Orig Life Evol Biosph
DOI 10.1007/s11084-015-9455-0

PREBIOTIC CHEMISTRY

The Activation of Free Dipeptides Promoted by Strong Activating Agents in Water Does not Yield Diketopiperazines

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Received: 8 June 2015 / Accepted: 13 July 2015

Abstract The activation of dipeptides was studied in the perspective of the abiotic formation of oligopeptides of significant length as a requirement for secondary structure formation. The formation of piperazin-2,5-diones (DKP), previously considered as a dead end when activating free dipeptides, was shown in this work to be efficiently suppressed when using strong activating agents (e.g., carbodiimides). This behaviour was explained by the fast formation of a 5(4*H*)-oxazolone intermediate at a rate that exceeds the time scale of the rotation of the peptide bond from the predominant *trans*-conformation into the *cis*-isomer required for DKP formation. No DKP was observed when using strong activating agents whereas phosphate mixed anhydrides or moderately activated esters were observed to predominantly yield DKP. The DKP side-reaction no longer constitutes a drawback for the C-terminus elongation of peptides. These results are considered as additional evidence that pathways involving strong activation are required to drive the emergence of living entities rather than close to equilibrium processes.

Keywords Peptide · Diketopiperazine · 5(4*H*)-oxazolone · Activation · Elongation

Introduction

The abiotic formation of α-amino acids has been demonstrated to take place under a diversity of abiotic conditions since its first description in the Miller experiment (1953) and the analyses performed on meteorites confirmed the occurrence of a synthetic pathway independent of

Electronic supplementary material The online version of this article (doi:10.1007/s11084-015-9455-0) contains supplementary material, which is available to authorized users.

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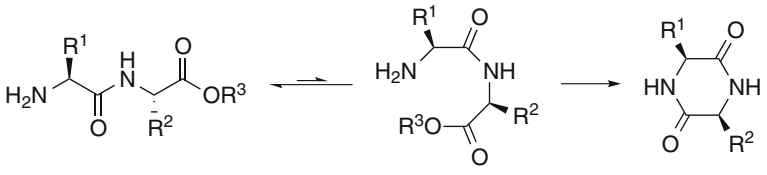
Published online: 24 July 2015

living organisms (Mullie and Reisse 1987; Pizzarello and Shock 2010). Consequently, their probable presence on the early Earth raises the issue of peptide elongation as one of the major directions of investigation in prebiotic chemistry (Brack 1987, 1993, 2007; Pascal et al. 2005; Lambert 2008; Danger et al. 2012b). Oligopeptides can be formed under close to equilibrium conditions by dehydration either in the presence of catalysts (Rode 1999; Rode et al. 2007; Jakschitz and Rode 2012) or at high temperature (Yanagawa and Kobayashi 1992; Imai et al. 1999; Kawamura et al. 2005; Cleaves et al. 2009) in order to facilitate peptide bond formation. An additional question ensuing from their abiotic formation is that of the potential role that the peptides could have played in the origin of life process. The ones that bear functional side-chain groups could be involved in catalytic activities or association with other components by hydrogen bonding or covalent interactions but any property resulting from the formation of tri-dimensional structures required much longer sequences (Barbier and Brack 1987; Luisi 2007). Moreover, the stability of peptide bonds is limited, as shown by apparent equilibrium constant values of c.a. 0.1 M^{-1} assigned to the binding of two peptide segments (Dobry et al. 1952). Therefore, the probability of forming peptides drops rapidly as length increases (Cleaves et al. 2009), so that the polymerization degree required for tertiary structure formation can only be reached with the assistance of activating agents or from activated α-amino acid monomers. In addition to non-favourable polymerization thermodynamics, the course of peptide elongation is hindered by side-reactions such as the formation of diketopiperazines (DKP) inducing a termination at the dipeptide stage (Brack et al. 1976; Rode 1999; Kawamura et al. 2009). However, diketopiperazines have also been proposed as intermediates of peptide polymerization at high temperature (Nagayama et al. 1990; Takaoka et al. 1991; Lambert 2008; Kawamura et al. 2005; Cleaves et al. 2009; Plasson et al. 2011), though, at least in part in this case, the length of peptides formed would be limited by thermodynamics. Additionally, the major pathway of cleavage of peptides occurs through cyclisation at the N-terminus yielding diketopiperazines (Steinberg and Bada 1983; Bujdak and Rode 2004; Danger et al. 2010).

All the data obtained through prebiotic chemistry investigations are consistent with information gathered by peptide chemists. The polymerization of α-amino acid esters in the presence of base (Greenstein and Winitz 1961) usually leads to the formation of a cyclic peptide called 2,5-diketopiperazine. The most straightforward path to these cyclic peptides involves heating a solution of dipeptide esters (Scheme 1). These cyclic peptides were considered as a drawback for the polymerization of α-amino acids in aqueous solution (Pascual et al. 2003) or as a factor of instability of peptides (Sepetov et al. 1991: Kertscher et al. 1993). A considerable amount of work has been dedicated to the diketopiperazine side-reaction occurring in peptide synthesis at the dipeptide stage because the C-terminal ester protection or the linkage to a solid support through an ester bond can be cleaved by cyclization to a significant extent (Gisin and Merrifield 1972; Nishino et al. 1992). An important parameter for the reaction is the prerequisite for a conversion of the usually largely predominant *trans* isomer of the peptide bond into a *cis* isomer



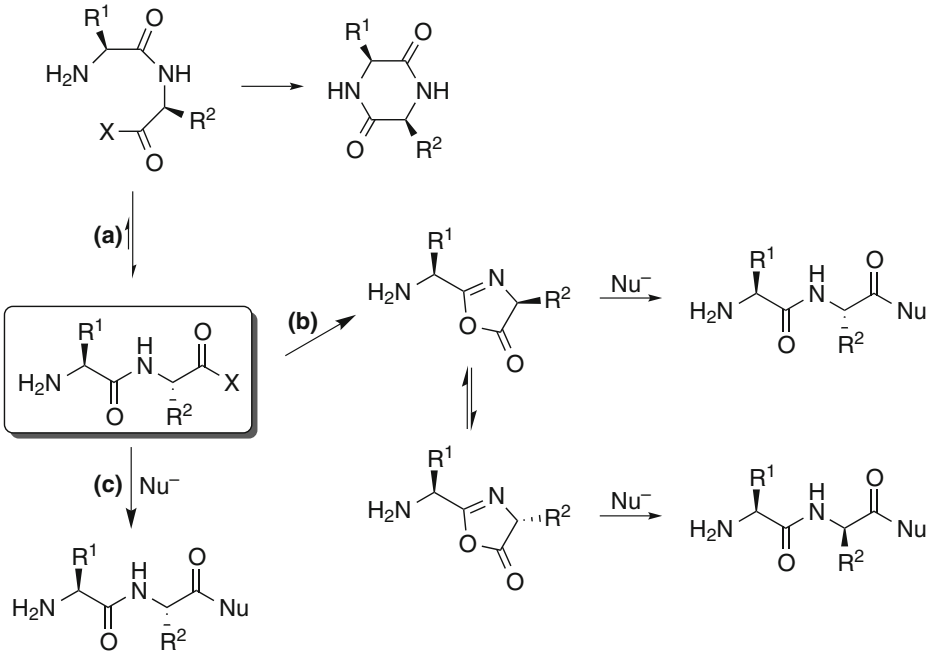
Scheme 1 Diketopiperazine formation via cyclization of dipeptide esters. A change in the stereochemistry of the prevailing *trans*-amide bond is required for reaction

(Fischer 2003), which is, on the contrary, favoured by alkylation of the peptide bond of the dipeptide (as with an N-terminal proline residue). Specific procedures involving a replacement of the alkyl ester moieties with hindered groups or alternative structures have been introduced to suppress this side-reaction (Akaji et al. 1990; Chao et al. 1994; Kochansky and Wagner 1992; Sola et al. 1996; Anne et al. 1998; Chiva et al. 1999; Gothe et al. 1999; Lundquist and Pelletier 2001; Sakamoto et al. 2002). On the other hand, a facilitated cleavage into diketopiperazines can constitute an advantage as for example by allowing the release of specific derivatives from a solid support under mild conditions (Burgess et al. 1997; Hulme et al. 1998; Orain and Bradley 2001; Chitku et al. 2001).

Our group has recently been involved in the identification of 5(4*H*)-oxazolones as intermediates of importance in prebiotic chemistry. They are formed upon the activation of the C-terminus in peptides using carbodiimides, such as EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), as strong activating agents or alternative reagents of prebiotic relevance such as cyanamide (Danger et al. 2013). This process is specific of acylated amino acids or more generally of the C-terminal residue of peptide chains so that the formation of 5(4*H*)-oxazolones strongly increases the rates of the reaction compared with related reactants lacking an acylamino group in the close proximity of the carboxyl group. We considered therefore that free dipeptides represent a specific case regarding this kind of activation since both the neighbouring oxygen of the amide group and the terminal amine constitute nucleophiles located at a favourable position for a fast intramolecular reaction (Kirby 1980). Though no explanation was given to these observations, the group of Luisi indeed observed that the activation of hydrophobic di-tryptophan in the presence of zwitterionic POPC (POPC=1-palmitoyl-2-oleoylphosphatidylcholine) vesicles (Blocher et al. 1999, 2000) or positively charged Arg-Trp in the presence of negatively charged mixed DOPA/POPC (DOPA=1,2-dioleyl phosphatidic acid) vesicles (Blocher et al. 2000; Hitz and Luisi 2000) yielded tetramers and higher oligomers rather than diketopiperazines that were marginally formed. We decided therefore to investigate the possibility that the pathway involving 5(4*H*)-oxazolones as intermediates could prevent DKP formation (Scheme 2). We suspected this issue to be related to the relative kinetic rates of **(a)** the *cis-trans* isomerization of the peptide bond required for the cyclization into DKP, **(b)** the cyclization into a 5(4*H*)-oxazolone prone to a fast epimerization preceding the nucleophillic attack, and **(c)** the direct attack of nucleophiles on the activated free dipeptide. This analysis was based on a prediction that any transformation of the 5(4*H*)-oxazolone activated species into DKP should be prevented by highly strained transition states and tetrahedral intermediates (Scheme 3). We report here the first results of these investigations confirming the efficiency of the peptide elongation pathway starting from dipeptides via 5(4*H*)-oxazolone and emphasizing the need of strong activating agents for facilitating the abiotic formation of long peptides by activation at the C-terminus.

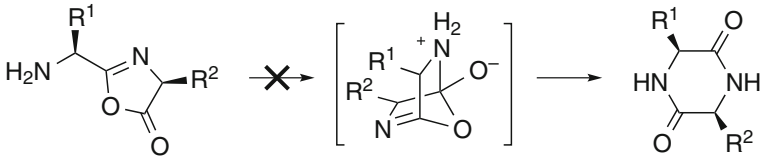
Methods

Reagents and solvents were purchased from Bachem, Aldrich and Acros and were used without further purification. NMR spectra (DMSO-d_6, $CDCl_3$ or D_2O solution) were recorded on a Bruker Avance 300 spectrometer at 300 MHz and 75.48 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts of solvents (DMSO: δ_H=2.54 ppm, δ_C=40.45 ppm; H_2O, δ_H= 4.79 ppm) served as internal reference. HPLC analyses were carried out on a Waters Alliance system including a 2690 separation module and a 996 photodiode array detector; Column



Scheme 2 The different pathways available for the reaction of free dipeptides activated by an electron withdrawing group X in the presence of nucleophiles: **a** side-reaction yielding diketopiperazines through the relatively slow and unfavourable isomerization into a *cis*-peptide bond; **b** cyclization into 5(4*H*)-oxazolone with a rate strongly depending of the X^- leaving group ability; **c** direct reaction with the nucleophile

Thermo Scientific BDS Hypersil C18 (3 μm, 2.1×50 mm); Method A: gradient of solvent A: 0.1 % TFA in H_2O and solvent B: 0.1 % TFA in CH_3CN; flow 0.2 mL/min, (starting from 5 % B to 15 % over 15 min, then 15 % B to 60 % over 10 min and finally 60 % B to 100 % in 1 min; UV detection at 273 nm; High-resolution mass spectra (HRMS) were recorded using a Waters Q-tof mass spectrometer using negative ion (ESI^-) or positive ion (ESI^+) electrospray ionisation mode. HPLC-ESI-MS analyses were carried out on a Waters Synapt G2-S system connected to a Waters Acquity UPLC H-Class apparatus equipped with an Acquity UPLC BEH C18, 1.7 μm 2.1×50 mm column; Method B: gradient of solvent A 0.01 % formic acid in H_2O and solvent B: 0.01 % formic acid in acetonitrile; flow rate: 0.5 mL/min; linear gradient 0 to 100 % B over 3 min. The starting materials and authentic samples of the peptide products needed for an identification purpose were prepared using standard methods of solution phase synthesis (Supplementary Material).



Scheme 3 Any direct conversion of dipeptide 5(4*H*)-oxazolones into DKP should be prevented by the strain of bicyclic tetrahedral intermediates involving a bridgehead carbon with sp^2 hybridisation

Results and Discussion

The model dipeptide H-Ala-Tyr(Me)-OH (L,L-**1**, 1 mM) was activated in 2-(*N*-morpholino)ethanesulphonic acid (MES) buffers at pH 5.5 in the presence of 1 mM EDC (Fig. 1). The moderately acidic pH value selected was earlier observed to correspond to a kinetic optimum for the reaction of EDC (Beaufils et al. 2014), was observed to limit the formation of side-products, and may be representative of aqueous prebiotic media at equilibrium with a CO_2-rich atmosphere. The reaction was monitored by HPLC (method A) using a procedure identical to that previously used for acetylated dipeptides Ac-Ala-Tyr(Me)-OH unable to give a diketopiperazine (Beaufils et al. 2014). Peaks corresponding to the two stereoisomers of DKP **2** (retention time=9.2 min and 10.5 min for authentic samples of heterochiral and homochiral forms, respectively) could be observed in almost undetectable amount (≤0.5 %). Instead of the stereoisomers of DKP **2**, the starting dipeptide L,L-**1** (retention time=11.9 min) yielded the stereoisomer L,D-**1** (retention time=15.4 min) produced by epimerisation. In addition to starting L,L-**1** and epimer L,D-**1**, minor amounts of a series of more hydrophobic products were also formed with retention times in the range 23–26 min. The presence of L,L-**1** and L,D-**1** was confirmed by HPLC-MS (*m/z*=267.13, ESI positive mode, method B) and peaks corresponding to the mass of the tetrapeptide H-(Ala-Tyr(Me))$_2$-OH (*m/z*=515.25) and its stereoisomers (representing overall 9.0 % of the absorption of tyrosine-containing products) were identified among the products with a longer HPLC retention time. Considering the 1 mM concentrations of dipeptide reactant and EDC in aqueous solution, the formation of non-negligible amounts of oligomers provides evidence for the efficiency of this

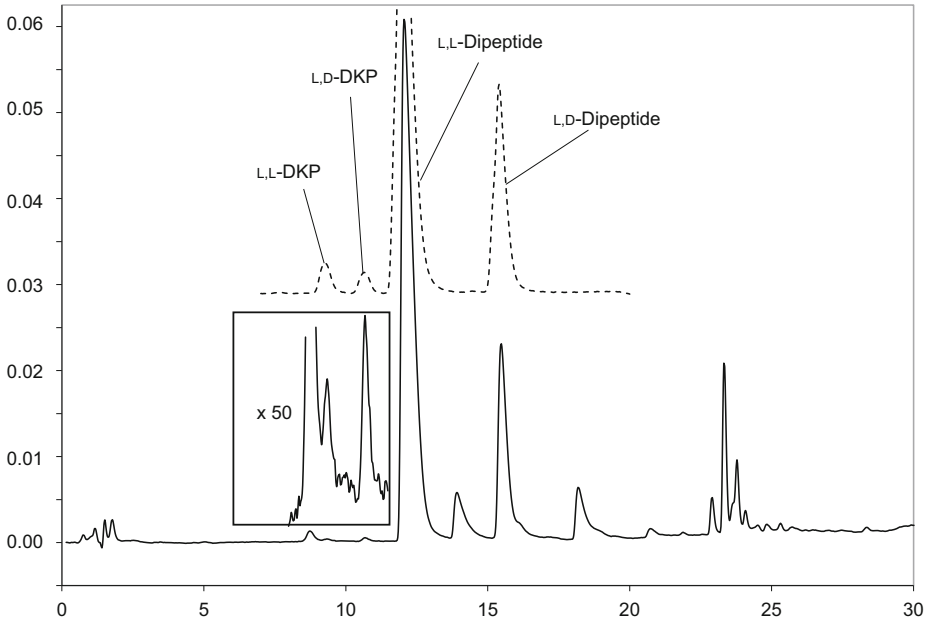
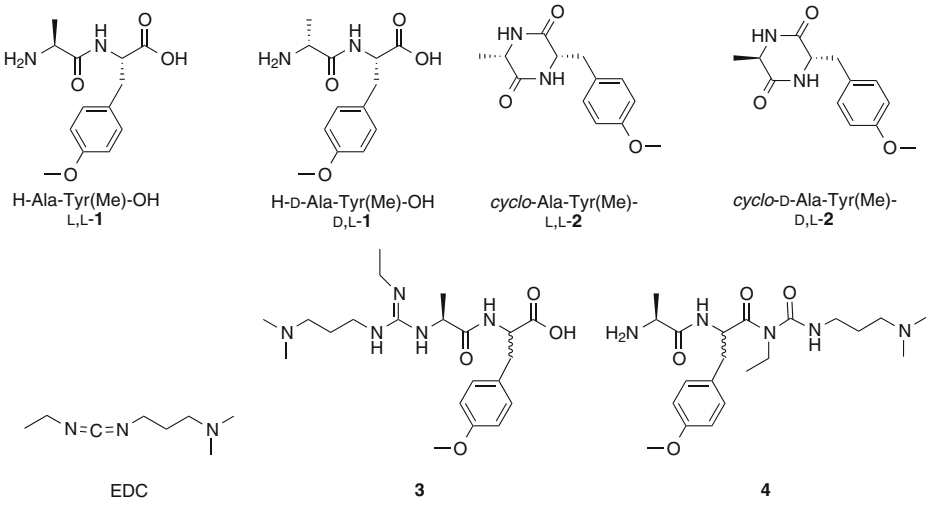


Fig. 1 Activation of H-Ala-Tyr(Me)-OH (1 mM) by EDC (1 equiv.) at room temperature in a pH 5.5 MES buffer (100 mM). Solid line: HPLC trace of an analysis of the reaction medium after 96 h of reaction (Method A, UV detection at 273 nm); the inset displays a 50-fold scale expansion of the UV absorbance of the peaks corresponding to L,L- and L,D-DKP; dotted line: HPLC trace corresponding to the injection of a mixture of pure dipeptides and DKPs

coupling method. Longer oligomers were not detected probably because of their low yield and possibly limited solubility. Additionally, EDC adducts of the dipeptide (retention time= 18.0 min, *m/z*=422.28) matching with the structure of either guanidinium **3** or *N*-acylurea **4** and possible subsequent degradation products were also observed in these reactions performed with 1 mM EDC. The fact that such side-products were not observed in earlier experiments starting from Ac-Ala-Tyr(Me)-OH, a substrate having an amino group protected by acetylation, strongly supports the formation of guanidinium derivative **3**. The final ratio of dipeptide diastereomers reached a value of 78/22. Owing to the extent of side-reactions further additions of EDC were not carried out to determine the final steady state ratio. The value of diastereomeric ratio (d.r.), reached at the steady state, might however not be very different from the 58/42 value in favour of the homochiral dipeptide observed earlier for the acetylated derivative Ac-Tyr(Me)-Ala-OH (Beaufils et al. 2014). We can therefore conclude that free dipeptides do not behave very differently from acylated derivatives (with the noticeable exception of the occurrence of side-reactions of the amino group) and that activation proceeds to a significant extent through the 5(4*H*)-oxazolone intermediate. This result confirms the assumption made in the introduction that the transient formation of this intermediate in the EDC-promoted reaction suppresses DKP formation during the reaction of dipeptides having a free amino group (Scheme 3). Similar results were obtained by replacing the starting material by its stereoisomer H-D-Ala-Tyr(Me)-OH (D,L-**1**) (Scheme 4).

To get additional information on the extent of epimerization during the activation step, we analysed the d.r. of peptide products formed through the reaction of 1 mM dipeptide **1** with 10 mM glycinamide as a model of achiral nucleophile in the presence of 1 equiv. of EDC in 100 mM MES buffers (pH 5.5). Since the products of the reaction (retention time 8.4 and 11.6 min for stereoisomers H-Ala-Tyr(Me)-Gly-NH_2 and H-Ala-D-Tyr(Me)-Gly-NH_2, respectively) can be monitored independently of the unreacted dipeptide L,L-**1** (11.8 min) and its isomer L,D-**1** (15.5 min), the stereochemical outcome of activation could be analysed independently of the presence of unreacted substrate. A d.r. value of 68/32 could be measured in favour of the homochiral stereoisomer of the tripeptide product after 4 days of reaction (Fig. 2).



Scheme 4 Structures of dipeptide (L,L-**1** and D,L-**1**), DKP (L,L-**2** and D,L-**2**) isomers, of EDC and of hypothesized dipeptide **1** EDC adducts **3** and **4**

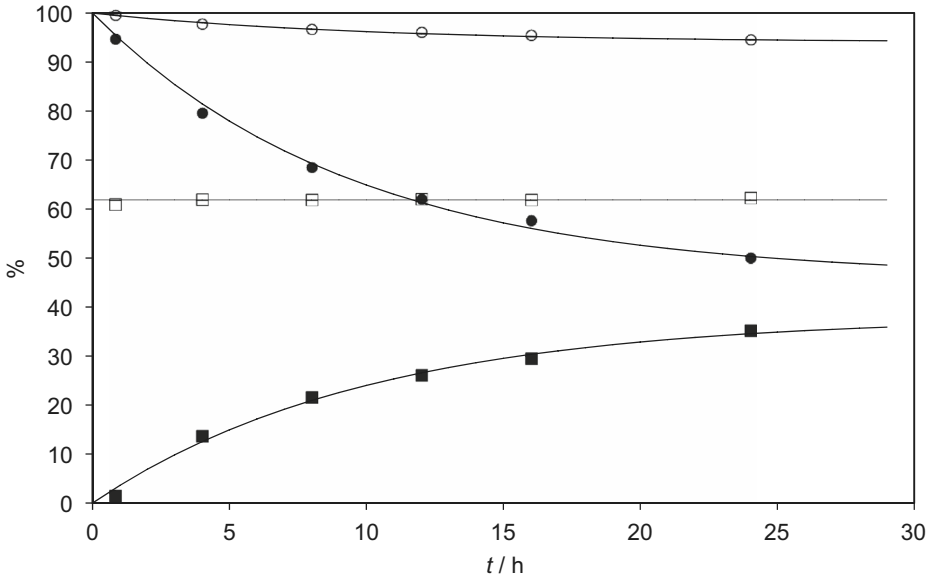


Fig. 2 Reaction of 1 mM H-Ala-Tyr(Me)-OH L,L-**1** with 10 mM H-Gly-NH_2 and 1 mM EDC in a pH 5.5 MES buffer (100 mM). Total unreacted dipeptide (L,L-**1**+L,D-**1**) measured as % of the total area of peaks at 273 nm (filled circles); diastereomeric composition (as the content of L,L-**1** diastereomer in dipeptide **1**) in the remaining starting material (open circles); total yield (filled squares) and diastereomeric composition (open squares as % H-Ala-Tyr(Me)-Gly-OH) in the diastereoisomeric mixture of the tripeptides

At the same time the epimerization of the unreacted material L,L-**1** remained limited to a value of 4 %. A similar experiment was carried out starting from the D,L-**1** isomer leading to d.r. values (homochiral/heterochiral) of 47/53 and 3/97 in the product and starting material, respectively. These experiments demonstrate that the epimerization of the C-terminal residue is the result of the activation process, which is likely to involve a 5(4*H*)-oxazolone intermediate in the same way as the acylated dipeptides. As a matter of fact, experiments carried out previously (Beaufils et al. 2014) starting from Ac-Tyr(Me)-Ala-OH and Ac-Tyr(Me)-D-Ala-OH resulted in d.r. values of 55:45 and 47:53, respectively. The similarity of these data suggests that the presence of a free amino group has no consequence on the stereochemistry in the product. This conclusion is also consistent with the results of the reactions of H-Ala-NH_2 and H-D-Ala-NH_2 (10 mM) with the two free dipeptides (L,L-**1** and D,L-**1**), which were additionally carried out in order to determine the influence of the chirality of the nucleophile on the configuration of the Tyr(Me) residue in the product. In these two experiments the homochiral configuration of the Tyr(Me) and C-terminal Ala residues was preferred with a d.r. of ca. 65/35. Attempts to perform the reactions at pH 6.5 led to a lower degree of conversion into tripeptides and slower reaction rates.

The investigations on the activation of free dipeptides by EDC demonstrate that 5(4*H*)-oxazolone formation prevents the cyclisation into DKP in spite of the fact that the N-terminal amine of the dipeptide (pK_a~8.14 for H-Ala-Ala-OH) (Perrin 1965) constitutes a much more efficient nucleophile than the amide oxygen (pK_A~0) (Olah et al. 1970). Although the formation of a five-membered ring may be easier than that of a six-membered one, the difference is unlikely to compensate for a change in pK_A of 8 units. The prevailing *trans* configuration of the amide bond and the kinetic barrier to rotation therefore constitute

reasonable explanations of the behaviour observed during the activation of dipeptides with EDC. The possibility of activating dipeptides with EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) within phospholipid bilayers, demonstrated by the group of Luisi (Blocher et al. 1999, 2000; Hitz and Luisi 2000), prompted us to undertake an analysis of the outcome of the use of other reagents capable of activating the free dipeptide substrate or directly of that of pre-activated forms (Table 1). In addition to being precursors of mixed anhydrides, reagents such as EEDQ or IBCF (*iso*-butyl chloroformate) are expected to yield urethanes owing to the nucleophilic character of the N-terminal amino group (pK_a~8). The reaction of the carboxyl group (pK_A~4) can however be favoured at slightly acidic pH values ensuring a protonation of the amino group. Experiments were carried out at pH 5.5 from the dipeptide L,L-**1** (Table 1). Although urethane formation was generally prevailing, significant degrees of epimerization were observed in most cases, which is indicative of the transient formation of a 5(4*H*)-oxazolone. EEDQ and IBCF are usual peptide synthesis reagents promoting peptide coupling via mixed anhydrides (Joullié and Lassen 2010), we present the results about the extent of epimerization and the yield of DKP using EDC and other reagents (Table 1). EEDQ and IBCF led to both activation (epimerisation) and amine reaction. The products of the reactions were identified by their retention time by HPLC (method A) as unchanged dipeptide L,L-**1** (11.8 min), the stereoisomer L,D-**1** (15.5 min) and the urethane by-products (22.4 and 25.0 min for the ethyl and isobutyl derivatives respectively). These attributions were confirmed by HPLC-ESI-MS (positive mode *m/z* 339.16 and 367.19 for $[M+H^+]$, respectively). By contrast, no epimerization was observed using cyanate as a potrential activating agent and the only product was the *N*-carbamoylated product (14.1 min, ESI-MS positive mode *m/z* 332.12 $[M+Na^+]$). This observation confirms the absence of reactivity of this reagent for the activation of protected peptides except C-terminal aspartyl peptides (Danger et al. 2012a). As regards pre-activated substrates, the reactivity of the methyl ester was sluggish at room temperature and yielded mostly the hydrolysis product. The more reactive phenyl ester yielded mainly DKP and hydrolysis products through a direct reaction (the ratio of DKP to dipeptide L,L-**1** was

Table 1 Formation of diketopiperazine from activated forms of the dipeptide L,L-**1**, either prepared in situ using activating agents or independently synthesized. Reactions carried out in 100 mM MES buffer (pH 5.5) at 20 °C

Reagent or activating agent[a]	Conversion extent / Reaction time	Yield of urethane	Yield of dipeptide	d.r. of dipeptide	Yield of DKP	d.r. of DKP
EDC	41 % / 96 h	5 %	n.a.[b]	78/22	≤0.5	7/3
EEDQ	29.5 % / 24 h	40 %	n.a.	98/2	n.d.[c]	n.a.
IBCF	14.5 % / 2 h	8.5 %	n.a.	98/2	n.d.	n.a.
KOCN / 5 mM	42 % / 120 h	42%[d]	n.a.	100/0	n.d.	n.a.
Methyl ester	4 % / 15 d	n.a.	4 %	100/0	<0.5 %	n.a.
Phenyl ester	99.5 % / 7 d	n.a.	7.7 %	100/0	91.8	100/0
Methyl phosphate mixed anhydride	100 % / 2 h	n.a.	≤5 %	n.a.[e]	≥95 %	n.a.[e]

[a] Concentration 1 mM unless otherwise mentioned

[b] Not applicable

[c] Not detected

[d] *N*-carbamoyl derivative

[e] unknown d.e. of starting material

independent of the reaction time). Lastly, a high yield in DKP was obtained from the mixed anhydride with methyl phosphate, which is relevant to any kind of polymerisation of α-amino acids mixed anhydrides such as aminoacyl adenylates. It definitely disqualify this process as a possible pathway for prebiotic peptide formation in addition to the observation of a fast CO_2-promoted conversion of aminoacyl adenylates into *N*-carboxyanhydrides (NCAs) preceding any possibility of polymerization (Liu et al. 2014).

The results presented in Table 1 show that the final yield in DKP is strongly dependent on the lifetime of the activated form of the dipeptide adduct. DKP was almost not detectable during EDC reaction, whereas it constitutes the major product observed from the phenyl ester or the methyl phosphate mixed anhydride. A rationale for this observation can be proposed by considering the degree of activation of the intermediate as a factor reducing the occurrence of the DKP reaction because it facilitates the 5(4*H*)-oxazolone pathway (Scheme 1, path **(b)**), whereas having a minor influence on the *cis-trans* isomerisation of the activated dipeptide.

Conclusion

The need of activating agents for promoting peptide formation has usually been considered in relation with the formation of oligomers exceeding lengths of a few residues to overcome the thermodynamic instability of the peptide bond in aqueous solution (Pascal et al. 2005; Brack 2007; Cleaves et al. 2009). The results presented here show that a further beneficial consequence of strong activation lies in the possibility of avoiding the termination of elongation at the dipeptide stage. The formation of diketopiperazine side-product is actually prevented by strain present in the bicyclic transition states and tetrahedral intermediates of the postulated pathway (Scheme 3). The limitation to the synthesis of chirally non-homogeneous polymers by the concomitant drawback of inducing epimerization at the C-terminal residue should be considered as unimportant regarding abiotic pathways starting from racemic mixtures. Within the context of abiotic peptide formation, a change of configuration of residues can on the contrary open the possibility of facilitating homochiral domain formation inducing a proper secondary structure. The stereoselectivity observed for the process (Beaufils et al. 2014) gives some support to this claim. The suppression of DKP formation constitutes another evidence in favour of the requirement for strong activating agents in the origin of life process. It is consistent with the requirement for free-energy-rich (and consequently low-entropy) energy carriers and gives support to the previously made analysis that any self-organizing system involving autocatalysis or replication processes (Pross 2009, 2012; Pross and Pascal 2013) must receive a supply from physical sources of energy with a free energy potential value close to 150 kJ per mole (Pascal and Boiteau 2011; Pascal 2012; Pascal et al. 2013). It also strengthens the need of efficient activation processes able to couple physical sources of energy with a peptide protometabolism (Danger et al. 2012b). The use of cyanamide as an activating agent (Danger et al. 2013) corresponds to this definition but it leads to slow kinetic rates. Other processes, such as the oxidation of thioacids (Hagan 2010) could be considered but no general activation process is widely accepted yet and this perspective should still constitute a major target of origins of life research.

Acknowledgments This work was supported by the Agence Nationale de la Recherche to the PeptiSystems project (grant ANR-14-CE33-0020) and by the Simons Foundation (Post-doctoral grant number 293065 to ZL). The authors benefited of fruitful scientific exchanges with European colleagues within the COST actions CM1304 "Emergence and Evolution of Complex Chemical Systems" and TD1308 "Life-Origins". We thank Cathy Carvalhosa for experimental assistance during the realisation of this work.

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