The Possible Influence of L-Histidine on the Origin of the First Peptides on the Primordial Earth

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One of the most unsettled problems of prebiotic evolution and the origin of life is the explanation why one enantiomeric form of biomolecules prevailed. In the experiments presented in this paper, the influence of L-histidine on the peptide formation in the Salt-Induced Peptide Formation (SIPF) reaction of the enantiomeric forms of valine, proline, serine, lysine, and tryptophan, and the catalytic effects in this first step toward the first building blocks of proteins on the primordial earth were investigated. In the majority of the produced dipeptides, a remarkable increase of yields was shown, and the preference of the L-amino acids in the peptide formation in most cases cannot be denied. In summary, our data provide further experimental evidence for the plausibility of the SIPF reaction and point at a possible important role of L-histidine in the chemical evolution on the primordial earth.

1. Introduction. – Biohomochirality is essential for the correct functioning of almost all of the biochemical mechanisms, for regulation and for replication of today's life-forms. All the vital biomolecules of life have the same handedness; for example, proteins comprise almost entirely 'left-handed' amino acids, while nucleic acids contain sugars that are of the D-form. The development of prebiotic biohomochirality on the early earth must be viewed critically. Why did life choose this way and not the mirror world? This selection process of nature is until now one of the great unsolved problems in science, but there are various theories so far, and many possibilities have been and still are being investigated, none of them having led to a conclusive explanation regarding the origin of biological homochirality [1][2] so far. With the possible exception of some interstellar processes, relating to the presence of circularly polarized radiation [3], prebiotic reactions should yield the same amount of L- and D-enantiomers. Since parity violation in weak nuclear interactions [4–6] has been discovered in the 1950s and has been connected with electromagnetic forces in the 'electroweak theory' in the 1980s [7–10], it is known that there is an inherent chirality of atoms, and a parity-violating energy difference between right- and left-handed enantiomers [11][12]. Therefore, an evidence for a natural preference for the homochiral selection exists, but, because of the very small energy difference (for amino acids in the order of 10^{-38} to 10^{-35} J), it cannot lead from an initially racemic mixture to a significant stereoselective differentiation, but still requires amplification factors to yield a full preference for one chiral form in prebiotic synthesis reaction pathways. The type of atoms involved in a synthetic reaction could play an important role, however, due to the Z^5 (Z being the atomic number) dependence of the parity-

violating energy difference, and thus attention in this aspect has to be paid on metal catalysis. It is known that metal centers with a specific complex geometry with achiral ligands can become chiral by themselves, and again this chirality can be amplified through induction by different chiral ligands [13][14]. The central atom of a complex with its inherent chirality can have an influence again on the ligand molecules, which could lead to a preference of one enantiomeric form.

The scope of our experiments was to exploit these circumstances, and, for this reason, we investigated the influence of a chiral catalyst like L-histidine on the stereoselective yield difference in prebiotic peptide formation with a model system consisting of five different amino acids in the SIPF reaction [15][16]. The experiments were conducted with the L-enantiomeric form of histidine, because previous studies had shown a major preference for the L-form of educt amino acids. We, thus, wanted to see whether this preference could be further increased by the use of an L-amino acid as catalyst. In further investigations, experiments with D-histidine will be carried out for comparison with the present data obtained in this work.

Under the rough conditions of the primordial earth, the SIPF reaction, which was discovered in the late 1980s, is one of the most facile and mild reaction pathways to explain the formation of the first peptides, eventually leading to the emergence of the first life on earth. It works with every amino acid investigated so far, especially with the biologically important α-amino acids, and provides substantial peptide yields within a wide range of conditions. The major reactants needed are amino acids, $CuCl_2$, and NaCl which, according to geochemical data [17–21], have been present in sufficient amounts on the primordial earth. *Ab initio* computer calculations [22] and *Monte Carlo* simulations [23] showed that, with a concentration of more than 3M in a NaCl solution, the first hydration shell of the Na^+ ion is not saturated, which can thus act as a dehydrating agent and lower the thermodynamic barrier of the peptide formation in aqueous solution. Cu^{II} acts as central ion for the formation of a complex with one chloride ligand, two H_2O molecules and the two amino acids. One of them chelates *via* its amino N-atom and the carboxylate O-atom, the other one only coordinates end-on *via* one of its carboxylate O-atoms. The steric and electronic conditions in the complex help to lower the kinetic barrier for the reaction. All these aspects have shown that the active species enabling the reaction is an amino acid–monochlorocuprate complex (see *Fig.*) [24].

The peptide formation as illustrated in *Scheme 1* has been experimentally confirmed by spectrophotometric and potentiometric titrations [25]. The fact that, with the help of catalytic effects [26][27] of glycine, the yields of L-L homodipeptides were increased, and that indications were found in previous studies that there is a stereoselective preference for one enantiomeric form of alanine [28] and for several other amino acids (valine, proline, tryptophan, lysine, and serine) [29] provoked the hypothesis that a chiral catalyst such as L-histidine could not only lead to a higher yield of peptides in general, but also further increase the yield difference between L-L and D-D peptides.

Besides this aspect, the catalytic power of L-histidine *via* the possible mechanism depicted in *Scheme 2* was investigated, comparing it with the results obtained in experiments with the catalyst glycine and diglycine [30][31].

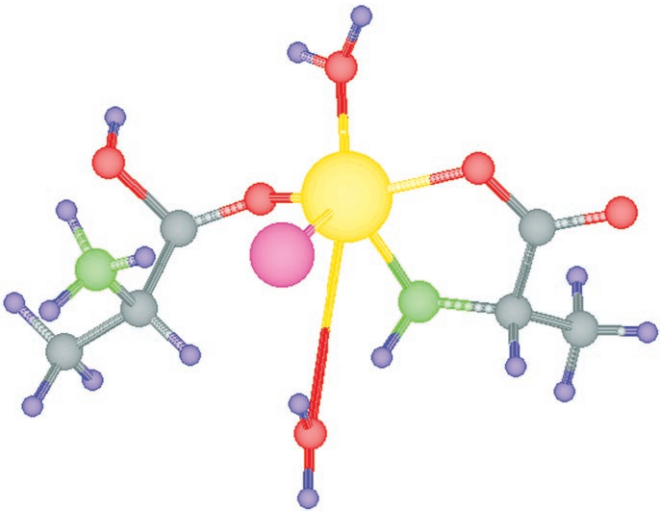
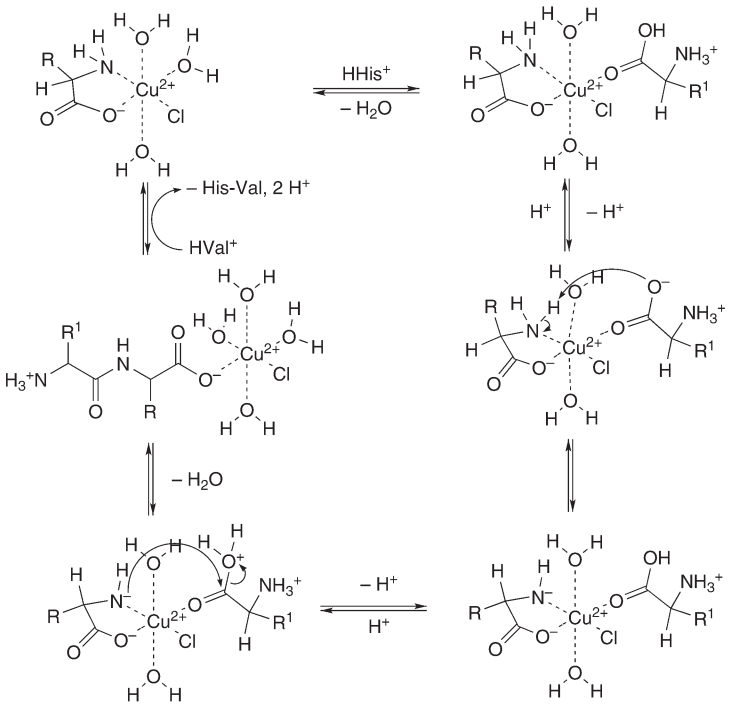
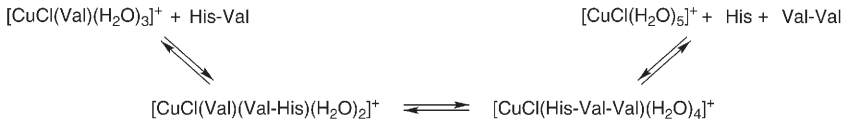


Figure. Ab initio *geometry-optimized structure of the tetrahedrally distorted SIPF complex* $[CuCl(ala)(alaH_2)(H_2O)_2]^+$

Scheme 1. *Postulated Mechanism for the Peptide Formation of ValHis in the SIPF Reaction (*R = ^{i}Pr, R^1 = (1*H*-imidazol-4-yl)CH_2 [24])



Scheme 2. *Reaction Scheme for Divaline Formation with Histidine Catalysis* [32]



Six independent evaporation cycle experiments, with 80, 40, and 20 mM starting concentration of valine, proline, lysine, and serine, and 40, 20, and 10 mM of tryptophan were performed in a way to mimick a coastal region with tidal zones and small lagunas, where the concentration of the reactants increases and decreases by the influence of sun, rain, and tidal changes. In the experiments, we simulated this scenario by heating the amino acid/$CuCl_2$/NaCl solution with/without addition of the catalyst L-histidine at 85°, by which all of the liquid evaporates in one day. For this reason, low initial reactant concentrations can be used; during an evaporation cycle they increase to the required values. The residue obtained was subsequently redissolved in pure H_2O, to start another wetting–drying cycle. Six independent evaporation-cycle series for every amino acid system, three each with chemicals from different suppliers (*Bachem*, *Senn*), were analyzed to compensate for effects of different preparation and to allow a statistical evaluation.

2. Results and Discussion. – The general difference of the homodipeptide yields in the SIPF reaction was correlated to the specific properties of the amino acids. For the different yields of dipeptides formed in the SIPF reaction, different factors, such as the complex-formation constant, the polarity, the size, the hydrophobicity, and the nucleophilicity and electrophilicity of the amino acids, are responsible. *Tables 1–3* show the statistical averages of the homodipeptide yields of the six independent evaporation series for every L- and D-amino acid with the effect of L-histidine catalysis. The comparison factor listed in the tables (yield comparison factor of L-L and D-D dipeptides) shows a possible stereoselective preference of the SIPF reaction for the L-amino acid.

When comparing the yield differences in the case of valine, the preference of the L-L homodipeptide form is apparent in almost every reaction system investigated. Examination of the diproline yields shows that, starting from low concentrations (40 and 20 mM of the amino acid) leads to a significantly higher L-L diproline yield. For lysine, the L-form appears also preferred over the D-form, and indications are found towards a preference of the L-enantiomeric form of lysine in most of the cases. In the case of serine, the D-form is slightly preferred in the formation of the homodipeptide, but the difference appears insignificant due to strong overlap of the statistical confidence intervals. The formation of ditryptophan is marginal, and the results for this system should thus be seen with some reservations. However, a preference for the L-form still seems recognizable, especially at lower starting concentrations. The reason for the generally low yields for ditryptophan is assumed to be the indole side chain of the amino acid, the steric hindrance of which can influence complex formation and thus lower the reactivity in the peptide formation. The different behavior of the five

Table 1. *Homodipeptide Yields with Standard Deviations* [%] *and Corresponding* L/D *Stereoselectivity Factors* R, *with Starting Concentration of 80 mM of Amino Acid* (40 mM Trp) *with 1/8 Concentration of* L-*Histidine as Catalyst*

		Reaction time		
		1 day	4 days	7 days
Val-Val	L-L	0.058 ± 0.011	0.43 ± 0.24	0.51 ± 0.01
	R	1.17	1.05	0.70
	D-D	0.05 ± 0.02	0.41 ± 0.02	0.74 ± 0.18
Pro-Pro	L-L	0.89 ± 0.03	1.19 ± 0.06	1.29 ± 0.04
	R	0.67	0.76	0.83
	D-D	1.33 ± 0.10	1.56 ± 0.37	1.55 ± 0.24
Lys-Lys	L-L	0.79 ± 0.30	2.52 ± 0.19	3.33 ± 0.26
	R	0.95	1.03	1.01
	D-D	0.83 ± 0.35	2.45 ± 0.30	3.31 ± 0.16
Ser-Ser	L-L	0.26 ± 0.049	0.35 ± 0.06	0.13 ± 0.02
	R	0.98	0.83	0.71
	D-D	0.27 ± 0.07	0.42 ± 0.07	0.18 ± 0.06
Trp-Trp	L-L	0.000	0.000	0.000
	R	1.00	1.00	1.00
	D-D	0.000	0.000	0.000

Table 2. *Homodipeptide Yields with Standard Deviations* [%] *and Corresponding* L/D *Stereoselectivity Factors* R, *with Starting Concentration of 40 mM of Amino Acid* (20 mM Trp) *with 1/8 Concentration of* L-*Histidine as Catalyst*

		Reaction time		
		1 day	4 days	7 days
Val-Val	L-L	0.11 ± 0.01	2.21 ± 0.37	2.23 ± 0.08
	R	2.41	1.04	1.29
	D-D	0.046 ± 0.052	2.13 ± 0.15	1.73 ± 0.09
Pro-Pro	L-L	1.60 ± 0.11	12.04 ± 0.52	7.93 ± 0.52
	R	1.40	1.59	2.33
	D-D	1.14 ± 0.22	7.59 ± 0.51	3.40 ± 0.82
Lys-Lys	L-L	1.34 ± 0.11	6.65 ± 0.11	8.11 ± 0.74
	R	1.15	1.03	0.99
	D-D	1.17 ± 0.88	6.44 ± 0.80	8.23 ± 0.53
Ser-Ser	L-L	0.40 ± 0.058	0.13 ± 0.02	0.07 ± 0.02
	R	0.90	0.83	0.92
	D-D	0.45 ± 0.05	0.15 ± 0.03	0.077 ± 0.032
Trp-Trp	L-L	0.000	0.000	0.000
	R	1.00	1.00	1.00
	D-D	0.000	0.000	0.000

investigated amino acids concerning the preference of one enantiomeric form in the formation of dipeptides might be ascribed to the different constitutions of these amino acids due to their very different side chains, which could influence the geometry of the active copper complex $[CuCl(aa)(aaH_2)(H_2O)_2]^+$ where aa represents an amino acid. The more the plane formed by the central ion, the chloride ligand, and the two amino

Table 3. *Homodipeptide Yields with Standard Deviations* [%] *and corresponding* L/D *Stereoselectivity factors* R, *with Starting Concentration of 20 mM of Amino Acid* (10 mM Trp) *with 1/8 Concentration of* L*-Histidine as Catalyst*

		Reaction time		
		1 day	4 days	7 days
Val-Val	L-L	0.057 ± 0.010	0.44 ± 0.11	0.71 ± 0.11
	R	1.08	1.05	1.10
	D-D	0.053 ± 0.010	0.42 ± 0.09	0.65 ± 0.10
Pro-Pro	L-L	6.94 ± 0.18	14.41 ± 0.72	8.24 ± 0.86
	R	4.47	1.26	1.43
	D-D	1.55 ± 0.09	11.44 ± 0.38	5.78 ± 0.85
Lys-Lys	L-L	1.51 ± 0.14	7.82 ± 0.21	8.36 ± 0.85
	R	0.92	1.01	0.96
	D-D	1.65 ± 0.15	7.77 ± 0.23	8.66 ± 1.17
Ser-Ser	L-L	0.86 ± 0.17	0.11 ± 0.03	0.043 ± 0.011
	R	0.77	1.33	1.84
	D-D	1.11 ± 0.08	0.077 ± 0.033	0.024 ± 0.01
Trp-Trp	L-L	0.000	0.011 ± 0.007	0.014 ± 0.004
	R	1.00	1.26	1.13
	D-D	0.000	0.008 ± 0.004	0.012 ± 0.003

acids is twisted towards a tetrahedral conformation, the more it gives rise to a central chirality at the Cu-atom. Based on this distorted complex, the considerable high inherent central chirality at Cu^{2+} which is due to the Z^5 dependence might be remarkably amplified by chiral induction due to the presence of chiral ligands like chiral L-histidine. Due to the fact that complexes then formed with the L- and D-forms of the amino acids are diastereoisomers, the complex-formation constants are different, which could explain the significant dipeptide yield differences observed.

Regarding the catalytic effect of histidine, it can be seen from the catalytic factor *P* (= dipeptide yield with catalyst/dipeptide yield without catalyst), that this effect is present with four of the five investigated amino acids (*Tables 4–8*). The reference dipeptide yields for the amino acids without catalyst were taken from former experiments under identical conditions [29].

Strong catalytic effects of L-histidine could be observed in particular in the case of valine, where L-Val-L-Val was obtained in higher yields in the presence of the catalyst for every starting concentration and every analyzed evaporation-cycle day. With proline, the only amino acid with a secondary amino group, the SIPF reaction with catalyst shows a doubling of the concentration of diproline in almost every reaction system. For lysine, a rather strong initial catalytic effect can be observed enhancing the dipeptide yields by factors of 3 to 13, especially at low starting concentrations of the amino acid. Except for the concentration of 80 mM lysine, a remarkable increase of the yields of all systems containing L-histidine could be detected after 1 day. In contrast to the experiments with valine, the catalytic efficiency of L-histidine in comparison to the system without catalyst is decreasing with further cycles. The strongly increased peptide yields of the three amino acid systems with valine, proline, and lysine reflect a specific ability of L-histidine to act as catalyst for the peptide formation of selected amino acids.

Table 4. *Average Yields of Divaline* [%] (six independent evaporation cycles) *from Different Starting Concentrations of* L- *and* D-*Valine, with and without* L-*Histidine after 1, 4, and 7 Days, and Corresponding Yield increase Factors* P (yields with catalyst/yields without catalyst)

		Reaction time			
		L-His	1 day	4 days	7 days
L-Val-L-Val	80	+	0.058	0.430	0.510
	P		1.26	6.06	7.39
	80	–	0.046	0.071	0.069
	40	+	0.110	2.210	2.230
	P		2.44	29.86	32.32
	40	–	0.045	0.074	0.069
	20	+	0.057	0.440	0.710
	P		1.43	5.30	8.07
	20	–	0.040	0.083	0.088
D-Val-D-Val	80	+	0.050	0.410	0.740
	P		50.00	205.00	246.67
	80	–	0.001	0.002	0.003
	40	+	0.046	2.130	1.730
	P		∞ [a])	710.00	192.22
	40	–	0.000	0.003	0.009
	20	+	0.053	0.420	0.650
	P		∞ [a])	∞ [a])	∞ [a])
	20	–	0.000	0.000	0.000

[a]) ∞ means infinite increase, because of division of zero.

Table 5. *Average Yields of Diproline* [%] (six independent evaporation cycles) *from Different Starting Concentrations of* L- *and* D-*Proline, with and without* L-*Histidine after 1, 4, and 7 Days, and Corresponding Yield Increase Factors* P (yields with catalyst/yields without catalyst)

		Reaction time			
		L-His	1 day	4 days	7 days
L-Pro-L-Pro	80	+	0.890	1.190	1.290
	P		3.18	1.37	0.64
	80	–	0.280	0.870	2.020
	40	+	1.600	12.040	7.930
	P		1.52	1.13	6.66
	40	–	1.050	10.670	1.190
	20	+	6.940	14.410	8.240
	P		0.93	1.13	1.06
	20	–	7.460	12.720	7.770
D-Pro-D-Pro	80	+	1.330	1.560	1.550
	P		4.75	1.77	0.78
	80	–	0.280	0.880	1.990
	40	+	1.140	7.590	3.400
	P		1.05	0.70	2.62
	40	–	1.090	10.880	1.300
	20	+	1.550	11.440	5.780
	P		0.24	1.04	0.77
	20	–	6.450	10.970	7.540

Table 6. *Average Yields of Dilysine* [%] (six independent evaporation cycles) *from Different Starting Concentrations of* L- *and* D-*Lysine, with and without* L-*Histidine after 1, 4, and 7 Days, and Corresponding Yield Increase factors* P (yields with catalyst/yields without catalyst)

		Reaction time			
		L-His	1 day	4 days	7 days
L-Lys-L-Lys	80	+	0.790	2.520	3.330
	P		3.67	0.81	0.73
	80	–	0.215	3.122	4.533
	40	+	1.340	6.650	8.110
	P		4.96	1.87	1.17
	40	–	0.270	3.554	6.961
	20	+	1.510	7.820	8.360
	P		13.02	3.89	1.97
	20	–	0.116	2.011	4.237
D-Lys-D-Lys	80	+	0.830	2.450	3.310
	P		3.43	0.79	0.64
	80	–	0.242	3.109	5.148
	40	+	1.170	6.440	8.230
	P		4.08	1.58	1.11
	40	–	0.287	4.073	7.413
	20	+	1.650	7.770	8.660
	P		11.87	3.46	1.96
	20	–	0.139	2.243	4.427

Table 7. *Average Yields of Diserine* [%] (six independent evaporation cycles) *from Different Starting Concentrations of* L- *and* D-*Serine, with and without* L-*Histidine after 1, 4, and 7 Days, and Corresponding Yield Increase Factors* P (yields with catalyst/yields without catalyst)

		Reaction time			
		L-His	1 day	4 days	7 days
L-Ser-L-Ser	80	+	0.260	0.350	0.130
	P		0.54	1.05	0.39
	80	–	0.483	0.334	0.334
	40	+	0.400	0.130	0.070
	P		0.96	0.41	0.36
	40	–	0.415	0.315	0.195
	20	+	0.860	0.110	0.043
	P		2.08	0.40	0.46
	20	–	0.414	0.274	0.093
D-Ser-D-Ser	80	+	0.270	0.420	0.180
	P		0.51	1.43	0.76
	80	–	0.526	0.293	0.237
	40	+	0.450	0.150	0.077
	P		0.93	0.51	0.43
	40	–	0.486	0.293	0.181
	20	+	1.110	0.077	0.024
	P		1.73	0.31	0.17
	20	–	0.643	0.247	0.142

Table 8. *Average Yields of Ditryptophan* [%] (six independent evaporation cycles) *from Different Starting Concentrations of* L- *and* D-*Tryptophan, with and without* L-*Histidine after 1, 4, and 7 Days, and Corresponding Yield Increase Factors* P (yields with catalyst/yields without catalyst)

		Reaction time			
		L-His	1 day	4 days	7 days
L-Trp-L-Trp	40	+	0.000	0.000	0.000
	P		∞ [a])	∞ [a])	∞ [a])
	40	−	0.000	0.000	0.000
	20	+	0.000	0.000	0.000
	P		∞ [a])	∞ [a])	∞ [a])
	20	−	0.000	0.000	0.000
	10	+	0.000	0.011	0.014
	P		0.00	1.83	2.80
	10	−	0.002	0.006	0.005
D-Trp-D-Trp	40	+	0.000	0.000	0.000
	P		0.00	∞ [a])	0.00
	40	−	0.001	0.000	0.001
	20	+	0.000	0.000	0.000
	P		0.00	0.00	0.00
	20	−	0.001	0.001	0.002
	10	+	0.000	0.008	0.012
	P		0.00	2.00	3.00
	10	−	0.001	0.004	0.004

[a]) ∞ means infinite increase, because of division of zero.

On the other hand, the serine dipeptide formation is not strongly influenced by addition of L-histidine, only some slight effects could be observed. This absence of a catalytic effect, also observed in the case of tryptophan, indicates that intermediate products, according to the proposed catalytic pathway [32], like di- or tripeptides with histidine are rather stable, interrupting thus the catalytic reaction cycle. The reason for the lack of a catalytic effect in the case of tryptophan could also be the large indole side chain, which causes a considerable steric hindrance.

3. Conclusions. – The results of the experiments presented in this paper add another puzzle piece to the very complex question about the origin of life, concerning the formation of the first peptides. Based on the SIPF reaction as an important process in the early stage of chemical evolution, the amino acid L-histidine could have played a key role due to its catalytic effect in the formation of peptides, but also as a promotor of stereoselectivity.

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Experimental Part

1. *General.* All utilized chemical reagents were of anal. grade. The amino acids (L-valine, L-proline, L-lysine, L-serine, L-tryptophan, and L-histidine) for the first three series, as well as all peptides standards, were obtained from *Bachem AG*, Switzerland, and the amino acids for the second three series were acquired from *Senn Chemicals AG*, Switzerland. NaCl and $CuCl_2 \cdot 2\ H_2O$ were purchased from *Merck*, D-Darmstadt. Na-hexanesulfonate and Na-heptanesulfonate, which act as ion-pairing agents were acquired from *Sigma-Aldrich GmbH*, Germany, as well as MeCN (super-gradient grade for HPLC). KH_2PO_4, conc. HCl, and conc. H_3PO_4 were obtained from *Fluka Chemie AG*, Switzerland. The aq. solns. were prepared with ultrapure water (18 MΩ) produced in-house in a water purification system (*Barnstead*).

2. *Evaporation-Cycle Experiments.* The previously discussed experiments to mimick a primordial scenario were performed as follows: reaction solns., with different starting concentrations of the amino acids (40, 20, and 10 mM for tryptophan, and 80, 40, and 20 mM for all other amino acids) were prepared independently by dissolving each amino acid and L-histidine as a catalyst at 1/8 of the concentration (5, 2.5, and 1.25 mM of L-histidine for tryptophan, and 10, 5, and 2.5 mM for all other amino acids) in the SIPF solution, consisting of 0.5M NaCl and 40 mM $CuCl_2$ in ultrapure H_2O. The concentration of tryptophan had to be lowered to half of the starting concentration of the other amino acids, because of its lower solubility. Besides that, an addition of 1–2 drops of conc. HCl per 50 ml was needed. The resulting soln. (1 ml) was transferred into a 2-ml HPLC vial and was evaporated in a time period of 24 h in a heating box at 85° to secure a slow increase of the concentration of the ingredients and to enable peptide formation.

For the next evaporation cycle, the residue was redissolved in 1 ml of ultrapure H_2O and was subjected to the same conditions as in the first cycle. Samples were taken for analysis after 0, 1, 4, and 7 days, and were filtered through a 0.2-μm hydrophilic polypropylene syringe filter (*GHP Acrodisc*, *Pall Gelman Laboratory*, USA) for HPLC analysis.

3. *HPLC Analysis.* The samples were analyzed by reversed-phase (RP) ion-pairing HPLC on an *Agilent 1100* series HPLC system with diode array and fluorescence detection, identifying and quantifying the peptide products by comparison of retention times, UV/VIS and fluorescence spectra, and response factors to pure reference substances in several concentrations. Solvent *A* consisted of 50 mM KH_2PO_4 and 7.2 mM $C_6H_{13}SO_3Na$ (as ion-pairing agent) in ultrapure H_2O, which was adjusted to pH 2.3 with phosphoric acid. Solvent *B* was super-gradient grade MeCN. Both solvents were filtered through 0.2-μm hydrophilic polypropylene membrane filters (*GH Polypro*, *Pall Gelman Laboratory*, USA) before use. For the analysis of the serine system, samples of a different solvent composition was used. 50 mM KH_2PO_4 with 10 mM sodium-heptane sulfonate, adjusted to pH 2.3 with phosphoric acid was used as solvent *A* and super-gradient grade MeCN as solvent *B*. The best conditions to analyze samples containing homodipeptides in the amino acid/L-histidine system were determined as the following: 1 μl of the sample was injected into an *Agilent Hypersil ODS* column (5 μm, 2.1 × 200 mm), equipped with a 20-mm precolumn of the same material and a precolumn filter. Only for the serine samples, a *ThermoHypersil-Keystone Hypercarb* porous graphitic carbon column (5 μm, 2.1 mm × 100 mm) with precolumn of the same material and pre-column filter was used.

Gradient Conditions. For the amino acid systems with valine, proline, serine, and lysine, diode array UV/VIS (detection at 200 nm, 4-nm bandwidth, reference wavelength 550- and 100-nm bandwidth) was used. For the tryptophan samples, fluorescence detection at an excitation wavelength of 280 nm and an emission wavelength of 350 nm with a PMT gain of 8 was utilized.

The following gradients were used: *Valine*/L-*histidine system:* an isocratic elution with 4% of solvent *B* was applied at a flow rate of 0.350 ml/min and a column temp. of 40° with a stop time of 30 min.

Proline/L-*histidine system:* a gradient was applied with 0 min 4% *B*, 4 min 25% *B*, 6 min 25% *B*, 9 min 1.5% *B*; stop time 20 min, flow rate of 0.400 ml/min, column temp. 35°.

Serine/L-*histidine system:* a gradient was applied with 0 min 1% *B*, 15 min 1% *B*, 16 min 15% *B*, 26 min 15% *B*, 30 min 1% *B*; stop time 50 min, flow rate of 0.150 ml/min, column temp. 20°.

Lysine/L-*histidine system:* a gradient was applied with 0 min 1.5% *B*, 1 min 1.5% *B*, 1.2 min 4.5% *B*, 2 min 4.5% *B*, 2.2 min 7.5% *B*, 3 min 7.5% *B*, 3.2 min 10.5% *B*, 4 min 10.5% *B*, 4.2 min 13.5% *B*, 5 min 13.5% *B*, 5.2 min 16.5% *B*, 6 min 16.5% *B*, 6.2 min 19.5% *B*, 7 min 19.5% *B*, 7.2 min 22.5% *B*, 8 min 22.5%

B, 8.2 min 25.5% *B*, 10 min 25.5% *B*, 13 min 1.5% *B*; stop time 16 min, flow rate of 0.350 ml/min, column temp. 35°.

Tryptophan/L-*histidine system:* a gradient was applied with 0 min 7% *B*, 4 min 27% *B*, 8 min 35% *B*, 12 min 37% *B*, 14 min 7% *B*; stop time 17 min, flow rate of 0.350 ml/min, column temp. 40°.

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