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Acceleration of amino acid racemization by isovaline: possible implications for homochirality and biosignature search

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Research Article

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Abstract

In nature, abiotically formed amino acids are usually racemic. However, this is not true for the α,α-dialkyl amino acid isovaline (Iva), which has an L-enantiomeric excess in some specimens of carbonaceous meteorites. On the early Earth and Mars, such meteorites were sources of amino acids, including Iva. Therefore, a connection may exist between the possible chiral influence of non-racemic Iva and the origin of biological homochirality. On the surface of a young terrestrial planet, amino acids can be chemically altered in many ways. For example, high temperatures from geothermal heating can lead to racemization. Four billion years ago, active volcanism and volcanic islands provided suitable conditions for such reactions and perhaps even for early microbial life on Earth. In the current study, we investigated the influence of D- and L-Iva on the thermal racemization of L-alanine (L-Ala) and L-2-aminobutyric acid (L-Abu) in a simulated hot volcanic environment. The amino acids were intercalated in the clay mineral calcium montmorillonite (SAz-1). While Iva was resistant to racemization, partial racemization was observed for Ala and Abu after 8 weeks at 150°C. The experimental results – for example, accelerated racemization in the presence of Iva and different influences of the Iva enantiomers – suggest that the amino acid molecules interacted with each other, possibly in hydrogen-bonded dimers. Accelerated racemization of amino acids could have been an obstacle to the development of homochirality. Besides, it is also detrimental to the use of homochirality as a biosignature, for example, in the search for microbial life on Mars.

Introduction

Almost 90 different amino acids of abiotic origin have been detected in the Murchison meteorite, which is one of the best studied carbonaceous chondrites (Meierhenrich, 2008). Among them, there are many α,α-dialkyl amino acids, such as the chiral isovaline (2-amino-2-methylbutanoic acid or 2-ethylalanine, Iva; Fig. 1). In contrast to α-H-α-amino acids (e.g. alanine (Ala); Fig. 1), α,α-dialkyl amino acids are remarkably resistant to chemical and thermal racemization (Fischer and von Grävenitz, 1914). This may explain why significant L-Iva enantiomeric excesses (L-ee) of up to 18.5% (Glavin and Dworkin, 2009) have survived in meteorites, whereas Ala and other α-H-α-amino acids are racemic. Small L-ee values of Iva and other α,α-dialkyl amino acids may have resulted from asymmetric photolysis of the initially racemic amino acids or their precursors by circularly polarized UV light (UV CPL) in the presolar nebula (Bailey *et al.*, 1998). Laboratory experiments with UV CPL confirmed that such an asymmetric photodecomposition can produce ees of up to 2.5% (Flores *et al.*, 1977). However, this is not sufficient to explain the L-ee values measured in meteorites. Therefore, it is assumed that additional unknown amplification processes occurred in asteroids, which are the parent bodies of the meteorites (Pizzarello *et al.*, 2003; Glavin and Dworkin, 2009). Indeed, much of the chemical composition of meteorites resulted from chemical and physical processes in the asteroids during an early stage, which is known as the aqueous alteration phase (MacDougall *et al.*, 1984; McSween *et al.*, 2002).

One thought that comes to mind in the context of aqueous alteration is asymmetric catalysis by slightly enantioenriched compounds. Pizzarello and Weber (2004, 2010) experimentally demonstrated that L-amino acids and LL-dipeptides, when present during carbohydrate synthesis (formose reaction), caused ees in the products. However, a large part of the initial asymmetric information of amino acids and peptides was lost. Thus, simple catalytic reactions were probably not responsible for enantiomeric amplification. However, the situation can be different when a chiral compound catalyses its own formation. Here, asymmetric autocatalysis can amplify ees. This is observed in the so-called Soai reaction, which is the only known chemical process in which a slight initial ee is continuously amplified until a high enantiomeric enrichment is reached (Soai *et al.*, 1995; Soai and Kawasaki, 2008). The basis of this exceptional behaviour appears to be the formation of dimers that act as catalysts (Ercolani and

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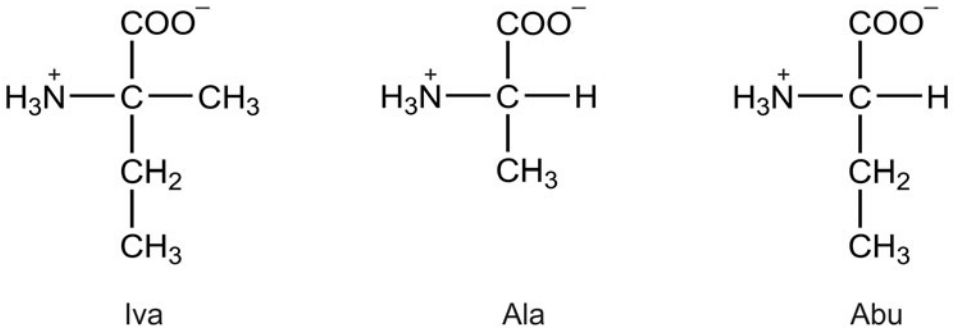

Fig. 1. Structures of the amino acids used in this study: Iva (2-amino-2-methylbutanoic acid), Ala (2-aminopropanoic acid) and Abu (2-aminobutyric acid). The L-enantiomers are shown.

Schiaffino, 2011). Though the Soai reaction itself is not directly relevant to the processes in asteroids, it nevertheless demonstrates that such mechanisms exist, and therefore other amplification reactions may be waiting to be discovered.

Howsoever the L-ee of meteoritic Iva originated, it is plausible that it reached the early Earth and became involved in prebiotic chemistry. Therefore, some assume that enantioenriched extraterrestrial compounds such as Iva could have triggered the evolution of biological homochirality, which is an essential feature of life as we know it (Barron, 2008). On the early Earth, rock pools on active volcanic islands could have been favourable locations for the formation of and interaction between prebiotic organic molecules (Fox and Strasdeit, 2013; Fox *et al.*, 2018; Pleyer *et al.*, 2018). Before permanent continents existed, volcanic islands were the only dry land, which probably allowed large amounts of organic compounds to accumulate in high concentrations. In contrast to this, the open ocean and the deep sea had a diluting effect. Volcanic islands also provided different types of gradients (e.g. temperature and pH) that could have driven chemical reactions. Moreover, minerals such as the phyllosilicate montmorillonite – a typical weathering product of basaltic material – could have affected the behaviour of organic molecules. For example, it is known that the intercalation of amino acids into phyllosilicates largely prevents the sublimation, which occurs for some amino acids at elevated temperatures (Dalai *et al.*, 2017).

Against this background, the experiments in the current paper were designed to simulate a geothermally heated rock pool that contained amino acids in montmorillonite. For this, a special apparatus was used, which allowed heating of samples under an inert atmosphere. The aim was to study the thermal racemization of α-H-α-amino acids in the presence of L- or D-Iva under intercalation conditions. Racemization is not only relevant to prebiotic chemistry, but also to the search for extraterrestrial life (e.g. on Mars), because it destroys enantiopurity. This is important since enantiopurity (i.e. 100% ee) is regarded as a strong biosignature, whereas the relatively small ees found in meteorites definitely do not indicate a biological origin (Fox and Strasdeit, 2017). So far, naturally occurring processes that generate enantiopurity without biological influence are unknown. Only in laboratory experiments, under special conditions, small ees were non-biologically amplified to near enantiopurity (>99% ee). This was achieved by asymmetric autocatalysis (see above; Soai *et al.*, 2018) or physical processes (Klussmann, 2012).

It is also worth mentioning that in the search for life, the absence of enantiopurity can be a false-negative observation. The reason is that the compound studied could have initially been enantiopure but has (partially) racemized. Racemization, especially of proteinogenic L-amino acids, is a well-known process (Bada and Schroeder, 1975; Bada 1985). In the current racemization study, experiments were performed in which a racemizable amino acid – either Ala or its next higher homologue 2-aminobutyric acid (Abu) (Fig. 1) – and the 'non-racemizable' Iva were employed simultaneously. The amino acids were immobilized in the interlayer space of montmorillonite. We focused on how the amino acid concentrations and the enantiomers of Iva affected the rates of racemization of the L-forms of Ala and Abu. In order to be able to see small effects, high initial ees were used.

Results and discussion

Non-racemization of Iva; chiral separation of amino acid derivatives and their mass-spectrometric analysis

Chiral analysis of the L- and D-forms of Iva showed that there were small but detectable amounts of the opposite enantiomer present before the heating experiments were started. However, the initial L to D ratios did not change during 8 weeks of heating at 150°C. In other words, there was no measurable racemization. Figure 2 shows the situation for L-Iva. This observation corresponds to the early findings of Fischer and von Grävenitz (1914) who studied Iva in aqueous solutions but not as an intercalate in a mineral matrix. Iva racemizes only under very harsh conditions, namely γ-irradiation, whereby considerable decomposition occurs (Bonner *et al.*, 1979). In our study, resistance to racemization of Iva ensured that throughout an entire experiment, the influence of the minor enantiomer was negligible because of its constant low amount.

In order to be able to prove by gas chromatography-mass spectrometry (GC-MS) that Iva did not racemize under our experimental conditions, baseline separation of the enantiomers was necessary. However, the enantiomeric separation of α,α-dialkyl-α-amino acids, such as Iva, is often challenging (see e.g. Fox *et al.*, 2015). Complete separation of the Iva enantiomers was achieved with the TFA methyl esters on a Lipodex E column. The same conditions allowed the baseline separation of the Ala and Abu enantiomers, which was very important because determining the development over time of the L to D ratios of these amino acids was central to this study. The separations achieved are exemplified in Fig. 2 for Iva and Ala. It can be seen that the peaks were nicely separated, including the pair D-Iva–D-Ala. The latter was particularly important in experiments where the formation of D-Ala from L-alanine (L-Ala) was quantified in the presence of enantiopure D-Iva.

When amino acids are heated at 150°C (the temperature used in this study), the possibility of decomposition and sublimation must be considered. These processes can reduce the amount of

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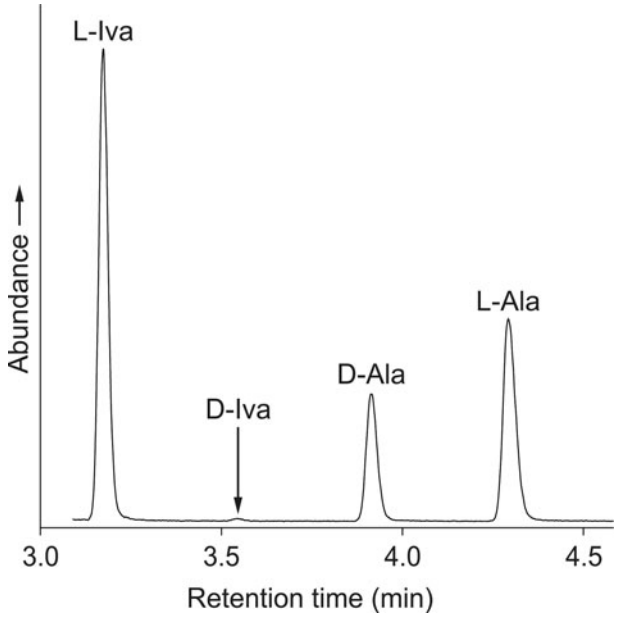

Fig. 2. Typical GC-MS chromatogram (total ion current) showing the baseline separation of the *N*-TFA-*O*-methyl esters of the Ala and Iva enantiomers on a Lipodex E column. The sample was obtained by heating the almost pure L-enantiomers in SAz-1 for 8 weeks at 150°C. Note the strong peak of D-Ala due to the racemization reaction.

amino acid present in the samples. If after 8 weeks only small amounts remained, the relevance of the racemization reactions studied here would be questionable from the outset. However, quantification by high-performance liquid chromatography (HPLC) showed that in all cases at least 67% of the initial amount were still present at the end of the experiment. Thus, the loss of material was not dramatic. Nevertheless, decomposition was an issue with the GC-MS measurements. This becomes evident when comparing the integration results for Ala obtained from the total ion current and the single ion current of the main fragment (Fig. 3). As can be clearly seen, the values from the total ion current are systematically lower and show substantially higher scattering, very probably because of interference from decomposition products. Therefore, instead of the total ion current, the ion currents of the major fragment ions of the TFA methyl esters were used to determine the enantiomeric ratios.

Partial racemization of L-Ala during loading into SAz-1

The commercial L-Ala used as a starting material had an ee of almost 100%, i.e. it was virtually enantiopure. After loading into montmorillonite SAz-1, however, the L-ee had decreased by a few percent (Figs 4(a) and 5). This effect was particularly pronounced when the mineral was loaded with only 0.5% Ala. In this case, the L-ee dropped to 86.7%. The values were between 92.7 and 95.1%, when Iva was additionally present (Fig. 5). Obviously, some slight racemization occurred during the loading process in which the amino acid–mineral suspension was repeatedly dried at 35°C for a short time. After the first drying, a layer of non-intercalated amino acid was clearly visible on the wall of the container. This material was washed back into the mineral with a small volume of water. Then the suspension was dried again. This procedure was repeated several times until the amino acid layer no longer occurred.

The relatively fast (partial) racemization of an amino acid at a temperature as low as 35°C might seem surprising. But, it is well known that wet–dry cycles, such as used in the loading process described here, can greatly accelerate chemical reactions (Fox *et al.*, 2018; Pleyer *et al.*, 2018). The presence of Iva apparently reduced the racemization rate of L-Ala to a moderate extent. However, this needs to be confirmed in a separate study because the number of cycles varied in different loading experiments and the heating times were not strictly controlled. In contrast to L-Ala, for L-2-aminobutyric acid (L-Abu) no racemization during the loading was observed with or without Iva (see initial L-ee values in Fig. 4(b)). This too may have resulted, at least in part, from differences in the loading procedures. In the heating experiments, L-Abu in fact showed a lower tendency to racemize compared to L-Ala, but the difference was not very large (Fig. 4).

Concentration dependence of the thermal racemization of intercalated L-Ala

Two samples of SAz-1 containing different concentrations of L-Ala (0.5 and 2.0%) were heated at 150°C for 8 weeks. The initial L-ee values were 86.7 and 96.7%, respectively. At the end of the experiment, the decrease of the ee differed greatly between the two samples. With the lower concentration, an L-ee of 61.0% was found, corresponding to a decrease of 25.7 percentage points. When the concentration was four times higher, the L-ee dropped by 50.5 percentage points to 46.2% (Figs 4(a) and 5(a)). Clearly, accelerated racemization occurred at the higher concentration. This observation could be explained by intermolecular interactions, such as the formation of hydrogen-bonded dimers, which enhance racemization. Alternatively, different binding sites may exist for the amino acid at the inner surfaces of the mineral. In this case, one would have to assume that at one of the binding sites L-Ala is less prone to racemization and that this site is the preferred one. A further assumption would be that this site is relatively rare. Therefore, with increasing concentration, an increasing number of molecules would have to bind to other sites which provide less protection against racemization. Analysis of the molecular basis of the concentration dependence of the racemization rate was beyond the scope of this study. However, the experiments described in the following section support the model of direct interactions between the amino acid molecules.

Accelerated racemization of L-Ala and L-Abu in the presence of L- or D-Iva

In a series of experiments, we studied the influence of different Iva-to-Ala molar ratios (1 : 1, 3 : 1 and 5 : 1) on the racemization of L-Ala. The limited intercalation capacity of SAz-1 made it necessary to reduce the L-Ala concentration from 2 to 0.5%. This ensured that the amino acids were completely intercalated also at high molar ratios. The general result of the experiments is that the racemization rate of L-Ala increases with increasing concentration of Iva. In the case of L-Iva, the final L-ee values of Ala were 47.6% (1 : 1 Iva-to-Ala molar ratio), 41.8% (3 : 1) and 29.9% (5 : 1), respectively (Fig. 5(a)). For comparison, in the absence of Iva, the L-ee of Ala was 61.0% at the end of the experiment. The acceleration effect is less pronounced for the D-form of Iva (Fig. 5(b)). Here, the final L-ee values of Ala were 51.0% (1 : 1), 45.8% (3 : 1) and 34.0% (5 : 1), respectively. The smaller effect of D-Iva compared to L-Iva was also observed with a 1 : 1 molar ratio and 2.0% loading, both for L-Ala and L-Abu (Fig. 4).

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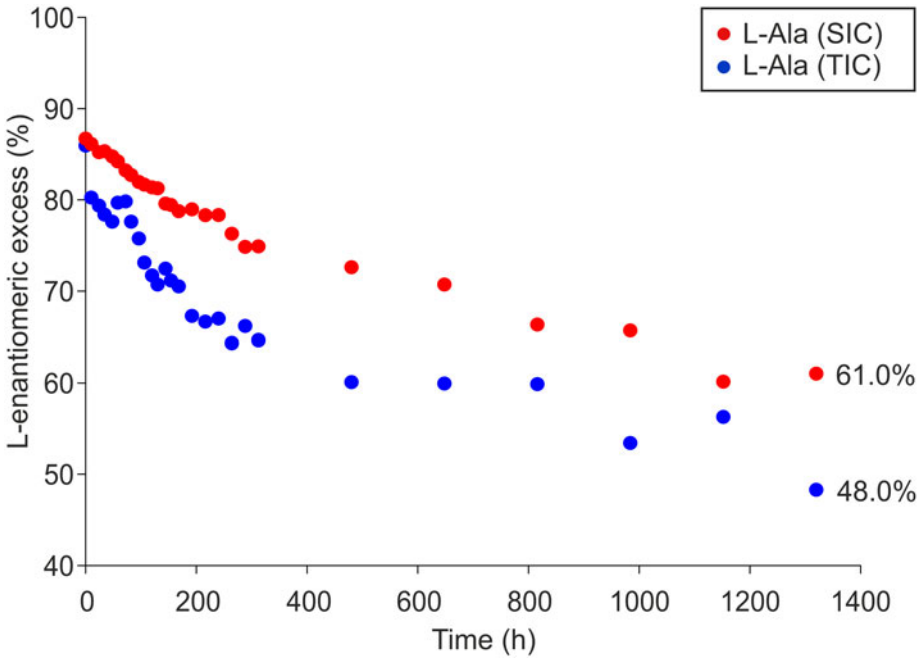

Fig. 3. Decrease of the L-ee of Ala in SAz-1 (0.5% loading) over an 8-week period at 150°C. Blue circles: from total ion current data, red circles: from single ion current data of the main fragment of Ala. The L-ee after 8 weeks is given on the right of each curve.

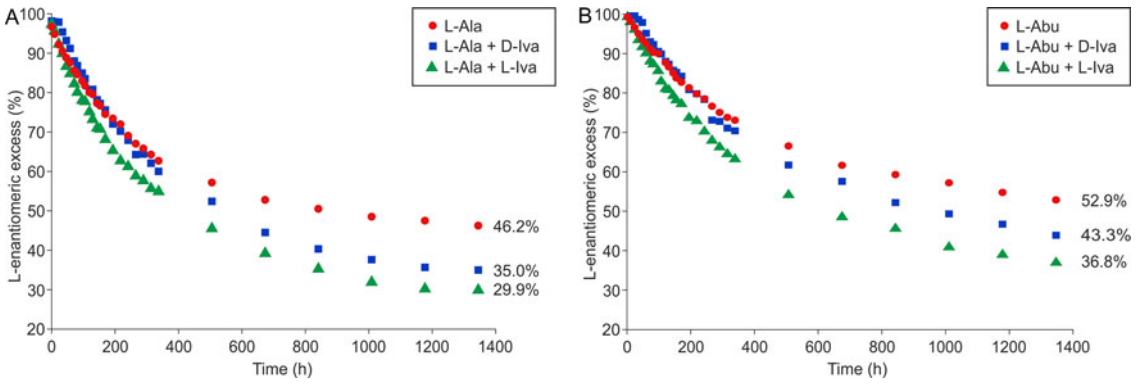

Fig. 4. Decrease of the L-ee of (A) Ala and (B) Abu in SAz-1 (2.0% loading) over an 8-week period at 150°C. The L-ee after 8 weeks is given on the right of each curve. Red circles: amino acid in the absence of Iva, green triangles: with L-Iva, blue squares: with D-Iva. The Iva content was 2.6% (Ala experiments) and 2.3% (Abu experiments), respectively, meaning that Iva was present in equimolar amount with respect to the other amino acid.

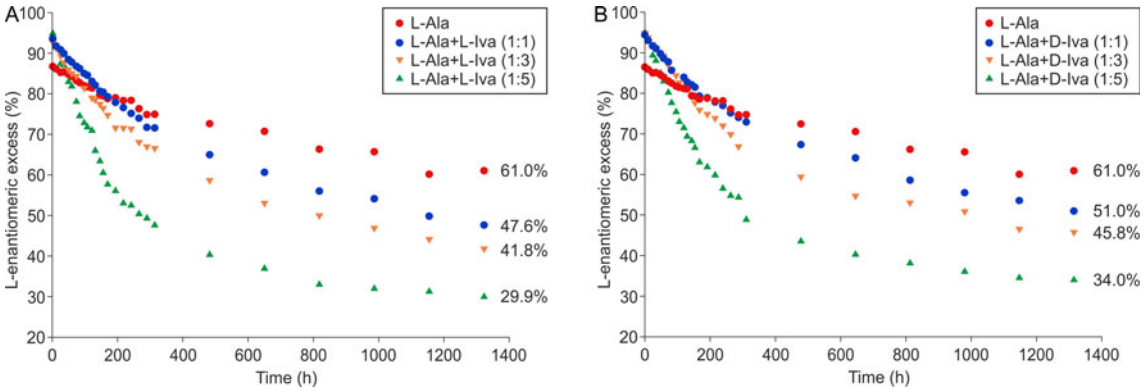

Fig. 5. Decrease of the L-ee of Ala in SAz-1 (0.5% loading) in the presence of (A) L-Iva and (B) D-Iva over an 8-week period at 150°C. The L-ee after 8 weeks is given on the right of each curve. Red circles: without Iva, blue circles: Ala : Iva = 1 : 1, orange triangles: Ala : Iva = 1 : 3, green triangles: Ala : Iva = 1 : 5 (molar ratios).

The acceleration of the racemization by higher amino acid concentrations can be explained by two different models described above. However, a model that is solely based on the existence of different binding sites in SAz-1 cannot account for the observed differences between L- and D-Iva, because SAz-1 is achiral. Thus, direct interaction between the amino acid

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molecules has to be assumed. Hydrogen-bonded L-Ala (L-Abu)–Iva dimers, for example, would exist as two diastereomers L–L and L–D. In this model, L-Ala and L-Abu would racemize faster when being part of the L–L dimer and/or for steric reasons the L–D dimer may form less readily than L–L.

In the experiments with 2.0% L-Ala and an equimolar amount of Iva, the final L-ee values of L-Ala were 29.9% (with L-Iva) and 35.0% (with D-Iva, Fig. 4(a)). These values were considerably lower than those obtained in analogous experiments with only 0.5% L-Ala and an equimolar amount of Iva. There, the final L-ee values were 47.6% (with L-Iva) and 51.0% (with D-Iva, Fig. 5), meaning that the racemization was slower. Again, this is consistent with the general acceleration effect that a higher overall amino acid concentration exerts on the racemization.

Furthermore, there is a small but significant difference between Ala and Iva in their ability to accelerate the racemization. This is apparent from comparison of the results of two experiments: (i) when a 5 : 1 Iva-to-Ala molar ratio and an L-Ala concentration of 0.5% were used, the final L-ee values were 29.9% (with L-Iva) and 34.0% (with D-Iva); (ii) with equimolar amounts of the two amino acids and an L-Ala concentration of 2%, the final L-ee values were 29.9% (with L-Iva) and 35.0% (with D-Iva, Figs 4(a) and 5). Thus, the degree of racemization was virtually the same in both experiments. The interesting thing is that in the second experiment the total amino acid amount was 2.8 mmol in 6.3 g of loaded SAz-1 (1.4 mmol each of L-Ala and Iva), whereas in the first experiment this amount was only 2.0 mmol (0.34 mmol of L-Ala and 1.7 mmol of Iva). This means that a 29% lower total amino acid concentration (including a four times lower L-Ala concentration) led to the same L-ee. Therefore, the cause must be that Iva has a higher efficiency in accelerating the racemization, as compared to Ala. However, the molecular basis of the different behaviour of Iva and Ala is unknown. The deprotonation at the α-C atom, which is an essential step in the most common mechanism of amino acid racemization, requires a Brønsted base. Although not particularly basic, the carboxylate group of a second (zwitterionic) amino acid may assist the deprotonation reaction. Thus, if we assume the existence of hydrogen-bonded dimers, the carboxylate group of the 'auxiliary' amino acid Iva in the dimer L-Ala–Iva may have a higher basicity and/or better proton accessibility than L-Ala in L-Ala–L-Ala.

Our results suggest direct contact between the intercalated amino acids. In addition, the amino acid molecules very probably interact with the inner surfaces and the interlayer Ca^{2+} cations of montmorillonite. Such interactions can occur via hydrogen bonding and complex formation, respectively. It remains to be determined to what extent they influence the racemization rate. Studies of the influence of different minerals on the racemization of amino acids are planned for the future.

Summary and conclusions

We showed that the α,α-dialkyl-α-amino acid Iva is resistant to thermal racemization when intercalated in the mineral calcium montmorillonite. No racemization was observed over an 8-week period at 150°C. By contrast, L-Ala and L-Abu were substantially racemized under the same conditions. In our study, enantiomeric ratios in mixtures of Iva with Ala or Abu were reliably determined by GC-MS. Baseline separation of the *N*-TFA-*O*-methyl esters was obtained for all enantiomers in Iva–Ala and Iva–Abu mixtures. At the temperature used in our experiments, alteration beyond racemization (i.e. degradation and condensation) must be considered for Ala and Abu. Some alteration did indeed occur, but in all cases at least 67% of the amino acid remained unaltered.

As a spin-off of this study, we found that L-Ala already partially racemized during loading of the montmorillonite at 35°C. The loading procedure consisted of multiple wetting and drying cycles. Obviously, wet–dry cycling and the presence of the mineral allowed partial racemization to take place under mild conditions in a relatively short period of time. However, the loss of ee during loading was much smaller than the one that occurred in the subsequent heating experiments.

The main result of this study is the finding that Iva accelerates the racemization of L-Ala and L-Abu. What is particularly interesting is that L-Iva has a stronger effect than D-Iva. Furthermore, the racemization can be accelerated by increasing either the L-Ala or the Iva concentration. These observations strongly suggest direct interactions between the amino acid molecules in the mineral. One possibility would be that covalently bonded amino acid dimers, i.e. dipeptides, form. However, in the quantification of the 2,4-dinitrobenzene derivatives by HPLC, no oligopeptides and particularly no dipeptides were detected. As the extraction method, the derivatization agent and the HPLC method that were used have already been successfully applied to the analysis of oligopeptides (Dalai *et al.*, 2017), we conclude that no detectable amounts of dipeptides were present. Another possibility is the formation of hydrogen-bonded dimers. If indeed L-Ala (L-Abu)–L-Iva and L-Ala (L-Abu)–D-Iva dimers form, this would imply that L-Ala and L-Abu racemize faster in the L–L dimers and/or the L–L dimers are more stable than L–D. We also observed that Iva was more efficient than Ala in accelerating the racemization of L-Ala. Thus, in the dimer model, L-Ala–L-Iva and L-Ala–D-Iva promote racemization more strongly than does L-Ala–L-Ala.

Our results show that under prebiotically plausible conditions, the racemization rate of an α-H-α-amino acid can increase with increasing concentration of the amino acid and that Iva can accelerate the racemization. It is evident that this could have counteracted both the origin and the preservation of homochirality on the early Earth but it is difficult to assess how important these effects really were. However, the role of abiotically formed amino acids goes beyond being carriers of ees. For example, they could have been starting compounds for other (pre-)biologically relevant molecules and nutrients for early microorganisms. The effects we observed in the current study may also be important in the search for extraterrestrial biosignatures. This is because accelerated loss of ee can rapidly obscure the biological origin of an amino acid and thereby increase the risk of false-negative conclusions.

Materials and methods

The following amino acids were used without further purification: L-Ala (Sigma-Aldrich, ≥99.5%), L-Iva monohydrate (Acros Organics, ≥99%), D-Iva monohydrate (Acros Organics, 97%; identified as the monohydrate by thermogravimetric analysis) and L-Abu (TCI, >99%). The Ca-montmorillonite SAz-1, a well-characterized clay mineral, was purchased from the Clay Minerals Society (The Clay Minerals Society, 2019).

Preparation of ≤2 μm montmorillonite particles and loading of the mineral

To obtain a size fraction of ≤2 μm, first a suspension of SAz-1 in double-distilled water was prepared in an agate grinding jar, agate

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crushing balls were added and the mineral was crushed to a powder in a planetary ball mill (Retsch PM 100 at 100 rpm) for 5 days. Then, the suspension was diluted, transferred to Atterberg cylinders and left undisturbed for an appropriate settling time. The height of fall of 2-µm-sized particles after the settling time was determined with the computer program 'Atterberg' (Krumm, 1994). The volume above this height was isolated and centrifuged. The centrifugation pellets were dried and pulverized, and the powder was stored at 51% relative humidity in a desiccator over saturated aqueous $Ca(NO_3)_2$ solution until needed for experiments. This $\leq$2 µm diameter fraction was used throughout the study.

The loading of SAz-1 with amino acid(s) was performed as follows: 6.0 g of the mineral powder was suspended in double-distilled water and the amino acid(s) was(were) dissolved in the suspension. The amino acids and amino acid quantities used depended on the particular experiment (see 'Results and discussion' section). The solution/suspension was thoroughly mixed and then evaporated to dryness at ca. 35°C. The residue was ground and the product stored at 51% relative humidity (see above) for at least 2 days prior to use.

Heating experiments

The heating experiments were performed in the apparatus described by Fox and Strasdeit (2013). The container with the amino acid-loaded mineral was placed in the apparatus, which was then purged with pure (99.999%) nitrogen gas for 2 days before heating was started. In all experiments, the samples were heated at 150°C under nitrogen for a total of 8 weeks and subsamples were taken periodically. The strict exclusion of air was a precautionary measure to prevent oxidation.

Amino acid extraction was performed as follows: the samples from the heating experiments were suspended in double-distilled water, vigorously shaken for 20 min, and then centrifuged at 13000 rpm (rotor radius: 73 mm). The supernatant was collected. This procedure was repeated nine times. The combined supernatants were evaporated to dryness at 50°C in a sand bath.

Analytical procedures and instrumentation

The dried extracts from the heating experiments were analysed by GC-MS, which was performed using a 7890B gas chromatograph and a 5977A mass selective detector from Agilent Technologies (Waldbronn, Germany). Prior to measurement, the amino acids were derivatized to the corresponding *N*-TFA-*O*-methyl esters as described previously (Fox *et al.*, 2015). For chiral separation, a capillary column Lipodex E (25 m length × 0.25 mm i.d.; Macherey-Nagel, Düren, Germany) was used. The Lipodex E column is coated with a solution of octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin in modified polysiloxane (0.25 µm film thickness). The carrier gas was helium (99.999%) at a pressure of 1.6 bar. A constant column temperature of 100°C was used, which gave a good compromise between enantiomer resolution and retention time. The major fragment ions of Ala and Abu at $m/z = 140$ and $m/z = 156$, respectively, were used for peak integration to determine the L-ees.

To quantify the survival of Ala and Abu at the temperature of the heating experiments, first the SAz-1 was loaded as described above and a heating experiment was performed. After 8 weeks at 150°C under nitrogen, the whole sample was recovered for analysis (i.e. no subsamples were taken). After extraction (see above), the amino acids were derivatized with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) for subsequent HPLC analysis. An HPLC system from Sykam (Fürstenfeldbruck, Germany) was used which consisted of reagent organizer S7121, solvent delivery system S1122, low pressure gradient mixer S8111, injector valve bracket S5111 and UV/Vis diode array detector S3210. Detection was at 340 nm. The derivatives were separated on a Nucleodur C18 Gravity column (150 mm length, 4.6 mm i.d., 3 µm particle size) from Macherey-Nagel (Düren, Germany). The column was kept at a constant temperature of 40°C in a column oven Jetstream II Plus from ERC (Riemerling, Germany). The mobile phase consisted of 0.01 mol l^{-1} trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). Elution was performed with a linear gradient from 25 to 50% acetonitrile over 50 min. A flow rate of 1 ml min^{-1} was used. The amino acid concentrations in the extracts were calculated using calibration curves.

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