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CHIRALITY

Dual Role of Hydrophobic Racemic Thioesters of α-Amino Acids in the Generation of Isotactic Peptides and Co-peptides in Water; Implications for the Origin of Homochirality

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Abstract Thioesters of α-amino acids are considered as plausible monomers for the generation of the primeval peptides. *DL*-Leucine-thioethyl esters (LeuSEt), where the *L*-enantiomer was tagged with deuterium atoms, undergo polycondensation in water or in bicarbonate or imidazole buffer solutions to yield mainly heterochiral (atactic) peptides and diketopiperazine, as analyzed by MALDI-TOF and ESI mass-spectrometry. In variance, when polymerization of *DL*(d_{10})-Leu, first activated with *N,N'*-carbonyldiimidazole, then initiated with ethanethiol or with *DL*(d_3)-LeuSEt yielded a library of peptides up to 30 detectable residues where those of homochiral sequence (isotactic) are the dominant diastereoisomers. At these conditions, racemic β-sheets are formed and operate as stereoselective templates in the process of chain-elongation. Isotopic *L*:*L*(d_{10})-Leu co-peptides were obtained in the polymerization of *L*(d_{10})-Leu with *L*-LeuSEt. By contrast, mixtures of oligo-*D*-Leu and oligo-*L*(d_{10})-Leu were obtained in the polymerization of mixtures of *D*-LeuSEt with activated *L*(d_{10})-Leu. Isotactic co-peptides containing Leu and Val residues were formed in the polymerization of mixtures of activated *DL*(d_8)-Val with *DL*(d_3)-LeuSEt in water, implying that the racemic β-sheets exert regio-enantio-selection but not chemo-selection. A reaction pathway is suggested, where LeuSEt operates both as initiator of the reaction as well as a multimer.

Keywords Homochiral oligopeptides · Desymmetrization of racemic isotactic oligopeptides · Racemic α-amino acid-thioesters · Origin of homochirality of peptides · MALDI-TOF MS

Dedicated to Prof. Dr. Helmut Ringsdorf on the occasion of his 80th anniversary.

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Introduction

The mist of the emergence, in the achiral prebiotic world, of proteins and nucleic acids composed from amino acids and sugars residues of a single handedness, remains still unsolved. One theory advocates that homochirality emerged only after the appearance of the first living system (Bada and Miller 1987), whereas another hypothesis maintains that it is imperative to generate first the homochiral biopolymers prior to the beginning of life (Avetisov and Goldanski 1996; Ehler and Orgel 1976; Goldanskii et al. 1986; Joyce et al. 1984). The feasibility of the last scenario has received experimental support by crystallization, polymerization, application of vortex and autocatalytic reactions. Since the pioneering report by Havinga (1954) on "mirror-symmetry breaking" by crystallization, this process has been proven for more than two dozen of compounds and it has been extended to an "absolute asymmetric synthesis" for achiral molecules packing in enantiomorphous (Addadi et al. 1982; Green et al. 1979; Penzien and Schmidt 1969; Toda 2005) or in centrosymmetric crystals (Weissbuch et al. 2005) as well by grinding conglomerates (Noorduin et al. 2008; Viedma 2005). Green et al. (1995) have demonstrated that polyalkyl isocyanates such as polyhexyl isocyanate form left- and right-handed helices, although the monomers themselves are achiral. Ribo et al. (2001) reported that achiral porphirin derivatives were successfully converted into homochiral *J*-aggregates by applying strong vortex. Furthermore, "mirror-symmetry" breaking was achieved in the elegant autocatalytic processes, by Soai and Kawasaki (2008) and, more recently, in Mannich and aldol-condensation reactions by Mauksch and Tsogoeva (2008; Mauksch et al. 2007).

In the present study, we addressed the feasibility to convert thioesters of racemic α-amino acids into polymers of homochiral sequence.

Polymerization of two-component systems, such as racemates, at ideal conditions, yields atactic polymers where the chains are composed from residues of both handedness. Experimentally, only small excess departure from a binomial distribution has been reported (Blocher et al. 2001; Hitz et al. 2001). Therefore, the generation of polymers of homochiral sequence (isotactic) from racemates requires the elaboration of synthetic non-linear polymerization pathways (Weissbuch et al. 2005).

Polycondensation of thioesters of racemic α-amino acids to yield isotactic peptides and co-peptides should have been of relevance to the prebiotic world, as proposed by de Duve (2002) and reviewed by Pascal et al. (2005) Amino acid thioesters (AA-SEt) might have served as the first high-energy molecules in the 'thioester world" as an intermediate stage between the abiotic synthesis of peptides and the "RNA world". This hypothesis is based on clues from present-day biology such as found in the synthesis of non-ribosomal peptides and coenzyme-A-thioester-linked reactions that involve thioesters as intermediates (Lipmann 1971). Indeed, sulfur derivatives, such as gaseous H_2S, COS or minerals such as FeS and NiS, are found near volcanic vents and laboratory experiments have demonstrated that they serve as plausible intermediates for the formation of organo-sulfur compounds such as thiols, thioacids and AA-SEt (Huber and Wächtershäuser 1997, 2003; Leman et al. 2004). Furthermore Weber (1998) reported a synthesis of AA-SEt starting from glycolaldehyde and formaldehyde, which are ingredients in the formose reaction. Enantiopure α-amino-thioesters as monomers for the peptides formation was reported by Wieland et al. (1955; Wieland and Bartmann 1956) when performing the polymerization of alanine thioester in a buffer of $NaHCO_3$, presumably via an NCA intermediate. Brack (1987; Bertrand et al. 2001) supported further that the thioesters react with CO_2 or bicarbonate to yield NCA as intermediates for the formation of peptides. More recently, Weber (2005) reported the formation of citrulline peptide thioesters, presumably via the

formation of AA-SEt as intermediates. Oligomerization reactions of *L*-α-thioglutamic acid (Maurel and Orgel 2000; Zepik et al. 2007) and *DL*-stearoyl-lysine thioethyl ester (Zepik et al. 2002) or *DL*-stearoyl-thiolysine (Weissbuch et al. 2002) have been also reported. Huang et al. (2008) reported a biomimetic catalysis of diketopiperazine via condensation of thioesters.

In our approach to the problem of chiro-biogenesis of peptides from racemates, we adopted principles from the field of crystallization, where molecules from isotropic media are converted into periodic crystals via a two-step process of crystal nucleation and crystal growth. Recently we reported the generation of isotactic peptides and co-peptides starting from mixtures of hydrophobic, activated racemic α-amino acids, via the formation of β-sheets intermediate architectures (Illos et al. 2008; Rubinstein et al. 2007, 2008; Weissbuch et al. 2009).

Here we report the generation of isotactic peptides and co-peptides from racemic α-amino acid-thioesters (AA-SEt) via racemic β-sheet templates formed in-situ from activated hydrophobic racemic α-amino acids.

Materials and Methods

Deuterated *L*-α-amino acids, AA, leucine *L*(d_{10})-Leu and *L*(d_3)-Leu, valine *L*(d_8)-Val, were purchased from Cambridge Isotope Laboratories USA, the corresponding *D*-α-AA from Sigma-Aldrich and the enantiopure *D*-Boc-AA-OH from Bachem.

Synthesis of Amino Acid Thioesters (H-AA-SEt)

Boc-AA-SEt All amino acid thioesters were synthesized by reacting *N*-*t*-butyloxycarbonyl amino acids (Boc-AA-OH) (4.98 g, 20 mmol) with ethanethiol (5.1 mL, 70 mmol) in the presence of a condensing agent (diphenyl phosphorazidate, DPPA) (6.63 g, 5.17 mL, 24 mmol) and triethylamine (2.4 g, 3.30 mL, 24 mmol), according to a published procedure (Yokoyama et al. 1977). The evaporated residue was purified by recrystallization with methylene chloride/hexane, the residue was dissolved in 30–50 mL methylene chloride and precipitated with hexane 400 mL after leaving the solution to stand overnight in the deep freeze to afford white needles of Boc-AA-SEt (3.85 g, 70%).

H-AA-SEt.HCl The deprotection of the *tert*-butyloxycarbonyl (Boc) group was carried out using HCl (4 M)/dioxane at 0°C and the mixture was then stirred for 30 min at room temperature (Han et al. 2001).

H-AA-SEt H-AA-SEt.HCl (0.115 g, 0.54 mmol) was added to a NH_3 (g)/methylene chloride solution (20 mL) with stirring at room temperature for 20 min. The precipitated ammonium chloride salt was removed by vacuum filtration with celite and the filtrate was evaporated under reduced pressure to yield a colorless oil (0.043 g, 45.4%).

Polymerization reaction Aqueous solutions of racemic AA, *DL*(d_{10})-Leu, 40 mM, *DL*(d_8)-Val, 40 mM, in which the *L*-enantiomer was enantioselectively labeled with deuterium, were reacted, in an ice-cooled bath with solid *N*,*N*′-carbonyldiimidazole, (1.25 or 2.5 mol/mol AA) for two minutes. Then, 0.25–1 mol/mol ethanethiol or AA-SEt was added to initiate the polymerization reaction that proceeded at room temperature with vigorous stirring. After 24 hours, the reaction mixture was freeze-dried under vacuum after centrifugation, decantation

and several washings with water of the precipitate. The diastereoisomeric distribution of the oligopeptides was determined by matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis. FTIR spectra of the solid products were measured in KBr pellets and the X-ray powder diffraction on the powders deposited on a horizontal holder.

MALDI-TOF MS, sample and matrix preparation Representative samples were measured in the analytical laboratories at the Weizmann Institute as well as in Paris VI. The MALDI-TOF MS analysis was performed on a Bruker Reflex II™ MALDI-TOF mass spectrometer (Bruker, Bremen, Germany), equipped with a 337 nm nitrogen laser and with the SCOUT source (delayed extraction and reflector). Each mass spectrum was generated from the signal average of 300 laser shots. Oligopeptide products were dissolved in 20 μl trifluoroacetic acid (TFA) and the solution was diluted with 80 μl THF. The matrix was prepared in the following way: 6 mg of dithranol were dissolved in 125 μl of chloroform and to the obtained solution 125 μl of a solution prepared from 17 mg of NaI dissolved in 1 ml THF were added. The final matrix solution was vortex for 1 min. at high speed. The best preparation for the MALDI-TOF MS analysis was achieved by the double deposit procedure: 1 μl of the matrix solution was deposited on the target holder then 1 μl of the sample solution was deposited on the matrix layer.

Results

Aqueous solutions of racemic leucine thioester (*DL*(d_3)-LeuSEt pH=8.5), where the *L*-enantiomer is tagged with three deuterium atoms, were vigorously stirred at room temperature for 24–48 h to yield small quantities of peptides up to dodecamers. Among the library of oligopeptide products, those of homochiral sequence (isotactic) were formed in small excess in comparison to a binomial distribution, Fig. 1, but not beyond octamers, as detected by MALDI-TOF MS. In the spectrum, the groups of signals correspond to diastereoisomeric chains of a given length n where the isotactic chains D_n and L_n appear at the wings of the group and the heterochiral ones D_hL_d ($h+d=n$) appear in between.

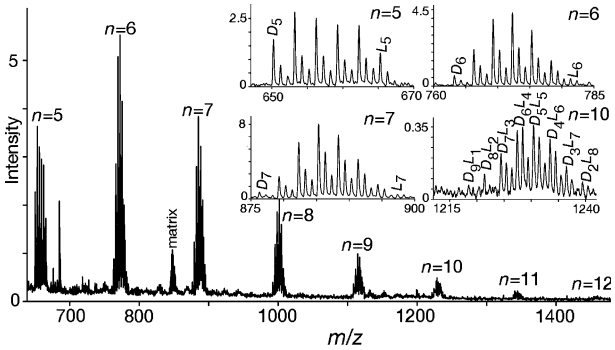


Fig. 1 MALDI-TOF spectra of Na^+ cationized oligopeptides obtained by polymerization of *DL*(d_3)-Leu-SEt (160 mM) in water at pH=8.5. The insert shows the penta-, hexa- hepta and deca-peptide regions of the spectrum. Signals D_n and L_n represent the isotactic oligo-Leu. Note that according to the *m/z* value, all the oligopeptides have a thioester (-CO-S-CH_2CH_3) group at the C-terminus of the chains

In a second attempt, we performed this reaction in solutions of (40 mM) bicarbonate buffer/solid CO_2 or (240 mM) imidazole buffer/solid CO_2 (Bertrand et al. 2001). At such conditions, the thioester should operate as an initiator of its polycondensation and also in the polymerization of *N*-carboxyanhydride (NCA) generated by conversion of part of the thioester. These reactions start in homogeneous conditions but, after 30–40 min., a precipitation (~30% wt yield) was observed. About 30–50% of the precipitate, that was soluble in methylene chloride, was found by electro-spay ionization (ESI) MS analysis to be diketopiperazine in the ratio 1:2:1 *DD:DL:LL*, accompanied by cyclo-tri- to cyclo-penta-peptide diastereoisomers (Kricheldorf 2006). The diastereoisomeric distribution of the longer oligopeptides, as determined by MALDI-TOF MS analysis (Fig. 2a, b), shows that isotactic chains were not found beyond octamers implying that, at these conditions, racemic β-sheet templates (Illos et al. 2008; Rubinstein et al. 2007, 2008) were not formed.

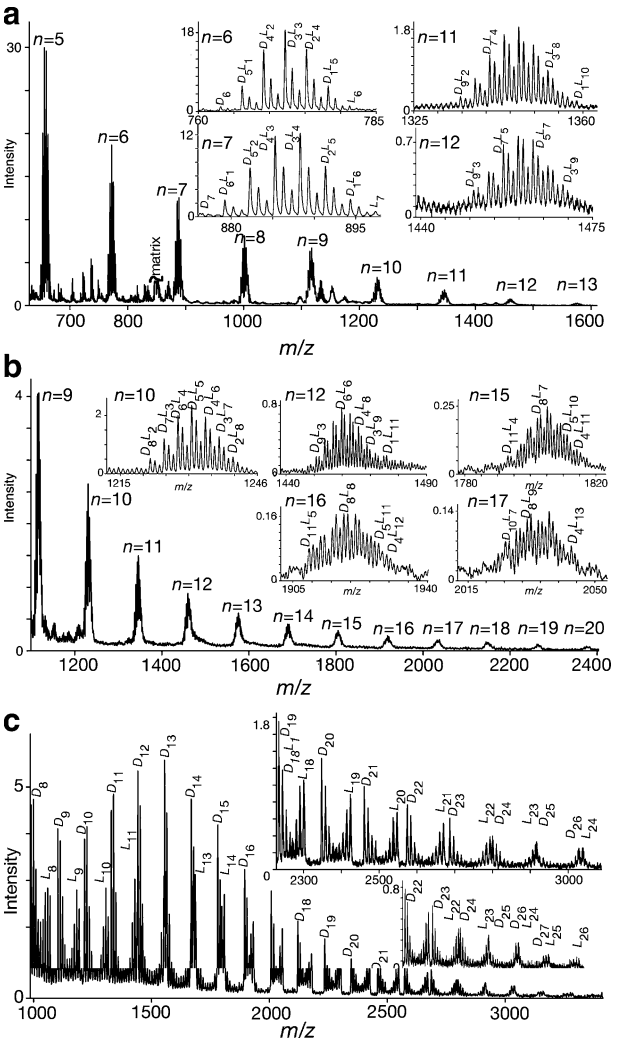


Fig. 2 MALDI-TOF spectra of Na^+ cationized oligopeptides obtained by polymerization of: **a**, **b** *DL*(d_3)-LeuSEt (80 mM) in imidazole buffer/solid CO_2 at pH 7.02 and $NaHCO_3$/solid CO_2 at pH=7.6–8.4 and **c** *DL*(d_{10})-Leu (40 mM) activated with solid CDI and initiated with 100% mol/mol CH_3-CH_2-SH. The insert shows the region where the strongest signal D_n and L_n represent the long isotactic oligo-LeuSEt. Note that according to the *m/z* value, all the oligopeptides have a thioester (-CO-S-CH_2CH_3) group at the C-terminus of the chains

In order to probe the feasibility to generate isotactic oligopeptides from α-amino acid-thioesters in the presence of separately generated β-sheet templates, we performed the polymerization of *DL*(d_{10})-Leu, first activated with solid *N*,*N*′-carbonyldiimidazole (CDI) and then initiated with ethanethiol. In variance to the polycondensation of pure *DL*(d_3)-

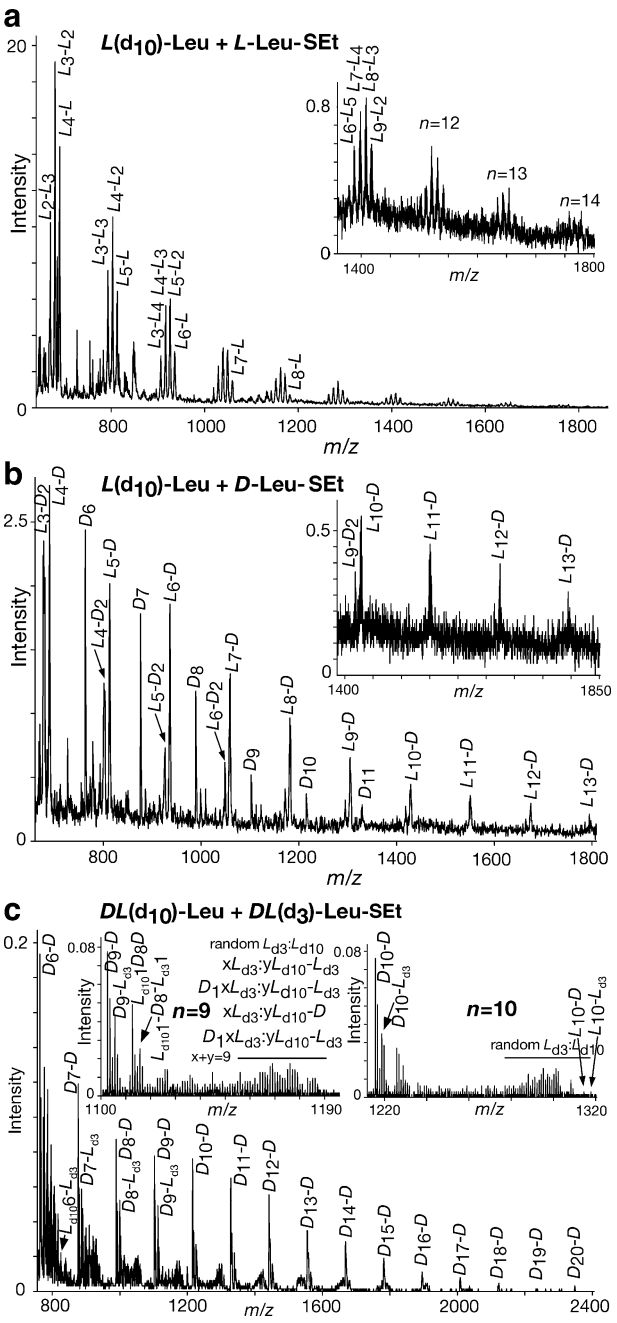


Fig. 3 MALDI-TOF MS analysis of Na^+ cationized oligopeptides obtained in the polymerization of *L*(d_{10})-Leu (40 mM) activated with CDI (50 mM) and initiated with 10 mM of: **a** *L*-LeuSEt and **b** *D*-LeuSEt; **c** polymerization of *DL*(d_{10})-Leu (40 mM) activated with CDI (50 mM) and initiated with *DL*(d_3)-LeuSEt (10 mM)

LeuSEt in water, under the above conditions the reaction started in homogeneous solution and after ~30–40 min. the solution became turbid with the formation of colloidal particles. The X-ray powder diffraction of the precipitate, separated by centrifugation, displays Bragg peaks at *d*-spacings of 12 Å and 4.9 Å and the corresponding FTIR spectrum displays peaks at 1,640 cm^{-1} and 1,540 cm^{-1} suggesting the formation of crystalline β-sheet-like motifs. A very different distribution of the diastereoisomeric oligopeptides (precipitate, ~33% wt yield) was found by MALDI-TOF MS analysis, Fig. 2c. All the oligopeptides contain a thioester (-SEt) group at the C-terminus of the chains. The spectra demonstrate that the isotactic oligopeptides, D_n and L_n, of length up to $n=27$ residues are the dominant products. Moreover, whereas the short peptides, till hexa- to octa-mers, appear as libraries of isotactic and atactic peptides, beyond this length the isotactic ones are formed as the dominant diastereoisomers. These results suggest that the β-sheet templates were formed at intermediate stages to engender enantioselection in the ensuing steps of chain elongation.

From the above results, it follows that thioesters were formed in the presence of ethanethiol and they operated as polymerization initiators since they are present at the C-terminus of each peptide chain. However, it is not clear whether the residues of the oligopeptide chains originate from both in-situ formed NCA and thioester. In order to provide additional insight to this mechanism, we performed the following experiments: polymerization of CDI activated *L*(d_{10})-Leu with either *L*- or *D*-LeuSEt and a double labeling experiment of polymerization of CDI activated *DL*(d_{10})-Leu with *DL*(d_3)-LeuSEt where the *L*-enantiomer of thioester was tagged with three deuterium atoms. The MALDI-TOF MS spectra of the three experiments are shown in Fig. 3a–c. When CDI activated *L*(d_{10})-Leu was reacted with *L*-LeuSEt the MALDI-TOF MS spectrum shows the formation of isotopic co-peptides containing *L*(d_{10})-Leu and *L*-Leu residues in a binomial distribution, although with unknown sequence, Fig 3a. On the other hand, when *L*(d_{10})-Leu was reacted with *D*-LeuSEt, the latter operated also as an initiator in the polymerization of *L*(d_{10})-Leu. However, the reaction proceeded enantioselectively since the oligopeptide product contain isotactic oligo-*D*-Leu with a SEt-group at the C-terminus and isotactic oligo-*L*(d_{10})- Leu containing a *D*-LeuSEt residue from the initiator, Fig 3b. The diastereoisomers $L_{n-2}D_2$ represent chains containing a *D*-Leu residue in addition to that from the initiator. Furthermore, the polymerization of the two racemates yielded primarily isotactic oligo-*D*-Leu peptides initiated by *D*-LeuSEt or *L*(d_3)-LeuSEt and a complex mixture of isotopic co-peptides derived from a random composition of *L*(d_{10})-Leu and *L*(d_3)-LeuSEt, Fig 3c. Some other diastereoisomers containing additional one or two residues of opposite handedness were also detected.

Table 1 Oligopeptides and co-peptides products obtained in the reactions shown in Fig. 3

Reaction	Products[a]				
	Isotopic Leu co-peptides L_h:$L(d_{10})_d$	Oligopeptides $L(d_{10})_n$	D_n	D_{n-1}-*L* (d_3)	Isotopic Leu co-peptides $L(d_{10})_m$- $L(d_3)_{n-m}$
L(d_{10})-Leu[b]+*L*-LeuSEt	yes	no	no	no	no
L(d_{10})-Leu[b]+*D*-LeuSEt	no	yes (+ *D*-initiator)	yes	no	no
DL(d_{10})-Leu[b]+*DL*(d_3)-LeuSEt	no	no	yes	yes	yes

[a] all the oligopeptide products have a thioester group from the initiator at C-terminus; $h+d=n$

[b] the α-amino acid was first activated with solid CDI at 0°C;

The above results (summarized in Table 1) indicate the feasibility to synthesize isotactic co-peptides by the polymerization of racemic hydrophobic α-amino acids and racemic thioesters derivatives. First, we describe the polymerization of *DL*(d_8)-Val initiated with *L*-LeuSEt that yielded, according to the MALDI-TOF analysis, the formation of a complex library of oligopeptides: (i) isotactic *L*-oligo-Leu, (ii) *D*-oligo-Val peptides containing the initiator *L*-LeuSEt and also an additional *L*-Leu residue, presumably located at the N-terminus as a terminator, (iii) isotactic co-peptides of oligo-*L*(d_8)-Val: oligo-*L*-Leu, Fig. 4a

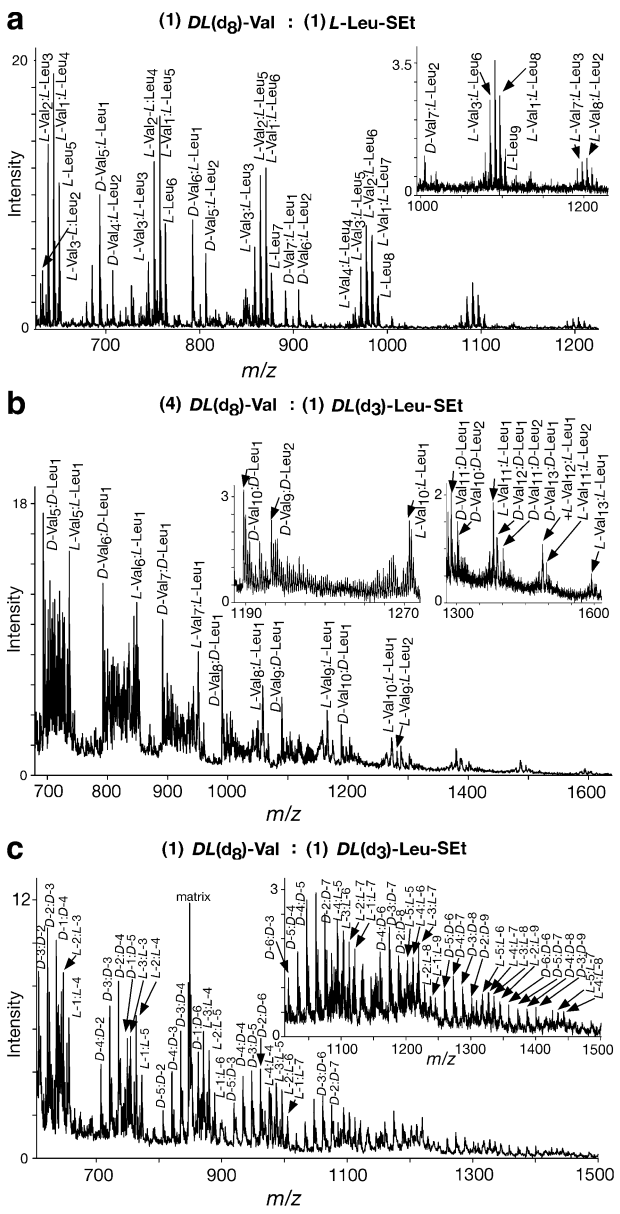


Fig. 4 MALDI-TOF MS of Na^+ cationized oligopeptides obtained in the polymerization of: **a** mixture of activated (1) *DL*(d_8)-Val (40 mM): (1) *L*-LeuSEt (20 mM); **b**, **c** (4): (1) and (1): (1) mixtures of activated *DL*(d_8)-Val (40 mM): *DL*(d_3)-LeuSEt. Note that in **c** the isotactic co-peptides are labeled *D-n*:*D-m* and *L-m*: *L-n* representing the *D*-Val:*D*-Leu and *L*(d_8)-Val-:*L*(d_3)-Leu number of residues, respectively

Table 2 Oligopeptides and co-peptides products obtained in the reactions shown in Fig. 4

Reaction	Products[a]				
	Val:Leu co-peptides $L_m(d_8)$-L_{n-m}	Val:Leu co-peptides D_h:$L(d_3)_d$	Oligo-Leu L_n	Oligo-Val D_n	Val:Leu co-peptides D_m-D_{n-m}
(1) $DL(d_8)$-Val[b]+(1) L-LeuSEt	yes	yes	yes	no	no
(4) $DL(d_8)$-Val[b]+(1) $DL(d_3)$-LeuSEt	yes	yes	no	no	yes
(1) $DL(d_8)$-Val[b]+(1) $DL(d_3)$-LeuSEt	yes	yes	no	no	yes

[a] all the oligopeptide products have a thioester group from the initiator at C-terminus; $h+d=n$

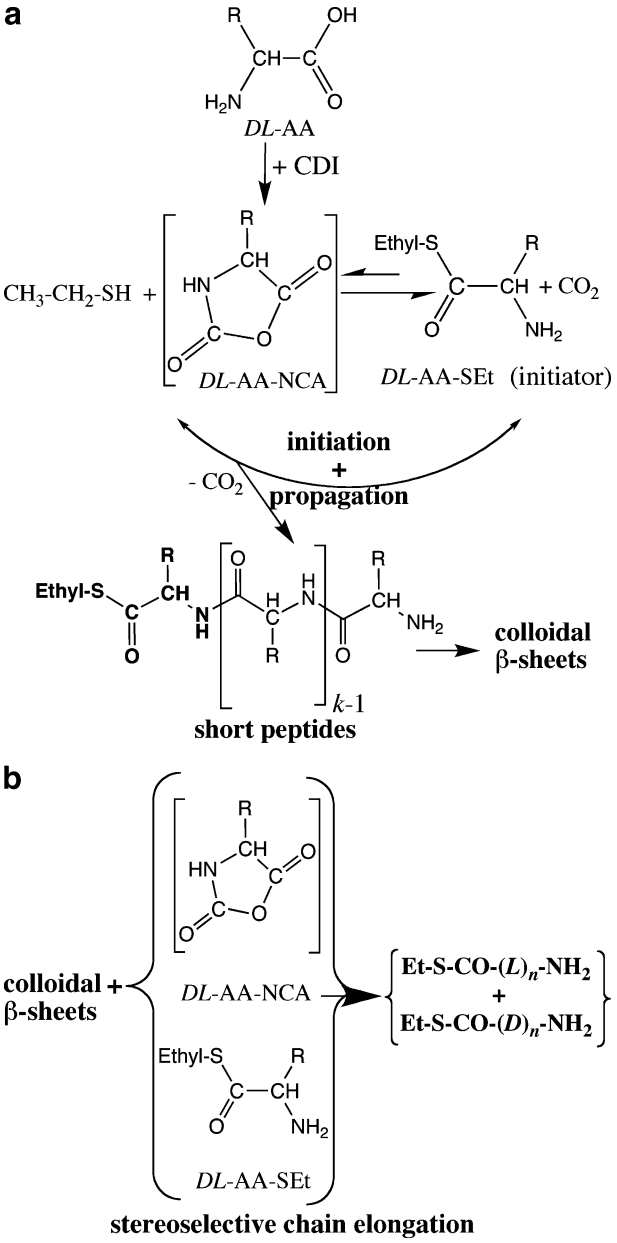
[b] the α-amino acid was first activated with solid CDI at 0°C;

and Table 2. Therefore, *L*-LeuSEt operated as an initiator of the enantioselective self-condensation, polymerization of $L(d_8)$-Val and that of *D*-Val as well as multimer in the chain elongation. As a consequence of this complex mechanism, only one of enantiomeric oligo-*L*-Val: oligo-*L*-Leu co-peptides were formed. Second, polymerization of CDI activated $DL(d_8)$-Val with $DL(d_3)$-LeuSEt (4:1 ratio) yielded a mixture oligo-*D*-Val with the *D*-LeuSEt initiator at C-terminus and oligo-$L(d_8)$- Val with the $L(d_3)$-LeuSEt initiator at the C-terminus, Fig 4b. Polymerization of the two racemates in 1:1 ratio yielded a complex mixture of isotactic oligo-*D*-Val: oligo-*D*-Leu and oligo-*L*-Val: oligo-*L*-Leu co-peptides as the dominant diastereoisomers, Fig 4c. These results are summarized in Table 2.

Discussion

In this study, we demonstrate that the polycondensation of racemic LeuSEt yields diketopiperazines and only low amounts of short atactic peptides since at these conditions β-sheets are not formed. On the other hand, when hydrophobic β-sheets are formed as in the polymerization of racemic α-amino acids activated by CDI and initiated with racemic LeuSEt, the isotactic oligopetides become the dominant diastereoisomers. The thioesters operate as initiators of the reaction since the thioester group is found at the C-terminus of all the oligopeptide chains. In addition, the enantioselective labelling polymerization experiments showed that the thioesters also operate as multimers during the chains elongation. Such insertion of the residues originating from thioesters might take place either by their prior conversion into NCA via their reaction with the released CO_2, as previously claimed (Wieland and Bartmann 1956; Bertrand et al. 2001), or by direct insertion into the growing sites of the β-sheets. Various reactions are taking place during the polymerization experiments, as shown in Schemes 1a and b. The thioesters can react with CO_2 to form NCA. The CO_2 originates both from its release during the polymerization of NCA as well as by decomposition of CDI. Indeed, when the thioesters were reacted in a solution where CDI was intentionally decomposed to imidazole and CO_2, the quantity of the formed oligopeptides was augmented.

This reaction releases ethanethiol, which can react with the NCA to form the thioester again, as demonstrated in the polymerization reaction of NCA with ethanethiol. The polymerization reaction is always initiated by the thioester. Once the β-sheets of the oligo-Leu are formed they exert regioenantioselection but not chemoselection in the reaction with valine. The dual role played by LeuSEt, as initiator and as a multimer, provides a most efficient route for desymmetrization of oligopeptides formed in the polymerization of *DL*-



Scheme 1 **a** Reactions pathways for the polycondensation of *DL*-AA into short peptides that self-assemble into colloidal β-sheets; **b** Stereoselective chain-elongation of the β-sheets into racemic isotactic oligopeptides

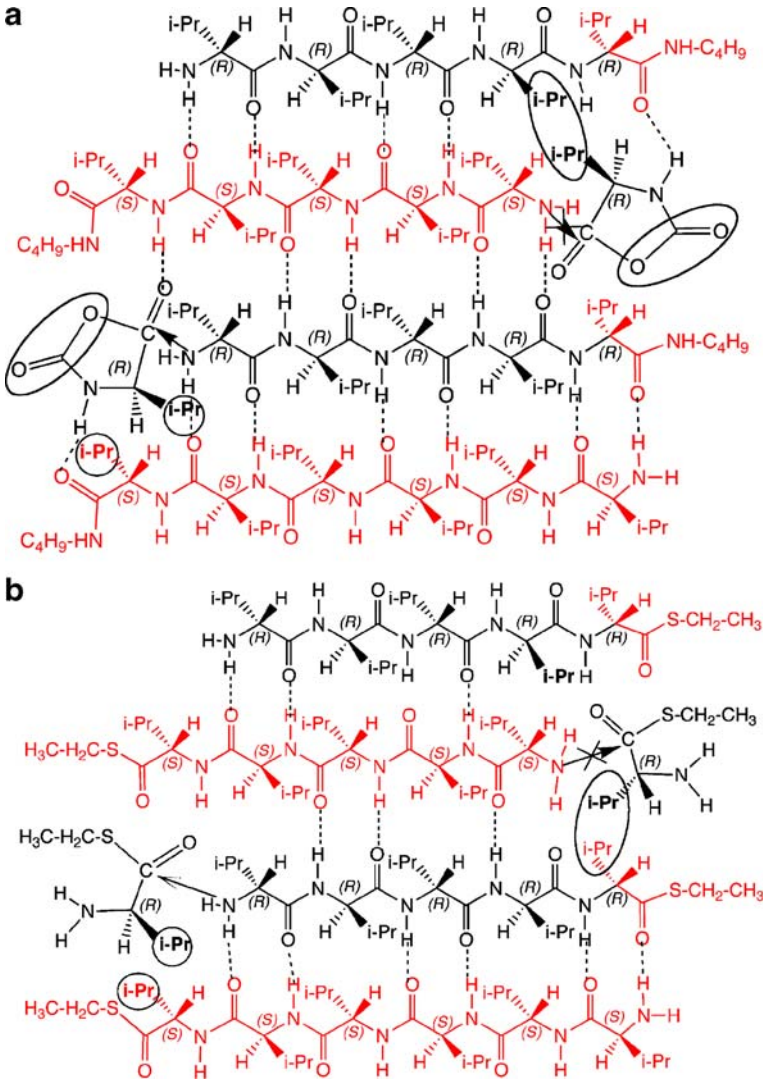
Val when initiated by *L*- or *D*-LeuSEt. The complex library of peptides contains oligo-*D*-Val with one or two residues of *L*-Leu at the two termini of the chains, and co-peptides of *L*-Val:*L*-Leu.

One of the open questions that arose during this study is whether the thioesters are inserted directly into the peptide chains or only via their prior conversion into NCA. A chemical model shows that both the NCA as well as the thioesters can be inserted enantioselectively within the growing sites of the β-sheets, thus supporting their ability to

undergo direct polymerization, Scheme 2a, b. The insertion of a thioester residue at the active site of the β-sheets should reduce, for steric reasons, the formation of the diketopiperazines.

Conclusions

In this study we demonstrate that β-sheets can operate as efficient templates for the regio-enantioselective polymerization of racemic thio-ethyl esters of α-amino acids. Moreover, they can be co-polymerized with CDI activated racemic α-amino acids to form co-peptides.



Scheme 2 Schematic representation of the enantio-regioselective chain-elongation of the oligopeptides by addition of **a** LeuNCA and **b** LeuSEt to the growing sites of the β-sheets

As a result of the dual role played by the thioesters, as initiators and as multimers, they can be considered as efficient systems for the de-symmetrization of oligo-peptides and co-peptides generated from racemic α-amino acids (Siegel 1998). The survival from hydrolysis of the thioester group at the C-terminus of the peptide chains provides a possible route for a chain elongation of the isotactic chains by chemical ligation (Payne et al. 2008; Wilken and Kent 1998; Wu et al. 2006). One may anticipate that among such libraries one may find, by combinatorial methods, homochiral co-peptides that display catalytic properties.

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