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A Reassessment of Prebiotically Relevant Chemical Agents for the Activation of α -Amino Acids and Peptides

Ghinwa Ajram,¹ Jean-Christophe Rossi,¹ Laurent Boiteau,¹ and Robert Pascal^{1*}

ABSTRACT

From an origin of life perspective, processes able to provide energy to feed self-organizing systems are required for the formation of their own components but also to maintain a far from equilibrium state essential for the system to remain under kinetic control. α -Amino acid *N*-carboxyanhydrides (NCAs) and 5(4*H*)-oxazolones represent activated intermediates of the chemistry of peptides that may be involved in the self-organization of life and in the evolution of translation. An experimental survey of previously published activating agents has been carried out using the epimerization associated with the transient formation of 5(4*H*)-oxazolones as a tool to monitor their potential to promote strong activation. None of reagents investigated in this survey proved to be totally satisfactory demonstrating that the identification of activation processes remains a crucial goal in this field.

Keywords: peptides; activating agents; self-organization; origin of life

Introduction

Life's complexity arose from a limited number of building blocks, including α -amino acids, nucleotides, or fatty acids. The abiotic polymerization or self assembly of these monomers or their derivatives gave birth to the three subsystems (peptides, nucleic acids, and lipid membranes) that are responsible for the main functions observed in living cells. Complex systems could therefore be formed from their interactions, which resulted eventually into the self-organization of the first living organisms [1]. In addition to the requirement for building blocks, polymers and supramolecular assemblies as material components for building the living entities, the emergence and evolution of life correspond to a state in which change is driven by a continuous research for increased Dynamic Kinetic Stability (DKS) [2]. This behaviour is expressed by entities able to reproduce themselves and

thus to grow exponentially when resources are not limited. Maintaining a drive towards higher DKS requires an environment able to provide energy and thus to keep the system in a far from equilibrium situation. An analysis of the kinetic requirements for the occurrence of DKS as a driving force in simple chemical systems (*i.e.* replicating entities, autocatalysts) led to the conclusion that the entities must be reproduced through a cycle that proceeds irreversibly [3–5]. As a result, chemical systems capable of evolving toward the living state would have to spend a cost of irreversibility by dissipating energy and making the reverse process slow enough compared to the time scale of the reproduction cycle (meaning that the flux of reagents crossing the higher activation barrier in the reverse direction becomes negligible) [3–6]. This approach suggests that energy must not only be provided to the system to allow for the synthesis of

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biomolecules or the polymerization of building blocks but must, for the most part, be dissipated without serving any other purpose than satisfying this requirement for kinetic irreversibility and that strong activation processes should be involved in the process of self-organization.

For that reason, sources of free energy are required to constrain self-organization as far as the scope of the chemical perception of the origin of life is concerned. This analysis is supported by considering peptide biosynthesis, shared with minute differences by all extant living organisms, which reveals one of the paradoxes of life's emergence and early evolution. Among the intermediates of the process, aa-AMPs, known as aminoacyladenylates (Scheme 1), are produced by a reaction of ATP with amino acids in the presence of a set of aminoacyl-tRNA-synthetases (aaRSs) the enzymes responsible for the reaction. aaAMPs possess free energies of hydrolysis values ($\Delta G^\circ = ca. -70 \text{ kJ mol}^{-1}$) among the highest of the metabolites present in the cells [7]. The aaRS enzymes not only increase the rate of formation of adenylates from ATP and free amino acids owing to their kinetic activity but also stabilize the intermediate that remains sequestered in the local environment provided by the enzyme, which therefore thermodynamically contributes to the reaction as well. Without that stabilizing contribution from the enzyme, the equilibrium values of the concentration of free aa-AMP formed from ATP and amino acids would be negligible [7], which raises the chicken-and-egg question of how this process could have emerged in the absence of enzymes. This observation strongly suggests that translation evolved in an environment providing strong activation processes in which adenylates or other mixed anhydrides could be available from alternative pathways [8,9]. Several groups, including ours, have provided evidence that α -amino acids and peptides, strongly activated in the forms of *N*-carboxyanhydrides (NCAs) [10-12] or 5(4*H*)-oxazolones [13-15], respectively, react spontaneously with inorganic phosphate and phosphate esters to give mixed anhydrides including aaAMPs at equilibrium in diluted neutral aqueous solution (Scheme 2). One of the most logical consequences of these observations is that the possible formation of peptides, involving for instance mild wetting/drying cycles [16-18] at early stages of life, does not prevent the need of processes involving strong reagents for life to emerge.

As far as strong activating agents are involved, *N*-carboxyanhydrides (NCAs) or 5(4*H*)-oxazolones are essential intermediates during the activation of α -amino acids or peptides, respectively [19]. Some

examples of prebiotically plausible processes for the formation of these activated intermediates have been reported in the literature. *N*-Carbamoyl amino acids yield NCAs by nitrosation in the dry state [20] or, more directly, by heating neutral aqueous solution [21]. Carbonyl sulfide (COS) is also efficient in producing NCAs from free amino acids in the presence of oxidizing or alkylating agents and transition metal ions. [22]. 5(4*H*)-Oxazolones are formed when acyl-amino acids or C-terminal residues in peptides are activated by strong activating agents like carbodiimides [23]. The latter reaction significantly increases the rate of polymerization of amino acids promoted by carbodiimides [23]. Though being considered as a drawback leading to a loss of chirality in the synthesis of peptides, 5(4*H*)-oxazolone formation has a very different outcome in prebiotic chemistry starting from racemic reagents since it may also be considered as a tool to induce a stereomer imbalance during polymerization [23]. However, carbodiimides are not likely activating agents in an origin of life perspective. Cyanamide constitutes an alternative but its reactions turned out to be tedious [23] even at 80°C. We therefore considered the published work on amino acid condensation agents [24,25]. Many other reagents including cyanamide, dicyanamide, dicyandiamide (DCDA), diaminomaleonitrile (DAMN, HCN tetramer), and cyanogen leading to peptide bond formation can be found in the literature, although the yields were usually limited [26]. However, general conclusions are not easily drawn from these studies involving different conditions, different analytical methods, different starting materials, and in some cases using elevated temperatures or extreme values of pH. We realized that the formation of oxazolones from dipeptides constitutes a simple and practical way to characterize strong carboxyl activating agents (Scheme 3). The chiral instability of oxazolones rapidly results in a partial change of the configuration of the α -carbon of the amino acid residue, which can easily be monitored by analytical methods (e.g. HPLC) through the formation of diastereomers thus revealing the effect of powerful activating agents. The aim of the present work is to use this tool as a mean of investigating the reactivity of a series of prebiotically plausible condensing agents (Scheme 4) under conditions compatible with the environment of the primitive earth.

Experimental

Reagents and solvents were purchased from Sigma-Aldrich or Bachem, and were used without further purification. Dicyclohexyl ammonium methyl

phosphate was prepared as described earlier [27]. All aqueous solutions were prepared using pure water (18 M Ω) produced with a Milli-Q (Merck-Millipore) apparatus. NMR spectra in either DMSO-*d*₆ or D₂O solution were recorded on a Bruker DPX 300 spectrometer (300 MHz) or on a Bruker Avance 400 spectrometer (400 MHz); chemical shifts δ_{H} are reported in ppm; coupling constants *J* are reported in Hz. In all the experiments, the pH was monitored using a Thermo Orion 3-STAR pH-meter with a VWR electrode. UPLC analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Standard system including an autosampler unit, a thermostated column compartment and a photodiode array detector, using UV absorbance detection (acquisition in the 200–400 nm range) and the reactants and product were monitored at $\lambda = 273$ nm, which corresponds to the maximum of absorption of the aromatic ring of tyrosine. UPLC/ESI-MS analyses were carried out on a Waters UPLC Acquity H-Class system including a photodiode array detector (acquisition in the 200–400 nm range), coupled to a Waters Synapt G2-S mass spectrometer, with capillary and cone voltage of 30 kV and 30 V respectively, source and desolvation temperature of 140 °C and 450 °C respectively. ESI⁺ and ESI⁻ refer to electrospray ionisation in positive and negative mode respectively. HRMS spectra were recorded on the same spectrometer, using the same source settings. HPLC conditions: Method A: Separations were carried out on a BDS-Hypersil C18 50×2.1 mm, 3 μm column, mobile phase made of solvent A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (5% B), 15 min (15% B), 25 min (60% B), 26 min (100% B), 31 min (100% B); Method B: Separations were carried out on a BDS-Hypersil C18 50×2.1 mm, 3 μm column, mobile phase made of solvent A: H₂O (0.1% TFA), B: CH₃CN (0.1% TFA); flow rate 0.2 mL/min; gradient: 0 min (5% B), 25 min (25% B), 30 min (100% B), 32 min (100% B), and 33 min (5% B). Method C (LC/ESI-MS): Kinetex C18 100 Å 100×2.1 mm, 2.6 μm column (Phenomenex), mobile phase made of solvent A: H₂O (0.1% formic acid), B: CH₃CN (0.1 % formic acid), flow rate 0.5 mL/min; linear gradient: 0 min (0% B) to 3 min (100% B).

Results

The activating agents shown in Scheme 4 were checked for strong activation using the dipeptide models **1-LL**

(R¹ = H) and **2-LL** (R¹ = Ac) as starting materials (HPLC retentions times 9.6 min and 16.2 min respectively, method A, Scheme 3). Any formation of 5(4*H*)-oxazolones from these substrates should result in the epimerization of the C-terminal alanine residue yielding the diastereomers **1-LD** (R¹ = H) and **2-LD** (R¹ = Ac) [28] (HPLC retentions times 16.5 min and 19.9 min respectively, cf. Figure 1). Monitoring products is facilitated by the presence of the aromatic side-chain chromophore of tyrosine ($\lambda_{\text{max}} = 273$ nm). Activation was carried out in a neutral to moderately acidic pH range considered as representative of aqueous environments of the primitive Earth with an atmosphere enriched in CO₂ under conditions chosen to remain close to those reported in the literature for such experiments. The efficiency of the method was checked using the EDC activation reagent (Table 1 entries 1 and 2) known to induce 5(4*H*)-oxazolone formation [28] even in the case of unprotected dipeptide **1-LL**, which does not yield diketopiperazine owing to specific stereochemical properties [29] though in this case hydrophobic products having 23.3 to 24.9 min retention times were observed and correspond very probably to longer oligomers formed by reaction of the oxazolone with the free dipeptide. The advantage of this procedure is that the detection of epimerization gives information on the activation step independently of subsequent processes that are dependent on the concentration of nucleophile and other parameters that should influence the fate of the system provided that activation works. The results of the activation experiments using different reagents and conditions are listed in Table 1. None of the reagents checked during our investigations proved to reproduce an activity reaching that of EDC and, in most cases, no activity could be detected. Significant epimerization of the starting material was observed using cyanamide (Figure 1, Table 1, entry 7), confirming earlier preliminary results [23], and the related dicyanamide (Table 1, entries 17 and 18). However, the process required heating of the mixture to 80–90 °C for long periods. Other reagents observed earlier to induce free amino acid polymerization in solution turned out to be inert with the acetylated dipeptide. This was indeed the case for cyanogen (Table 1, entries 3 and 21) formed in situ by the reaction of CuSO₄ and KCN [30] and diaminomaleonitrile (DAMN, entries 5, 6 and 22). The acetylated dipeptide **2-LL** remained unchanged in these experiments revealing that the activity observed previously should result from a pathway different from direct carboxyl group activation. The replacement of

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the acetylated derivative by the dipeptide having a free amine gave an indication about the process of activation observed in the literature. A partial carbamoylation of the free amino group of dipeptide 1-LL was observed through the observation of an additional HPLC peak with a retention time of 14.4 min (Method A) and signals at m/z 310.14 $[M+H^+]$ and 332.12 $[M+Na^+]$ in the HPLC-ESI-MS (positive mode) analysis of the mixture, which was particularly well established from the reaction of $CuSO_4$ and KCN. Similar signals were observed from DAMN. In fact, any hydrolysis of cyanogen formed during the reaction should lead to cyanate [30] and then to the carbamoylation of free amines [31]. The activation observed in the literature should therefore result from the formation of carbamoyl-amino acids and their further conversion into NCAs as demonstrated by a re-examination of the activation of amino acids by cyanate [21]. The result obtained from DAMN may be accounted for in a similar way since it was suggested in the literature that it can disproportionate into cyanogen and amino acetonitrile [32]. No other combination of reagents proved to be effective. Interestingly, even the procedure reported recently [33] to improve the rate of formation of cyclic phosphodiester by a catalytic cycle based on the reactivity of glyoxylate or pyruvate with cyanamide at moderate temperature proved to be ineffective (Table 1, entry 11) demonstrating that, in this case, the increase in rate is brought about at the expense of the strength of the activating agent. In the same way, the use of a nucleophilic methyl phosphate buffer expected to react with cyanamide to give a reactive adduct proved to be ineffective (Table 1, entry 9). Further attempts to add metal ions to the reaction mixture, with the aim of binding both the reagent and the activating agent and bringing reactive centres in close proximity in the coordination sphere of the metal failed.

A theoretical analysis of the conditions required for a steady state based on DKS led to the conclusion that energy has to be provided to the system with a potential equivalent or exceeding than that of visible light [3,4]. It would have been logical to investigate potential activation processes based on visible or UV irradiation of solutions containing our reagents and a series of potential activating agent precursors. However, preliminary experiments demonstrated that the dipeptide models used in the present study were not stable under irradiation at 254 nm. These investigations will require the development of a more sophisticated methodology avoiding a light-sensitive substrate.

Conclusions.

The presence of strong activating agents or of energy-rich carriers is a crucial parameter to induce self-organization as a consequence of a selection process among variants of entities capable of reproducing themselves [2, 34–36]. In addition to the need of unlimited possibilities of variations that can be brought about by mutations in the sequence of a polymer, approaches based on the requirements for establishing DKS states emphasize the need for sources of energy capable of feeding these systems and holding them in a state that is sufficiently far from equilibrium for a reproduction loop to work in an irreversible way [37]. The results of the present study indicate that a process able to activate the C-terminal end in a peptide in a way consistent with this approach is not available yet. Indeed, carbodiimides are not likely prebiotic reagents and cyanamide does not constitute an appropriate alternative because of its sluggish reaction even at high temperature. The only established processes leading to strong activation in prebiotically plausible environments are at present those based on the formation of *N*-carboxyanhydrides of amino acids [20–22]. The occurrence of a presently unknown catalytic process similar to that already established with oxoacids [33] but that do not reduce the potential of cyanamide reagent could be a possibility. In the absence of a catalytic pathway, the lack of reactivity of cyanamide and other nitriles is a drawback but it must be acknowledged that limited spontaneous breakdown rates are also necessary for the emergence of a metabolism in which reactants must be protected by kinetic barriers [38,39]. Compounds containing triple bonds including nitriles and cyanamides are very likely candidate for this role [6]. The kinetic barriers protecting them from spontaneous hydrolysis actually represent a property allowing them to migrate from the location of their formation to that of a self-organizing system potentially requiring a different context. This hypothesis is however also dependent on the occurrence of efficient catalytic systems that could trigger the transfer of their energy potential into the metabolism of a self-reproducing entity. In a systems chemistry approach to the origin of life, a very attractive possibility for the conversion of the free energy of nitriles could involve an autocatalytic process able to contribute to a steady state behaving in conformity with a DKS drive and associated with possibilities of variations allowing the initiation of an open-ended evolutionary process. Finally, this observation means that the difficulty in detecting any possibility of strong activation of C-terminus in peptide must not be considered as a definitive conclusion since

the height of the kinetic barriers associated with nitrile reactions may turn out to become an advantage and should encourage further investigations in this direction [40], provided that catalysts are found that conserve the strong activation character. This potential of nitriles or other simple species bearing triple bonds in activation might be on the contrary considered as in line with the role of hydrogen cyanide that has been recently uncovered in the formation of a wide range of biochemical building blocks through photocatalytic redox cycles [41-43].

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Author's contributions

GA and JCR designed and performed the experiments. RP supervised the project. GA, JCR, LB and RP analyzed results and wrote the paper.

Competing interests

The authors declare that they have no competing interests.

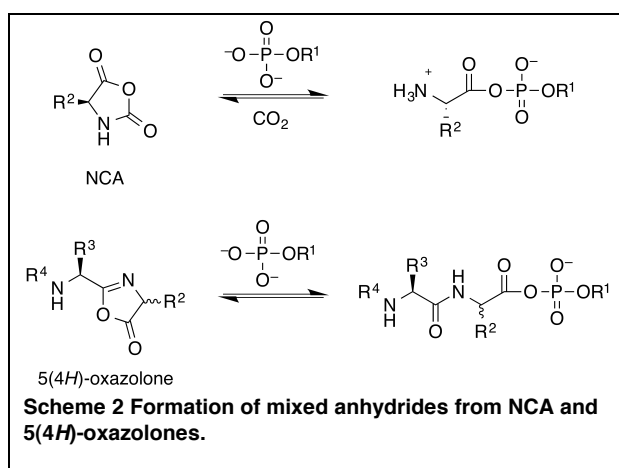
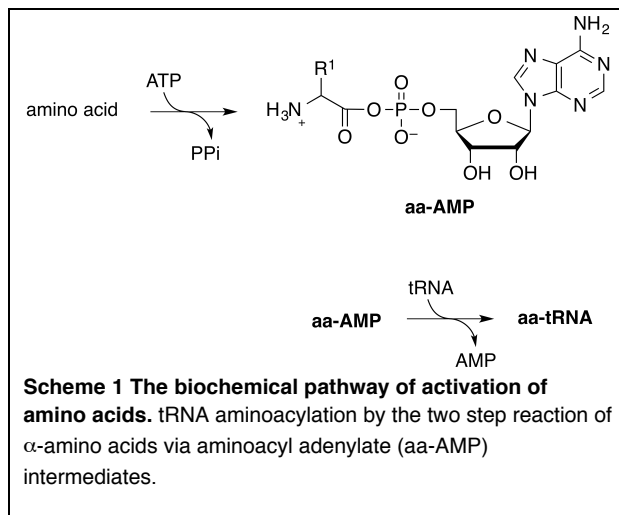
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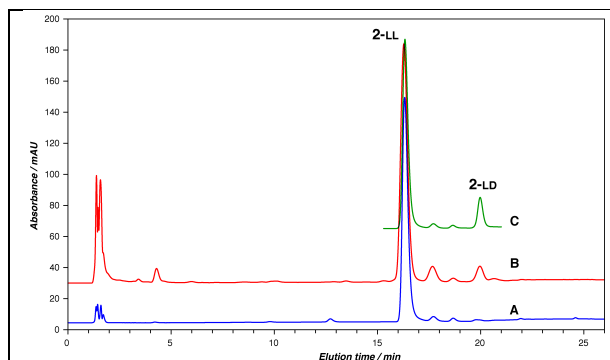
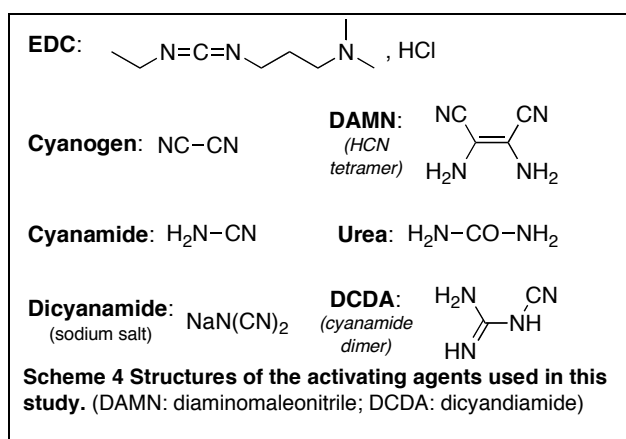
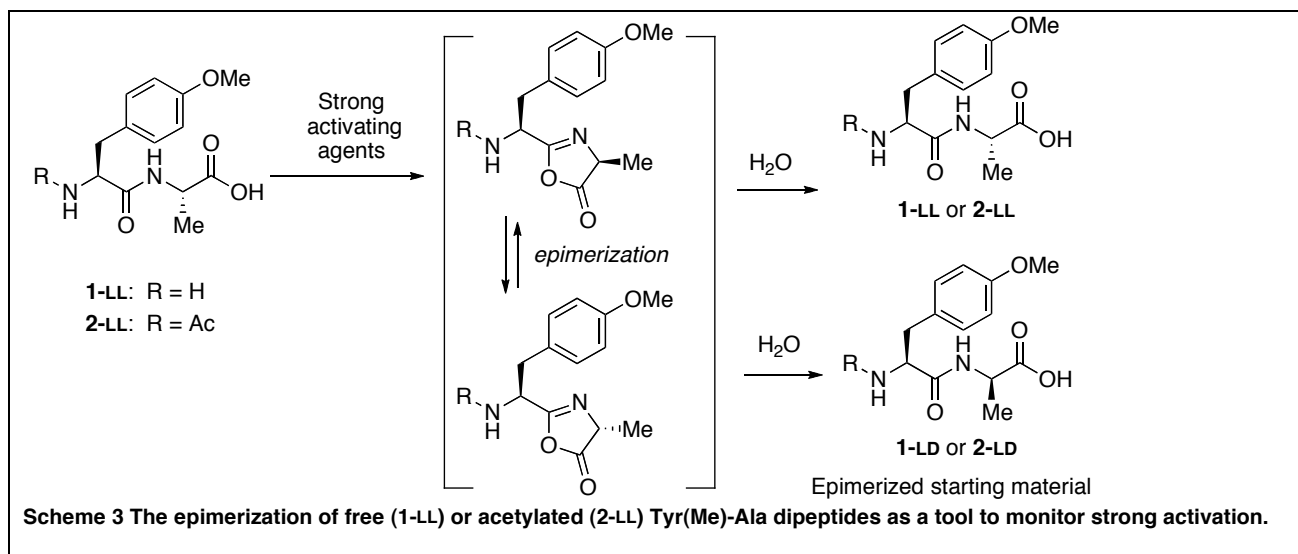


Figure 1 Reaction of 1 mM Ac-Tyr(Me)-Ala dipeptide, 2-LL, with 40 mM cyanamide at pH 5.5 (100 mM MES buffer) and 80°C. HPLC chromatograms (method A, UV detection 273 nm) **A:** starting material 2-LL in MES buffer; **B:** reaction of 2-LL with cyanamide after 20 days; **C:** comparison with the mixture of dipeptide epimers 2-LL and 2-LD obtained from the reaction of 1 mM 2-LL with 1 mM EDC in 100 mM MES buffer pH 6.5 at after 3 days.

Additional files

Additional file 1 — Supporting Information (pdf format) including the description of the preparation of starting materials and reagents and the detailed description of the experiments corresponding to the data provided in Table 1.

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Table 1 Screening of a variety of activating agents and reaction conditions to detect the epimerization of the two dipeptides H-Tyr(Me)-L-Ala-OH **1-LL** and Ac-Tyr(Me)-L-Ala-OH **2-LL** in buffered aqueous solution (100 mM MES unless otherwise mentioned). The solutions resulting from the activation experiments were analyzed by HPLC (method A, detection 273 nm unless otherwise mentioned) and potential products were characterized by mass spectrometry or NMR. Abbreviations: s.m. and n.d. stand for starting material and not detected, respectively.

Entry	Substrate	Activating agent and/or catalyst	Duration, temperature, pH	Result of activation (products, amounts)	Identification	Fig. ^a
1	1 mM 1-LL	1 mM EDC	4 d, <i>r.t.</i> , pH 6.5	Partial epimerization s.m. 1-LL + 3.7% 1-LD ^b + side products	HPLC retention time identical to pure 1-LD	S1
2	1 mM 2-LL	1 mM EDC	3 d, <i>r.t.</i> , pH 6.5	Partial epimerization s.m. 2-LL + 12.8% 2-LD ^b	HPLC retention time identical to pure 2-LD	S2
3	1 mM 2-LL	5 mM CuSO ₄ , 10 mM KCN	18 d, <i>r.t.</i> , pH 6.5	n.d., unchgd. s.m. 2-LL		S3
4	1 mM 2-LL	5 mM CuSO ₄ , 10 mM KCN in 50 mM methyl phosphate buffer	4 d, <i>r.t.</i> pH 7.1	n.d., unchgd s.m. 2-LL		S4
5	1 mM 2-LL	10 mM DAMN ^c	11 d, <i>r.t.</i> pH 6.5	n.d., unchgd s.m. 2-LL		S5
6	10 mM 2-LL	20 mM DAMN	25 d, 90 °C, pH 6.5	n.d., unchgd s.m. 2-LL		S6
7	1 mM 2-LL	40 mM Cyanamide	20 d, 80 °C, pH 5.5	Partial epimerization s.m. 2-LL + 2-LD 5.4%	LC-MS ESI ⁺ : <i>m/z</i> 309.15 [M+H] ⁺ , 331.13 [M+Na] ⁺	1
8	1 mM 2-LL	10 mM Cyanamide in 5 mM imidazole buffer	7 d, <i>r.t.</i> , pH 6.68	n.d., unchgd s.m.		S7
9	1 mM 2-LL	10 mM Cyanamide in 50 mM methyl phosphate buffer	13 d, <i>r.t.</i> , pH 7.1	n.d., unchgd s.m.		S8
10	1 mM 2-LL	15 mM Cyanamide, 1 mM CuCl ₂	5 d, <i>r.t.</i> , pH 6.5	n.d., unchgd s.m.		S9
11	1 mM 2-LL	5 mM Cyanamide/ 5 mM glyoxylic acid	5 d, 40 °C, pH 5.0	n.d., unchgd s.m.	HPLC method B	S10
12	1 mM 2-LL	20 mM Urea	20 d, 80 °C, pH 5.5	n.d., unchgd s.m.		S11
13	1 mM 2-LL	5 mM Dicyanamide	6 d, <i>r.t.</i> , pH 5.5	n.d., unchgd s.m.		S12
14	1 mM 2-LL	5 mM Dicyanamide/ 5 mM Cu(OAc) ₂	6 d, <i>r.t.</i> , pH 5.5	n.d., unchgd s.m.		S12
15	1 mM 2-LL	5 mM Dicyanamide/ 5 mM NiSO ₄	6 d, <i>r.t.</i> , pH 5.5	n.d., unchgd s.m.		S12
16	1 mM 2-LL	5 mM Dicyanamide/ 5 mM MnSO ₄	6 d, <i>r.t.</i> , pH 5.5	n.d., unchgd s.m.		S12
17	1 mM 2-LL	5 mM Dicyanamide	11 d, 85 °C, pH 5.5	Partial epimerization s.m. 2-LL + 2-LD 12.8%		S13
18	10 mM 2-LL	30 mM Dicyanamide	3 d, 85 °C, pH 5.5	Partial epimerization s.m. 2-LL + 2-LD 15.4%	LC-MS ESI ⁺ : <i>m/z</i> 309.15 [M+H] ⁺ , 331.13 [M+Na] ⁺	S14
19	1 mM 2-LL	10 mM DCDA ^d	3 d, <i>r.t.</i> , pH 5.5, dark	n.d., unchgd s.m.		S15
20	1 mM 2-LL	10 mM DCDA	22 d, 80 °C, pH 5.5, dark	Limited epimerization s.m. 2-LL + 2-LD 2.1% ^a	HPLC retention time identical to 2-LD	S16
21	1 mM 1-LL	5 mM CuSO ₄ + 10 mM KCN	5 d, <i>r.t.</i> , pH 6.5	n.d., unchgd s.m. 1-LL + <i>N</i> -carbamoyl dipeptide	LC-MS ESI ⁺ : <i>m/z</i> 310.14 [M+H] ⁺ , 332.12 [M+Na] ⁺ ; NMR ^e	S17
22	1 mM 1-LL	10 mM DAMN	7 d, <i>r.t.</i> , pH 6.5	n.d., unchgd s.m. 1-LL + <i>N</i> -carbamoyl dipeptide + non-identified side product ^f	LC-MS ESI ⁺ : <i>m/z</i> 310.14 [M+H] ⁺ , 332.12 [M+Na] ⁺ ; ESI ⁻ : 308.20 [M-H] ⁻	S18

^a For figures S1 to S18, see Supporting Information; ^b Relative to all species absorbing at 273 nm; ^c DAMN: diaminomaleonitrile (HCN tetramer); ^d DCDA: dicyandiamide (cyanamide dimer); ^e NMR analysis of separated products; ^f LC-MS ESI⁺: *m/z* 418.18 for the unidentified side-product.