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Amino acid analyses of Antarctic CM2 meteorites using liquid chromatography–time of flight–mass spectrometry

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Abstract–Amino acid analyses of the Antarctic CM2 chondrites Allan Hills (ALH) 83100 and Lewis Cliff (LEW) 90500 using liquid chromatography–time of flight–mass spectrometry (LC-ToF-MS) coupled with UV fluorescence detection revealed that these carbonaceous meteorites contain a suite of indigenous amino acids not present in Antarctic ice. Several amino acids were detected in ALH 83100, including glycine, alanine, β-alanine, γ-amino-*n*-butyric acid (γ-ABA), and α-aminoisobutyric acid (AIB) with concentrations ranging from 250 to 340 parts per billion (ppb). In contrast to ALH 83100, the CM2 meteorites LEW 90500 and Murchison had a much higher total abundance of these amino acids (440–3200 ppb). In addition, ALH 83100 was found to have lower abundances of the α-dialkyl amino acids AIB and isovaline than LEW 90500 and Murchison. There are three possible explanations for the depleted amino acid content in ALH 83100: 1) amino acid leaching from ALH 83100 during exposure to Antarctic ice meltwater, 2) a higher degree of aqueous alteration on the ALH 83100 parent body, or 3) ALH 83100 originated on a chemically distinct parent body from the other two CM2 meteorites. The high relative abundance of ε-amino-*n*-caproic acid (EACA) in the ALH 83100 meteorite as well as the Antarctic ice indicates that Nylon-6 contamination from the Antarctic sample storage bags may have occurred during collection.

INTRODUCTION

The investigation of organic compounds in primitive carbonaceous chondrites provides a record of the chemical processes that occurred in the early solar system. In particular, amino acids have been shown to be potential indicators in tracing the nature of carbonaceous chondrite parent bodies (Ehrenfreund et al. 2001). The delivery of amino acids by carbonaceous chondrites to the early Earth could have also been an important source of the Earth's prebiotic organic inventory (Oró 1961; Anders 1989; Delsemme 1992; Chyba and Sagan 1992). The Murchison meteorite, a non-Antarctic CM2 type carbonaceous chondrite that fell in Australia in 1969, has been analyzed extensively for amino acids over the past 35 years using a variety of techniques (Kvenvolden et al. 1970; Pereira et al. 1975; Cronin et al. 1979a; Cronin and Pizzarello 1983; Pizzarello et al. 1994; Engel and Macko 1997; Ehrenfreund et al. 2001). Over 80 different amino acids have been detected in the Murchison meteorite, and they comprise a mixture of C_2 to C_8 cyclic and acyclic monoamino alkanoic and alkandioic acids of nearly complete structural diversity, many of which are nonexistent in the terrestrial biosphere (Cronin and Pizzarello 1983; Cronin and Chang 1993; Botta and Bada 2002a; Sephton 2002). The α-dialkyl amino acids, α-aminoisobutyric acid (AIB), and isovaline, which are extremely rare amino acids on the Earth, are two of the most abundant amino acids found in the Murchison meteorite (Kvenvolden et al. 1971).

The subsequent discovery of a large number of meteorites in Antarctica since 1969 has provided additional opportunities to search for organic compounds in CM type carbonaceous chondrites. Analyses of several Antarctic CM2 carbonaceous meteorites have demonstrated that many of these samples contain a similar abundance and distribution of amino acids compared to non-Antarctic CM2s such as the Murchison and Murray meteorites (Cronin and Moore 1971; Kotra et al. 1979; Shimoyama et al. 1979; Cronin et al. 1979a; Botta and Bada 2002a). One exception is the Antarctic CM2

meteorite Yamato (Y-) 791198, which contains the largest total abundance of AIB in any carbonaceous chondrite studied to date at approximately ten times the total concentration of AIB in Murchison (Shimoyama et al. 1985; Shimoyama and Ogasawara 2002). In contrast, subsequent analyses of the Y-793321 and Belgica (B-) 7904 meteorites revealed that these Antarctic CM2 meteorites were depleted in amino acids relative to other CM2s suggesting loss due to aqueous metamorphism on the parent body or leaching in the Antarctic ice (Shimoyama and Harada 1984). Although a relatively large sample size exists for the Antarctic CM2 meteorites Allan Hills (ALH) 83100 and Lewis Cliff (LEW) 90500 compared to other Antarctic CM2s, these two meteorites have not been studied as extensively for amino acids.

The most common techniques that have been used for amino acid analyses of carbonaceous meteorites are reverse phase high-performance liquid chromatography with UV fluorescence detection (HPLC-FD) and gas chromatography-mass spectrometry (GC-MS). Although these analytical techniques have been very useful for the characterization of complex amino acid mixtures found in carbonaceous meteorites (Kvenvolden et al. 1970; Cronin et al. 1982; Ehrenfreund et al. 2001; Glavin and Bada 2001), both techniques have their limitations. For example, HPLC-FD has a much lower detection limit for amino acids (~10^{-14}–10^{-15} mol) than traditional GC-MS (~10^{-12} mol) detection, but relies on the identification of amino acids by fluorescence retention time only (Lindroth and Mopper 1979; Cronin et al. 1979b; Roach and Harmony 1987). Therefore, misidentification or inaccurate amino acid quantification by HPLC-FD due to the presence of interfering and/or coeluting peaks in meteorite extracts can occur. With traditional GC-MS, amino acids in meteorite extracts can be identified by both retention time and by their characteristic mass fragmentation pattern (Cronin and Pizzarello 1983). However, the mass fragments of the parent ions generated by electron impact (EI) ionization can often be difficult to interpret when dealing with unknown compounds. In addition, there is reduced sensitivity in EI ionization GC-MS compared to other soft ionization liquid chromatography-mass spectrometry (LC-MS) techniques such as electrospray ionization (ESI) (e.g., Whitehouse et al. 1985; Fenn et al. 1989). In ESI, nitrogen gas is used to help nebulize the liquid from the HPLC and evaporate the solvent to impart a charge on the analyte by the addition (ES+ mode, $M + H^+$) or removal (ES− mode, $M - H^+$) of a hydrogen ion with extremely low fragmentation of the parent ion (M). The advantage of using a time of flight mass spectrometer (ToF-MS) over quadrupole and ion trap mass spectrometers that are typically used in gas chromatography is that ToF-MS provides an exact molecular mass (RMS error ≤ 3 ppm) that can be used to help determine the molecular formula of an unknown parent compound without significant mass fragmentation and the sensitivity is not affected by coeluting compounds (Verentchikov et al. 1994; Krutchinsky et al. 1998).

Recently, the combination of HPLC-FD with ESI-MS has been used to analyze amino acids and amine standards after *o*-phthaldialdehyde/*N*-acetyl-L-cysteine (OPA/NAC) derivatization (Kutlán et al. 2002; Mengerink et al. 2002; Hanczkó et al. 2004; Gyimesi-Forrás et al. 2005). For this study, we have for the first time combined traditional HPLC-FD with atmospheric ESI-ToF-MS for the identification of OPA/NAC amino acid derivatives in meteorite extracts by fluorescence retention time and exact mass simultaneously. Here we report on the first analyses of amino acids in the CM2 carbonaceous meteorites ALH 83100, LEW 90500 and Murchison using this new LC-ToF-MS analytical configuration. Amino acid analyses of any kind have not previously been reported for the ALH 83100 carbonaceous meteorite.

MATERIALS AND METHODS

Chemicals and Reagents

All of the chemicals used in this study were purchased from Sigma-Aldrich and Fisher. A stock amino acid solution (~10^{-5} M) was prepared by mixing individual standards (97–99% purity) in Millipore (18.2 MΩ) or double-distilled (dd) water. The OPA/NAC reagent used for amino acid derivatization was prepared by dissolving 4 mg OPA in 300 μl methanol (Fisher Optima), and then adding 685 μl 0.1 M sodium borate buffer (pH 9) and 15 μl 1 M NAC. A 0.1 M hydrazine (NH_2NH_2) solution was prepared by double vacuum distillation of anhydrous hydrazine (98% purity) and subsequent dilution in water. The HCl was double-distilled, and the ammonium formate buffer used in the LC-ToF-MS analyses was prepared by NH_4OH titration of a 50 mM formic acid solution to pH 8. A 5 μM phenolphthalein solution in acetonitrile with 0.1% formic acid was used for internal mass calibration of the ToF-MS.

Samples

The Antarctic CM2 carbonaceous chondrites ALH 83100 (split 246, parent 26, mass 5.6 g) and LEW 90500 (split 69, parent 1, mass 5.0 g) analyzed in this study were interior fragments selected by the meteorite sample curator at the NASA Johnson Space Center (JSC). These Antarctic meteorite samples were collected during the 1983 and 1990 Antarctic Search for Meteorites (ANSMET) field seasons, respectively. Several individual interior fragments (>2 cm from fusion crust) of the CM2 meteorite Murchison (USNM 6650.2, mass 6.3 g) that fell in southeastern Australia in 1969 were provided by the Smithsonian National Museum of Natural History. A sample of crushed serpentine (a hydrated magnesium silicate mineral present in these meteorite

samples) that had been heated at 500 °C for 3 h was used as a procedural blank. In addition, a 4.5 kg block of ice extracted from the La Paz region of Antarctica during the 2003–04 ANSMET field season (La Paz 16704) was used as a control for the Antarctic meteorite analyses. Antarctic ice samples from the 1983 and 1990 field seasons in Allan Hills and Lewis Cliffs were not available for study (R. Harvey, personal communication). A sterile nylon bag of the type used to store the Antarctic meteorite samples and ice after collection was also analyzed in parallel (provided by K. Righter, JSC meteorite sample curator).

Extraction and Processing Procedures

All glassware and sample handling tools were rinsed with Millipore water, wrapped in aluminum foil, and then heated in an oven at 500 °C overnight. The extraction protocol used in this study was based on the extraction procedure used to isolate amino acids from the Murchison meteorite over 30 years ago (Kvenvolden et al. 1970). However, in this investigation we included an improved high temperature acid vapor hydrolysis procedure that was shown in previous studies to be much faster and cleaner than the traditional acid liquid hydrolysis protocol (Tsugita et al. 1987; Keil and Kirchman 1991; Glavin et al. 1999; Glavin and Bada 2001).

The meteorite sample vials were opened in a positive pressure (1 μm filtered air) clean room at the Scripps Institution of Oceanography (SIO). Individual meteorite fragments were then crushed using a mortar and pestle, and a portion of the powdered samples transferred into clean pre-weighed glass test tubes (16 × 125 mm) and weighed again. The CM2 meteorites ALH 83100 (126 mg), LEW 90500 (205 mg) and Murchison (140 mg) and the serpentine (253 mg) were flame-sealed inside individual test tubes containing 1 ml of water and then heated for 24 h in a heating block set at 100 °C. A small piece of nylon was cut from the top of the Antarctic meteorite storage bag (47 mg), transferred to a test tube, and then carried through the identical hot water extraction procedure as the meteorites. In addition, the same nylon bag was extracted in water at room temperature by adding 5 ml of water directly to the interior of the bag. After 24 h, the water was removed from the nylon bag and processed in the same manner as the meteorite samples described below.

After hot water extraction, the tubes were rinsed with dd water to remove exterior surface contamination, broken open, and then centrifuged for 5 min to separate sample particulate from the water supernatant. One half of the water supernatant from each tube was transferred to a new test tube (10 × 75 mm), dried under vacuum, and then hydrolyzed under 6 M HCl vapor by flame-sealing each small test tube inside a larger test tube (20 × 150 mm) containing 1 ml dd 6 M HCl, and then by placing the large sealed tubes in an oven at 150 °C for 3 h. The remaining water supernatants were not acid-hydrolyzed in order to determine the concentration of free amino acids in the extracts. After vapor hydrolysis, the large tubes were broken open and the small interior tubes removed and dried under vacuum to remove any residual HCl from the hydrolyzed sample. Both the hydrolyzed and unhydrolyzed residues were redissolved in 3 ml water and desalted via a cation exchange column (AG 50W-X8, 100–200 mesh, hydrogen form, BIO-RAD). The nylon bag water extracts were not desalted. During column loading, a D,L-norleucine internal standard was added to each sample to estimate the amino acid recoveries from desalting and derivatization. Norleucine was selected since this amino acid had a very late HPLC retention time and did not interfere with most of the meteoritic amino acids of interest. After elution of the anionic species with water, the amino acids were obtained by elution with 3 ml aqueous 2 M NH_4OH. The NH_4OH eluate was dried under vacuum, resuspended in 20 μl 0.1 M sodium borate buffer (pH 9) and derivatized with 5 μl OPA/NAC (Zhao and Bada 1995) in glass vials. The derivatization reaction was then quenched after 1 or 15 minutes at room temperature with 75 μl of 50 mM sodium acetate buffer (SIO) or 75 μl of 0.1 M hydrazine hydrate (NASA Goddard Space Flight Center [GSFC]), and the solution was loaded into the auto sampler carousel at 4 °C prior to HPLC-FD and LC-ToF-MS analysis (GSFC).

The ~4.5 kg block of Antarctic ice (La Paz 16704) was removed from the nylon sample bag, wrapped with clean aluminum foil that had been heated at 500 °C overnight, and then rinsed with dd water in order to remove surface contaminants (the washing procedure removed approximately 10% of the total ice by volume). A previous analysis of Allan Hills, Antarctic ice at SIO has shown that significant amino acid contamination from both nitrile and latex gloves can occur from direct exposure of the ice block to the gloves during the rinsing procedure (unpublished data from SIO). Therefore, direct contact with the La Paz Antarctic ice sample using nitrile or latex gloves was avoided. The La Paz ice sample was melted overnight in a large glass beaker that had been heated at 500 °C overnight, and the top of the beaker was covered with aluminum foil. The following day 4 μl of the melted ice water was concentrated using rotary evaporation at 45–50 °C and the dried residue dissolved in dd water (100 μl). One tenth of the unfiltered ice extract was derivatized with OPA/NAC and then analyzed using HPLC-FD and LC-ToF-MS (GSFC). A blank consisting of one liter dd water was carried through the identical rotovap processing procedure as the Antarctic ice.

HPLC-FD and LC-ToF-MS Detection

Amino acid derivatives and their enantiomeric ratios in the meteorite and ice samples were independently analyzed after derivatization by reverse phase HPLC-FD at SIO and at GSFC. For the HPLC-FD measurements at SIO, amino acids

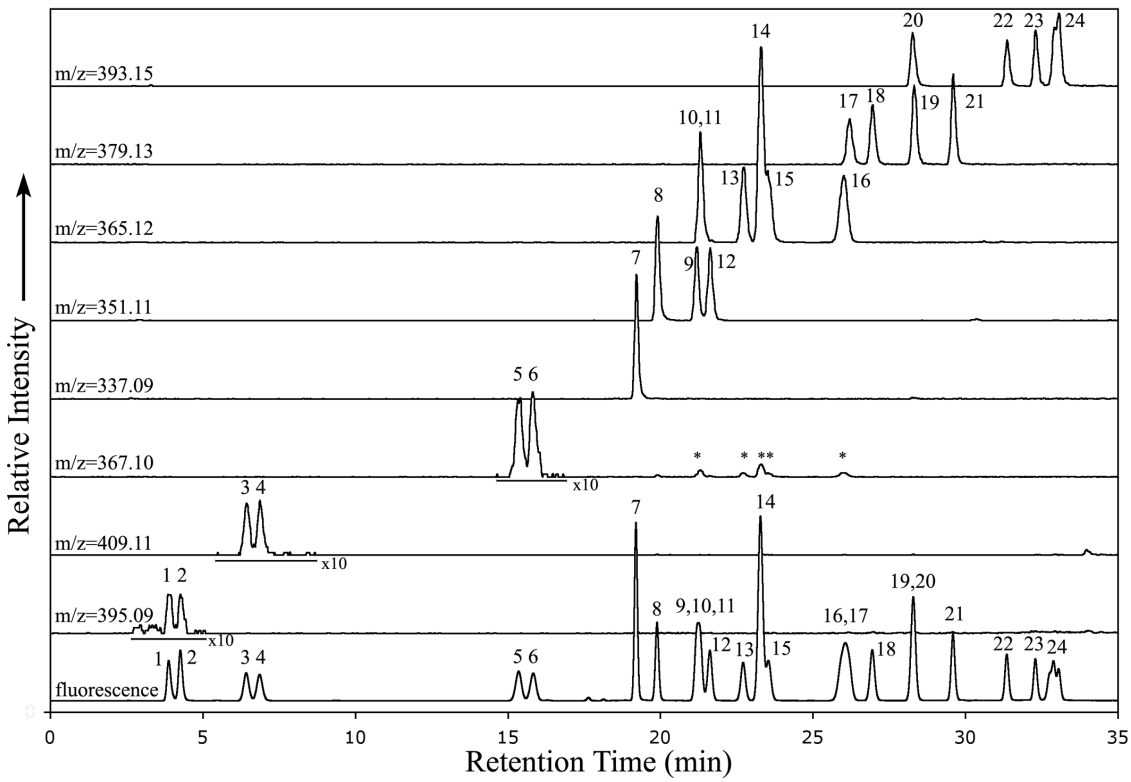

Fig. 1. The 0–35 min region of the HPLC-FD and LC-ToF-MS chromatograms obtained from a single injection of a mixture of OPA/NAC labeled (15 min derivatization) amino acid standards (~10 pmol of each compound). Amino acid peak identifications are given in Table 1. The UV fluorescence trace is shown at the bottom and the exact mass traces were obtained simultaneously in positive electrospray mode for the individual amino acid standards. A detection limit for these amino acids using both UV fluorescence and ToF-MS was determined to be about 10^{-15}–10^{-16} mol (S/N ~30). The conditions for amino acid separations for the mobile phase at 30 °C were as follows: flow rate, 1 ml/min; solvent A (water); solvent B (methanol); solvent C (50 mM ammonium formate, 8% methanol, pH 8.0); gradient 0–5 min, 100% C; 5–15 min, 0–83% A and 0–12% B; 15–22 min, 83–75% A and 12–20% B; 22–35 min, 75–35% A and 20–60% B. Peaks 1–6 were magnified tenfold due to suppression of the ionization efficiency under highly aqueous conditions during this stage of the HPLC gradient. The asterisks in the 367.10 m/z mass trace correspond to OPA/NAC labeled aminobutyric acids (peaks 10, 11, 13–16) with two naturally occurring ^{13}C atoms. A similar figure was obtained from negative electrospray mode (data not shown).

were eluted from a Phenomenex Synergi 4 μm Hydro-reverse phase column (4.6 × 250 mm) at 1 ml/min with a binary gradient of 50 mM sodium acetate buffer (8% methanol, pH 5.5) and methanol and then detected using a Shimadzu RF-530 fluorescence detector (λ_{ex} = 340 nm and λ_{em} = 450 nm). At GSFC, the HPLC-FD measurements were made with a Waters Alliance 2695 HPLC system controlled by MassLynx 4.0 coupled to a 2475 multi-λ fluorescence detector at the same excitation and emission wavelengths as SIO. Amino acid separation was achieved with a Phenomenex Luna 5 μm reverse phase phenyl-hexyl column (4.6 mm × 250 mm) and maintained at 30 °C; elution was performed using a ternary gradient with 50 mM ammonium formate buffer (8% methanol, pH 8) and increasing amounts of water and methanol at a flow rate of 1 ml/min (Fig. 1).

In addition to UV fluorescence detection, the unfragmented masses of the amino acid OPA/NAC derivatives that eluted from the column were detected by orthogonal acceleration reflectron time of flight mass spectrometry using a Waters LCT Premier ToF-MS instrument at GSFC. The mass spectrometer was run in "W optics mode" which employs three reflectrons to provide a FWHM mass resolution of greater than 8000 in the monitored mass range of 100–1000 m/z. For all runs the column eluant (flow rate: 1 ml/min) was split 85% to the FD and 15% to the ToF-MS. The ToF-MS was configured for ESI with a 1 s integration time in each ionization mode and alternated between ES+ and ES− every 0.2 s during the acquisition. The ESI settings were: desolvation gas (N_2) temperature: 250 °C, 600 l/h; capillary voltage: 3.8 kV (ES+) and 4.2 kV (ES−); cone voltage: 20 V (ES+) and 35 V (ES−). The instrument was optimized for maximum sensitivity of OPA/NAC amino acid derivatives in the 300–450 m/z range with detection limits in the subfemtomole (~10^{-15} to 10^{-16} mol) range.

Mass calibration of the ToF-MS during the acquisition was maintained by monitoring a reference compound (phenolphthalein, $C_{20}H_{14}O_4$, monoisotopic mass (M) = 318.0892 Da, 5 μM in acetonitrile and 0.1% formic acid) every 10 s which was delivered to the MS via a separate pump

Table 1. Peak identifications and abbreviations for amino acids detected in the standard and meteorite samples.

Peak	Amino acid	Peak	Amino acid
1	D-aspartic acid	13	D-β-amino-*n*-butyric acid (D-β-ABA)
2	L-aspartic acid	14	α-aminoisobutyric acid (AIB)
3	L-glutamic acid	15	L-β-amino-*n*-butyric acid (L-β-ABA)
4	D-glutamic acid	16	D,L-α-amino-*n*-butyric acid (D,L-α-ABA)
5	D-serine	17	D-isovaline
6	L-serine	18	L-isovaline
7	Glycine	19	L-valine
8	β-alanine	20, X	ε-amino-*n*-caproic acid (EACA)
9	D-alanine	21	D-valine
10	γ-amino-*n*-butyric acid (γ-ABA)	22	D-isoleucine
11	D,L-β-aminoisobutyric acid (D,L-β-AIB)	23	L-isoleucine
12	L-alanine	24	D,L-leucine

at 10 μl/min to an independent and alternating ESI sprayer. This allows the exact mass of OPA/NAC amino acid derivatives to be determined within ± 0.001 Da (~3 ppm). Under these conditions, the sensitivity for ToF-MS detection of the OPA/NAC amino acid derivatives was higher in ES+ mode compared to ES– mode, therefore the ^{12}C monoisotopic mass of the protonated OPA/NAC-amino acid molecular ion was used for quantification. Peak areas were obtained by performing two 4 channel Savitzky-Golay smoothing of the spectrum and then plotting a chromatogram of a given mass at FWHM (typically 7–9 channels or about ± 0.02 Da around the center of the peak). No significant difference in area was found by a twofold alteration of the smoothing parameters. The peaks generated in this chromatogram were manually integrated in MassLynx.

RESULTS AND DISCUSSION

The amino acid data from this study are based on the HPLC-FD and LC-ToF-MS techniques described above. Amino acid peak identifications for all chromatograms are given in Table 1. A sample chromatogram demonstrating HPLC separation and detection of a standard mixture of amino acids by both UV fluorescence and exact mass using the LC-ToF-MS instrument at GSFC is shown in Fig. 1. The amino acid concentrations for the CM2 carbonaceous meteorites and Antarctic ice are the average of multiple independent analyses of the same extracts at SIO and at GSFC. Each peak in the chromatograms was identified by comparison of its UV fluorescence retention time and exact molecular mass with those of authentic amino acid reference standards.

Amino Acid Analyses of the CM2 Carbonaceous Meteorites

The HPLC-FD chromatograms of the 6 M HCl-hydrolyzed, hot water extracts from the GSFC analyses of the CM2 meteorites ALH 83100, LEW 90500 and Murchison and the serpentine blank are shown in Fig. 2. Similar HPLC-FD chromatograms for these meteorites were obtained by SIO (data not shown). Simultaneous LC-ToF-MS mass data in both ES+ and ES– modes were also collected for these meteorites at GSFC. The relative distributions of amino acids in the Murchison and LEW 90500 chromatograms were found to be similar but not identical (Fig. 3), which is consistent with previous analyses of these two meteorites (Ehrenfreund et al. 2001; Botta and Bada 2002b). The most abundant amino acids detected in Murchison and LEW 90500 were α-aminoisobutyric acid (AIB) and isovaline (1300 to 3200 ppb), with lower levels of aspartic and glutamic acids, glycine, β-alanine, α-ABA, β-ABA, γ-ABA, and valine (Table 2). The α-dialkyl amino acids AIB and isovaline are extremely rare on Earth, and thus characteristic of amino acids of apparent extraterrestrial origin. The amino acid serine was also identified in the Murchison and LEW 90500 acid-hydrolyzed hot-water extracts by HPLC-FD at SIO, but this amino acid was not detected by HPLC-FD or LC-ToF-MS analyses at GSFC (Fig. 2); therefore only an upper limit for serine is reported in Table 2. It should be emphasized that there are other unknown amino acid and primary amine peaks present in the HPLC-FD chromatograms of these meteorite extracts that were not identified by comparison with known standards (Fig. 2) for this study.

The chromatographic separation of γ-ABA from β-AIB by HPLC was not achieved under the conditions used at GSFC. In addition, since γ-ABA and β-AIB have identical molecular masses, we were unable to differentiate between these two amino acids using LC-ToF-MS; therefore the total concentration of these amino acids is reported as a sum in Table 2. β-AIB has previously been reported to be present at lower concentrations (~150–340 ppb) compared to γ-ABA (~720–1330 ppb) in the CM2 meteorites Murchison and Murray (Ehrenfreund et al. 2001). Trace amounts (<10 ppb) of L-aspartic acid, L-serine, glycine, β-alanine, L-alanine, and L-valine in the serpentine blank could be detected by standard HPLC-FD and LC-ToF-MS (Fig. 2), which indicates that only very low levels of amino acid contamination of the samples occurred during the processing procedure. However, the low abundance of amino acids in the serpentine blank does not

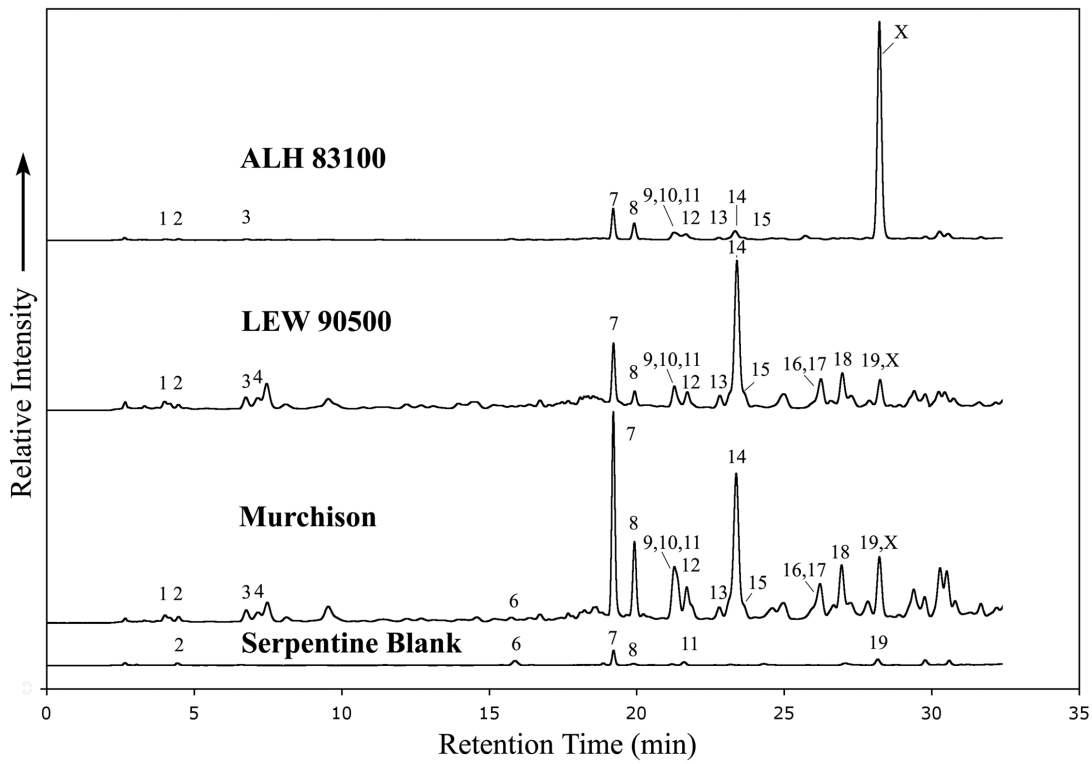

Fig. 2. The 0–32 min region (only the norleucine internal standard peak appeared outside of this region) of the HPLC-FD chromatograms from the GSFC analyses. Similar chromatograms were obtained from SIO and are available upon request. OPA/NAC derivatization (15 min) of amino acids in the 6M HCl-hydrolyzed hot-water extracts from the CM2 carbonaceous chondrite Murchison, the Antarctic CM2 meteorites LEW 90500 and ALH 83100, and the serpentine blank. The peaks were identified by comparison of the retention time and exact molecular mass to those in the amino acid standard run on the same day. All of the coeluting amino acids were separated and quantified by their exact masses with the exception of peak numbers 10 + 11 which correspond to γ-ABA and D,L-β-AIB (mass data for ALH 83100 shown in Figs. 4 and 6).

rule out the possibility of amino acid contamination of the meteorites during collection, storage or handling of the samples.

Although the distribution of amino acids in Murchison and LEW 90500 is similar, the total concentration of amino acids in the acid-hydrolyzed hot-water extract of Murchison is ~14,600 ppb compared to ~9000 ppb for LEW 90500 (Table 2). These total amino acid concentrations should be considered lower limits since not all of the amino acids present in these meteorite extracts were identified and quantified. A similar set of free amino acids was also detected in the unhydrolyzed water extracts of Murchison and LEW 90500 (Table 2). From the data in Table 2, we calculate that the ratio of free amino acids to total amino acids (free + bound) in Murchison (0.45 ± 0.08) is identical to the ratio in LEW 90500 (0.43 ± 0.13). The low free to total amino acid ratio in ALH 83100 (0.08 ± 0.02) compared to Murchison and LEW 90500 is due to a high concentration of peptide bound "compound X" in ALH 83100 (detailed discussion of compound X in later section).

The large increase in amino acid concentration in both Murchison and LEW 90500 after acid hydrolysis indicates that amino acids in these meteorites occur as both free amino acids and as derivatives and/or acid labile precursors that can be converted to amino acids after acid hydrolysis (Cronin 1976a). However, it has been reported that acid labile precursors including amino acid dipeptides and diketopiperazines (cyclic dimers of amino acids) account for only a small percentage (0.05–2.0%) of the acid-liberated amino acids in Murchison (Cronin 1976b; Shimoyama and Ogasawara 2002), and that most of the precursors in Murchison are low molecular weight amino acid derivatives including mono- and dicarboxylic acid amides, hydroxy acid amides, lactams, carboxylactams, lactims, N-acetylamino acids, and substituted hydantoins (Cronin 1976a; Cooper and Cronin 1995). Analyses of these linear and cyclic aliphatic amides in the LEW 90500 and ALH 83100 meteorites have not been reported.

The D/L enantiomeric ratios calculated for several amino acids in Murchison, LEW 90500 and ALH 83100 are reported in Table 3. Since racemic mixtures of amino acids (D/L ~1) are predicted for reactions (e.g., Strecker-cyanohydrin synthesis) believed to be active on CM2 meteorite parent bodies (Peltzer et al. 1984; Lerner et al. 1993; Ehrenfreund et al. 2001), the relatively high D/L ratios (0.8–1.0) measured for the protein amino acids aspartic acid, glutamic acid, and

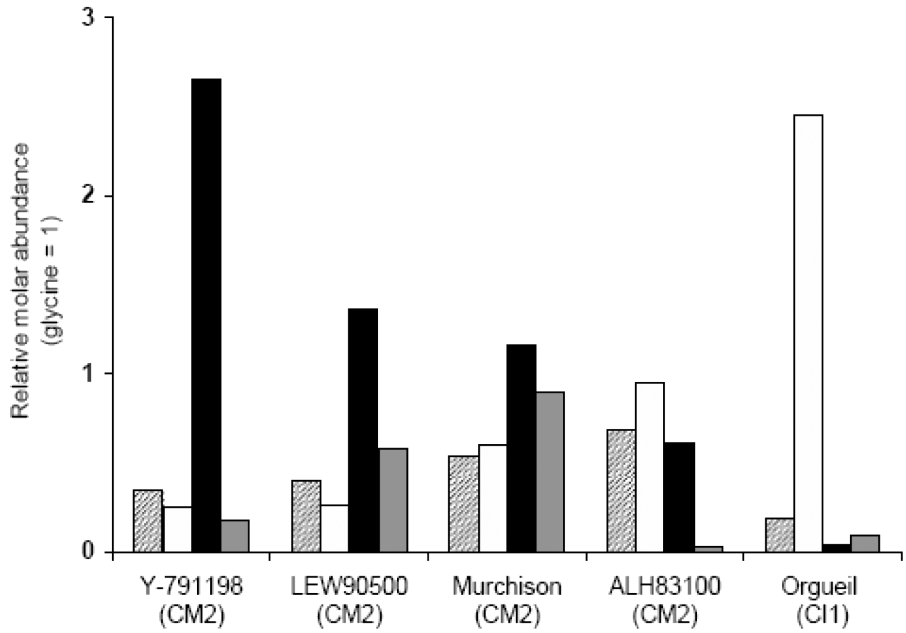

Fig. 3. A comparison of the relative molar amino acid abundances (glycine = 1.0) of alanine (stripes), β-alanine (white), α-aminoisobutyric acid (black), and isovaline (gray) in the acid-hydrolyzed hot-water extracts of several carbonaceous chondrites. The relative amino acid abundances for the CM2 meteorites Murchison, LEW 90500, and ALH 83100 are from this study only. The data for the Y-791198 and Orgueil meteorites were calculated from previous analyses (Shimoyama and Ogasawara 2002; Ehrenfreund et al. 2001).

alanine, and the nonprotein amino acid β-ABA in the CM2 meteorites Murchison and LEW 90500 (Table 3), suggests that a large fraction of these amino acids are indigenous in origin. Analyses of the Murchison meteorite less than two years after its fall to Earth revealed that several protein amino acids including alanine, glutamic acid, valine, proline, and the non-protein amino acids ABA, β-AIB, and norvaline were nearly racemic (D/L ~1) with minimal terrestrial contamination (Kvenvolden et al. 1970; 1971). Racemic mixtures of protein and nonprotein amino acids have also been detected in the Antarctic CM2 chondrites Y-74662 and Y-791198 (Shimoyama et al. 1979; Shimoyama and Ogasawara 2002). The calculated D/L ratios for the amino acids in ALH 83100 ranged from 0.7 for aspartic acid to 1.0 for β-ABA (Table 3). Terrestrial L-amino acid contamination of these meteorites after their fall to Earth, especially for valine (D/L ~0.5 for Murchison and LEW 90500, Table 3), may have lowered the initial D/L ratios of these protein amino acids to the D/L ratios presently observed. Previous analyses of Murchison have also shown that L-enantiomeric excesses of the nonprotein amino acid isovaline range from 0–15.2%, with significant variation between meteorite fragments (Pizzarello and Cronin 2000; Pizzarello et al. 2003). Since isovaline is an extremely rare amino acid on Earth, the contribution of terrestrial contamination to the L-isovaline excess observed in Murchison is unlikely (Pizzarello and Cronin 2000). The enantiomeric ratios for isovaline detected in Murchison and LEW 90500 are not reported here, but are discussed elsewhere (Glavin and Dworkin 2006).

Amino Acid Depletion in ALH 83100

In contrast to Murchison and LEW 90500, the ALH 83100 meteorite contained much lower abundances of most amino acids with total concentrations ranging from <1 to 338 ppb (Table 2). One notable difference was the low relative and total abundances of AIB (250 ppb) and isovaline (<10 ppb) in ALH 83100 compared to Murchison and LEW 90500 (Table 2; Fig. 3). One possible explanation for the depleted amino acid content of ALH 83100 relative to other CM2 meteorites is a loss of water-extractable amino acids due to ice meltwater exposure in Antarctica. The Antarctic CM2 carbonaceous chondrites Y-793321 and B-7904 were also found to be depleted in amino acids relative to other Antarctic CM2 meteorites (Shimoyama and Harada 1984). Given a residence time in the Antarctic ice of 11.6 ka for ALH 83100 (Jull et al. 1998), it is possible that some fraction of the original amino acids were leached from the meteorite during this time. Experiments designed to study the leaching of amino acids from the Murchison carbonaceous chondrite after exposure of the meteorite to cold water (4 °C), indicate that about half of the free amino acids including AIB and isovaline could be leached from the meteorite after only 6 weeks, while the remaining more strongly bound amino acids were extracted only after heating the meteorite in hot water (100 °C) for 24 h (Kminek et al. 2002). Since the terrestrial residence time of LEW 90500 has not been reported, it is difficult to draw any correlation between Antarctic meteorite exposure time and amino acid leaching at this time.

Table 2. Summary of the average blank-corrected amino acid concentrations in the unhydrolyzed (free) and HCl acid hydrolyzed (total) hot-water extracts of the CM2 type carbonaceous meteorites Murchison, LEW 90500, and ALH 83100, and an unhydrolyzed Antarctic ice extract as measured by two separate analyses, one at SIO and the other at GSFC[a].

Amino acid detected	CM2 carbonaceous meteorites						Antarctic ice
	Murchison		LEW 90500		ALH 83100		La Paz 16704
	Free (ppb)	Total (ppb)	Free (ppb)	Total (ppb)	Free (ppb)	Total (ppb)	Free (ppt)
D-aspartic acid	18 ± 2	120 ± 16	15 ± 3	127 ± 24	7 ± 2	29 ± 4	<0.3
L-aspartic acid	23 ± 3	132 ± 15	23 ± 5	151 ± 73	10 ± 3	43 ± 6	1.3 ± 0.3
D-glutamic acid	10 ± 4	343 ± 44	9 ± 3	317 ± 55	2 ± 1	21 ± 7	<0.1
L-glutamic acid	26 ± 2	357 ± 42	22 ± 4	316 ± 55	4 ± 2	23 ± 6	<0.1
D-serine	<4	<138[b]	<8	<219[b]	<4	<4	0.3 ± 0.2
L-serine	<5	<173[b]	<14	<235[b]	<5	<5	3.7 ± 0.1
Glycine	345 ± 27	1995 ± 122	520 ± 32	1448 ± 682	117 ± 12	300 ± 75	9.6 ± 2.4
β-alanine	302 ± 18	1419 ± 157	161 ± 21	442 ± 238	104 ± 8	338 ± 31	0.2 ± 0.1
γ-amino-*n*-butyric acid + D,L-β-AIB[c]	437 ± 30	1460 ± 213	132 ± 12	164 ± 21	155 ± 25	308 ± 68	<0.1
D-alanine	162 ± 10	623 ± 6	198 ± 10	343 ± 171	38 ± 6	110 ± 44	0.5 ± 0.1
L-alanine	171 ± 8	659 ± 84	203 ± 9	352 ± 161	43 ± 8	134 ± 19	2.5 ± 0.8
D-β-amino-*n*-butyric acid	91 ± 6	233 ±17	61 ± 7	155 ± 16	19 ± 4	33 ± 8	<0.1
L-β-amino-*n*-butyric acid	93 ± 11	256 ± 15	62 ± 7	172 ± 40	27 ± 5	33 ± 12	<0.1
α-aminoisobutyric acid (AIB)	2349 ± 404	3182 ± 620	1364 ± 188	2706 ± 377	115 ± 27	250 ± 40	46 ± 5
D,L-α-amino-*n*-butyric acid[c]	284 ± 95	403 ± 156	243 ± 98	431 ± 159	11 ± 4	19 ± 5	<0.1
D,L-isovaline	1872 ± 48	2796 ± 298	625 ± 21	1306 ± 83	<5	<10	<0.1
ε-amino-*n*-caproic acid (EACA)[d]	264 ± 138	268 ± 123	228 ± 161	386 ± 169	146 ± 60	8480 ± 953	886 ± 118
D-valine	37 ± 2	103 ± 9	21 ± 2	48 ± 12	<1	<1	<0.1
L-valine	73 ± 9	218 ± 23	46 ± 10	102 ± 16	<1	<1	<0.1
Total	6600	14,600	3900	9000	800	10,100	950

[a]The values are reported in parts per billion (ppb) for the meteorites and parts per trillion (ppt) for the Antarctic ice on a bulk sample basis. Quantification of the amino acids included background level correction using a serpentine blank and a comparison of the peak areas with those of an amino acid standard. The final values were normalized using the desalting and derivatization recoveries of an internal D,L-norleucine standard (recoveries ranged from 60–75% for the meteorite samples). The uncertainties (δx) are based on the standard deviation of the average value of between three and six separate measurements (N) with a standard error, $\delta x = \sigma_x \cdot (N - 1)^{-1/2}$. For all UV fluorescence data, co-eluting amino acid peaks and/or compounds with interfering peaks were not included in the average.

[b]These values must be considered upper limits since they were detected at SIO, but were not detected at GSFC.

[c]Amino acids and/or enantiomers could not be separated under the chromatographic conditions used.

[d]Major component of Nylon-6 identified as peak X in meteorite and ice samples; also known as 6-aminohexanoic acid.

Another possibility for the depleted amino acid levels in ALH 83100 is aqueous alteration on the meteorite parent body. Shimoyama and Harada (1984) have previously suggested that low temperature and/or aqueous metamorphism on the parent bodies of Y-793321 and Belgica-7904 could have been responsible for the amino acid depletion observed in these meteorites relative to other less altered Antarctic CM2 meteorites. Analyses of Mg-rich phyllosilicates and Ca-Mg carbonates in ALH 83100 suggest that this Antarctic CM2 meteorite has been extensively altered by parent body processes (Zolensky and Browning 1994). Therefore, it is possible that ALH 83100 originated on a parent body that was depleted in amino acids and/or their precursors because of more thorough aqueous processing. In contrast to ALH 83100, examination of the fine-grained mineralogy of Y-791198 indicate that this Antarctic meteorite may be the most weakly altered CM meteorite analyzed to date (Chizmadia and Brearley 2003). A comparison of the relative amino acid abundances in the CM2 meteorites Y-791198, Murchison, LEW 90500, ALH 83100 and the more extensively altered CI1 carbonaceous meteorite Orgueil (Zolensky and McSween 1988) may indicate some trends in amino acid distribution with respect to the degree of parent body alteration (Fig. 3). In general, the relative abundance of β-alanine relative to glycine appears to be higher in meteorites that have experienced more extensive aqueous alteration such as the CM2 ALH 83100 and the CI1 Orgueil, while the relative abundance of AIB in these meteorites is lower than in the other less altered CM2 meteorites. However, the relative abundance ratios in these meteorites should be interpreted with caution since these amino acid ratios were not corrected for the potential contribution of terrestrial glycine contamination, which could vary between meteorite samples. Additional studies of more CM meteorites may help reveal whether or not the relative distribution of amino acids in carbonaceous chondrites is influenced by the degree of aqueous alteration on the parent body.

It is also possible that ALH 83100 originated on a parent body that was chemically distinct from the other CM2 meteorites. The absolute and relative abundances of the α-dialkyl amino acids AIB and isovaline are lower in ALH 83100 than in other CM2 meteorites including Murchison, LEW 90500, and Y-791198 (Fig. 3). If Strecker-cyanohydrin synthesis was the predominant pathway for the formation of α-amino acids on these CM2 meteorite parent bodies (Peltzer et al. 1984; Ehrenfreund et al. 2001), then the parent body of ALH 83100 may have been depleted in acetone and 2-butanone required for the formation of AIB and isovaline by the Strecker pathway. However, alternative sources for amino acids in carbonaceous meteorites have also been proposed (Bernstein et al. 2002). For example, β-amino acids cannot be produced by the Strecker-cyanohydrin pathway, but must be formed by a different synthetic pathway than α-amino acids. It has previously been suggested that the synthesis of β-alanine in carbonaceous meteorites could proceed by Michael addition of ammonia to cyanoacetylene, followed by hydrolysis (Miller 1957; Cronin and Chang 1993; Ehrenfreund et al. 2001).

Table 3. Amino acid enantiomeric ratios in the CM2 carbonaceous meteorites.

Amino acid	Enantiomeric ratio (D/L)[a]		
	Murchison	LEW 90500	ALH 83100
Aspartic acid	0.91	0.84	0.67
Glutamic acid	0.96	1.00	0.91
Alanine	0.95	0.97	0.82
Valine	0.48	0.47	b.d.[c]
β-amino-*n*-butyric acid[b]	0.91	0.90	1.00
Isovaline[b]	n.d.[d]	n.d.[d]	b.d.[c]

[a]D/L ratios calculated from the total concentrations reported in Table 2. The uncertainties ranged from ± 0.05 to ± 0.2 and are based on the absolute errors shown in Table 2.
[b]Nonprotein amino acid.
[c]b.d. = below detection.
[d]n.d. = not determined.

Identification of Compound X in ALH 83100

The most striking difference between ALH 83100 and the other CM2s was the large unidentified compound (labeled peak 'X' in Fig. 2) present in the acid hydrolyzed hot water extract of ALH 83100 at a much higher concentration (8480 ppb) than detected in the Murchison (268 ppb) and LEW 90500 (386 ppb) meteorite extracts (Table 2). Peak X was only detected at a very low concentration (146 ppb) in the water extract of ALH 83100 prior to acid hydrolysis, which indicated that compound X was present in predominantly bound or peptide form. In a previous investigation of the Antarctic martian meteorites ALH 84001 and MIL 03346, an unidentified compound with a similar retention time as peak X was also observed in the acid hydrolyzed water extracts, however the mass of the peak could not determined under the analytical conditions used (Bada et al. 1998; Glavin et al. 2005).

Since the abundance of peak X in the ALH 83100 extract was much higher than in previous analyses of Antarctic meteorites, we were able to measure the mass of the OPA/NAC derivative of the unknown compound ($M_x + H^+ = 393.148$) by LC-ToF-MS (Figs. 4 and 6). Although the retention time of peak X is identical to L-valine under our chromatographic conditions, peak X cannot be L-valine, which has a different mass $M_{val} + H^+ = 379.133$ (Figs. 1 and 2). The mass of peak X was identical to leucine, isoleucine, and the norleucine internal standard that are routinely tested, however the retention time of peak X was substantially different from these amino acid standards (Fig. 4). Since OPA/NAC only reacts with primary amines to form a fluorescent derivative, compound X must contain a primary amine group with a molecular weight identical to our norleucine internal standard. We determined that the area of

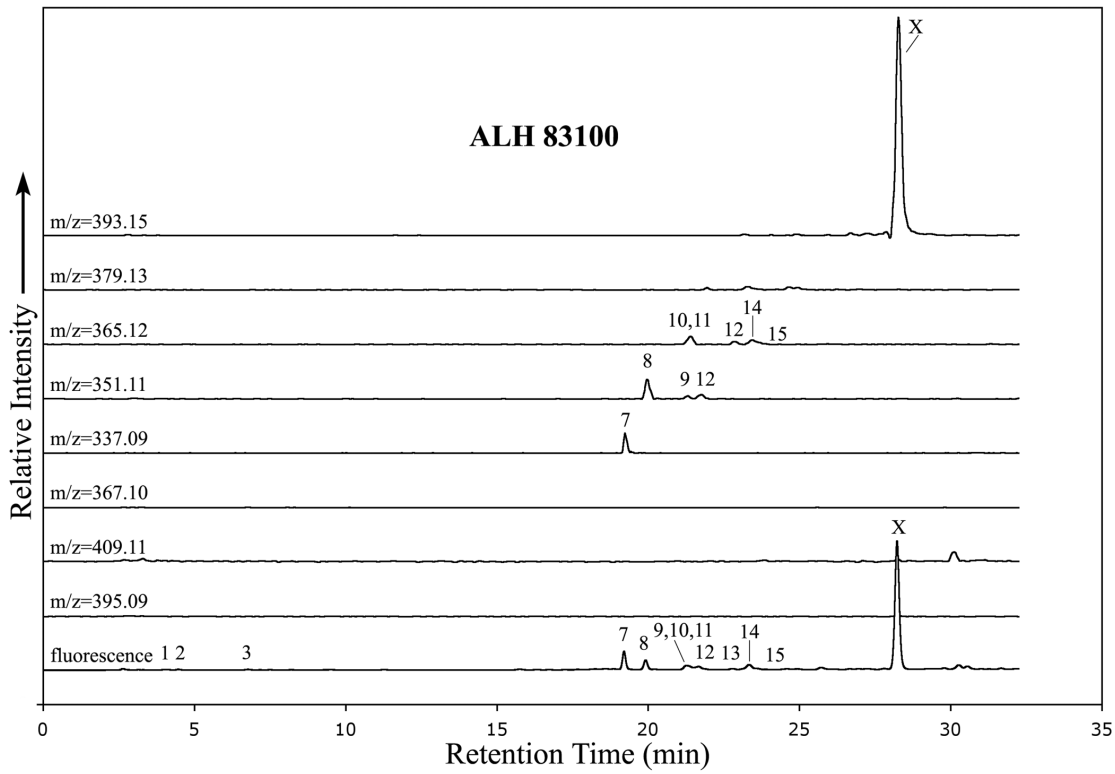

Fig. 4. The 0–32 min region of the HPLC-FD and ToF-MS chromatograms of the ALH 83100 meteorite acid hydrolyzed water extract. The peaks were identified by comparison of the retention time and exact molecular mass to those in the amino acid standard run on the same day. Aspartic and glutamic acids (peaks 1–3) could not be identified by exact mass due to a low abundance and poor ionization of these compounds during the analysis.

peak X was lower after 15 min versus 1 min OPA/NAC reaction times. This indicates that compound X, unlike AIB and isovaline, does not contain two alkyl groups in the α-position to the amine (Zhao and Bada 1995). Furthermore, we have observed that the same decrease in peak area after 15 min OPA/NAC derivatization compared to a 1 min derivatization that we see with peak X will occur with amino acids when a CH_2 group is in the α-position to the amino group (e.g., glycine, β-alanine, γ-ABA). This decrease in peak intensity with OPA/NAC derivatization time has previously been observed with amino acids containing a $-CH_2-NH_2$ moiety and is due to the reaction of the initially formed OPA/NAC derivative with one additional OPA molecule (Mengerink et al. 2002; Hanczkó et al. 2004). Among the most plausible compounds for peak X was the straight chained C_6 amino acid, 6-aminohexanoic acid or ε-amino-*n*-caproic acid (EACA). We determined that the retention time of EACA was consistent with compound X, and we verified that it is compound X by co-injection of the ALH 83100 extract with an equal concentration of authentic EACA.

Analysis of the La Paz Antarctic Ice Sample

LC-ToF-MS analysis of the La Paz Antarctic ice sample revealed trace levels of aspartic acid, serine, glycine, β-alanine, and alanine above procedural blank levels with free concentrations ranging from 0.2 to ~10 parts per trillion (ppt) in the unfiltered ice extract (Table 2). These levels are a factor of ~10^4 lower than those observed in the Antarctic meteorite samples (Table 2). Moreover, several amino acids that were detected in the Antarctic CM2 meteorites LEW 90500 and ALH 83100 including glutamic acid, α-ABA, β-ABA, γ-ABA, isovaline, and valine, were not detected in the ice above the 0.1 ppt level. A similar distribution of amino acids was also detected in a previous HPLC-FD analysis of ice collected from the Allan Hills region of Antarctica (Bada et al. 1998). Therefore, it is highly unlikely that the Antarctic ice is the source of the majority of amino acids detected in the Antarctic CM2 meteorites ALH 83100 and LEW 90500.

Perhaps one of the most intriguing findings in the La Paz 16704 Antarctic ice sample is the relatively large amount of AIB (46 ppt) present in the extract (Table 2). The identification of AIB in the ice sample was confirmed using HPLC-FD and LC-ToF-MS by comparison of the fluorescence retention time and exact mass to an authentic AIB standard. Moreover, a large increase of the peak in the La Paz ice sample was observed after a 15 min OPA/NAC derivatization of the ice extract as compared to a 1 min derivatization. This provided additional evidence for the

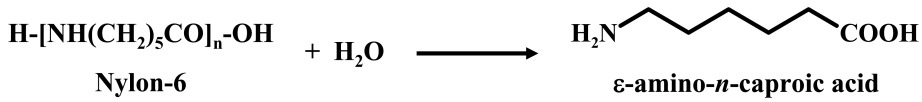

Fig. 5. Nylon-6 is a polymer (peptide) of ε-amino-*n*-caproic acid (EACA) which readily hydrolyzes to the monomer.

presence of AIB, since the reaction of the α-dialkyl amino acid AIB with the OPA/NAC reagent requires longer to reach completion (Zhao and Bada 1995). AIB was not detected in a sample of Antarctic ice from the Allan Hills region above the 2 ppt level (Bada et al. 1998), which suggests that the distribution of AIB in Antarctic ice is heterogeneous.

Since AIB has now been identified in several Antarctic CM2 chondrites (Cronin et al. 1979a; Kotra et al. 1979; Shimoyama et al. 1985; Botta and Bada 2002a; this study) and also in Antarctic carbonaceous micrometeorites (Brinton et al. 1998; Matrajt et al. 2004), it is possible that AIB may have leached from carbonaceous meteorites present in the ice (Kminek et al. 2001). However, there were no carbonaceous meteorites found in the vicinity of the La Paz ice sample 16704 at the time of collection. Although the source of the AIB in the La Paz ice could not be determined from this study, one possible explanation could be Antarctic micrometeorites (AMMs) present in the ice. It has been well established that micrometeorites represent the main source of extraterrestrial material accreted by the Earth each year (Chyba and Sagan 1992; Love and Brownlee 1993). Although most AMMs do not contain AIB (Glavin et al. 2004), even a small amount of AIB derived from micrometeorites present in the ice, would have been concentrated during the melting and roto-evaporation processing procedures. The La Paz 16704 ice water extract was not filtered to isolate micrometeorites prior to LC-ToF-MS analysis, so this hypothesis cannot be confirmed.

Nylon Contamination of the Antarctic Meteorite and Ice Samples

The most abundant amino acid present in the La Paz 16704 ice water extract was EACA. With an EACA concentration of 886 ppt, this amino acid accounted for 93% of the total mass of identified free amino acids present in the ice (Table 2). Since this ice sample was sealed in a nylon bag at the time of collection, it is likely that the EACA detected in the ice sample is derived from the nylon material.

To test this hypothesis, a nylon bag of the type used to store Antarctic meteorite samples was carried through the meteorite extraction procedure. We found that EACA was the most abundant water extractable amino acid constituent of the nylon storage bag with a total concentration of ~23,000 parts per million (ppm) after hot water (100 °C) extraction and acid hydrolysis. A much lower concentration of the free EACA monomer was detected by LC-ToF-MS in the unhydrolyzed fraction (~13 ppm), which indicates that the dominant water extractable form of EACA is a peptide of Nylon-6. Using LC-ToF-MS we were able to identify peptides in the unhydrolyzed nylon water extract up to the EACA heptamer $M + 2H^+ = 536.330$ (data not shown). A high yield of EACA was also obtained from water extracts of the nylon bag kept at room temperature. These results are not surprising since Nylon-6 is a peptide of EACA (Fig. 5). Acid hydrolysis of Nylon-6 will therefore yield large quantities of free EACA. Only trace levels of other amino acids including glycine, β-alanine, γ-ABA, and alanine were detected in the nylon bag acid-hydrolyzed water extract (~0.1–0.3 ppm).

The identification of EACA by retention time and exact mass in the ALH 83100 meteorite, the Antarctic ice and nylon bag extracts using LC-ToF-MS (Figs. 4 and 6), demonstrates that this compound is a potential amino acid contaminant for all Antarctic meteorite samples or ice collected using nylon storage bags. Given the extremely high water extractable concentration of EACA in Nylon-6, and the fact that Antarctic meteorites are sometimes covered with ice or snow when sealed inside the nylon storage bags in the field, it is not surprising that a high concentration of EACA (8480 ppb) was detected in ALH 83100. Assuming EACA was derived entirely from nylon, nylon contamination in ALH 83100 accounts for roughly 85% of the total abundance of amino acids in the meteorite (Table 2).

In contrast to ALH 83100, a much lower concentration of the EACA was detected in LEW 90500 (386 ppb) which indicates that the extent of EACA contamination from nylon in Antarctic meteorites may be highly variable. It is possible that the LEW 90500 meteorite sample contained only limited exterior snow or ice when collected in Antarctica or the interior fragment of LEW 90500 that we analyzed was less susceptible to nylon contamination than exterior pieces. The Murchison meteorite sample, which was stored in low density polyethylene (LDPE) bags at the Smithsonian National Museum of Natural History (L. Welzenbach, personal communication), contained the lowest abundance of EACA (268 ppb) of the meteorite samples analyzed (Table 2). In addition, the ratios of free to total EACA in Murchison (1.0) and LEW 90500 (0.6) are much higher compared to ALH 83100 (0.02), providing additional evidence that most of the EACA in Murchison and LEW 90500 is not derived from Nylon-6, but is instead indigenous to these meteorites as has been found for other C_6 amino acids in Murchison (Cronin et al. 1981). It is also worth noting that only very low levels (~210 ppb) of predominantly free EACA (free/total = 0.8) were reported in the Y-91198 Antarctic meteorite sample that was collected and stored in a Teflon bag (Shimoyama and Ogasawara 2002).

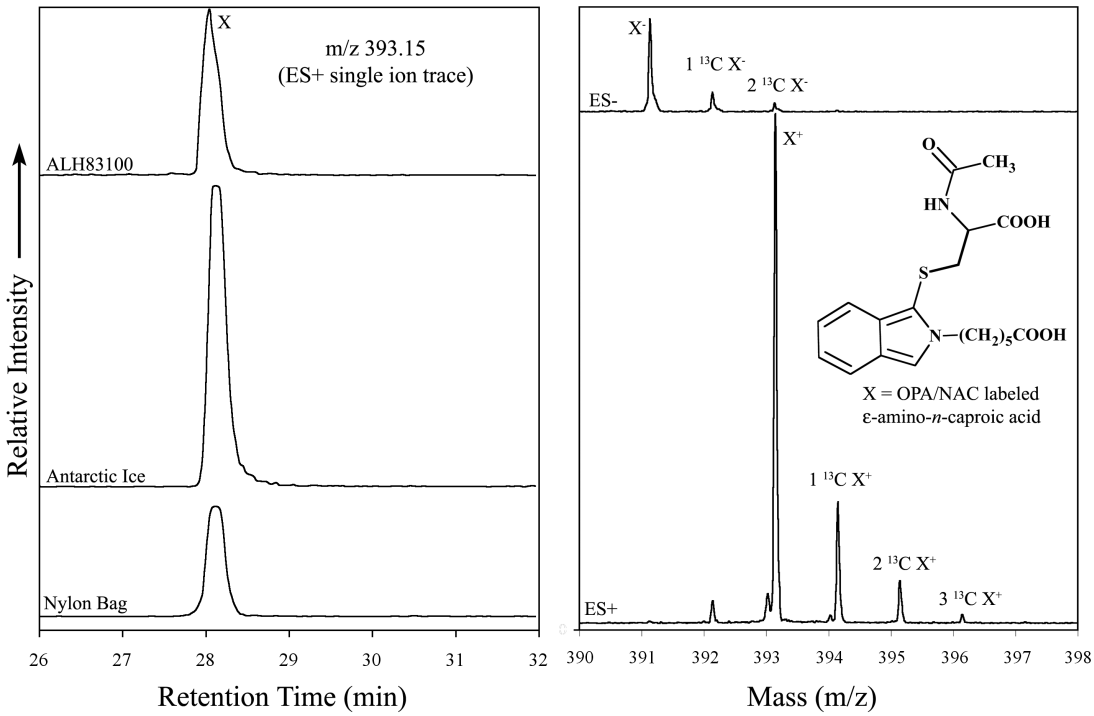

Fig. 6. a) Single ion LC-ToF-MS trace at m/z 393.15 in ES+ mode of compound X in the ALH 83100 meteorite, La Paz Antarctic ice sample, and nylon bag water extracts. b) Peak X was identified as ε-amino-*n*-caproic acid (EACA) based on the exact mass and retention time of the OPA/NAC derivative in both ES+ and ES− modes. In addition, peaks corresponding to the OPA/NAC labeled EACA compound containing one, two, and three naturally occurring ^{13}C atoms were also detected in these extracts.

CONCLUSIONS

In this study we have demonstrated that liquid chromatography–time of flight–mass spectrometry (LC-ToF-MS) can be a useful tool for the identification of amino acids in meteorite and ice samples. LC-ToF-MS coupled with HPLC-FD fluorescence detection is a very powerful combination with a detection limit for amino acids that is at least three orders of magnitude lower than traditional gas chromatography-mass spectrometry (GC-MS) techniques. Using this new analytical technique, we were able to identify a total of 20 different amino acids and their enantiomers in the Antarctic CM2 meteorites ALH 83100 and LEW 90500, as well as the non-Antarctic CM2 meteorite Murchison. Many more amino acids were observed in Murchison and LEW 90500 but have not yet been identified using this technique. The high D/L amino acid ratios in these samples as well as the presence of a variety of structural isomers for C_3 to C_5 amino acids suggest that most of the amino acids are indigenous to these meteorites. ALH 83100 was depleted in amino acids with a strikingly different amino acid distribution compared to the CM2 meteorites LEW 90500 and Murchison. We cannot rule out the possibility that some amino acids were leached from the ALH 83100 meteorite during its residence time in the Antarctic ice. The unique distribution of amino acids in ALH 83100 may indicate that this meteorite originated from a chemically distinct parent body from the CM2 meteorites Murchison and LEW 90500 and/or the ALH 83100 parent body was depleted in amino acid precursor material due to a higher degree of aqueous alteration.

One amino acid that has previously been detected in the Antarctic Martian meteorites ALH 84001 and MIL 03346 but has eluded identification, was also detected in ALH 83100 and determined by LC-ToF-MS to be ε-amino-*n*-caproic acid (EACA). EACA is a likely amino acid contaminant derived from the nylon bag used to store the Antarctic meteorite samples. Fortunately Nylon-6 leaches only trace levels of other amino acids, however future meteorite collection efforts in Antarctica should consider other types of sterile sample storage bags such as Teflon or LDPE as an alternative to Nylon-6. Finally, due to the coelution of EACA with L-valine under most HPLC separation schemes, researchers should be careful to avoid misidentification using this technique.

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