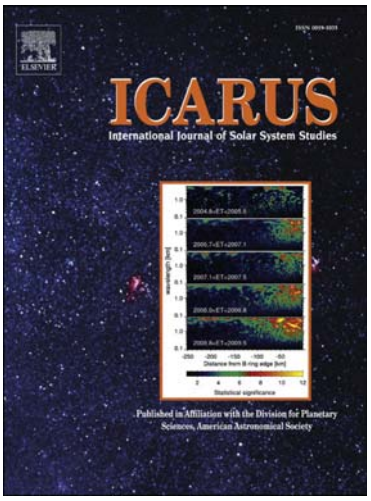

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Amino Acids Generated from Hydrated Titan Tholins: Comparison with Miller-Urey Electric Discharge Products

H. James Cleaves II[1,2,3,4*], Catherine Neish[5], Michael P. Callahan[6], Eric Parker[4,7], Facundo M. Fernández[4,7] & Jason P. Dworkin[6]

[1] Earth Life Science Institute, Tokyo Institute of Technology, Minato-ku, Tokyo, Japan

[2] Institute for Advanced Study, 1 Einstein Dr., Princeton, NJ 08540, USA

[3] Blue Marble Space Institute for Science, Washington, DC 20008, USA

[4] Center for Chemical Evolution, Georgia Institute of Technology, Atlanta, GA 30332, USA

[5] Florida Institute of Technology, 150 West University Boulevard, Melbourne, FL 32901, USA

[6] Solar System Exploration Division, NASA Goddard Space Flight Center, Greenbelt, MD 20771, USA

[7] School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA

*To whom correspondence should be addressed: Email: cleaves@elsi.jp, Tel. 858-366-3049.

Abstract

Various analogues of Titan haze particles (termed 'tholins') have been made in the laboratory. In certain geologic environments on Titan, these haze particles may come into contact with aqueous ammonia (NH_3) solutions, hydrolyzing them into molecules of astrobiological interest. A Titan tholin analogue hydrolyzed in aqueous NH_3 at room temperature for 2.5 years was analyzed for amino acids using highly sensitive ultra-high performance liquid chromatography coupled with fluorescence detection and time-of-flight mass spectrometry (UHPLC-FD/ToF-MS) analysis after derivatization with a fluorescent tag. We compare here the amino acids produced from this reaction sequence with those generated from room temperature Miller-Urey (MU) type electric discharge reactions. We find that most of the amino acids detected in low temperature MU $CH_4/N_2/H_2O$ electric discharge reactions are generated in Titan simulation reactions, as well as in previous simulations of Triton chemistry. This argues that many processes provide very similar mixtures of amino acids, and possibly other types of organic compounds, in disparate environments, regardless of the order of hydration. Although it is unknown how life began, it is likely that given reducing conditions, similar materials were available throughout the early Solar System and throughout the universe to facilitate chemical evolution.

Keywords: Atmospheres, chemistry – Astrobiology – Titan - Organic chemistry - Prebiotic chemistry

1. Introduction

It is generally believed that life on Earth originated from environmentally supplied organic precursors (Oparin 1924; Haldane 1929; Miller and Orgel 1974; Cleaves 2008). A variety of possible sources have been invoked for such an "Oparin-Haldane" type origin of life, including atmospheric synthesis (Miller 1953; Schlesinger 1983; Schlesinger 1983), extraterrestrial delivery from meteorites and comets (Chyba and Sagan 1992), and geothermal synthesis (Wächtershäuser 1988; Steele, McCubbin et al. 2012).

Although the composition of the atmosphere during the time life originated on Earth is debated (Tian, Toon et al. 2005; Zahnle, Schaefer et al. 2010; Trail, Watson et al. 2011), other bodies in the Solar System have atmospheres which may be more conducive to atmospheric organic synthesis. One example is Saturn's moon Titan, which has an upper atmosphere consisting of ~98 % N_2 and 2 % CH_4, along with minor amounts of other species including H_2, HCN, CO and organics such as ethane, ethylene, acetylene and cyanoacetylene (Niemann, Atreya et al. 2005). However, due to Titan's low temperature (~95 K), most of its water is present as ice in the crust and mantle, acting similar to bedrock on the Earth. In contrast, the early Earth was likely above the freezing point of water for long periods of time, with possible early excursions above and below the conditions at which water can exist as a liquid (Kasting and Pollack 1984; Bada, Bigham et al. 1994).

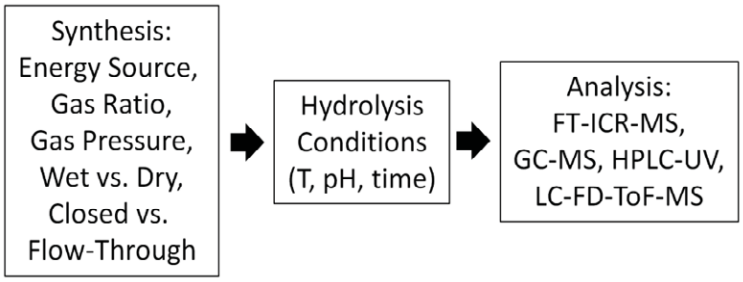
Titan's atmosphere is exposed to various types of energetic processing, producing higher molecular weight organic polymers. When produced under laboratory conditions, these polymers are collectively known as tholins (Sagan and Khare 1979). Similar materials have also been suggested to be formed in the atmospheres of Triton and Pluto (McDonald, Thompson et al. 1994). On Titan, it has been proposed that as the organic haze particles precipitate from the atmosphere they may come into contact with eutectic brines highly enriched in NH_3, in short-lived 'oases' of liquid water formed through impact melting or cryovolcanism (Thompson and Sagan 1992). These interactions could lead to reactions producing molecules of biological interest, for example amino acids and nucleobases (Neish, Somogyi et al. 2008; Neish, Somogyi et al. 2009; Neish, Somogyi et al. 2010).

The energetic processing of Titan's present atmosphere is likely somewhat different than that which acted upon the primitive Earth's (Chyba and Sagan 1992), or that which contributed to interstellar ice grain organic formation (Meierhenrich, Muñoz Caro et al. 2005; Öberg, Garrod et al. 2009). For example, the Earth has always received a significantly higher flux of solar radiation than Titan, and the flux in interstellar environments is attenuated distinctly

due to the differences in path lengths, composition and density of the medium. However, it was noted almost 30 years ago that electric discharge reaction products and organic molecules found in carbonaceous chondrites are very similar with respect to their amino acid contents (Wolman, Haverland et al. 1972). The inference is that there is some similarity in the mechanism of formation in both cases (Peltzer, Bada et al. 1984), for example *via* Strecker-like synthesis from small reactive organic precursors such as ketones or aldehydes (Miller 1957). Analytical studies since the 1970's have revealed a much richer molecular complexity in many carbonaceous chondrites (Schmitt-Kopplin, Gabelica et al. 2010; Burton, Stern et al. 2012). It is worth bearing in mind the various degrees of post-accretional processing the natural samples have undergone over ~ 4.5 Ga compared to the laboratory samples, and that, with few exceptions (Johnson, Cleaves et al. 2008; Vuitton, Bonnet et al. 2010), laboratory samples have not received the same degree of scrutiny.

A number of laboratories have modeled the synthesis of Titan haze particles using CH_4/N_2 gas mixtures at low temperature using various energy sources, such as electric discharge and UV light (Ferris 1981; Khare, Sagan et al. 1986; Thompson, Henry et al. 1991; McDonald, Thompson et al. 1994; Ehrenfreund, Boon et al. 1995; Kobayashi, Kaneko et al. 1997; Neish, Somogyi et al. 2008; Cable, Hörst et al. 2012). A subset of these laboratories also investigated the products of tholin hydrolysis, which is the subject of this paper.

There are numerous variables involved in these simulations which could render comparison difficult, for example the conditions under which the tholins are synthesized, and their products processed and analyzed (Scheme 1); nevertheless, as we will show, the detected products remain remarkably similar despite these variables.



Scheme 1. Some variables involved in the preparation of amino-acid yielding tholins reported in the literature.

For example, during tholin synthesis, a wide-range of ratios of N_2 to CH_4 (from ~ 1000:1 to 1:1), total gas pressures (from ~400 mm Hg to 1 mm Hg) and reaction temperature (from 296 K to 195 K) have been used. Some use flow reactors while others use static ones. Workup has ranged from high temperature (>100 °C) aqueous acid to extremely low temperature (-20 °C) aqueous base over widely varying time ranges. Lastly, a variety of analytical techniques have been used to measure the amino acid products of these reactions.

A short historical note regarding the terminology used here is useful for clarifying this discussion. While there was some experimental work on the generation of discrete low molecular weight organic compounds, such as amino acids, using electric discharges acting on various gas mixtures prior to Miller's pioneering 1953 publication (Miller 1953) (see for example (Löb 1913)), no formal name was applied by Miller or his predecessors to the complex organic mixtures which result from such experiments. It should be stressed that Miller's experiments were produced in the context of a reducing early Earth atmosphere, which adds a level of intentional contextualization to the materials produced. Furthermore, Miller recognized that discrete organic compounds were isolated from hydrolysis of the products of the electric discharge reaction, which could include a mixture of those synthesized directly in the discharge, those made in aqueous phase reactions from intermediates produced in the gas phase, and those derived from hydrolysis of larger more complex organic polymers produced in either phase. Collectively these were termed "electric discharge products"†

Thus the principle practical distinctions between MU electric discharge products and tholins, are that MU products can be considered as alternatively 1.) organic materials derived from the action of electric discharges acting on reduced gases, 2.) organic materials derived from energetic (be it *via* electric discharge, UV, or high energy particle) processing of gas mixtures (reducing or otherwise) simulating planetary atmospheric chemistry, or 3.) chemistry as described in 2.) in the presence of a liquid phase. Tholins, in contrast are generally considered to be the resulting non-volatile organic products derived from reduced gases *via* any type of energetic processing simulating solar system environments at temperatures below the freezing point of water. In both cases, the materials are considered to be precursors which require some work-up for the release of biomolecules, and in neither case is the potential importance of the molecular weight of the precursors considered significant, *i.e.* either or both might liberate small molecules in water, such as HCN, NH_3 and aldehydes and ketones, which may undergo other types of secondary reactions, such as the Strecker reaction, to give the final observed small molecule products such as amino acids.

The salient points we wish to make are that there is considerable similarity between tholins and MU discharge products when derived from methane and molecular nitrogen, with

respect to the amino acids, and potentially other products, produced regardless of the temperature of reaction or the presence of liquid water during energetic processing. This suggests that the gas-phase chemistry of non-oxygen-containing species dominate the amino acid synthesis pathways, and that as these are highly transient species the temperature may be of little importance (Khare, Sagan et al. 1984). This point may have considerable consequence for the types of organic materials which can be expected to be delivered to almost any reducing planetary environment throughout the universe.

Among the various other studies of these materials (Pernot, Carrasco et al. 2010; Vuitton, Bonnet et al. 2010), it has often been of interest to measure the amino acids produced from these simulations. As mentioned above, the paucity of water or other O-containing species in Titan's atmosphere means that the carboxyl and side chain oxygen must either be derived from a step simulating some sort of aqueous surface chemistry, or *via* a laboratory hydrolysis step.

Recent advances in mass spectrometry have allowed unprecedented high resolution accurate mass analysis of complex prebiotic mixtures, for example FT-ICR-MS studies of the Murchison meteorite and Titan tholins (Sarker, Somogyi et al. 2003; Pernot, Carrasco et al. 2010; Schmitt-Kopplin, Gabelica et al. 2010). Amino acids have been detected in several studies modeling these types of processes, though the techniques used have variable sensitivity for confident identification and quantification. For example, Khare et al. (1984) detected 16 amino acids using GC-MS, while MacDonald et al. (1994) found a partially overlapping set of 15 using HPLC with UV detection. More recently, Neish et al. (Neish, Somogyi et al. 2010) found species with the exact masses of 18 of the coded amino acids not containing sulfur using FT-ICR-MS, but only four of these were confirmed as the correct structural isomer using tandem mass spectrometry. Hörst et al. (2012) found evidence for the presence of 14 of the non-sulfur coded amino acids, though only the two simplest, alanine and glycine, could be confirmed using GC-MS.

We report here, for the first time, on the application of high sensitivity liquid chromatography with tandem fluorescence detection and mass spectrometry for the analysis of concentrated aqueous NH_3-processed tholins and their comparison with the products of a Miller-Urey $CH_4/N_2/H_2O$ electric discharge experiment. We find a large number of amino acid and amine products. The distribution of amino acids in these samples is remarkably similar, though there are some differences which likely speak to nuances in these molecules' modes of formation. The importance of the differences and similarities is discussed here.

2. Materials and Methods

2.1 Reagents

All reagents used in the analyses reported here were purchased from Fisher Scientific or Sigma-Aldrich, and were of reagent grade or higher unless otherwise stated. All tools used to handle the samples were cleaned using Millipore Direct Q10 H_2O (18.2 MΩ·cm, < 3 ppb total organic carbon, hereafter mQH_2O) before wrapping them in aluminum foil and heating them in air overnight at 500 ˚C.

2.2 Titan Tholins

Titan tholins were generated as described in Neish *et al.* (2009) by subjecting a mixture of 2% CH_4 and 98% N_2 to an AC electrical discharge at a pressure of 880 Pa and 195 K in a glass reaction vessel. 50 mg of tholins were dissolved in 2 mL of acetonitrile, and 100 µL of this solution was further diluted into 2 mL of a 1:1 mixture of 14 N aqueous NH_4OH (EMD Chemicals Inc.) and doubly deionized water. This solution was allowed to sit at room temperature for 2.5 years. As this sample did not contain significant amounts of inorganic salts it was derivatized and analyzed directly after hydrolysis (*i.e.* without desalting *via* IEC). However, we found that it was helpful to include a room temperature vacuum drying step prior to derivatization to remove excess NH_3, which often led to a large derivatized NH_3 peak which interfered with analysis.

2.3 Miller-Urey Electric Discharge Polymers

Miller-Urey electric discharge polymers were generated as described in Ring *et al.* (1972), except the ammonia buffer was replaced with a 0.225 M sodium bicarbonate buffer adjusted to pH 8.7 which was degassed prior to introduction of the gas mixture. These were analyzed after desalting by Ion-Exchange Chromatography IEC as described in (Johnson, Cleaves et al. 2008) both directly and after vapor phase acid hydrolysis.

Table 1 shows a comparison of previously reported results using different methods of synthesis and processing.

Study	MU – NH_3(aq)[a,b]	MU – no NH_3[c]	Titan Tholin[d]	Titan Tholin[e]	Triton Tholin[f]	Titan Tholin[g]
Gas Mixture (pressure, mm Hg)	N_2 (200), CH_4 (200), H_2O (14)	N_2 (200), CH_4 (200), H_2O (14)	N_2 (6.3), CH_4 (0.13)	N_2 (0.135), CH_4 (0.015)	N_2 (0.999), CH_4 (0.001)	N_2 (0.735), CH_4 (0.015)
Solution Phase[h]	0.0675 M NH_4Cl, pH 8.7[i]	0.225 M $NaHCO_3$ pH 8.7[i]	None	None	None	None
Energy Source	Electric Discharge	Electric Discharge	Electric Discharge	Electric Discharge	Electric Discharge	Electric Discharge
Experiment Temperature	298 K	298 K	Gas Phase: 195 K	298 K	298 K	170 K
Workup	3 N HCl hydrolysis 373 K24 h.	Straight/6 N HCl vapor hydrolysis 373 K 24 h.	15 wt % aq. NH_3 pH ~ 11.5, 253 K	6 N HCl 373 K 20 h IEC	HCl/propionic acid 150 °C 85 min.	25 wt % aq. NH_3, 253 K
Derivatization		OPA/NAC	OPA/NAC		Waters Picotag	
Analysis	GC-MS	LC-FD-ToF-MS	FT-ICR-MS/LC-FD-ToF-MS	GC-MS	HPLC-UV	GC-MS

Table 1. Comparison of the various experimental conditions and analytical techniques which have been used to study MU reaction products and Titan tholins. a, b. as reported in (Ring, Wolman et al. 1972; Wolman, Haverland et al. 1972). c. This paper. d. This paper, also reported in (Neish, Somogyi et al. 2010). e. As reported in (Khare, Sagan et al. 1984). f. As reported in (McDonald, Thompson et al. 1994). g. As reported in (Poch, Coll et al. 2012). h. If present, exposed to gas during energetic gas processing. i. Initial pH. GC-MS: gas chromatography-mass spectrometry; LC-FD-ToF-MS: liquid chromatography with fluorescence detection and time-of-flight mass spectrometry; FT-ICR-MS: Fourier-transform ion cyclotron resonance mass spectrometry; IEC: ion exchange chromatography; OPA/NAC: *o*-phthaldialdehyde/N-acetyl-L-cysteine.

2.4 Standard Analysis

Stock amino acid solutions (~10^{-3} M) were produced by mixing individual amino acid powders (97-99% purity) with mQH_2O. Three reagents were prepared to derivatize the samples for analysis by a Waters ACQUITY UPLC and fluorescence detector and a Waters LCT Premier time-of-flight mass spectrometer (UHPLC-FD/ToF-MS): 1) 0.4 M sodium borate (pH 9.4), 2) 0.1 M hydrazine hydrate, and 3) OPA/NAC, a fluorescent reagent used to tag primary amino groups. These three reagents were prepared using the methods detailed in (Glavin, Dworkin et al. 2006). We had available as standards the 18 non-sulfur-containing coded amino acids, as well as various others including DL α-hydroxymethylalanine, α-hydroxymethylserine (α-HMS), β-hydroxyaspartic acid, DL ornithine, DL α-amino adipic acid, DL homoserine, DL-2-methylglutamic acid and DL isoserine.

The ammonium formate buffer used for UHPLC-FD-ToF-MS analyses was made by titrating 50 mM formic acid to pH 8 with NH_4OH and adding methanol to a final concentration of 8 volume percent.

2.5 LC-FD-ToF-MS Analysis

Sample extracts were analyzed by LC-FD/ToF-MS, as described in (Johnson, Cleaves et al. 2008). Sample extracts were prepared as described above and derivatized with the following modification. The samples were derivatized for 1 minute after which the reaction was quenched with 75 μL of hydrazine. Samples were then immediately placed into the LC-FD/ToF-MS autosampler and injected. Details of the ToF-MS settings and the amino acid quantification methods used for these analyses are detailed elsewhere (Glavin, Dworkin et al. 2006). The LC-FD-ToF-MS technique had a detection limit in the low femtomole range for amino acids.

Products were identified based on three criteria: 1.) the fact that they must be amine-functional group containing molecules to produce fluorescence signals, 2.) the correspondence of their chromatographic retention time with that of a known standard, and 3.) their protonated molecular (parent) ions in the mass spectrum.

3. Results and Discussion

Examination of the chromatograms obtained reveals a complex suite of fluorescent products (Figure 1). These should contain primary amino groups as to be fluorescent given the excitation and emission wavelengths should require the presence of an OPA tag. However, in the case of samples which were not subjected to IEC there may also be other untagged compounds present in these chromatograms which are inherently fluorescent under these

conditions.

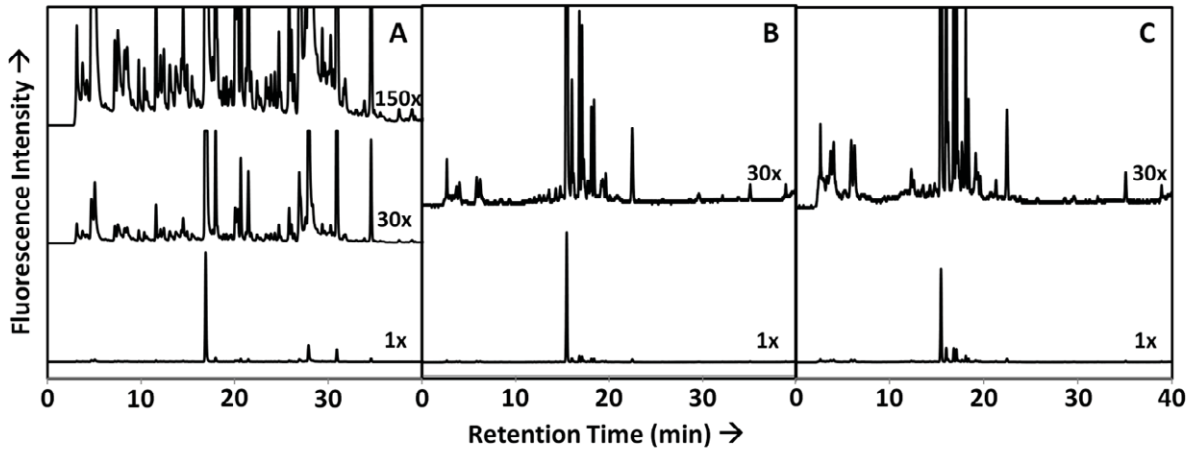


Figure 1. LC-FD traces showing the abundance of likely OPA/NAC tagged peaks of A.) the low temperature ammonialyzed tholin; B.) the HCl vapor hydrolyzed carbonate-buffered spark product; and C.) the H_2O hydrolyzed carbonate-buffered spark product, showing magnifications. B and C are to scale.

Closer examination of this data reveals even greater amine diversity (Figure 1A, middle trace, Figure 1B/C, top traces). Zooming in further on the baseline an even larger diversity of amines is noticeable (Figure 1A, top trace). The diversity is almost fractal, and its visualization may be limited by chromatographic peak capacity. The baseline complexity of the Titan tholin chromatogram is not completely mirrored in the MU samples, however some of this may be due to volatile components lost during the IEC and workup of the MU samples such as small primary amines, and others may be inherently fluorescent molecules which are not retained by IEC (Hodyss, McDonald et al. 2004).

Examination of the extracted ion chromatograms (EICs) allows more confident identification of these amino acids, including their enantiomers (Figure 2).

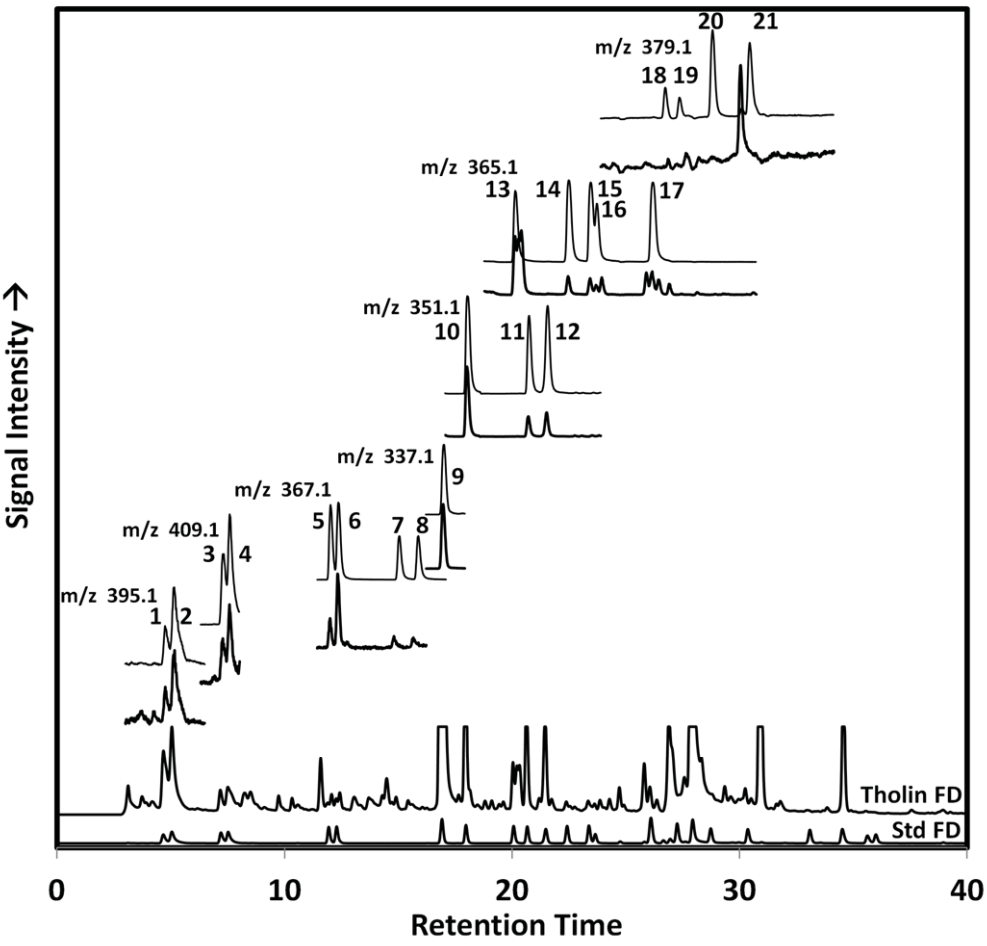


Figure 2. Fluorescence traces and EICs of various amino acids searched for in the Titan tholin products and an authentic standard. 1, 2 DL-aspartic acid, 3,4 DL-glutamic acid, 5,6 DL-serine, 7,8 DL-isoserine, 9 glycine, 10 β-alanine, 11, 12 DL-alanine, 13 γ-aminobutyric acid, 14 D-β-aminobutyric acid, 15 L-β-aminobutyric acid, 16 α-aminobutyric acid , 17 DL-α–aminobutyric acid, 18, 19 DL-isovaline, 20, 21 DL-valine. Bottom: fluorescence trace, amino acid standard, above that, fluorescence trace of the Titan tholin analogue. Shown floating above are the EICs of the OPA/NAC traces, with the standard trace above the Tholin trace. All EICs are traces of the protonated molecular ion of the given OPA/NAC labeled amino acids with a ± 0.1 amu mass window.

Among the coded amino acids detected in the tholin sample were glycine (gly), DL-alanine (ala), DL serine (ser), DL-aspartic acid (asp), DL-glutamic acid (glu), DL-asparagine (asn) and DL-glutamine (gln). Among the non-coded amino acids detected in the tholin sample were

DL-isoserine (iser), β-alanine (βala), γ-aminobutyric acid (γaba), DL-β-aminobutyric acid (βaba), α-aminoisobutyric acid (αaib), DL-α–aminobutyric acid (αaba) and DL-isovaline (ival). Among the coded amino acids not detected within the detection limit of this analysis were valine (val), leucine (val), isoleucine (ile), histidine (his), arginine (arg), phenylalanine (phe), tyrosine (tyr), tryptophan (trp) and lysine (lys) (Figure 3). These results show some similarities to those of Neish et al. (2010), who detected Asn, Asp, Glu, and Gln in the sample, but lacked the capability to detect species as small as Gly, Ala, and Ser.

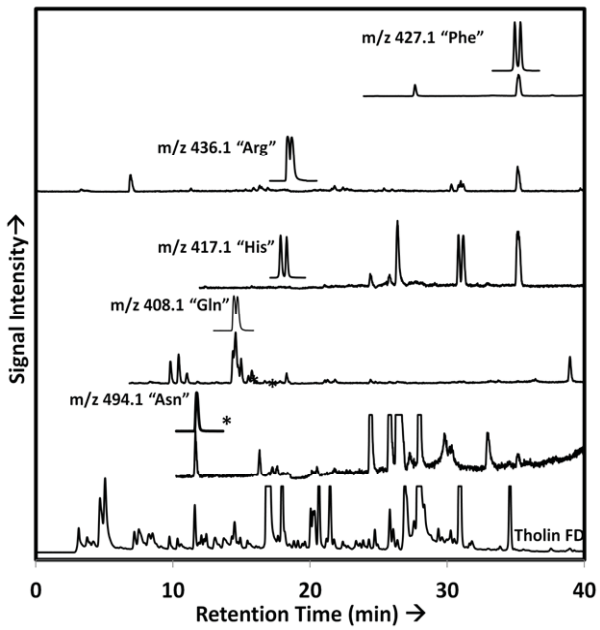


Figure 3. Fluorescence chromatogram and EICs of Gln, Asn and other coded amino acids searched for, along with EICs from authentic standards. His, Arg and Phe were not detected, nor were DL-Trp or DL-Tyr (data not shown).

Gln, Asn and Trp are not typically detected after high temperature (100 °C) acid hydrolysis, due to their hydrolytic instability (Wong and Bronskill 1979), which explains their scarcity in the acid hydrolyzed MU samples. However Asn and Gln survive the low-temperature hydrolysis conditions to which the Titan tholin was exposed, and the hot water hydrolysis conditions in the case of the MU sample. The failure to detect the others could be due to their being present in quantities below the detection limit of our analysis or their absence. This work emphasizes the importance of chromatographic analyses beyond the simple infusion and identification of the exact mass of a species through FT-ICR-MS, since isobaric structural

isomers may be present. For example, in the case of His, we observe what appears to be an enantiomeric pair, suggesting this species shares the molecular formula of His, one stereocenter, and a single primary amino group (or it would derivatize twice, giving it a different parent mass), but likely also one free carboxyl group (otherwise it would not elute on the time-scale observed), though many other primary amine-containing structural isomers are possible. This agrees with the results of Neish et al. (2010), who found evidence for a structural isomer of His with a different MS/MS fragmentation pattern than the pure substance.

Comparison of peak areas with those of known standards allows quantification, which is shown in Table 2.

Sample	**Titan Tholin[a]**		**$NaHCO_3$ Buffered MU[b]**		**$NaHCO_3$ Buffered MU[c]**	
Compound	**Mole Ratio[d]**	**D/L[e]**	**Mole Ratio[d]**	**D/L**	**Mole Ratio[d]**	**D/L**
Glycine	1	-	1	-	1	-
DL Aspartic Acid	7×10^{-2}	1.00	7×10^{-2}	0.77	3×10^{-2}	0.60
DL Alanine	6×10^{-2}	1.05	0.3	1.07	9×10^{-2}	1.01
β-Alanine	4×10^{-2}	-	0.2	-	3×10^{-2}	-
DL Asparagine	2×10^{-2}	nd	1×10^{-2}	nd	5×10^{-4}	nd
DL Glutamic Acid	2×10^{-2}	1.24	0.1	0.73	4×10^{-2}	1.02
γ-Aminobutyric Acid	1×10^{-2}	-	6×10^{-5}	-	2×10^{-3}	-
DL Glutamine	1×10^{-2}	0.85	0.2	1.03	0	nd
DL Isoserine	1×10^{-2}	1.15	1×10^{-2}	1.02	2×10^{-2}	0.78
DL Serine	9×10^{-3}	0.75	3×10^{-2}	1.27	9×10^{-3}	0.78
DL α-Aminobutyric Acid	8×10^{-3}	nd	6×10^{-2}	nd	3×10^{-2}	nd
α-Aminoisobutyric Acid	6×10^{-3}	-	3×10^{-2}	-	1×10^{-2}	-
DL β-Aminobutyric Acid	6×10^{-3}	1.00	1×10^{-2}	1.29	3×10^{-3}	1.16

Table 2. Yields of amino acids from aqueously ammonialyzed Titan tholin analogue and water and acid hydrolyzed electric discharge polymer. a. – Indicates the compound in question is achiral. nd = not determined, as the enantiomers were not chromatographically resolved. 1. The recovery of glycine was 9.1 mg per g of polymer. b. H_2O hydrolyzed. The recovery of glycine was 1.13 mmoles per mole of input methane. c. HCl hydrolyzed. d. Relative to Gly = 1. The

recovery of glycine was 1.44 mmoles per mole of input methane. Note the recovery of glycine from the HCl hydrolyzed MU experiment was 1.27x that of the H_2O hydrolyzed sample. The relatively greater recovery of glycine may account for some of the apparent reduction in relative yields of other amino acids for the HCl hydrolyzed MU experiment. e. Ratio of the detected D and L enantiomers, where measurable. A dash indicates there are no stereoisomers for the compound, nd indicates the enantiomers were not chromatographically separated.

Also identified in all of these reactions were significant amounts of iser and various low molecular weight amines including methylamine, ethylamine and ethanolamine. Since some of these are volatile and some may have been lost during the evaporation steps during workup, they could not be meaningfully quantified and compared here. Comparison of data from previous measurements of amino acids from tholins (Khare et al. 1984; Neish et al. 2010) and electric discharge reactions (Ring, Wolman et al. 1972; Wolman, Haverland et al. 1972) and our current data is presented in Figure 4. We have focused on a subset of the amino acids generated, which are among the most abundant in all samples examined. This subset is amenable to primary amine analysis using OPA/NAC. This technique does not allow for the analysis of secondary amines such as sarcosine and proline, even though these are sometimes significant components of such samples. For example sarcosine is produced in ~13 % and proline in 0.3 % of the relative molar abundance of glycine in some low temperature MU experiments (Ring, Wolman et al. 1972). Furthermore, it can be difficult to compare the results of studies using different analytical techniques. It should also be borne in mind that there may be species which are abundant in these samples that are not readily detected by our or other techniques, which might could make various samples appear similar to different degrees. For example, proline and sarcosine and 1,2-diamines are not detectable using our techniques. Raulin and colleagues detected three amino acids (Gly, Asp and Ala) after MSTFA derivatization of similar materials using GC-MS analysis (Poch, Coll et al. 2012). These three compounds are also detectable using the present methodology, which is considerably more sensitive. Gly was easily detected as were a large suite of other amino acids, including Ala, Asn, Gln, Asp, Glu, Ser, and Iser. Additionally our method allows the separation of the enantiomers of these amino acids, thus we can determine their potential degree of terrestrial contamination.

For the comparison below we report the yields of amino acids from various tholin syntheses binned by the number of carbon atoms per molecule (C2: Gly; C3: DL-Ser, DL-Isoser, DL-Ala, βAla; C4: DL-Asp, γABA, αAIB, DL-βABA; DL-αABA C5: DL-Glu).

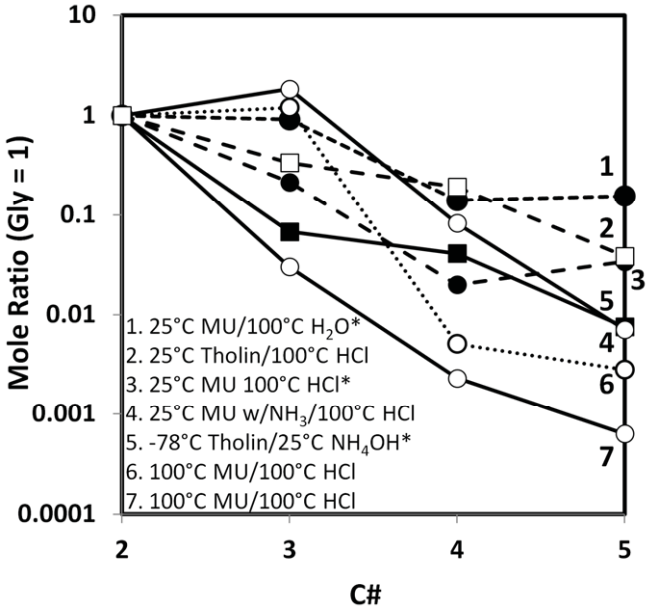


Figure 4. Relative yields of amino acids as a function of number of carbon atoms for a variety of CH_4/N_2 gas experiments simulating abiotic synthesis conditions. Closed symbols, this paper; open symbols, literature data; circles "MU" experiments; squares "Titan tholin" experiments. The inset information provides reference to the experimental details (Reaction Temperature/Hydrolysis Conditions). The asterisk indicates data from the present analysis. 1. Room temperature (RT, 298 K) MU experiment, H_2O hydrolyzed (this study); 2. RT dry Titan tholin (Khare et al. 1984); 3. RT MU experiment, HCl hydrolyzed (this study); 4. RT MU experiment using single chambered apparatus buffered with NH_3, HCl hydrolyzed (Ring et al 1972); 5. Titan tholin, cold NH_3(aq) hydrolyzed (Neish et al. 2010) (data this study); 6. Hot MU using double chambered "volcanic" apparatus buffered with NH_3, HCl hydrolyzed (Johnson et al. 2008) 7. Hot MU using double chambered "silent discharge" apparatus buffered with NH_3, HCl hydrolyzed (Johnson et al. 2008).

The most notable differences are in the ratios of C2:C3 amino acids, with the major contributor being alanine. Relative yields are similar in many ways as evidenced by several studies. Modifications of the original MU apparatus set up, for example those including H_2S or nebulized water vapor, produce amino acid mixtures that are even more similar than that used for Peltzer *et al.*'s comparison (Johnson, Cleaves et al. 2008; Parker, Cleaves et al. 2010; Parker, Cleaves et al. 2011). While these variations have not been systematically explored, we suggest here that multiple mechanisms could explain the similarity of one to another. These molecules are very reminiscent of the molecules produced from MU type experiments, suggesting there are similarities in their modes of formation. The laboratory experiments of Khare et al. (1984)

and McDonald et al. (1994) were carried out at room temperature; however, these authors argued that because of the large difference in effective temperature between the energy source for molecular dissociation and the ambient temperature, both in the laboratory and on Titan, the choice of reaction temperature may not significantly affect the results.

There are significant differences in how these laboratory materials were formed. The Miller-Urey electric discharge samples were made from CH_4 and N_2 at room temperature in the presence of water vapor buffered with aqueous $NaHCO_3$, while the Titan tholin samples were made from a high frequency discharge at low temperature in the absence of water and then hydrated in aqueous NH_3 at low temperature. Electric discharge experiments starting from N_2 or NH_3, CH_4 and H_2O likely initially generate various radical species such as N and CH_3 which recombine to give species such as HCN, acetylene and cyanoacetylene (Miller and Urey 1959; Sanchez, Ferris et al. 1966). The deposition of these species into aqueous solutions containing NH_3 allows various Strecker and other reactions, which can directly yield amino acids (Miller 1957).

Strecker- and Michael-addition type reactions proceed directly in the aqueous phase starting with the organic precursors such as HCN, aldehydes, acrylonitrile and cyanoacetylene. Aqueous HCN chemistry itself may produce some higher carbon skeletons such as those of Asp and Glu (Ferris, Wos et al. 1974), although the mechanism for the generation of Glu from HCN does not have a good mechanistic explanation yet (Harada 1967).

If these species are derived from non-oxygenated species, mechanistic pathways for their synthesis must exist, which must be distinct from classically proposed ones such as the Strecker syntheses, as aldehydes and ketones cannot be initial precursors for their formation. This does not mean that processes which include aldehydes or ketones cannot also contribute to these processes, but that both types of synthesis contribute to the formation of the same types of compounds (He, Lin et al. 2012).

Despite some early arguments to the contrary (Matthews 1982), tholins are clearly different materials than HCN polymers, although both contain considerable amounts of nitrile functional groups. Tholins also appear to be better matches in general for extraterrestrial organic materials, both spectrally and with regard to their components. They are typically much more C rich and N poor than HCN polymers, and have rather different mass distributions (Vuitton, Bonnet et al. 2010).

Interestingly, room temperature hydrous spark discharge reactions are evidently good analogues of hydrated Titan tholins. Rather than being merely serendipitous, it seems likely that CH_4 and N_2 when excited recombine to give very similar organic products, which hydrate

similarly regardless of hydrolysis temperature. It also seems likely that among any one of these samples, multiple mechanisms of formation may be operative, as evidenced by isotopic labeling studies (Elsila, Dworkin et al. 2008). Regardless of the precise mechanism of synthesis, it does seem that there is a cohesive mechanism at some level in all of these syntheses.

Miller's pioneering work showed how surprisingly easily compounds of biological relevance, for example urea, Gly, Ala, Asp, *etc.*, could be made in simple prebiotic simulations (Miller 1953). Consequently there has been a general trend to look for more compounds of biological relevance in these reactions. While several more have been found, it must be borne in mind that organic structural isomerism leads to there being a very large number of compounds with identical formulas, even among the subset of those which are amino acids (Table 3).

Amino Acid	Molecular Formula	Number of Isomers		
		All*	No 3- or 4-rings	With αAA Backbone**
Gly	$C_2H_5NO_2$	84	53	1
Ala	$C_3H_7NO_2$	391	244	1
Ser	$C_3H_7NO_3$	1,391	857	2
Cys	$C_3H_7NO_2S$	3,838	2,422	2
Thr	$C_4H_9NO_3$	6,836	4,242	4
Asp	$C_4H_7NO_4$	65,500	25,036	14
Asn	$C_4H_8N2O_3$	210,267	81,702	45
Pro	$C_5H_9NO_2$	22,259	8,462	3 (6)[1]
Val	$C_5H_{11}NO_2$	6,418	3,973	2
Met	$C_5H_{11}NO_2S$	86,325	54,575	10
Glu	$C_5H_9NO_4$	440,821	172,617	71
Gln	$C_5H_{10}N_2O_3$	1,360,645	539,147	207
Leu, Ile	$C_6H_{13}NO_2$	23,946	14,866	4
Lys	$C_6H_{14}N_2O_2$	257,122	162,054	31
His	$C_6H_9N_3O_2$	89,502,542	13,563,099	902
Arg	$C_6H_{14}N_4O_2$	88,276,897	36,666,235	3,563
Phe	$C_9H_{11}NO_2$	277,810,163	25,316,848	571 (6)[2]
Tyr	$C_9H_{11}NO_3$	2,132,674,846	209,838,248	8,309 (43)[2]
Trp	$C_{11}H_{12}N_2O_2$	1,561,538,202,786	64,968,283,073	559,128 (1,770)[2]

Table 3: Numbers of isomers for the 20 biologically encoded protein forming α-amino acids. *"All" isomers denotes all structures which satisfy Lewis electron pairing rules for a given formula, the following column reports this number of isomers minus those structures which

include 3 or 4 membered rings. ** The total number of formula structural isomers which also contain the α-amino acid backbone motif. The numbers in parentheses refer to isomers which contain 1. a secondary α -amino acid backbone motif or 2. a primary α-amino acid motif and an aromatic ring in the side-chain. Adapted from (Meringer, Cleaves et al. 2013).

Although the excellent mass resolution and mass accuracy of FT-ICR-MS allow for unambiguous elemental formula assignment with minimal sample cleanup (for example directly from organic solvent extraction (Ehrenfreund, Boon et al. 1995; Schmitt-Kopplin, Gabelica et al. 2010)), a single formula can represent many structures (Table 3), zwitterions may not electrospray well in either positive or negative mode, and ion suppression in such complex samples is likely. Additionally, compounds with the same molecular formula derived from the same isotopically enriched starting materials can reasonably be expected to have similar isotopic enrichments. Thus, while FT-ICR-MS is an extremely powerful method for finding molecular formulas, it alone cannot provide unambiguous identification, and further dimensions of analysis, such as liquid or gas-phase separations and fragmentation are required.

All analytical methods have their limitations when analyzing amino acids. Amino acids lacking chromophores are difficult to detect *via* HPLC, and thus derivatization is typically required, commonly targeting amino groups (Lunn and Hellwig 1998). While UV detection is typically robust, many extraneous compounds may absorb in near UV wavelengths which are typically monitored. Fluorescence detection overcomes this to some extent, as specific excitation and emission wavelengths allow peaks lacking the derivatization reagent to be filtered out. Derivatization also renders amino acids less polar, and more amenable to analysis *via* reversed phase chromatography.

GC-MS allows for simple detection and fairly unambiguous identification *via* EI fragmentation spectra, with numerous derivitization reagents allowing volatilization of polar compounds. One drawback, however, is that large polar compounds with multiple tags may not volatilize well, or they may thermally decompose before eluting from the GC column. On the positive side, fragmentation spectral libraries usually facilitate compound identification, but require comparison with authentic standards to further validate candidate compound identities.

LC-FD-MS is very sensitive, retention times are diagnostic, and a mass peak must correlate with a fluorescence peak: if there is no FD peak then the mass detected does not likely correspond to a primary amine. ToF-MS allows for good mass resolution, which means one can be fairly certain of molecular formula identification, and the addition of the OPA/NAC moiety constrains possible formulas. The downside is that obtaining fragmentation spectra requires more sophisticated hybrid Q-TOF instrumentation, and OPA/NAC does not tag some

classes of compounds including 1, 2-diamines (*e.g.* 2, 3-diaminopropionic acid, 2, 4-diaminobutyric acid, and ornithine) and secondary amines (*e.g.* proline and sarcosine). How similar these samples appear when a larger set of compounds is considered is the subject of ongoing work in our laboratories.

The LC-FD-MS techniques used here are very sensitive, highly unambiguous due to their multi-dimensionality, and are standardized and readily available, though they have blind spots, such as the inability to detect certain molecular weight amines of potential prebiotic interest, such as secondary amines and vicinal diamines. Ideally, an LC-MS-compatible derivitization method should be developed which allows for separation and detection of primary, secondary and vicinal diamines and their enantiomers. The development of such derivitization techniques should be possible, and would be useful for future solar system exploration programs.

4. Conclusions

Room temperature MU experiments conducted in the presence of water, with or without initial aqueous NH_3, hydrolyzed under various conditions, and various anhydrous Titan tholin hydrolysis experiments produce many of the same amino acid products. However, we note that considerable care must be exercised in their identification, as neither chromatographic retention time nor mass alone are fully reliable detection techniques. Our results suggest there may be a remarkable cohesiveness in the types of amino acids which can be produced in various primitive planetary environments given a reducing atmosphere.

Footnote

†It was not until Sagan and co-workers recognized the potential parallels between MU type reactions and the chemistry of the atmospheres of the Jovian planets and their satellites that the term “tholin” was coined, which Sagan derived from the Greek root “tholos” (“θολοσ”). It appears Sagan intentionally chose a Greek root which could be alternatively interpreted as being derived from the word meaning “muddy” or “unclear”, referencing both the color and molecular complexity and heterogeneity of these materials, as well as from the root meaning “dome”, referencing their astronomical significance. In a personal communication from Miller to Sagan discussing Sagan’s suggested name, Miller, tongue-in-cheek, suggested “tholin” was a poor choice of terminology, partly because the English voiceless dental fricative “Θ” (“th-“) would be difficult to pronounce in German (where it would be pronounced as “/d/”) (Miller, personal communication to HJC).

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Highlights from: Amino Acids Generated from Hydrated Titan Tholins: Comparison with Miller-Urey Electric Discharge Products

H. James Cleaves II, Catherine Neish, Michael P. Callahan, Eric Parker, Facundo M. Fernández & Jason P. Dworkin

- Titan tholin amino acids were measured using multi-dimensional chromatographic analysis.
- We compared the tholin amino acids with those generated from Miller-Urey (MU) reactions.
- The amino acids detected in MU reactions are similar to those generated in the Titan simulation.
- Many solar system environments may thus provide similar organic compounds.
- Given reducing conditions, similar materials should be available throughout the universe.