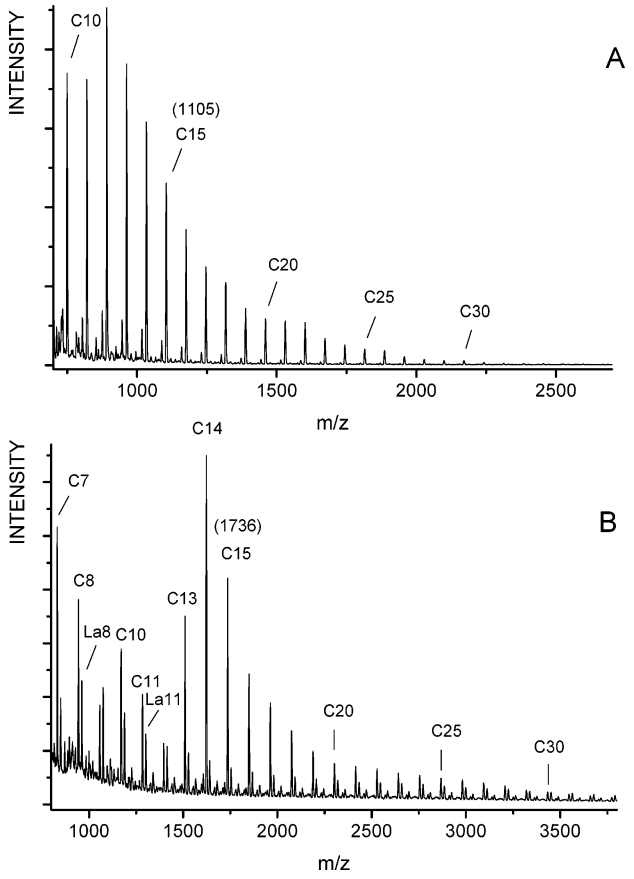


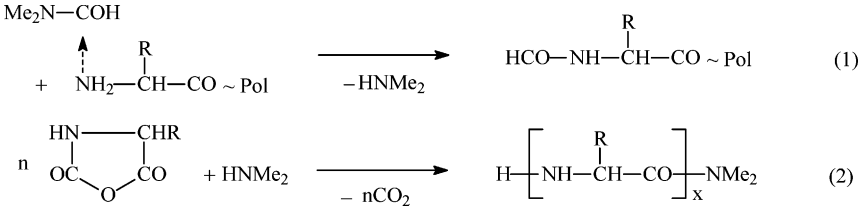
Figure 4. MALDI−TOF mass spectra of (A) poly(L-Ala) prepared in DMSO at 20 °C (no. 2, Table 2) and (B) poly(DL-Leu) prepared in DMSO at 60 °C (no. 3, Table 2). The weaker peaks result from H^+ and Na^+ doping.

The results obtained from all the experiments listed in Tables 1 and 2 suggest the following mechanistic interpretation.The solvent reacts certainly as nucleophile and electron donor and generates a zwitterionic species as reactive intermediate (eq 3). This zwitterion can in principle maintain a zwitterionic polymerization (Scheme 2), or it can possibly initiate the nonionic polymerization mechanism outlined in Scheme 3. However, the mechanism outlined in Scheme 3 is incompatible with the structure of Sar-NCA, and thus, the solvent-induced polymerization of Sar-NCA proves the existence of a zwitterionic polymerization mechanism for Sar NCA. For other NCAs at least the initiation step involves the formation of zwitterions, but it is not clear to what extent the chain growth mechanism follows the zwitterionic course of Scheme 3 or the "*N*-acyl-NCA mechanism" of Scheme 4. The zwitterionic polymerization can, in principle, follow two different kinetic courses. The chain growth consists either of ring-opening polymerization involving a chain growth kinetic (eqs 4 and 8) or of polycondensation steps involving a step growth kinetic (eqs 5 and 9). Both kinetic courses produce identical chains and end groups, but the molecular weight distributions should be different. Relatively narrow distributions with polydispersities below 2.0 are typical for most chain growth polymerizations.[11] In so-called living polymerizations the polydispersities may be as low as 1.05−1.10.[11] Also characteristic for polycondensates is a frequency distribution with an absolute maximum at the linear or cyclic dimer and a steady exponential decrease of the frequencies for higher degrees of polymerizations (DPs).[12,13] The frequency distribution found in this work have maxima between DPs of 10 and 15 and are in better agreement with a chain growth kinetic than with a step growth kinetic. Because of the partial precipitation of oligomers in the β-sheet conformation (secondary structure), a narrow frequency distribution with PDs below 1.2 cannot be expected anyway. Unfortunately, the insolubility of the polypeptides in common solvents such as THF or chloroform prevented SEC measurements.

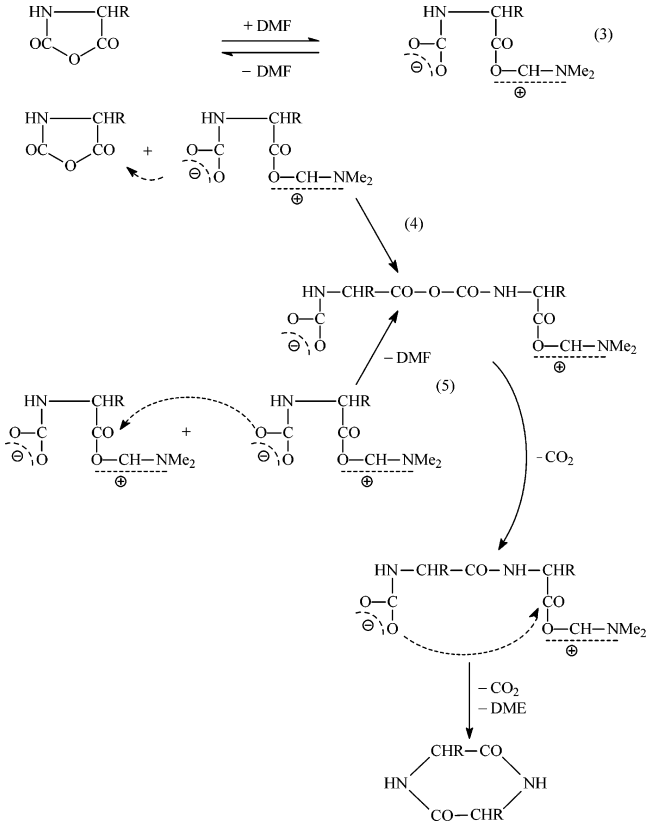
Finally, it should be mentioned that, in principle, a further version of the zwitterionic polymerization mechanism may exist, which is outlined in eq 11. In the case of N-unsubstituted polypeptides, the cationic chain end may undergo a "backbiting reaction" with the formation of a dioxopiperazine ring. In combination with the decarboxylation of the carbamate chain end, **Le** chains will be formed which can continue the chain growth by reaction of the amino end group with NCAs according to the normal "amine mechanism" also formulated in eq 8. These **Le** chains are isomers of the cyclopeptide and thus cannot be identified by MALDI−TOF mass spectrometry. To prove the presence or absence of **Le** chains, model reactions with previously synthesized[8,9,14] benzylamine-initiated linear chains having amino end groups were performed. It was found that heating of poly(L-Ala),[8] poly(DL-Leu),[14] and poly(DL-Phe)[9] with acetic anhydride in DMF at 60 °C resulted in a quantitative acetylation of the amino end group (eq 12). This acetylation was identified by ^{1}H NMR spectroscopy and by MALDI−TOF mass spectrometry. The MS also proved that the frequency distribution of the starting materials did not change. When the cyclopeptides listed in Tables 1 and 2 were subjected to such an acetylation procedure, the ^{1}H NMR and MALDI−TOF mass spectra did not change. In other words, the cyclopeptides possess, indeed, the expected structure labeled **C** in Scheme 1, and **Le** chains were absent.

Secondary Structure and Ring Size. It is well-known[15] from numerous syntheses of monodisperse cyclic oligopeptides having degrees of polymerization (DPs) ≤ 10 that the cyclization tendency depends very much on the chain lengths due to conformational constraints. This aspect is also evident from the MALDI−TOF MS of poly(DL-Phe) (Figure 2A) and poly(DL-Leu) (Figure 2B), which indicate that the formation of C10 and C11 was considerably more favored than that of C7−C9. However, for DPs ≥ 14 no preferential formation of a certain ring size was observed, regardless of

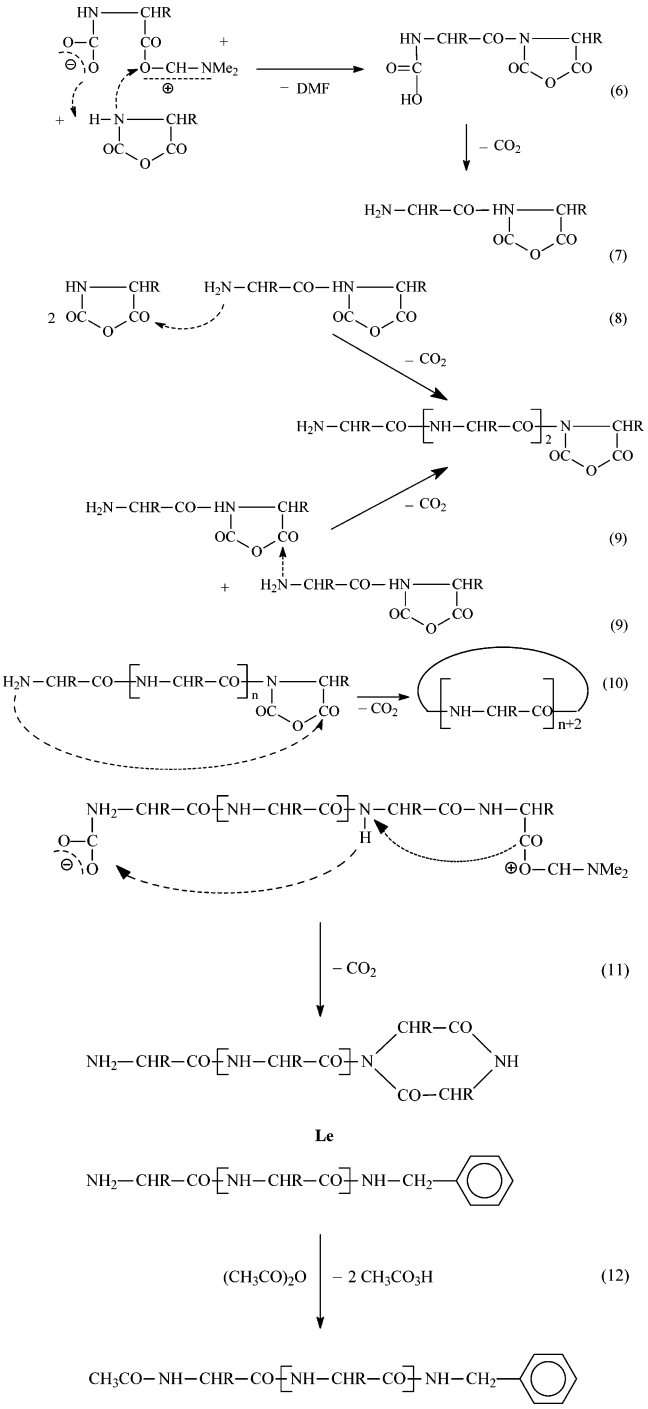
Scheme 2. Potential Side Reactions of Growing Peptide Chains with DMF



Scheme 3. Exemplary Illustration of the DMF-Initiated Zwitterionic Polymerization and Cyclization



Scheme 4. Zwitterion-Initiated Chain Growth and Cyclization via Amino End Groups

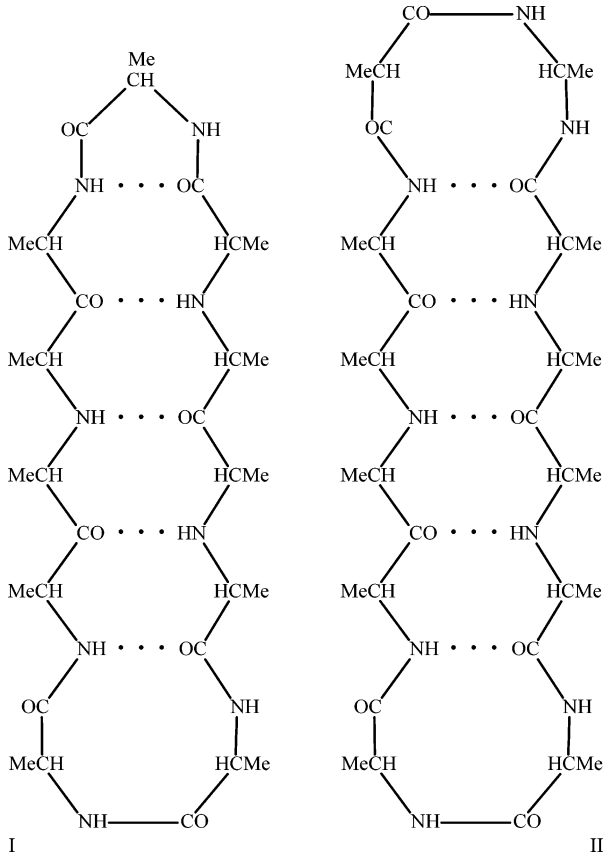


the polypeptide. (The same trend is true for cyclic poly(γ-benzyl-L-glutamate)s as will be reported in a future publication.) This finding is best explained by rapid chain growth and cyclization of dissolved polypeptides having random chain conformations. Such a situation is obvious for poly(Sar) because the absence of NH groups enables the simultaneous presence of cis- and trans-amide groups and prevents fixation and precipitation of certain secondary structures.[16,17]

In the case of poly(Phe) and poly(Leu), secondary structure and solubility depend very much on the stereosequence. Poly(L-Phe) and poly(L-Leu) are the poly(α-amino acid)s with the lowest solubility in inert organic solvents. They are even insoluble in neat trifluoroacetic acid. However, a random sequence of D- and L-units considerably destabilizes β-sheet and α-helix structures and greatly enhances the solubility in polar organic solvents. ^{13}C NMR spectra of the poly(DL-Phe) and poly(DL-Leu) samples prepared in this work proved that both polypeptides possessed nearly random stereosequences.[14,18] Hence, it is obvious that rapid cyclization resulted from solvated flexible polypeptide chain prior to precipitation.

A particularly interesting result is the almost quantitative formation of cyclic poly(L-alanine)s up to DPs around 30. From oligo(L-Ala) it is known that at DPs $\leq 10 \pm 1$ (depending on the solvent) the β-sheet structure is preferred, whereas at higher DPs the α-helix is more stable in the solid state. Therefore, a poly(L-Ala) prepared by primary amine-initiated polymerization with

Scheme 5. Most Likely Conformations of Cyclic Poly(L-alanine)s in the Solid State



a monomer/initiator ratio of 10 contains 50% β-sheet structure due to oligomers having a DP > 10 and 50% α-helix structure due to chains with a DP > 10.[19,20] In contrast to this scenario, a ^{13}C NMR CP/MAS spectrum of the cyclic poly(L-Ala) of this work revealed that at least 90% of this sample existed in the β-sheet structure (the usefulness of ^{13}C NMR CP/MAS spectroscopy for a qualitative and quantitative evaluation of solid secondary structures was reported previously).[20,21] In other words, all the cyclic poly(L-Ala)s should have the secondary structure outlined in Scheme 5 for odd- (I) and even-membered (II) cyclopeptides. The β- and γ-loops presented in Scheme 5 are known from monodisperse cyclic oligopeptides.[15] The result is again best explained by a kinetic scheme consisting of a slow initiation and a rapid propagation plus cyclization, so that the cycles of long poly(L-alanine)s (DP > 10) are formed before α-helices begin to precipitate. Both the kinetics and the thermodynamics of the precipitation process depend on the concentration, and thus, a low actual concentration of growing chains favors cyclization before precipitation sets in. A low actual concentration of growing chains is the characteristic consequence of a slow initiation combined with rapid propagation.

Conclusion

The results of this work demonstrate that solvent-induced "spontaneous" polymerizations of α-amino acid NCAs are not limited to DL-Phe-NCA. The solvents with the highest nucleophilicity and donicity proved to be the best catalysts. This finding and the "spontaneous" polymerization of Sar-NCA indicate that at least the initiation process involves the formation of zwitterions. The frequency distributions reflected in the MALDI−TOF mass spectra suggest that the majority of the propagation steps result from anionic ring-opening polymerization (eq 3). Particularly characteristic for the solvent-induced zwitterionic polymerization of NCAs is the formation of cyclic oligo- and polypeptides, which may reach 100% yield under water-free conditions. In the case of cyclic poly(L-alanine)s it was found that 90% or more exist in the β-sheet structure, although the majority of the cycles has a DP above 10. This finding and other observations suggest that a slow initiation process is followed by rapid propagation and cyclization, so that cyclic poly(L-alanine)s are formed prior to a stabilization and precipitation of α-helices.

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