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Understanding Titan's Prebiotic Chemistry: Synthesizing Amino Acids Through Aminonitrile Alkaline Hydrolysis

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ABSTRACT: Titan is an ocean world with a plethora of organic material in its atmosphere and on its surface, making it an intriguing location in the search for habitable environments beyond Earth. Settled aerosols will mix with transient surface melts following cryovolcanic eruptions and impact events, driving hydrolysis reactions and prebiotic chemistry. Previous studies have shown that the hydrolysis of laboratory-synthesized Titan organics leads to the production of amino acids and other prebiotic molecules. The exact molecular structure of Titan aerosols remains unclear, yet aminonitriles have been hypothesized to be among the organic components. This laboratory study tested three reaction pathways that could potentially lead to the formation of amino acids: aminoacetonitrile → glycine, 2-aminopropanenitrile → alanine, and 4-aminobutanenitrile → γ-aminobutyric acid. Liquid chromatography mass spectrometry (LCMS) is used to quantify the abundance of amino acids over a 6-month period. We conclude that ammonia plays a key role in the synthesis of amino acids from aminonitriles, while the inclusion of salts (1 wt %) and minerals (25 mg/mL) did not have a significant effect on amino acid formation compared to ammonia. Rate constants (k) for alkaline hydrolysis of the aminonitriles were calculated. Our results suggest that if Titan's surface melts have a composition, including at least 5% ammonia in water, and if aminonitriles are present in Titan's organic aerosols, then amino acids will likely form. These results are highly relevant to the Dragonfly mission to Titan, which will sample impact melt material at Selk crater to search for prebiotic molecules.

KEYWORDS: *titan, hydrolysis, prebiotic chemistry, amino acids, alkaline*

Titan's Selk Crater from Cassini Radar

Aminonitrile Alkaline Hydrolysis Pathway to Amino Acids
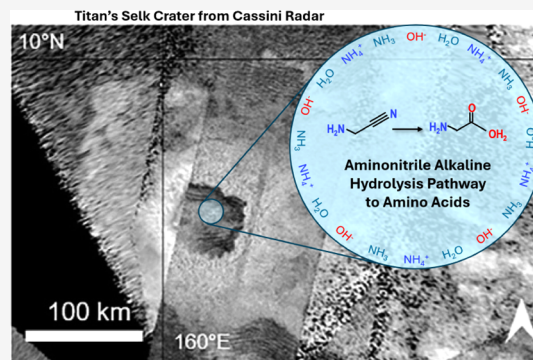
1. INTRODUCTION

Saturn's largest moon, Titan, has a predominant nitrogen atmosphere with a few percent methane and other trace species.[1] The breakdown of these atmospheric molecules by photons and energetic molecules from Saturn's magnetosphere leads to the synthesis of complex $C_xH_yN_z$ molecules that then conglomerate into aerosols and gravitationally settle through the atmosphere.[2] This organic matter ultimately deposits on Titan's surface[3,4] where it is available to interact with surface liquids.

Transient liquid water environments are expected to persist in cryovolcanic flows and impact melt pools[5] (Figure 1)[6,7] for hundreds to possibly tens of thousands of years prior to refreezing.[8−11] Numerous impact craters and several candidate cryovolcanic features have been identified on Titan's surface by the Cassini spacecraft[7,12−15] (Figure 1); thus, transient surface melts of predominately liquid water have existed on Titan in the past and will occur again. Ammonia ($\leq$14 wt %)[16−18] and salt (1 wt %)[19] have been hypothesized to be present in Titan's interior ocean. Therefore, ammonia and salt could also be mixed into Titan's surface melts, particularly if sourced from the interior ocean via cryovolcanic flows or a breach of Titan's crust via an impactor.[20,21] Impacts are also believed to have aided in the origin of life on Earth[22] and impactor materials have been shown to facilitate organic synthesis.[23]

The initial cryovolcanic flow temperature is likely dependent on the composition of the interior ocean, ranging from ~0 °C in liquid water to −23 °C with 14% NH_3, with decreasing temperatures until solidification occurs. Impact melt on Titan may be significantly warmer, with hypotheses ranging from a few degrees above 0 °C[9−11] to an initial impact shock temperature surpassing the boiling point of water.[8] Though, it is reasonable to suggest the majority of the impact melt will likely spend the largest interval of time at lower temperatures during refreeze.
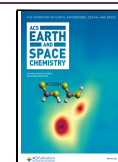
Prior to refreezing of the melt pools, the present organic molecules interact with the liquid melt, driving hydrolysis reactions. A number of studies have tested such potential hydrolysis reactions in the laboratory.[24−34] These studies were all initiated with the synthesis of Titan-like analogs in the
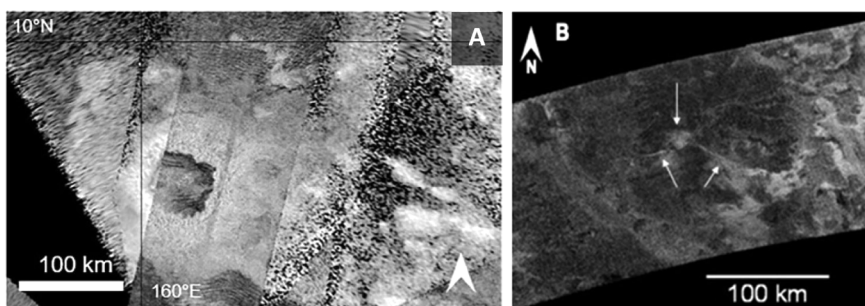
Figure 1. Geologic formations, such as impact melt pools and cryovolcanic flows, are locations where transient water may persist on Titan's surface. Impact craters (A) and cryovolcanic domes (B), as pictured here on Titan, may have hosted prebiotic chemistry prior to freezing. (A) Titan's Selk crater from Cassini synthetic aperture radar (SAR). Selk crater is the target location for NASA's Dragonfly mission (JMARS; Reprinted from, Elsevier, Farnsworth et al., Chapter 10 Titan, Enceladus, and Other Icy Moons of Saturn. In Soare et al. (Eds.), Ices in the Solar System, first edition, 315−356, Copyright (2024), with permission from Elsevier). (B) Cassini SAR observation of Titan's Ganesa Macula, a hypothesized cryovolcanic dome or shield. Arrows highlight Ganesa's central caldera and channels (Reprinted from Icarus, 186(2), Lopes, R. M. C., et al., Cryovolcanic features on Titan's surface as revealed by the Cassini Titan Radar Mapper, 395−412, Copyright (2007), with permission from Elsevier).

Table 1. The Five Most Frequently Identified Products Detected in Nine Tholin Hydrolysis Studies[24−26,29−34]

		Khare 1986	Raulin 2007	Nguyen 2007 †	Neish 2010	Ramírez 2010	Poch 2012	Taniuchi 2013	Cleaves 2014	Brassé 2017
The 5 Most Frequently Identified Products	Glycine[a]	✓	✓	✓	n.d.	✓	✓	✓	✓	✓
	Alanine[a]	✓	✓	✓	n.d.	✓	✓	✓	✓	✓
	Aspartic Acid	✓	✓	n.d.	✓	n.d.	✓	✓	✓	✓
	Urea	✓	✓	✓	n.d.	✓	✓	n.d.	n.d.	✓
	Aminobutyric acid[a]	✓+	✓	✓	n.d.	n.d.	n.d.	✓	✓+	✓+
Tholin Hydrolysis Parameters	pH	Acidic	Neutral	Acidic & Neutral	Alkaline	Alkaline	Alkaline & Neutral	Acidic	Alkaline	Alkaline
	Temperature	373 K	343 K	n/a	253, 293 K	96, 253, 277 K	253, 279 K	383 K	298 K	293, 279, 253 K
	Time	20 hrs	48 hrs	n/a	1 yr	10 w	10 w	24 hrs	2.5 yrs	10 w

†As reported in Brassé et al., 2017
+γ-ABA was detected

[a]Green rows highlight the amino acid products explored in this study.

laboratory, called tholins.[35] Tholins are believed to be similar to Titan aerosols, with complex $C_xH_yN_z$ molecular structures, yet their exact molecular structures remain unknown and laboratory tholin structure likely varies with the starting gas phase composition and varying production mechanisms and instrumentation.[35] In these prior works, tholins were mixed with water solutions under varying temperatures and reaction times to study the potential products of such reactions. Over 30 oxygenated products have been identified in these studies, several of which are amino acids.[24−26,29−34] The top five most frequently reported products were glycine, alanine, aspartic acid, urea, and aminobutyric acid (Table 1). Based on the identified products, several key molecular classes in tholins have been hypothesized to be likely precursors: aminonitriles, alkenes, heterocyclic rings, imines, nitriles, and amines.[27,28,31−33]

One potentially important reaction pathway for the formation of amino acids on Titan involves aminonitriles as reactants via alkaline hydrolysis. This mechanism was proposed by Brassé et al. (2017) and Poch et al. (2012) (Figure 2) to explain their observations of amino acid products after reaction of tholins in ammonia hydroxide solutions. This weak base reaction has similarities to the commonly known Strecker synthesis. However, unlike the heated (i.e., above room temperature) acid hydrolysis step in the traditional Strecker synthesis, in alkaline hydrolysis, ammonia is expected to act as a catalyst in a basic solution, and the reaction may proceed without heating. Alkaline environments on Titan are thought to be more likely

than acidic environments given the potential presence of ammonia and other organic species. Titan's low temperature and basic conditions make the alkaline hydrolysis reaction more favorable than the traditional Strecker synthesis on Titan.

In this study, we test the proposed aminonitrile alkaline hydrolysis reaction under various Titan-relevant impact melt pool environmental parameters: temperature (−22 °C, 3 °C, and 21 °C), ammonia concentration (0, 5, 10, and 15% NH_3), the inclusion of salts and minerals (1 wt % salt and 25 mg/mL olivine/pyroxene), and time (up to 6 months). Our study aims to further constrain the conditions necessary for the formation of amino acids in Titan's surface melts.

2. METHODS

2.1. Materials. An overview of the aminonitriles and their amino acid products explored in this study can be found in Table 2. The three aminonitrile reactants tested in this study are aminoacetonitrile (Thermo Scientific, purity: 98+%, CAS: 6011-14-9), 2-aminopropanenitrile (Ambeed, 95%, CAS: 72187-91-8), and 4-aminobutanenitrile (Biosynth Carbosynth, >95%, CAS: 1601190-8) (all delivered as hydrochloride for stability; 1:1 stoichiometric ratio). The aminonitriles are referred to in this manuscript as AAH, 2AH, and 4ABH, respectively. The ammonia hydroxide solution (Honeywell Fluka, 28.0−30.0% NH_3, ACS Reagent) was diluted with DI
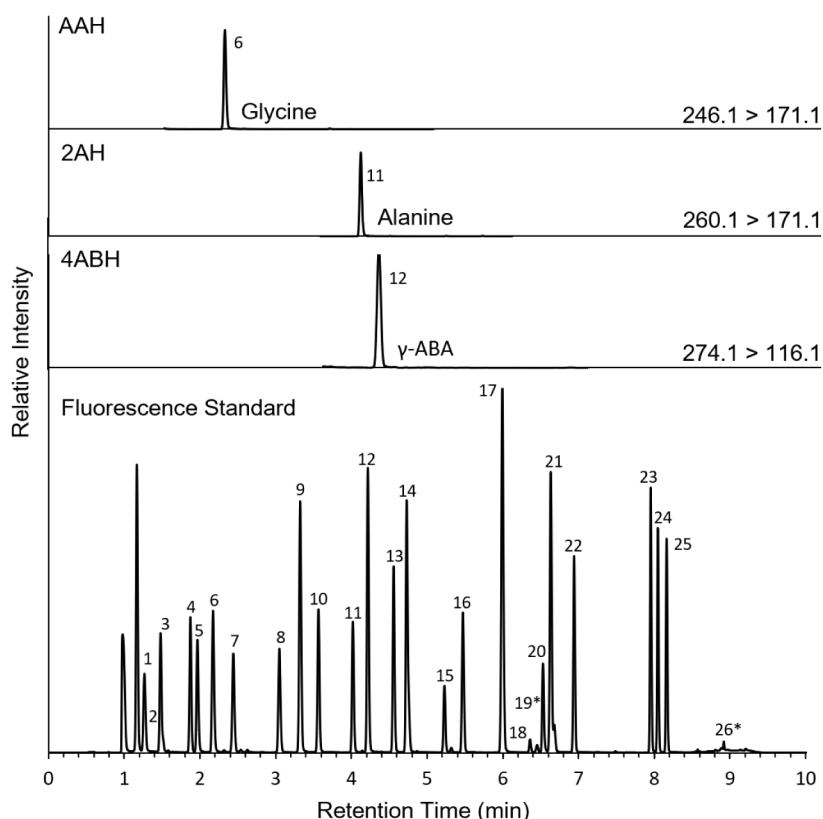
Figure 2. LC-FD-MS chromatograms of the AccQ·Tag derivatized amino acid products in our samples. Shown here are the 5% NH$_3$ samples at 3 °C with a reaction time of 3 months for AAH (top), 2AH (top-middle), 4ABH (bottom-middle), and the amino acid standard (bottom). The multiple reaction monitoring (MRM) mass transitions are provided on the right side of each chromatogram. Amino acid peak numbers are as follows: (1) histidine; (2) asparagine; (3) glutamine; (4) serine; (5) arginine; (6) glycine; (7) aspartic acid; (8) glutamic acid; (9) β-alanine; (10) threonine; (11) alanine; (12) γ-amino butyric acid (γ-ABA); (13) DL-β-amino butyric acid (DL-β-ABA); (14) DL-β-amino isobutyric acid (DL-β-AIB); (15) proline; (16) α-amino butyric acid (α-ABA); (17) α-amino isobutyric acid (α-AIB); (18) cysteine; (19*) tyrosine; (20) lysine; (21) methionine; (22) valine; (23) leucine; (24) isoleucine; (25) phenylalanine; and (26*) tryptophan. All derivatized amino acids were observed in the fluorescence trace except for tyrosine and tryptophan (indicated by asterisks), which were identified by MRM transitions. MRM transitions and parameters used to target the 26 analytes are provided in Table S1.

Table 2. Aminonitrile Reactants and their Amino Acid Products Explored in this Study

Reactant (formula)	Molecular Weight (g/mol)	Reactant Molecular structure	Amino Acid Product	Molecular Weight (g/mol)	Amino Acid Molecular Structure
Aminoacetonitrile (C$_2$H$_4$N$_2$) (i.e., AAH)	56.07		Glycine	75.07	
2-aminopropanenitrile (C$_3$H$_6$N$_2$) (i.e., 2AH)	70.09		Alanine	89.09	
4-aminobutanenitrile (C$_4$H$_8$N$_2$) (i.e., 4ABH)	84.12		γ-aminobutyric acid (γ-ABA)	103.12	

H$_2$O (Fisher Scientific, NERL reagent grade) to 5%, 10%, and 15% NH$_3$, with a systematic error of ±0.5%.

Salts and minerals were added to a subset of samples to test their potential catalytic role in the alkaline hydrolysis reaction. Salt that is hypothesized to be in Titan's interior ocean[19] was used at a concentration of 1 wt % total[19] and include NaCl (Sigma-Aldrich, ACS reagent, ≥99.0%), MgSO$_4$ (VWR, reagent grade), and KCl (Sigma-Aldrich, anhydrous ACS reagent,

≥99%). A weight of 13.3 ± 0.8 mg of each of the three salts was added to each 4 mL vial for a total mixture of ~10 mg/mL or 1.00 ± 0.04 wt % salt to water.

San Carlos olivine was used as the impactor simulant in this study to test the potential catalytic role of olivine and pyroxene minerals. Crushed San Carlos olivine was added to each sample, along with salt, at a concentration of 25.0 ± 0.7 mg/mL. San Carlos olivine was crushed using a mortar and pestle and then

Table 3. Experimental Parameters Explored in this Study and their Scientific Motivation[a]

Reactant	Solvent	Temperature	Parameter Motivation
AAH (0.1 M)	100% H$_2$O	3 °C, 21 °C	<u>Simulation:</u> amino acid formation in Titan's surface melt (e.g., impact melt pools and cryolava).
	5% NH$_3$	3 °C	<u>Reactants:</u> precursors to three of the most frequently identified amino acids in tholin hydrolysis
	10% NH$_3$	3 °C	studies.[24−26,29−34]
	15% NH$_3$	−22 °C, 21 °C	<u>Solution Concentration:</u> Water is the composition of Titan's crust,[3] and the predominant
2AH (0.07 M)	100% H$_2$O	3 °C, 21 °C	component of Titan's interior ocean. We chose three ammonia concentrations encompassing the hypothesized composition in the interior ocean (≤14 wt %).[16−18]
	5% NH$_3$	3 °C	
	10% NH$_3$	3 °C	<u>Temperature:</u> near the solvent freezing points, ensuring the sample remains in the liquid phase.
	15% NH$_3$	−22 °C, 21 °C	Room temperature is used as a "warm melt" scenario.[8,11]
4ABH (0.05 M)	100% H$_2$O	3 °C	<u>Salt and mineral additions:</u> NaCl, MgSO$_4$, and KCl are hypothesized to be present in Titan's
	5% NH$_3$	3 °C	interior ocean (up to 1 wt % salt).[19] Olivine/pyroxene minerals are components of carbonaceous
	10% NH$_3$	3 °C	chondrites and comets, and thus likely to be brought in by an impactor.[40−42]
	15% NH$_3$	−22 °C	
Salt and Mineral (NaCl, MgSO4, KCl; 1 wt % total) (Olivine/Pyroxene Mix; ~25 mg/mL)	100% H$_2$O	3 °C	
	5% NH$_3$	3 °C	
	10% NH$_3$	3 °C	
	15% NH$_3$	1 °C	

[a]The salt and mineral sample set was prepared for the three reactants. All samples were made in triplicate.

sieved to a size fraction of <150 μm. A composition of 75% olivine and 25% pyroxene was confirmed via X-ray diffraction analysis (XRD). XRD analysis methods are provided in the Supporting Information. The crushed sample was then wrapped in aluminum foil and ashed (heated in air at 500 °C for 8 h) to remove organic contaminants.

Prior to use, all glassware was ashed to eliminate organic contaminants; if glassware was used previously (e.g., beakers), the laboratory standard solvent rinse was performed prior to ashing (rinsed in order: DI H$_2$O, methanol, dichloromethane/methanol, hexanes).

2.2. Sample Preparation. The aminonitriles and salts/minerals (where used) were weighed and added to 4 mL glass vials. Reactant concentrations of 0.1 ± 0.003, 0.07 ± 0.002, and 0.05 ± 0.002 M were added of AAH, 2AH, and 4ABH, respectively. At the time of the study, the reactant 4ABH had limited availability and was hygroscopic, making it difficult to weigh and handle in the laboratory; therefore, the reactant concentration used was less than that for AAH and 2AH. We account for the relative reactant concentration in our calculations (see Results). Reactants were prepared in separate sample sets, and each set was prepared in triplicate to avoid reaction competition and account for reaction variability. Next, 4 mL of solvent [100% H$_2$O (0% NH$_3$), 95% H$_2$O (5% NH$_3$), 90% H$_2$O (10% NH$_3$), and 85% H$_2$O (15% NH$_3$)] was added to each vial. Vials were then capped, vortexed, and placed in one of three temperature environments: −22 °C, 3 °C, and 21 °C.

While Titan's ambient temperatures are much colder than the freezing point of water, the conditions within slowly cooling melt pools are of interest to this study. Therefore, we held our sample with the reactants AAH, 2AH, and 4ABH at temperatures near the solvent freezing points, ensuring the sample remained in the liquid phase. Samples with 0−10% NH$_3$ reacted at 3.3 ± 0.7 °C to explore this scenario (refrigerator set to 4 °C), while those with 15% NH$_3$ were subjected to a temperature of −21.6 ± 1.7 °C (freezer set to −20 °C). The most productive reactants based on our initial experiments at lower temperatures (AAH and 2AH) were chosen to simulate a warmer impact melt.[8,11] For the warm samples, a set of end members (100% H$_2$O and 15% NH$_3$) was tested at room temperature (21.1 ± 0.7 °C). Temperatures were monitored and recorded via Fisherbrand Traceable memory-card thermometers placed next to

the samples, which were stored inside a fume hood (only used for this project) or in the refrigerator and freezer. Table 3 illustrates the temperature regimes, reaction times, and ammonia concentrations for each sample. An overview of each sample, including the weights of the aminonitrile reactant, salt, and minerals, can be found in Tables S2 and S3. An additional error discussion is provided in the Supporting Information text.

All samples reacted for a total of 6 months (26 weeks) and were vortexed for 5 s every 2-weeks to minimize concentration gradients. An aliquot of 875 μL was removed from each sample at each time interval, with 125 μL dedicated to LCMS analysis. An aliquot without salt/mineral was removed for testing at 1 month (4 weeks), 3 months (12 weeks), and 6 months (26 weeks). Within a few hours of retrieval, aliquots were dried for 1−2 h via a Labconco CentriVap concentrator at 30 °C and stored in the −20 °C freezer until analysis.

Aliquots of salt/mineral samples were removed at 6 months (26 weeks) only to compare the amino acid formation to the samples without salt/mineral. After removal, salt/mineral samples were centrifuged in the CentriVap concentrator (while capped) for 10 min, then decanted, dried, and stored in a −20 °C freezer until analysis. The 15% NH$_3$ samples with salt/mineral at the −22 °C reaction temperature were frozen or formed a slush after 2 weeks and were therefore moved to the 4 °C refrigerator to ensure the samples remained in the liquid phase. Samples without salt/mineral remained a liquid in the −20 °C freezer and therefore remained under that temperature to test the coldest temperature regime. As a result, the time-weighted average temperature of the 15% NH$_3$ salt/mineral samples is slightly lower than that of the other refrigerated samples, at 1.4 ± 0.8 °C.

Procedural blanks were prepared alongside aminonitrile samples. A set of procedural blanks (8 total) were prepared for 100% H$_2$O, 5% NH$_3$, 10% NH$_3$, and 15% NH$_3$, with and without salt/minerals. Procedural blanks were utilized to track contamination through the end-to-end sample handling procedure (e.g., solution preparation, aliquot removal, dry down, and derivatization process). All temperature regimes had the same sample handling procedure, and blanks were stored at 21 °C. Respective procedural blanks followed the same sample procedure and retrieval described above.

Table 4. Experiment Overview: Amino Acid Formation in %Yield Over Time[a]

		Amino acid concentration (%yield)					
		−22 °C	3 °C			21 °C	
Reactant	Time (weeks)	15% NH$_3$	100% H$_2$O	5% NH$_3$	10% NH$_3$	100% H$_2$O	15% NH$_3$
AAH	0	0.025 ± 0.002	0.025 ± 0.002	0.025 ± 0.002	0.025 ± 0.002	0.025 ± 0.002	0.025 ± 0.002
	4	0.077 ± 0.018	0.062 ± 0.008	0.85 ± 0.02	0.90 ± 0.11	0.054 ± 0.005	20.7 ± 3.7
	12	0.069 ± 0.005	0.060 ± 0.023	1.9 ± 0.04	2.6 ± 0.2	0.060 ± 0.015	60.7 ± 12.1
	26	0.046 ± 0.003	0.031 ± 0.003	2.7 ± 0.02	6.2 ± 0.9	0.031 ± 0.004	59.5 ± 5.6
2AH	0	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001
	4	1.9 ± 0.6	0.057 ± 0.002	8.2 ± 0.2	7.1 ± 1.4	0.044 ± 0.003	29.1 ± 4.9
	12	2.1 ± 1.0	0.11 ± 0.01	16.4 ± 4.4	19.0 ± 1.5	0.080 ± 0.005	65.0 ± 11.3
	26	3.3 ± 0.7	0.027 ± 0.006	39.0 ± 3.0	34.2 ± 1.2	0.029 ± 0.009	83.3 ± 10.8
4ABH	0	$1.3 \times 10^{-4} \pm 9.4 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 9.4 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 9.4 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 9.4 \times 10^{-5}$	-	-
	4	0.0033 ± 0.001	$5.3 \times 10^{-4} \pm 9.4 \times 10^{-5}$	0.21 ± 0.01	0.18 ± 0.02	-	-
	12	0.0071 ± 0.0027	$6.0 \times 10^{-4} \pm 1.6 \times 10^{-4}$	0.63 ± 0.07	0.65 ± 0.05	-	-
	26	0.022 ± 0.005	$0.0021 \pm 7.0 \times 10^{-4}$	1.7 ± 0.1	1.9 ± 0.2	-	-

[a]Time zero (t_0) data points were measured once separately and therefore, each reactant has the same value (see Methods section).

Table 5. Experiment Overview: Amino Acid Formation After 6 Months for Samples with Added Salts and Minerals

		Amino acid concentration (%yield)			
Reactant	Time (weeks)	100% H$_2$O	5% NH$_3$	10% NH$_3$	15% NH$_3$
AAH	26	0.027 ± 0.006	5.3 ± 0.9	8.0 ± 0.5	6.4 ± 0.4
2AH	26	0.015 ± 0.001	31.8 ± 1.7	30.3 ± 7.4	22.1 ± 2.2
4ABH	26	0.014 ± 0.002	0.78 ± 0.11	0.94 ± 0.07	0.73 ± 0.13

Time-zero (t_0) samples were prepared separately to understand whether amino acids may already be present in the reactants prior to hydrolysis. The t_0 samples were prepared as follows: 100% DI H$_2$O was added to the solid reactant, the sample vortexed, and 10 μL was immediately derivatized for liquid chromatography mass spectrometry (LCMS) analysis (<10 min in H$_2$O prior to derivatization). See Section 2.3 for LCMS instrumentation details and the derivatization procedure.

2.3. Liquid Chromatography Mass Spectrometry (LCMS) Analysis: Sample Preparation and Derivatization Method. Samples were analyzed using AccQ·Tag derivatization coupled with LCMS analysis; a robust technique frequently used to detect amino acids in meteorites and prebiotic samples.[36−39] The AccQ·Tag-derivatized amino acid extracts were analyzed using a Waters Acquity H-class PLUS coupled with a Waters Xevo TQ-S Micro triple quadrupole equipped with an electrospray ionization source (positive ion mode) using multiple reaction monitoring (MRM), located in the Astrobiology Analytical Laboratory (AAL) at the NASA Goddard Space Flight Center. Amino acid products were confirmed by comparing three chromatographic retention times to those of the amino acid standard, including one UV fluorescence chromatogram and two MRM mass transition chromatograms. The amino acid products were quantified using peak areas from the most abundant MRM mass transition. See the Supporting Information for LCMS instrument and quantification methods.

An internal standard of 3-fluoro-DL-valine (Sigma-Aldrich, ≥99.0%, CAS: 43163-94-6) was used to account for instrument variability over time, derivatization efficiency, and varying ammonia concentrations present in the samples (see the Supporting Information for additional information). A volume of 10 μL of a 100 μM solution of 3-fluoro-DL-valine in H$_2$O was added to each sample prior to derivatization.

The 125 μL samples were resuspended in 100% H$_2$O (Millipore Milli-Q Integral 10 ultrapure water; 18.2 MΩ·cm,

≤3 ppb total organic carbon), and 10 μL was derivatized for analysis. The AccQ·Tag derivatization process was performed by (1) adding 60 μL of borate buffer, 10 μL of sample, 10 μL of internal standard, and 20 μL of AccQ·Tag derivatization agent to a total recovery vial, (2) heating at 55 °C for 10 min, and (3) injecting 1 μL into the LCMS using the conditions described in the Supporting Information. If the initial analysis revealed that the samples were too concentrated for accurate quantitation (>125 μM), they were diluted once (dilution 1:10 μL in 1000 μL H$_2$O) and a second time as necessary (dilution 2:10 μL of dilution 1 in 100 μL H$_2$O). Procedural blanks and t_0 samples were obtained with the same dilution factors. Between runs, original samples were either dried and stored in a −20 °C freezer or kept in water frozen in a −80 °C freezer.

3. RESULTS

This work explored the environmental factors that influence amino acid synthesis via alkaline hydrolysis of three aminonitriles: AAH, 2AH, and 4ABH. These reactants were chosen because they are precursors to three of the most frequently identified amino acids in tholin hydrolysis studies (glycine, alanine, and γ-aminobutyric acid, respectively—see Table 1). The amino acid formation was tracked over 6 months and reported in %yield ($M_P/M_R \times 100$; where M is the molarity, M_P is the concentration of the amino acid product, and M_R is the original reactant concentration). Tables 4 and 5 provide the detected abundances of amino acids as a function of the experimental parameter for samples without salt/mineral (Table 4), and samples with salt/mineral added (Table 5). An example set of LCMS chromatograms is presented in Figures 2 and 3. Figure 2 shows the fluorescence chromatogram for the amino acid standard (bottom) and the MRM chromatograms with peaks that correspond to #6 glycine (top), #11 alanine (middle), and #12 γ-ABA (middle-bottom). Figure 3 shows fluorescence chromatograms for t_0, 1 m, 3 m, and 6 m (bottom to top) for the
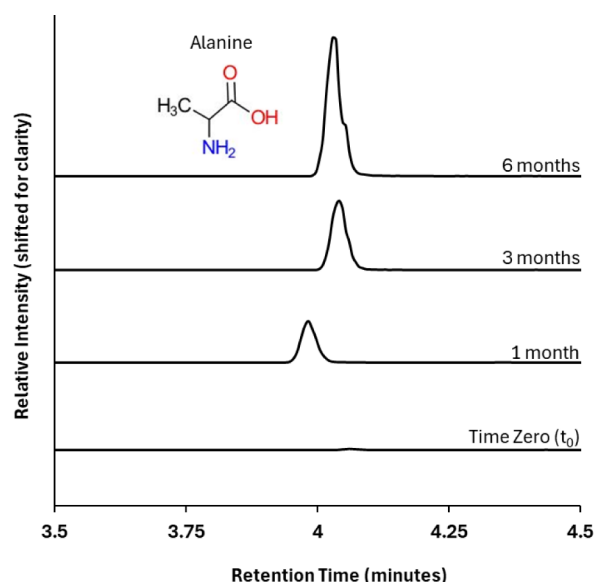
Figure 3. LC-FD-MS fluorescence chromatograms of the 2AH reactant with 5% NH_3 at 3 °C. Reaction time increases from bottom to top: initial (bottom), 1 month (bottom-middle), 3 months (top-middle), 6 months (top). Notice the increase in peak area over time, indicating an increase in alanine formation. Chromatograms are shifted vertically for visual aid.
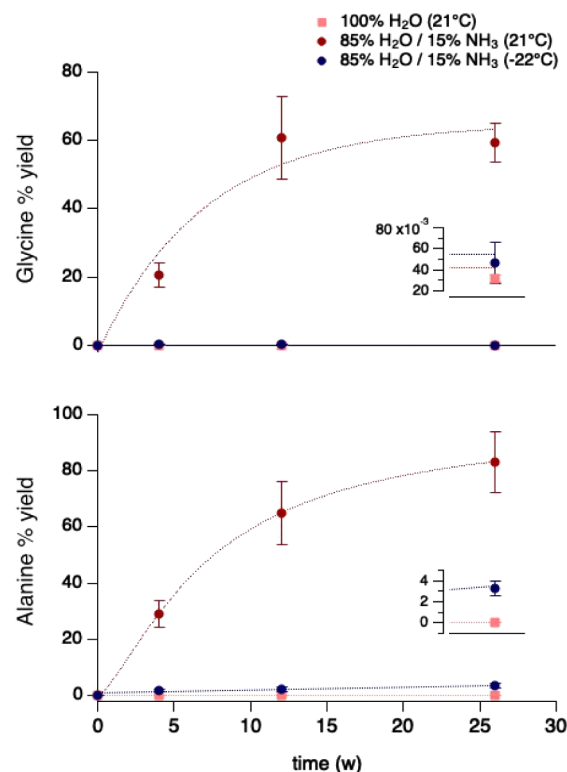

Figure 4. Abundance of glycine (top) and alanine (bottom) over time as measured in the AAH and 2AH samples, respectively. The endmembers of temperature and ammonia concentrations explored in this study are shown here (100% H_2O at 21 °C and 15% NH_3 at 21 °C and −22 °C). Samples with 15% NH_3 and 100% H_2O are indicated by a circle and square data points, respectively. Best fits shown here are not used for the calculations presented in Section 4. This plot highlights the increase in amino acid formation when ammonia is present.

2AH sample and illustrates the change in alanine concentration over time. Amino acid formation as a function of time, temperature, ammonia concentration, and the inclusion of salts and minerals is discussed herein.

3.1. The Influence of Ammonia and Temperature on Amino Acid Production.

Figure 4 examines the effects of ammonia concentration versus temperature at the extreme parameter limits of this study (100% H_2O at 21 °C, 15% NH_3 at 21 °C, and 15% NH_3 at −22 °C) on the formation of glycine (top) and alanine (bottom). Samples with 15% NH_3 at the warmer temperature produced the largest quantity of amino acids after 6-months, with ~60 and 80 %yield for glycine and alanine, respectively. There is a logarithmic increase in amino acid abundance with time in this warmer regime (reaction rates in Section 4.1). These results suggest that the addition of ammonia has a stronger influence on the reaction rate and product yield than the temperature, as seen by amino acid formation in 15% NH_3 at −22 °C and the lack of amino acid formation in 100% H_2O at 21 °C. Glycine and γ-ABA had a similar trend in formation in samples with 15% NH_3 and −22 °C, where formation increased slightly after 6 months, yet their yields remained below 0.1%. Alanine increased more significantly, with ~3 %yield under the same conditions. In the presence of ammonia, however, temperature is more important than ammonia concentration, as seen in the vast increase of amino acid abundance in the 15% NH_3 samples at 21 °C versus −22 °C. Under these conditions, glycine increased from <0.1 % yield to ~60 %yield, while alanine increased from 3 %yield to 80 %yield.

Figure 5 examines the amino acid yields for the cold temperature samples (3 °C) as a function of ammonia concentration for glycine (top), alanine (middle), and γ-ABA (bottom). At all ammonia concentrations, alanine had the highest yield after 6-months, followed by glycine and γ-ABA. When ammonia is present (5 and 10% NH_3), there is generally a positive linear dependence in amino acid abundance over time

in the cold temperatures. Samples with 5% and 10% NH_3 at 3 °C have the same yield within error for alanine and γ-ABA, resulting in ~40 and ~2% yield after 6 months, respectively. Glycine resulted in a larger variation between 5 and 10% NH_3 after 6 months, with the production at 10% NH_3 more than twice that of 5% NH_3 (6.2 and 2.7 %yield, respectively). Samples with solvents of 15% NH_3 and a reaction temperature of −22 °C, have a much lower amino acid yield than the 5% and 10% NH_3 samples at 3 °C. This variation is likely a kinetic temperature effect, where the reaction proceeds faster at warmer temperatures. Additionally, without ammonia present, at temperatures ≤21 °C, temperature did not play a role, as amino acid formation beyond the initial concentration (t_0) was not observed in either temperature regime (3 and 21 °C).

Small quantities of amino acids were identified in the time zero sample for all three aminonitriles (<0.03 %yield). This initial amino acid concentration may be the result of contamination in the aminonitrile reactant or the result of nearly instantaneous hydrolysis (<10 min). The relative consistency of the amino acid concentration over time in the pure water samples favors the contamination hypothesis, yet instantaneous hydrolysis cannot be ruled out. The t_0 value is reported in Table 4.

3.2. Influence of Salts and Minerals.

Salts (1 wt %) and minerals (~25 mg/mL) were added to a subset of samples to test their potential catalytic role in the alkaline hydrolysis reaction. Salts have been hypothesized to be present in Titan's
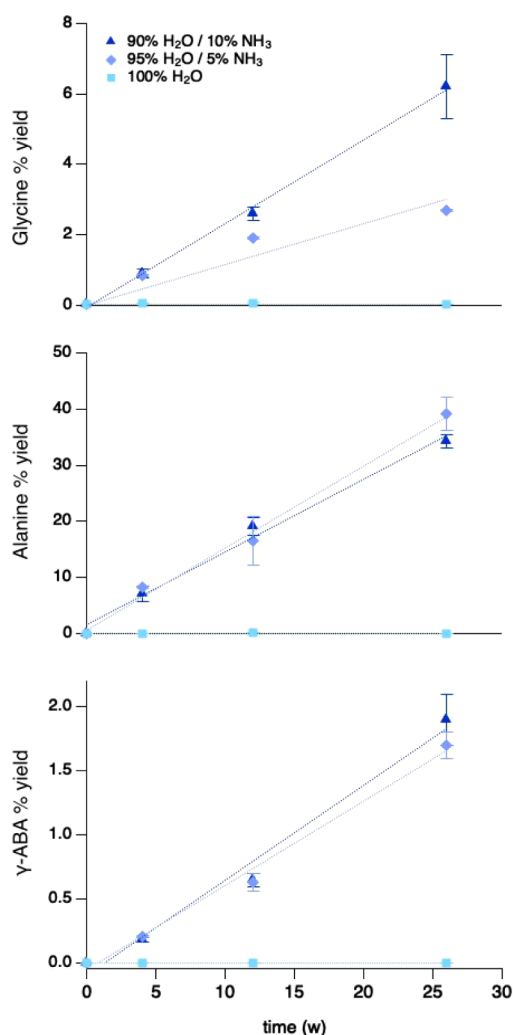
Figure 5. Abundance of amino acids over time at 3 °C for glycine (top), alanine (middle), and γ-ABA (bottom), as measured in the AAH, 2AH, and 4ABH samples, respectively. Here, 100% H_2O, 5% NH_3, and 10% NH_3 are represented by the symbols light-blue square, lavender diamond, and royal-blue triangle, respectively. Note the significant increase in amino acid production when ammonia is introduced. There is a linear dependence of production over time in the cooler temperatures. Linear best fit trendlines for 5% and 10% NH_3 were used to calculate the aminonitrile concentration in previous studies (see Section 4.2.1).

interior ocean at a concentration of 1 wt %[19] and may be present in cryolava flows or impact melt pools where the impactor breaches the ice crust. The salts chosen for this study are species hypothesized to be within Titan's subsurface ocean[19] and include NaCl, $MgSO_4$ and KCl.

Minerals can be delivered by an impactor and may play an important role in organic synthesis.[23] Ordinary chondrites—the most common type of meteorite—are predominately composed of chondrules rich in olivine and pyroxene with minor silicates, oxides, sulfides, and metal.[40,41] Olivine and pyroxene are also components of cometary materials.[42] San Carlos olivine (75% olivine and 25% pyroxene) was chosen as our impactor simulant in this study to test the potential catalytic role of olivine and pyroxene minerals.

The subset of samples that included salt/mineral reacted for 6 months. A comparison of the amino acid quantity with and without salts/minerals after 6 months is shown in Figure 6. Samples with solvents of water only, 5% NH_3, and 10% NH_3, reacted at a temperature of 3 °C, while samples with a solvent of 15% NH_3 reacted at a time-weighted average temperature of 1 °C (see methods section for additional details).

The results suggest that the inclusion of salts/minerals increased glycine production and decreased the production of alanine and γ-ABA. Glycine increased by ~50% in the 5% NH_3 samples when the salts/minerals were present (total increase of ~2.5 %yield) and increased ~25% in the 10% NH_3 samples (total increase of ~2 %yield). Alanine decreased by ~20% (total decrease of ~7 %yield) in the 5% NH_3 samples when the salts/minerals were present, and the values were within error for the 10% NH_3 samples. γ-ABA decreased by ~50% in both the 5% and 10% NH_3 samples (total decrease of ~1 %yield). All salt/mineral samples with 15% NH_3 had less amino acid production than samples with 5% NH_3 and 10% NH_3. The decrease in production may be due to the slight decrease in reaction temperature, or excess ammonia may slightly hinder the production of amino acids. Amino acid formation was unaffected in the 100% H_2O samples, with formation remaining at <0.1 %yield.

Freezing point suppression was observed in the 15% NH_3 solution with the addition of salts/minerals. The salt/mineral samples became frozen or slush under the −22 °C conditions, while samples without salt/mineral remained in the liquid phase. Impurities are known to assist in heterogeneous nucleation,[43] and therefore the minerals likely acted as a nucleating agent or provided nucleation sites,[44] ultimately increasing the freezing point of the sample.
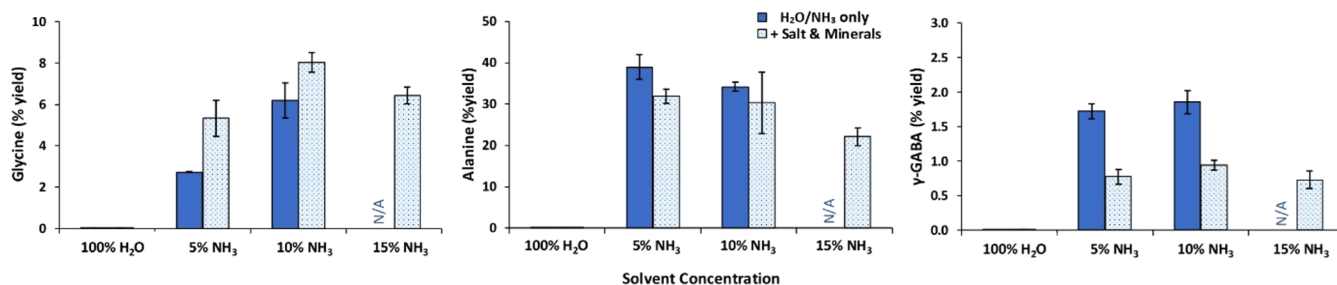

Figure 6. Amino acid abundance after 6 months for glycine (left), alanine (middle), and γ-ABA (right), with salts/minerals added (dotted blue) and without (solid blue). Samples are separated on the *x*-axis by their solvent concentration. Samples with salts/minerals added show an increase in glycine formation and a decrease in alanine and γ-ABA formation. Samples with solvents of water only, 5% NH_3, and 10% NH_3 reacted at a temperature of 3 °C, while 15% NH_3 reacted at a time-weighted average temperature of 1 °C (see the Methods Section for additional details).

4. DISCUSSION

4.1. Kinetics: Reaction Rates and Rate Constants.

Reaction rate plots are shown in Figure 7 and the reaction rate constant (k) for each of the ammonia solution samples is reported in Table 6 and presented in Figure S4. In this study, samples with 15% NH_3 at 21 °C (alanine and glycine) proceeded to completion as a first-order reaction. Samples with 5 and 10% NH_3 at 3 °C, and 15% NH_3 at −22 °C, likely did
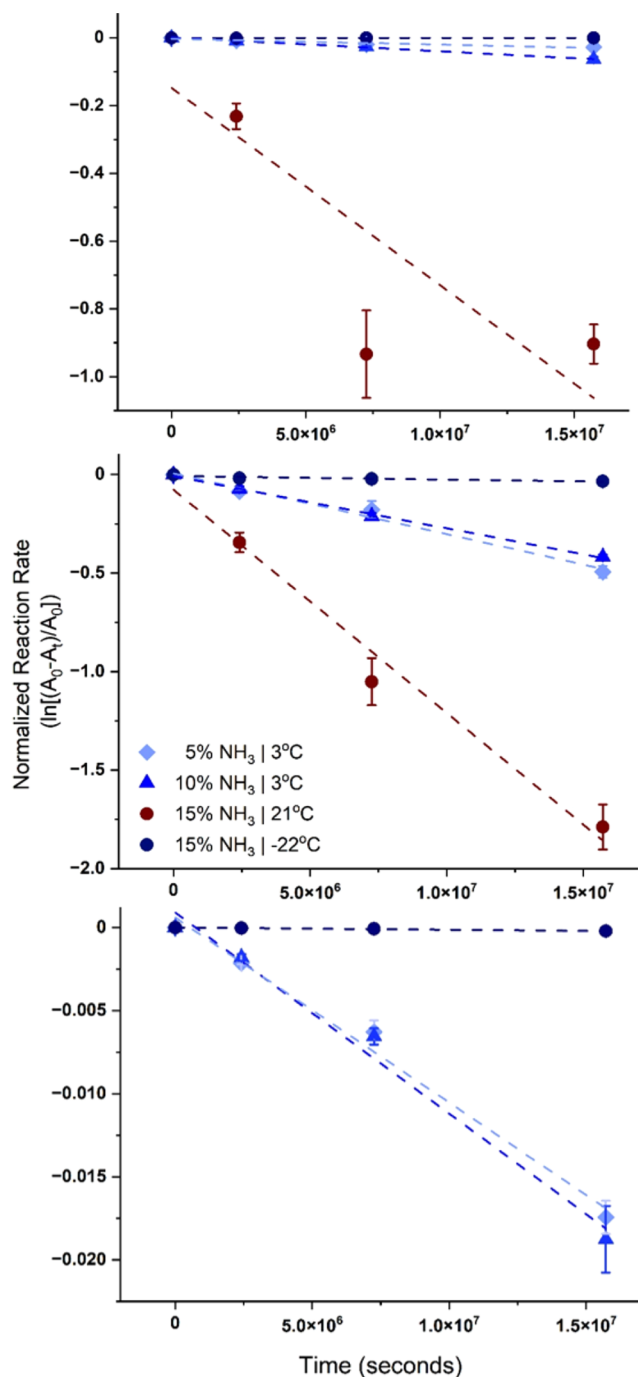

Figure 7. Normalized reaction rates for the three aminonitriles in this study are AAH (top), 2AH (middle), and 4ABH (bottom). Reaction rates for samples with a solvent of 5% NH_3 at 3 °C (lavender diamond), 10% NH_3 at 3 °C (royal-blue triangle), 15% NH_3 at −22 °C (navy-blue circle) and 15% NH_3 at 21 °C (red circle) are presented. The linear best fit lines are used to calculate the rate constant k (see Table 6).

not proceed to completion after 6 months, presenting a pseudo zero-order reaction. If given a longer reaction time interval, it is likely that the samples would reach completion with a first-order reaction. Therefore, all of the reactions were treated as first-order reactions to calculate the reaction rate constants for the alkaline hydrolysis of the three aminonitriles.

To calculate the rate constant, we assumed that the desired amino acid is the only product, based on the large yield of the single amino acid upon reaction completion and the lack of detection of additional amino acids products. Therefore, the increase in amino acid quantity is assumed to be equivalent to the decrease in the aminonitrile quantity. Although this may not be a completely valid assumption, it allows us to calculate a rate constant that is lower than the limit for the conversion of the reactant. The analysis of other potential reaction products is outside the scope of this paper.

First, we calculated the normalized reaction rate. Here, we subtracted the amino acid quantity (in molarity) at time t (A_t) from the initial molarity of the reactant (A_0) to obtain the time-dependent aminonitrile concentration. Next, we took the natural log of the normalized concentration, as expressed in eq 1.

$$\text{Rate} = \ln[(A_0 - A_t)/A_0] \tag{1}$$

Where, A_0 is the initial concentration of the aminonitrile and A_t is the amino acid concentration at time t. The rate constant, k, is the negative of the slope of the linear trend of the values, as seen in Figure 7. We comment herein on samples that have positive k values and R^2 values >0.5.

The first-order rate constant of 2AH with the inclusion of ammonia is higher than those of AAH and 4ABH in all ammonia concentrations and temperatures tested. The rate constants for 15% NH_3 at 21 °C has the highest values, in the range of 10^{-7} s^{-1} for 2AH and 10^{-8} s^{-1} for AAH. For all three aminonitriles (AAH, 2AH, 4ABH), the rate constants for the 5% and 10% NH_3 reactions at 3 °C agree within error for the same reactant. 2AH, however, has a rate constant value an order of magnitude higher than that of AAH and 4ABH, in the range of 10^{-8} s^{-1} and 10^{-9} s^{-1}, respectively. The rate constant for 2AH with 15% NH_3 and −22 °C is in the range of 10^{-9} s^{-1}, 2 orders of magnitude less than that of the 21 °C samples with the same concentration of ammonia. The rate constant for 4ABH, however, is 2 orders of magnitude lower under the same conditions, 10^{-11} s^{-1}.

4.2. Comparison to Previous Work: Alkaline Hydrolysis of Titan-like Tholins. *4.2.1. Ammonia Concentration and Amino Acid Yields.* Here, we compared the amino acid yields in our experiments with those produced in prior tholin hydrolysis studies to constrain the potential aminonitrile composition of the Titan tholin analogs. In our study after 12 weeks, we observed an increase of 1.9, 16.4, and 0.6 %yield, for glycine, alanine, and γ-ABA, respectively, at conditions of 5% NH_3 and 3 °C. These values correspond to a resulting concentration relative to the starting reactant mass of 2.5, 21, and 0.8 wt %, respectively. The Brassé et al. (2017) tholin hydrolysis study detected various amino acid products, including glycine, alanine, and γ-ABA. They report yields of 0.0058, 0.00085, and 0.0057 wt %, respectively, relative to the initial tholin sample mass, after reacting for 10 weeks at 6 °C in a 5% ammonia solution.[33] Note that these yields are updated relative to those initially reported in Table 8 of Brassé et al., 2017 (personal communication). Poch et al. (2012) conducted tholin hydrolysis experiments at the same temperature as Brassé et al. (2017), but at a higher ammonia concentration, 25% NH_3. Poch et al. (2012), report yields of glycine and alanine of ∼0.35 and

Table 6. Rate Constant (k) Calculated for the Aminonitriles Investigated in this Study[a]

Sample	AAH			2AH			4ABH		
	k (s^{-1})		R^2	k (s^{-1})		R^2	k (s^{-1})		R^2
5% NH$_3$ | 3 °C	1.68×10^{-9}	$\pm 3.1 \times 10^{-10}$	0.94	3.18×10^{-8}	$\pm 2.7 \times 10^{-9}$	0.99	1.11×10^{-9}	$\pm 9 \times 10^{-11}$	0.99
10% NH$_3$ | 3 °C	4.05×10^{-9}	$\pm 1.6 \times 10^{-10}$	0.99	2.65×10^{-8}	$\pm 9 \times 10^{-10}$	0.99	1.21×10^{-9}	$\pm 1.0 \times 10^{-10}$	0.99
15% NH$_3$ | −22 °C	-		-	1.78×10^{-9}	$\pm 5.9 \times 10^{-10}$	0.82	1.38×10^{-11}	$\pm 1.6 \times 10^{-12}$	0.98
15% NH$_3$ | 21 °C	5.82×10^{-8}	$\pm 2.51 \times 10^{-8}$	0.73	1.13×10^{-7}	$\pm 1.1 \times 10^{-8}$	0.98	N/A		

[a]Note: Rate constants are reported for positive k values and $R^2 > 0.5$.

~0.025 wt %, respectively, and do not report the detection of γ-ABA. Our values are several orders of magnitude higher after 12 weeks compared to both the Brassé et al. (2017) and Poch et al. (2012) studies.

Brassé et al. (2017) hypothesized that the aminonitriles explored in this study were the precursors for glycine, alanine, and γ-ABA detected in their study. Given that the increase in yield in our study was significantly larger than that observed in Brassé et al. (2017) and Poch et al. (2012), it is still plausible that aminonitriles could be the precursors to the observed amino acid products in these two studies but that the quantity of aminonitriles in the original tholin sample was very small. Given the wide array of molecules identified in tholin samples,[45] this is not surprising, as tholins have been shown to be diverse and molecularly complex.

By calculating the moles of amino acid products detected in Brassé et al. (2017) and Poch et al. (2012), and using the reaction rates calculated in this study, the wt % of aminonitriles in the starting tholin material can be calculated. Here, we assume that all amino acid products originated from aminonitriles, providing an upper limit on the aminonitrile concentration. To compare our study to Brassé et al. (2017), we calculated the yield after 10 weeks with 5% NH$_3$ in our study to be 1.2, 15.2, and 0.6% for glycine, alanine, and γ-ABA, respectively. Similarly, to compare our study to Poch et al. (2012), we calculated the yield after 10 weeks at 10% NH$_3$ in our study to be 1.3, 13.9, 0.6% for glycine, alanine, and γ-ABA, respectively. The concentrations of AAH, 2AH, and 4ABH in the starting tholin material are found to be 0.37, 0.004, and 0.76 wt % for Brassé et al. (2017), and AAH and 2AH to be 11.3 and 0.1 wt % for Poch et al. (2012), resulting in a total aminonitrile abundance of approximately 1 and 11 wt %, respectively. The order of magnitude variation in aminonitrile abundance in Poch et al. (2012) compared to Brasse et al. (2017) could be the result of a greater aminonitrile abundance in the starting tholin material created during the tholin production process, or an increase in reaction rate from the increase in NH$_3$ concentration in Poch et al. (2012).

Moreover, our study showed that 2AH → alanine was the most productive reaction of the three tested and produced the largest yields. Interestingly, Poch et al. (2012) detected a greater abundance of glycine than alanine in their sample, and Brassé et al. (2017) also had the greatest abundance of glycine in their sample, followed by γ-ABA and alanine. Cleaves et al. (2014) detected the greatest abundance of glycine in their samples (2.5 years at 25 °C, and 14% NH$_3$), with a relative yield to glycine abundance of 1 mol % for both alanine and γ-ABA. This suggests that if aminonitrile hydrolysis was the primary formation pathway for amino acids in the tholin sample, AAH is a favored product of tholin production compared to 2AH and 4ABH. Also note that glycine is known to have high backgrounds and contamination rates in the laboratory environment, which may also play a role in elevating the reported glycine values.

Additionally, we do not observe an increase above t_0 values in the 100% H$_2$O samples over time, yet we do observe a significant increase in amino acid production with the addition of 5% NH$_3$. The ammonia is expected to be a catalyst in this reaction and is not consumed. Therefore, the difference in production is likely due to pH, where 5% NH$_3$ has a pH of ~11, and 0.1 M of the aminonitrile in water has a pH of ~3, as measured in our lab. Interestingly, a pH of 3 does not appear sufficient to drive the acid hydrolysis of the aminonitriles at temperatures ≤21 °C after a 6-month reaction time, as observed in our study. Similarly, other tholin hydrolysis studies found a significant increase in products and amino acid production in the presence of ammonia.[27−29,31] For example, Poch et al. (2012) observed an order of magnitude increase in glycine production with the presence of 25% NH$_3$ (at 6 °C after 10 weeks). Moreover, in Poch et al. (2012) alanine was only detected in the presence of ammonia under the same experimental conditions, just as in this study. Our results confirm the observations in the tholin hydrolysis studies: alkaline pH is favored over a neutral pH in the formation of amino acids from aminonitriles at cooler temperatures. Future work should establish the lower limit of ammonia concentration necessary for aminonitrile alkaline hydrolysis at Titan-relevant temperatures.

4.2.2. Reaction Rates and Oxygen Incorporation. In this study, the reaction of aminonitriles with ammonia proceeds much slower than observed in the tholin hydrolysis experiments performed by Neish (2008) and (2009). Additionally, we did not observe amino acid formation over our experimental duration in 100% H$_2$O, while Neish et al. (2008) observed tholin hydrolysis (but not necessarily amino acid formation) under similar conditions. Neish et al. (2008) calculated the reaction rates for tholin hydrolysis in 100% H$_2$O to be much faster than our study, with rate constants in the range 10^{-5} to 10^{-7} s^{-1} as a function of temperature from 0−$^+$40 °C. Neish et al. (2009) furthered this effort and found the rate constant increased with the addition of 14% NH$_3$ and at temperatures between −20 to +24 °C, with rate constants as high as 10^{-2} s^{-1}. These reactions ran to completion in less than 1 month. It must be noted that Neish et al. (2008) and (2009) calculated the rate of oxygen addition to select ions identified in a mass spectrometer, but the molecular structure and reaction pathways leading to the observed hydrolysis product were not identified. Due to the complex nature of tholins, there are many potential reactants and reaction pathways that could lead to the observed hydrolysis products listed in the Neish studies.

Additionally, we calculated the weight % oxygen incorporation over time in our study using the yields after 6 months at warm temperatures (~60 %yield of glycine and ~80 %yield of alanine). See the Supporting Information for calculation details. We find a maximum value of 28 and 31 wt % oxygen incorporation, respectively. Using an estimated concentration of ~1 wt % of aminonitriles, an upper limit derived from the calculation in the previous section (excluding the high AAH

value from Poch et al., 2012), this would be observed as a 0.03% oxygen by mass in the total sample. Neish et al. (2009) reported a higher percentage, and an additional 2% oxygen by mass was incorporated into the bulk hydrolyzed tholin samples.

The rate constants and the amount of oxygen incorporated into the amino acids measured in our study compared to Neish et al. (2008) and (2009), indicates that aminonitriles are a small component of the tholin material and aminonitrile hydrolysis alone is not the primary mechanism that led to oxygen incorporation. While aminonitrile hydrolysis could still be a productive pathway in tholin experiments with ammonia present, alternative reactions dominate the net hydrolysis of tholins in those works, especially in the water-only solvents.

4.3. Salt/Mineral Inclusion. Our results show an increase in glycine production and a decrease in alanine and γ-ABA production with the inclusion of salts (NaCl, MgSO$_4$, and KCl; 1 wt % total) and minerals (olivine/pyroxene mix; $\sim$ 25 mg/mL). Minerals are known to readily adsorb amino acids onto their surfaces,[46,47] and the subject is an important current topic for the origin of life and the production of important molecules such as peptides, proteins, and polymers.[46,47] The difference in adsorption efficiency has been linked to various factors, such as mineral properties and the amino acid R-group charge.[46,47] Additionally, a Titan tholin hydrolysis study found a decrease in glycine, alanine, and γ-ABA with the inclusion of carbonates in the sample.[33]

To test if the decrease in amino acid concentration was influenced by the presence of minerals (e.g., adsorption), we conducted a mineral adsorption test, where the mineral was spiked with an amino acid standard of varying concentrations from 0.313−62.5 μM (see Supporting Information for mineral test methods). By comparing the peak areas of glycine, alanine, and γ-ABA, we found that the peak area of the amino acid standard remained unaffected and within error when the mineral was added to the standard (Figures S1 and S2). Therefore, the amino acids did not adsorb to the surface of the minerals within the few minutes of our test experiment, nor did the minerals affect derivatization.

If amino acid adsorption onto the minerals transpires over a longer time frame (i.e., >10 min), adsorption may still explain the decrease in alanine and γ-ABA abundance with the inclusion of salts/minerals as seen in our study. Alternatively, glycine experienced an increase with salt/mineral inclusion, even though the salts/minerals procedural blanks had low glycine contamination (<0.25 μM; lower than the lowest standard). Therefore, it is likely that the salt/mineral indeed increased glycine production, and if amino acids do indeed adsorb to olivine/pyroxene over the longer time frame, glycine may not adsorb as strongly as alanine and γ-ABA. It is also possible the salts/minerals hindered the production of alanine and γ-ABA, yet enhanced glycine formation. While out of the scope of this study, exploring the adsorption efficiency of San Carlos olivine would be an intriguing undertaking and important for *in situ* sample analysis where olivine/pyroxene minerals are thought to be present.

Next, to understand the effects of salt on the derivatization efficiency, we tracked the peak area of the derivatized internal standard, 3-fluoro-DL-valine, in our samples with and without salts/minerals (see Supporting Information). The internal standard should derivatize similarly to the amino acids explored in this study, and therefore, their behavior with the influence of salt should be similar. We found that the internal standard peak areas between the same samples with and without salts/minerals

were within error (Figure S3). There was, however, a discrepancy between the samples with a solvent of 100% H$_2$O and samples containing ammonia as well as between runs over time. This discrepancy illustrates the importance of including an internal standard, as performed in this study, to normalize the sample abundances when comparing sample analysis over time or comparing samples with varying solvent compositions or pH.

4.4. Implications for Titan. This work suggests that small amounts of ammonia ($\sim$5%) are necessary to drive aminonitrile alkaline hydrolysis in cooler temperatures and that in a matter of months aminonitriles can be converted to amino acids with very high yields provided ammonia is present. However, the amount of ammonia present in Titan's surface melts, crust, and interior ocean is currently unknown. Ammonia is expected to exist in Titan's interior ocean ($\leq$14 wt %),[16−18] and there is strong evidence that ammonia is the source of Titan's thick nitrogen atmosphere.[2,48] Similarly, another one of Saturn's ocean worlds, Enceladus, has an ammonia concentration in its interior ocean of $\sim$1%, measured in the ejecta of Enceladus' plumes with Cassini.[49] Therefore, it is likely that Titan's interior includes a small percent of ammonia as well. It should be noted, however, that Sotin et al. (2021) suggests that Titan's density can be explained without the presence of ammonia in Titan's subsurface water ocean. Thus, while ammonia is likely on Titan, it is unclear how much ammonia may be present in Titan's interior ocean or surface melts.

Even if a very small percentage of ammonia is present in Titan's melts, as the melt freezes the relative abundance can increase in the aqueous environment over time, thus transitioning into an environment that is more conducive to amino acid formation. Ammonia will also act as an antifreeze agent and prolong the melt refreeze,[9,11] allowing hydrolysis reactions to persist for longer time scales. Alternatively, the presence of salts/minerals may decrease the freezing point of the impact melt pool even in the presence of ammonia (as seen in our study), ultimately leading to faster refreeze.

Our results also suggest that if Titan's surface melts have at least 5% NH$_3$ and temperatures $\geq$3 °C, aminonitrile hydrolysis will readily occur, leading to the production of amino acids. Alternatively, if Titan's melts lack ammonia and have temperatures <21 °C, aminonitriles within the organic aerosols may remain unhydrolyzed. Similarly, if the melts are too cold $\leq$ −22 °C, even with 15% NH$_3$ only small amounts of 2AH might be hydrolyzed, resulting in a small amount of alanine but would lack glycine and γ-ABA. It is possible that over the course of the melt refreeze−hundreds of years−the hydrolysis of aminonitriles may transpire, though competing reactions within Titan aerosols may hinder production. Moreover, if melt pools become warmer than hypothesized, then hydrolysis may occur in 100% H$_2$O melt environments, though future work would be necessary to confirm the required reaction conditions.

Our reaction rates and high %yield of amino acid formation, along with the vast quantity of hydrolysis products identified in tholin-hydrolysis studies, indicate that aminonitriles are likely only a small portion of the tholin molecule. Therefore, other reaction pathways may result in organic synthesis in tholins and may also proceed on Titan's surface and in Titan's atmosphere. One such proposed pathway for amino acid formation on Titan includes the hydrolysis of HCN.[27] HCN has been proposed to make up $\sim$5 wt % of Titan's solid organics,[50,51] and therefore would likely be available to interact with surface liquids. Amino acids may also form in Titan's atmosphere via the photo-dissociation and recombination of nitrogen, methane, and

carbon monoxide.[52] While oxygen is scarce in Titan's atmosphere, trace levels of oxygen have been detected (~50 ppm),[53] allowing a pathway for oxygen incorporation into hydrocarbon molecules without hydrolysis. Experiments conducted by Hörst (2012) confirmed the atmospheric pathway and detected amino acids, including glycine and alanine, yet the amino acid yields of such reactions remain unknown. Since the concentration of the organics that reach Titan's surface are not fully known, multiple pathways need to be examined in order to characterize Titan's potential for prebiotic chemistry.

Our results are highly relevant to the Dragonfly mission to Titan, which will search for prebiotic molecules in the impact melt material.[54] If amino acids are found in the once-liquid impact melts on Titan, the results from this work will provide much needed data to evaluate the possible reaction pathways and postimpact conditions that contributed to the amino acid formation. Furthermore, if amino acids are not found on Titan, these data will help to shed light on the reasons they may not be present in abundance. This work also provides an abiotic background that is useful in understanding biosignatures in the search for extraterrestrial life.

5. CONCLUSIONS

This work simulates abiotic organic synthesis that may occur in Titan's surface melt and tests a potentially important reaction pathway in such an environment: aminonitrile alkaline hydrolysis at cool temperatures. Here, we track the amino acid quantity over 6 months and analyze our samples using LCMS. Our work confirms amino acid synthesis by means of the alkaline hydrolysis pathway. We find that 2AH → alanine is the most productive reaction, followed by AAH → glycine and 4ABH → γ-ABA. All three aminonitrile hydrolysis reactions proceed similarly with varying reaction rates. All three reactions with ammonia present resulted in amino acid formation that increased over time and had a positive dependence with temperature. Alternatively, in the water-only samples, amino acid formation beyond the initial concentration (t_0) was not observed. The high amino acid yields found in this study suggest the reaction may be a productive source of amino acids in Titan surface melts, if ammonia and aminonitriles are indeed present.

Key takeaways from this study include:

1. Ammonia of at least 5% in the ammonia−water solution plays a critical role in amino acid synthesis over time.

2. An increase in ammonia from 5% to 10% did not significantly affect amino acid synthesis at 3 °C.

3. The addition of 0.05−0.1 M aminonitrile (pH ∼ 3) and the inclusion of salts and minerals was not sufficient to drive aminonitrile hydrolysis in 100% water at cooler temperatures (≤21 °C).

Compared to the hydrolysis of Titan-like tholins in previous work, our study shows that the aminonitrile reaction rates are a few orders of magnitude slower, with amino acid abundance a few orders of magnitude higher. This is not surprising, as the tholins contain a wide variety of molecules, many that have remained unidentified, and thus, one reaction pathway alone is unlikely to replicate the results of a hydrolysis experiment on such material. Rather, with this type of systematic study, we gain specific knowledge that will contribute to a library of relevant chemical reactions that have the potential for generating molecules of prebiotic relevance under the conditions expected in ocean worlds in our solar system.

■ ASSOCIATED CONTENT

Data Availability Statement

The data for the LCMS figures (Figures 2,3) are provided on Zenodo with the following DOI: 10.5281/zenodo.13294737. All other information, including the data underlying this study, is available in the published article and its online Supporting Information.

ⓈⒾ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspace-chem.4c00114.

Additional figures, tables, uncertainty discussion, and methods (PDF)

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Nixon, C. A. The Composition and Chemistry of Titan's Atmosphère. *ACS Earth Space Chem.* **2024**, *8*, 406−456.

(2) Hörst, S. M. Titan's atmosphere and climate. *J. Geophys. Res.: planets* **2017**, *122*, 432−482.

(3) Janssen, M. A.; Le Gall, A.; Lopes, R. M.; et al. Titan's surface at 2.18-cm wavelength imaged by the Cassini RADAR radiometer: Results and interpretations through the first ten years of observation. *Icarus* **2016**, *270*, 443−459.

(4) Malaska, M. J.; Radebaugh, J.; Lopes, R. M.; et al. Labyrinth Terrain on Titan. *Icarus* **2020**, *344*, 113764.

(5) Neish, C. D.; Lorenz, R. D.; Turtle, E. P.; Barnes, J. W.; Trainer, M. G.; Stiles, B.; Kirk, R.; Hibbitts, C. A.; Malaska, M. J.; et al. Strategies for Detecting Biological Molecules on Titan. *Astrobiology* **2018**, *18* (5), 571−585.

(6) Farnsworth, K. K.; Dhingra, R. D.; Ahrens, C. J.; Nathan, E. M.; Magaña, L. O. Chapter 10 Titan, Enceladus, and Other Icy Moons of Saturn. In *Ices in the Solar System*; Soare, R. J.; Williams, J.-P.; Ahrens, C.; Butcher, F. E. G.; El-Maarry, M. R. ed., 1 st ed.: Elsevier, ISBN, 2024, pp. 315-356.

(7) Lopes, R. M. C.; Mitchell, K. L.; Stofan, E. R.; Lunine, J. I.; Lorenz, R.; Paganelli, F.; Kirk, R. L.; Wood, C. A.; Wall, S. D.; Robshaw, L. E.; Fortes, A. D.; et al. Cryovolcanic features on Titan's surface as revealed by the Cassini Titan Radar Mapper. *Icarus* **2007**, *186* (2), 395−412.

(8) Artemieva, N.; Lunine, J. Cratering on Titan: Impact melt, ejecta, and the fate of surface organics. *Icarus* **2003**, *164* (2), 471−480.

(9) O'Brien, D. P.; Lorenz, R. D.; Lunine, J. I. Numerical Calculations on the Longevity of Impact Oases on Titan. *Icarus* **2005**, *173*, 243−253.

(10) Hedgepeth, J. E.; Buffo, J. J.; Chivers, C. J.; Neish, C. D.; Schmidt, B. E. Modeling the distribution of organic carbon and nitrogen in impact crater melt on Titan. *Planet. Sci. J.* **2022**, *3* (51), 51−1.

(11) Wakita, S.; Johnson, B. C.; Soderblom, J. M.; Shah, J.; Neish, C. D.; Steckloff, J. K. Modeling the Formation of Selk Impact Crater on Titan: Implications for Dragonfly. *Planet. Sci. J.* **2023**, *4* (3), 51.

(12) Wood, C. A.; Lorenz, R.; Kirk, R.; Lopes, R.; Mitchell, K.; Stofan, E.; The Cassini RADAR Team. Impact craters on Titan. *Icarus* **2010**, *206* (1), 334−344.

(13) Lopes, R. M. C.; Kirk, R. L.; Mitchell, K. L.; LeGall, A.; Barnes, J. W.; Hayes, A.; Kargel, J.; Wye, L.; Radebaugh, J.; Stofan, E. R. Cryovolcanism on Titan: New results from Cassini RADAR and VIMS. *JGR Planets* **2013**, *118* (3), 416−435.

(14) Hedgepeth, J. E.Impact Craters on Titan: Finalizing Titan's Crater Population. In *Electronic Thesis and Dissertation Repository*, Western Libraries 2018, 5618. https://ir.lib.uwo.ca/etd/5618.

(15) Wood, C. A.; Radebaugh, J. Morphologic Evidence for Volcanic Craters Near Titan's North Polar Region. *JGR Planets* **2020**, *125* (8), 8−1.

(16) Tobie, G.; Grasset, O.; Lunine, J. I.; Mocquet, A.; Sotin, C. Titan's internal structure inferred from a coupled thermal-orbital model. *Icarus* **2005**, *175* (2), 496−502.

(17) Tobie, G.; Gautier, D.; Hersant, F. Titan's Bulk Composition Constrained by Cassini-Huygens: Implication for Internal Outgassing. *Astrophys. J.* **2012**, *752* (125), 125−1.

(18) Sotin, C.; Kalousová, K.; Tobie, G. Titan's Interior Structure and Dynamics After the Cassini-Huygens Mission. *Annu. Rev. Earth Planet. Sci.* **2021**, *49* (1), 579−607.

(19) Leitner, M. A.; Lunine, J. I. Modeling early Titan's ocean composition. *Icarus* **2019**, *333*, 61−70.

(20) Zahnle, K. J.; Korycansky, D. G.; Nixon, C. A. Transient Climate Effects of Large Impacts on Titan. *Icarus* **2014**, *229*, 378−391.

(21) Crosta, A. P.; Silber, E. A.; Lopes, R. M. C.; Johnson, B. C.; Bjonnes, E.; Malaska, M. J.; Vance, S. D.; Sotin, C.; Solomonidou, A.; Soderblom, J. M. Modeling the Formation of Menrva Impact Crater on Titan: Implications for Habitability. *Icarus* **2021**, *370*, 114679.

(22) Osinski, G. R.; Cockell, C. S.; Pontefract, A.; Sapers, H. M. The role of meteorite impacts in the origin of life. *Astrobiology* **2020**, *20* (9), 1121−1149.

(23) Bizzarri, B. M.; Saladino, R.; Delfino, I.; Garcia-Ruiz, J. M.; Di Mauro, E. Prebiotic Organic Chemistry of Formamide and the Origin of Life in Planetary Conditions: What We Know and What Is the Future. *Int. J. Mol. Sci.* **2021**, *22*, 917.

(24) Khare, B. N.; Sagan, C.; Ogino, H.; Nagy, B.; Er, C.; Schram, K. H.; Arakawa, E. T. Amino Acids Derived from Titan Tholins. *Icarus* **1986**, *68*, 176−184.

(25) Nguyen, M.-J. Nouvelles contraintes sur la nature physico-chimique des aérosols de Titan: analyse des données de la mission Cassini-Huygens et simulation expérimentale en laboratoire Ph.D thesis; Université de Paris-Val-de-Marne: France, 2007.

(26) Raulin, F.; Nguyen, M.-J.; Coll, P. Titan: an astrobiological laboratory in the Solar System. In *Instruments, Methods, and Missions for Astrobiology X*, SPIE; 2007, Vol. *6694*, pp. 193−204.

(27) Neish, C. D.; Somogyi, Á.; Imanaka, H.; Lunine, J. I.; Smith, M. A. Rate Measurements of the Hydrolysis of Complex Organic Macromolecules in Cold Aqueous Solutions: Implications for Prebiotic Chemistry on the Early Earth and Titan. *Astrobiology* **2008**, *8* (2), 273−287.

(28) Neish, C. D.; Somogyi, Á.; Lunine, J. I.; Smith, M. A. Low temperature hydrolysis of laboratory tholins in ammonia-water solutions: Implications for prebiotic chemistry on Titan. *Icarus* **2009**, *201*, 412−421.

(29) Neish, C. D.; Somogyi, A.; Smith, M. A. Titan's Primordial Soup: Formation of Amino Acids via Low-Temperature Hydrolysis of Tholins. *Astrobiology* **2010**, *10* (3), 337−347.

(30) Ramírez, S. I.; Coll, P.; Buch, A.; Brassé, C.; Poch, O.; Raulin, F. The fate of aerosols on the surface of Titan. *Faraday Discuss.* **2010**, *147*, 419−427.

(31) Poch, O.; Coll, P.; Buch, A.; Ramírez, S. I.; Raulin, F. Production yields of organics of astrobiological interest from H2O−NH3 hydrolysis of Titan's tholins. *Planet. Space Sci.* **2012**, *61* (1), 114−123.

(32) Cleaves, J. H., II; Neish, C.; Callahan, M. P.; Parker, E.; Fernandez, F. M.; Dworkin, J. P. Amino acids generated from hydrated Titan tholins: Comparison with Miller-Urey electric discharge products. *Icarus* **2014**, *237*, 182−189.

(33) Brassé, C.; Buch, A.; Coll, P.; Raulin, F. Low-Temperature Alkaline pH Hydrolysis of Oxygen-Free Titan Tholins: Carbonates Impact. *Astrobiology* **2017**, *17*, 8−26.

(34) Cable, M. L.; Hörst, S. M.; Hodyss, R.; Beauchamp, P. M.; Smith, M. A.; Willis, P. A. Titan Tholins: Simulating Titan Organic Chemistry in the Cassini-Huygens Era. *Chem. Rev.* **2012**, *112* (3), 1882−1909.

(35) Taniuchi, T.; Takano, Y.; Kobayashi, K. Amino acid precursors from a simulated lower atmosphere of Titan: Experiments of cosmic ray energy source with 13C- and 18O-stable isotope probing mass spectrometry. *Anal. Sci.* **2013**, *29*, 777−785.

(36) Dworkin, J. P.; Adelman, L. A.; Ajluni, T.; et al. OSIRIS-Rex Contamination Control Strategy and Implementation. *Space Sci. Rev.* **2018**, *214* (19), 19−1.

(37) Vinogradoff, V.; Remusat, L.; McLain, H. L.; et al. Impact of Phyllosilicates on Amino Acid Formation under Asteroidal Conditions. *ACS Earth Space Chem.* **2020**, *4*, 1398−1407.

(38) Aponte, J. C.; McLain, H. L.; Simkus, D. N.; Elsila, J. E.; Glavin, D. P.; Parker, E. T.; Dworkin, J. P.; Hill, D. H.; Connolly, H. C., Jr; Lauretta, D. S. Extraterrestrial Organic Compounds and Cyanide in the CM2 Carbonaceous Chondrites Agus Zarcas and Murchison. *Meteorit. Planet. Sci.* **2020**, *55* (7), 1509−1524.

(39) Giese, C.-C.; Ten Kate, I. L.; van den Ende, M. P. A.; Wolthers, M.; Aponte, J. C.; Camprubi, E.; Dworkin, J. P.; Elsila, J. E.; Hangx, S.; King, H. E.; et al. Experimental and Theoretical Constraints on Amino Acids Formation from PAHs in Asteroidal Settings. *ACS Earth Space Chem.* **2022**, *6* (3), 468−481.

(40) Mason, B. Olivine composition in chondrites. *Geochim. Cosmochim. Acta* **1963**, *27*, 1011−1023.

(41) Russell, S. Chondrites. In *Encyclopedia of Geochemistry*, White, W. M., Ed.; Springer, 2018.

(42) Leone, G.; Tanaka, H. K. M. Igneous Processes in the Small Bodies of the Solar System I Astroids And Comets. *iScience* **2023**, *26*, 107160.

(43) Cantor, B. Impurity effects on heterogeneous nucleation. *Mater. Sci. Eng.: A* **1997**, *226−228*, 151−156.

(44) Maeda, N. Brief Overview of Ice Nucleation. *Molecules* **2021**, *26* (2), 392.

(45) Moran, S. E.; Hörst, S. M.; He, C.; Radke, M. J.; Sebree, J. A.; Izenberg, N. R.; et al. Triton haze analogs: The role of carbon monoxide in haze formation. *J. Geophys. Res.: planets* **2022**, *127*, No. e2021JE006984.

(46) Zaia, D. A. M. A review of adsorption of amino acids on minerals: Was it important for origin of life? *Amino Acids* **2004**, *27*, 113−118.

(47) Cruz-Hernández, A. E.; Colin-Garcia, M.; Ortega-Gutierrez, F.; Mateo-Marti, E. Komatiites as Complex Adsorption Surfaces for Amino Acids in Prebiotic Environments, a Prebiotic Chemistry Essay. *Life* **2022**, *12*, 1788.

(48) Mandt, K. E.; Mousis, O.; Lunine, J.; Gautier, D. Protosolar Ammonia as the Unique Source of Titan's Nitrogen. *Astrophys. J. Lett.* **2014**, *788* (L24), L24−1.

(49) Waite Jr, J. H.; Lewis, W. S.; Magee, B. A.; et al. Liquid Water on Enceladus from Observations of Ammonia and 40Ar in the Plume. *Nature* **2009**, *460*, 487−490.

(50) Krasnopolsky, V. A. A photochemical model of Titan's atmosphere and ionosphere. *Icarus* **2009**, *201* (1), 226−256.

(51) Neish, C.; Malaska, M. J.; Sotin, C.; Lopes, R. M. C.; Nixon, C. A.; Affholder, A.; Chatain, A.; Cockell, C.; Farnsworth, K. K.; Higgins, P. M.; et al. Organic Input to Titan's Subsurface Ocean Through Impact Cratering. *Astrobiology* **2024**, *24* (2), 177−189.

(52) Hörst, S. M.; Yelle, R. V.; Buch, A.; Carrasco, N.; Cernogora, G.; Dutuit, O.; Quirico, E.; Sciamma-O'Brien, E.; Smith, M. A.; Somogyi, A.; et al. Formation of Amino Acids and Nucleotide Bases in a Titan Atmosphere Simulation Experiment. *Astrobiology* **2012**, *12* (9), 809−817.

(53) de Kok, R.; Irwin, P. G. J.; Teanby, N. A.; Lellouch, E.; Bézard, B.; Vinatier, S.; Nixon, C. A.; Fletcher, L.; Howett, C.; Calcutt, S. B.; Bowles, N. E.; Flasar, F. M.; Taylor, F. W. Oxygen compounds in Titan's stratosphere as observed by Cassini CIRS. *Icarus* **2007**, *186*, 354−363.

(54) Barnes, J. W.; Turtle, E. P.; Trainer, M. G.; et al. Science Goals and Objectives for the Dragonfly Titan Rotorcraft Relocatable Lander. *Planet. Sci. J.* **2021**, *2*, 130.