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**Vertical distribution of amino acids and chiral ratios in
deep-sea hydrothermal sub-vents of the Suiyo Seamount,
Izu-Bonin Arc, Pacific Ocean**

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Abstract

A high-temperature deep-sea hydrothermal system related to dacitic arc-volcanism has been drilled using a tethered, submarine rock-drill system as a part of the Archaean Park Project. The benthic multi-coring system (BMS) employed allows for direct sampling of microorganisms, rocks and fluids beneath hydrothermal vents. The samples examined in this study are from sites APSK 08, 09 and 10 on the Suiyo seamount of the Izu-Bonin Arc in the Pacific Ocean. Based on the vertical distribution and stereochemistry of amino acids in this vigorous sub-vent environment, a model of deep-sea subterranean chemistry and biology is proposed, describing optimal microbial activities rather than abiotically synthesized amino acids components. Total hydrolyzed amino acids in the hydrothermal sub-vent core samples are on the order of 10^1 – 10^2 nmol/g-rock. The ratios of β -alanine/aspartic acid and γ -aminobutyric acid/glutamic acid are low, consistent with a large microbial population and fresh subterranean bioorganic compounds. The D/L ratios of chiral amino acids such as aspartic acid, glutamic acid and alanine in these rock samples were also quite low overall. Large enantiomeric excesses of L-form amino acids also support the existence of a vigorous subjacent microbial oasis in this seamount hydrothermal sub-system. The present findings represent crucial evidence that sub-vent regions are a previously unknown extreme-environment biosphere, extending the known subterranean habitable spaces.

Keywords: deep-sea hydrothermal sub-vent, amino acids, stereo chemistry, subterranean biosphere

Introduction

Deep-sea hydrothermal systems are natural laboratories for the study of organic geochemistry regarding microbial habitats on extreme environments. The historical discovery of the Galapagos deep-sea hydrothermal systems (Corliss et al., 1979) has lead many researchers to consider that deep-sea hydrothermal systems are suitable environments for chemical evolution, with possible implications for the origins of life on Earth (e.g. Holm, 1992). The extremophilic phenomena of these environments have attracted interest from many scientific viewpoints, including geology, oceanography, biology, chemistry and physics (e.g. Holm, 1992). Anomalous concentrations of glycine in the Red Sea may also support the notion of deep-sea chemical evolution and biogeochemistry (Ingmanson et al., 1980). In fact, a number of submarine ecological colonies have been recognized near black or clear smokers and the associated organic-rich seafloor mats (Gold, 1992; Craggs and Parkes, 1994).

The biological environment of extreme ocean-floor vents can be well characterized by bioorganic compounds, particularly amino acids, which are common components of all organisms and constitute a major fraction of organics (Henrichs and Farrington, 1979). There have been several reports dealing with amino acid distributions in surface sediments of coastal regions (Shimoyama and Ponnampereuma, 1975; Burdige and Martens, 1988, 1990; Cowie and Hedges, 1994; Henrichs and Farrington, 1987; Burdige, 2001), of deep-sea sediment (Henrichs et al., 1984; Horsfall and Wolff, 1997; Rittenberg et al., 1963; Whelan, 1977), and in deep-sea sediment cores (Aizenshtat et al., 1973; Bada and Man, 1980). However, the amino acid distributions in deep-sea sediments from areas of hydrothermal activity have been studied at only a few locations. For example, Haberstroh and Karl (1989) investigated the total dissolved free amino acid concentrations in high-temperature smoker fluids and in interstitial waters of sediments recovered from a sediment-covered hydrothermal vent system located in the southern Guaymas Basin, Gulf of California; Silfer et al. (1990) isolated the water- and acid-extractable amino acid fractions of sediments affected by hydrothermal processes in the Bransfield Strait, Antarctic Peninsula; and Kawahata and Ishizuka (1989) determined the vertical distribution of amino acids in interstitial waters of ridge-flank sediments from the Galapagos spreading center.

Sub-vents, subjacent to seafloor hydrothermal vents, are of significant interest as a

new scientific frontier (Urabe *et al.*, 2001). While the existence of a deep bacterial
80 biosphere in oceanic sediment has been reported (e.g. Parkes *et al.*, 1994; Fredrickson
and Fletcher, 2001), the hydrothermal sub-vent world has yet to be described. The
purpose of this study is to investigate the vertical distribution and stereochemistry of
amino acids in deep-sea sediments affected by low- to high-temperature hydrothermal
activity. The samples analyzed in this study are deep-sea sediments recovered from
85 the subjacent hydrothermal system of the Suiyo seamount, Izu-Bonin Arc, Pacific
Ocean.

Amino acids associated with life, particularly those in proteins, exist as L-form only,
that is, chiral. The process of amino acid racemization in various geochemical samples
from terrestrial (e.g. Takano *et al.*, 2003b) and marine environments (e.g. Harada and
90 Handa, 1995) has been widely applied in geochemical research. Racemization of
amino acids is primarily dependent on the age and temperature of the environment
(Bada and Schroeder, 1975). As racemization in hydrothermal environments is
expected to progress via kinetic control based on environmental temperature (Bada,
1972), the D/L ratios of samples can be used as indicators of the extent of organic
95 matter alteration and the coincidence of subterranean microbial activity.

In the present study, the D-form and L-form contents of aspartic acid (Asp),
glutamic acid (Glu), and alanine (Ala) together with absolute and relative concentrations
of 17 hydrolyzed amino acids were determined in core samples from a deep-sea
hydrothermal sub-vent on the Suiyo seamount. This report describes the vertical
100 distribution and stereochemistry of amino acids under these extreme conditions, leading
to a model of deep-sea subterranean chemistry and biology. The chemical aspects of
abiotic synthesis of amino acids (e.g. Yanagawa and Kobayashi, 1988; Islam *et al.*,
2001) and their oligomerization processes (e.g. Imai *et al.*, 1999; Islam *et al.*, 2003)
have been investigated using simulated submarine hydrothermal systems, and the
105 stereochemistry of amino acids is of notable interest in the process of chemical
evolution (Takano *et al.*, 2001; Takano *et al.*, 2002). Biologically, however, it is
considered that hyperthermophiles (e.g. Blochl *et al.*, 1997) or barophiles with affinity
for high pressure (e.g. Prier, 1992) are likely to be widespread in regions immediately
below hydrothermal vents.

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Geological location and hydrothermal fluid discharge

The Izu-Bonin Arc lies on the eastern rim of the Philippine Sea plate. This arc is about 1,200 km long, extending from the Izu Peninsula (35 °N, 139 °E) to Minami-Iwojima Island (24 °N, 141 °E). This arc belongs to the circum-Pacific island-arc system and is adjacent to the Northeast Japan Arc to the north and the Mariana Arc to the south. Many volcanic islands and submarine volcanoes run parallel to the Izu-Bonin trench and form the volcanic front of this intra-oceanic island-arc system. The southern Izu-Bonin Arc, which is divided by the Sofugan tectonic line from the northern Izu-Bonin Arc (Tsunogai *et al.*, 1994) (Fig. 1-a), is thought to have become active at around 42 Ma (Yuasa, 1992). The Shichiyo seamount chain forms a volcanic front (Fig. 1-b) around which the arc crust is inferred to become thinner than that in the northern part (Hino, 1991). The Suiyo Seamount, one of the volcanoes in the Shichiyo chain, has two major peaks, located on the eastern and western sides of the seamount. The Suiyo Seamount is an active submarine volcano, and vigorous hydrothermal activity has occurred on the caldera floor atop the west peak (Watanabe and Kajimura, 1994; Yuasa *et al.*, 1991). Dacitic rocks of a calc-alkaline rock series and low-potassium andesites have been recovered from this area (Watanabe and Kajimura, 1993), and seafloor hydrothermal alteration at Suiyo has been preliminarily reported from the point of view of geochemical and mineralogical characteristics (Marumo *et al.*, 2002; Marumo *et al.*, 2003; Marumo *et al.*, 2004).

As to terrestrial origin of organics on Suiyo seamount, it was reported that total fatty acid compositions in surface sediments obtained from Suiyo hydrothermal system, Izu-Bonin Arc were not significant components of sedimentary organic matter (Yamanaka *et al.*, 2001; Yamanaka and Sakata, 2004). Analytical results of the surface sediments indicated very low contributions of terrestrial sediments. In addition, age determination of unaltered dacite by Ar-Ar method yields $9,000 \pm 8,000$ yrBP, suggesting zero age (Marumo *et al.*, 2003; Marumo *et al.*, 2004). The caldera floor is predominantly covered with sandy sediment and hydrothermal precipitations and lacks any evidence of muddy pelagic sediment.

The vertical variations in the mineral assemblages of these cores are presented in Figure 2. The core profile is characterized by dacitic lava and/or pyroclastic rocks at

the surface underlying unconsolidated volcanic sands and pumice fragments; a sheath of clay minerals and anhydrite cement with minor pyrite and other sulfide minerals that acts as a cap rock of the geothermal system; and end-member fluid ponding beneath the sheath. Extensive hydrothermal alteration is observed in the sedimentary unit and the upper fraction of the volcanic rocks. Numerous short black smokers and clear smokers were observed on the sandy floor. Hydrothermal circulation reaches the region adjacent to the magma source, and volatile constituents are extracted by water-rock interaction (Urabe *et al.*, 2001; Urabe *et al.*, 2003).

Experimental

Sampling

The deep-sea hydrothermal sub-vent core samples were collected as part of the Archaean Park Project in a cruise over the Suiyo Seamount (28° 33 'N, 140° 39 'E) in the Pacific Ocean in June, 2002. The deep-sea subterranean biosphere and geochemical interaction were examined by taking core samples using a fixed seafloor benthic multi-coring system (BMS) for pinpoint drilling in the caldera (Urabe *et al.*, 2001). The maximum depth of coring at sites APSK 08, 09 and 10 were 3.807 m, 7.035 m and 8.993 m below the sea floor, respectively. The hydrothermal fluid temperature measured using a custer-type thermometer (Ikeuchi *et al.*, 1998) was 69.2 °C at APSK 08, and 89.3 °C at APSK 10. The fluid temperature at APSK 09 could not be measured.

Extraction of amino acids

Rock core samples were carefully obtained and quickly sealed with a pack of dehydrating and deoxygenation reagent (AGELESS, Mitsubishi Gas Chemicals Co.). The sample was powdered and freeze-dried. Then, ca. 1.00 g of the sample was placed in a Teflon tube that had been cleaned by soaking it in 7 M HNO₃ overnight and rinsed in Milli-Q-water (Millipore Corp.). 10 ml of 5 M HF-0.1 M HCl mixture was poured in the Teflon tube, which was placed in a metal holder. It was continuously heated at 110 °C for 16 hours in order to release organics from the silicate matrix. After HF-HCl degradation, Teflon tubes were placed on a hot plate in a draft chamber to evaporate acids. The organic residues were extracted with pure water with

ultra-sonication. The aqueous fraction was filtered with a GF/A 1.6 μm glass filter, and then freeze-dried in a glass test tube. 2 ml of 6 M HCl was added to each of the test tubes to obtain total hydrolyzed amino acid fraction (THAA). The test tube was sealed and placed in a block heater and heated for 2 hours at 110 $^{\circ}\text{C}$. The hydrolysates were then dried *in vacuo* using a diaphragm pump. After dryness, the portions were adjusted to pH 1 with 0.1 M HCl, followed by desalting with a AG-50W-X8 (200-400 mesh) cation exchange resin column (Bio-Rad Lab.). Before application of the sample to the column, the resin had been cleaned by passing 1 M HCl, H_2O , 1 M NaOH and H_2O successively through the column. Just before applying the sample the resin was reactivated with 10 ml of 1 M HCl and rinsed with 10 ml of H_2O . The amino acid fraction was eluted with 10 ml of 10 % NH_3 . The eluate was freeze-dried and redissolved in 1.0 ml of 0.1 M HCl before injection into a liquid chromatographic system.

All reagents used were ultra-pure HPLC grade. Deionized water was further purified with a Millipore Milli-Q LaboSystemTM and a Millipore Simpli Lab-UVTM (Japan Millipore Ltd., Tokyo, Japan) to remove both inorganic ions and organic contaminants. All glassware was heated for 2 hours in high temperature oven (Yamato DR-22) at 500 $^{\circ}\text{C}$ in prior to use in order to eliminate any possible organic contaminants.

Determination of hydrolyzed amino acids

The concentration of THAA was determined with an ion-exchange HPLC system, which was composed of two high performance liquid chromatograph pumps (Shimadzu LC-6A), a cation exchange column (Shimpack ISC-07/S1504, 4 mm i.d. $\times$ 150 mm), a post column derivatization system with *o*-phthalaldehyde (OPA) and N-acetyl-L-cysteine (N-AcCys), and a Shimadzu RF-535 fluorometric detector (excited wavelength: 355 nm and emission wavelength: 435 nm) (Kobayashi *et al.*, 1991). The temperature of the column was maintained at 55 $^{\circ}\text{C}$. Gradient elution was performed by using the following eluents: A: 0.07 M sodium citrate perchloric acid (pH 3.20) with 7 % ethanol, B: 0.2 M sodium citrate boric acid-NaOH (pH 10). The flow rate of the carrier was 0.3 ml/min. The amino acid standards, OPA and N-AcCys were obtained

205 from WAKO Chemical Co.

Separation of D- and L- enantiomer

The determination of D- and L-amino acid enantiomers was achieved with an RP-HPLC system, which was composed of high performance liquid chromatograph pumps (Tosoh CCPM II), a reversed phase column (YMC-pack Pro C18 4.6 mm i.d. × 250 mm), a precolumn derivatization system with OPA and N-AcCys, and a Tosoh FS 8020 detector (Takano *et al.*, 2003a; Takano *et al.*, 2003b). An aliquot of desalted and redissolved amino acid extract was mixed in a glass vial with OPA and N-AcCys. Then the derivatives were extracted by solid phase extraction with a TOYOPACK ODS 215 column to eliminate hydrophobic impurities. The extract was injected to the RP-HPLC system to separate amino acid enantiomers. Gradient elution was applied using the following eluents; A: 40 mM sodium acetic acid buffer (pH 6.5), B: 100% methanol. Gradient program was performed as follows: 10 min (Eluent B: 0 %) – 25 min (Eluent B: 10 %) – 65 min (Eluent B: 20 %) – 80 min (Eluent B: 20 %) – 85 min (Eluent B: 40 %) – 115 min (Eluent B: 60 %) – 120 min (Eluent B: 80 %) – 135 min (Eluent B: 0 %) (Kudo *et al.*, 2003).

Result and discussion

Vertical profiles of total hydrolyzed amino acids

225 Blank analysis of amino acids during laboratory handling gave trace levels of glycine. Figure 3 shows a typical chromatogram of the hydrolyzed amino acid composition of the APSK 10-1-01 sample. Seventeen kinds of protein and non-protein amino acids were quantitatively determined. The concentrations of amino acids in the sub-vent core samples at the three sites are summarized in Table 1. Glycine was the 230 most abundant amino acid on the average, followed by alanine. The concentration of glycine ranged between 2.9 and 31.5 nmol/g-rock. Other protein amino acids such as valine, aspartic acid and glutamic acid were also among the major constituents, as shown in Figure 4. The relative abundance of each amino acid did not vary significantly with depth.

235 Figure 5 shows the vertical distribution of THAA and the relative abundances of

aspartic acid and β -alanine at the three sites. The THAA content was on the order of 10^1 – 10^2 nmol/g-rock for all samples.

Methionine is known to be highly susceptible to hydrolytic loss during acid hydrolysis. Therefore, the measured concentration of methionine in the sediments may be more than the actual concentration. Histidine, an essential amino acid, could not be detected because the detection peak was obscured by the background after 49 min retention time.

Concentrations of total organic carbon (TOC) and total nitrogen (TN) in the core samples were in the order of 10^1 to 10^2 μ g C/g-rock and 10^1 to 10^3 μ g N/g-rock at another hydrothermal area, respectively (Takano *et al.*, 2004). Although the C/N ratio for bacteria-inhabited hydrothermal vent environments is not known, the present values are noticeably rich in nitrogen. Fourier transform infrared (FT-IR) analysis of clay minerals in the core samples suggests that ammonium ions are present at interlayer sites (Marumo *et al.*, 2002). Ammonia is present in hydrothermal fluid at concentrations from 10 to 20 μ M and is an important source of nitrogen nutrition for the sub-vent ecosystem (Umeki *et al.*, 2002). This trend is similar to the distribution of total amino acids, suggesting that the organic carbon and amino acids are derived from in situ organisms, viable and/or nonviable (Takano *et al.*, 2004).

Alteration of dicarboxylic amino acids to ω -amino acids

Non-protein amino acids such as β -alanine, γ -aminobutyric acid and δ -aminovaleric acid were also detected as minor constituents in the core samples. Hydrothermal alteration may cause decomposition of protein amino acid *via* decarboxylation at the α -carbon (Ratcriff *et al.*, 1974), for example, aspartic acid and glutamic acid will alter to β -alanine and γ -aminobutyric acid. Although the coring sites were located in vigorous hydrothermal systems, the biogeochemical markers β -alanine and γ -aminobutyric acid were detected as minor constituents. The ratio of β -alanine to aspartic acid, β -ala/Asp, varied from 0.03 to 1.10 (mean 0.21), and the ratio of γ -aminobutyric acid to glutamic acid, γ -ABA/Glu, varied from 0.02 to 3.98 (mean 0.37). The presence of β -alanine and γ -aminobutyric acid as even minor constituents is considered evidence of the existence of bioorganic compounds derived from deep-sea

sub-vent microbial activity. An increase in the abundance of β -alanine and γ -aminobutyric acid with depth have been found in marine sediments from the Vema Fracture Zone at the Mid-Atlantic Ridge (Aizenshtat *et al.*, 1973), in marine clays from the Pacific Ocean (Schroeder and Bada, 1976), and in recent research conducted within the International Ocean Drilling Program (ODP) for sediment cores Leg 139 and 168 (Andersson *et al.*, 2000). As β -alanine and γ -aminobutyric acid comprise only a minor percentage of the total amino acid content of living organisms, it has been proposed that the decomposition of aspartic acid and glutamic acid, respectively, are the most likely source of β -alanine and γ -aminobutyric acid in marine sediments (Cowie and Hedges, 1992, 1994; Schroeder, 1975).

It is well known that organic matter including hydrolyzed amino acids becomes scarcer with depth in simple sedimentation in marine sea-floor environments (e.g. Andersson *et al.*, 2000) and terrestrial environment (Takano *et al.*, 2003b) by diagenetic alteration. In a previous study (Takano *et al.*, 2003b), total organic carbon (TOC), THAA, and the microbial cell density in terrestrial core samples were found to be greatest at the surface and to decrease rapidly with depth, with correlation coefficients (r) for TOC and THAA versus microbial cell density of 0.97 and 0.98, respectively. As seen in Figure 5, however, variable concentrations of amino acids were observed in some middle parts of the core samples. In fact, the highest concentration of amino acids was observed not at the surface but in the sub-seafloor of unconsolidated volcanic sands and pumice fragments. This may be due to fluids that migrate upward from deeper levels in the sub-vent, supplying energy and organic compounds. The movement of hydrothermal fluid may form veins toward the seafloor and/or black smoker chimneys.

Possibility of abiotic formation of amino acids

Amino acids in extreme hydrothermal environments may have been formed abiotically. Since the pioneering experiment of Miller (1953) established on the idea of chemical evolution, numerous laboratory experiments concerning the formation of biologically interesting organic compounds have been performed. Among these, amino acids were detected in the product of the Strecker-type reactions under

supercritical hydrothermal water flow conditions simulating submarine hydrothermal systems (Islam *et al.*, 2001). Oligomerization in a high-pressure and high-temperature flow reactor (Imai *et al.*, 1999; Alargov *et al.*, 2001; Islam *et al.*, 2003) have also been reported. Among the products of the Strecker-Type reaction, peculiar ω -amino acids such as β -alanine, γ -aminobutyric acid, and δ -aminovaleic acid were detected under high-temperature and high-pressure flow conditions (Islam *et al.*, 2001). It has thus been convincingly demonstrated that ω -amino acid analogs may be abiotic markers under hydrothermal conditions (Islam *et al.*, 2003).

Amino acids and microbial activities

The lack of evidence of abiotically synthesized amino acids of ω -amino acid specimens and the abiotic tendency of products support the existence of a vigorous subjacent microbial oasis, which extends the known terrestrial habitable zone (Takano *et al.*, 2004). Since the discovery of hyperthermophilic microbial activity in hydrothermal fluids recovered from smoker vents on the East Pacific Rise, a number of anaerobic sulfur-dependent heterotrophic hyperthermophiles have been isolated from high-temperature and high-pressure environments such as terrestrial hot springs and submarine hydrothermal systems. Most of the marine forms belong to the domain *Archaea*, with the exception of the genus *Thermotoga* (Jannasch *et al.*, 1988). Heterotrophic organisms utilize a wide variety of organic compounds including carbohydrates, amino acids, organic acids, and alcohols as sole carbon and energy sources. Isolated hyperthermophilic heterotrophic archaea and the requisite amino acid assemblages have been characterized experimentally (Hoaki *et al.*, 1994). Among these, the genus *Thermococcus* has been grown on protein mixtures, casamino acids (amino acid components hydrolyzed by casein), and purified proteins (e.g. casein and gelatin), but not on carbohydrates or organic acids (Hoaki *et al.*, 1994). Most of the S_0 -dependent hyperthermophilic heterotrophs isolated from marine hydrothermal vents require a complex proteinaceous substrate (e.g. Jannasch *et al.*, 1988; Jannasch *et al.*, 1992). Thus, microbacterial communities in hydrothermal areas favor not only free amino acid analogs but also combined proteinaceous substrate. This may indicate the consumption and reuse of the biological source of amino acids by subterranean

microorganisms to form a habitable zone. As noted earlier, the correlation coefficients
330 (r) for TOC and THAA versus microbial cell density are 0.97 and 0.98, respectively
(Takano *et al.*, 2003b). Therefore, the concentration of amino acids may be closely
related to the distribution of deep-sea subterranean microbial activity.

Stereochemistry of chiral amino acids

335 Amino acids may be synthesized by hydrothermal abiotic processes based on a
chemical evolution scenario (e.g. Yanagawa and Kobayashi, 1992; Islam *et al.*, 2001).
If the amino acids are formed abiotically, the D/L ratio converges to 1.0 in laboratory
experiments such as hydrothermal simulations (e.g. Yanagawa and Kobayashi, 1992) or
primitive atmospheric simulations (e.g. Takano *et al.*, 2001). However, amino acids
340 associated with life, particularly those forming proteins, are only L-form. Hence, low
D/L ratios are considered good evidence of biological activity, and a negative
correlation between microbial cell density and D/L ratio has been preliminarily reported
(Takano *et al.*, 2003b).

As shown in Table 2, the D/L ratios of aspartic acid (Asp), glutamic acid (Glu) and
345 alanine (Ala) indicate that only slight racemization from L-form to D-form occurred in
this environment. Among these three chiral amino acids, aspartic acid has the highest
rate constant of racemization during diagenesis (Takano *et al.*, 2003b). As mentioned
earlier, a monotonic decrease in L content, indicating racemization to D-form, has been
observed in simple sedimentation environments due to diagenetic alteration (e.g.
350 Andersson *et al.*, 2000; Takano *et al.*, 2003b). As shown in Table 2, however, the D/L
ratio in the present sub-vent environment is quite low on average. The highest D/L
ratio of aspartic acid at sites APSK 08, 09 and 10 was 0.11, 0.08, 0.12, respectively. The
D/L ratio of aspartic acid, glutamic acid, and alanine varied from 0.00 to 0.12 (mean
0.04), 0.00 to 0.06 (mean 0.03), and 0.00 to 0.21 (mean 0.05), respectively. In some
355 parts of the sub-vent column, the D/L ratios of glutamic acid and alanine were
predominant over aspartic acid. In the abiotic formation of amino acids, the D/L ratio of
amino acids converges to 1.0 due to the formation of a racemic mixture (e.g. Yanagawa
and Kobayashi, 1992; Takano *et al.*, 2001). The large enantiomeric excess of L-form
amino acids observed in the present study may indicate that the amino acids were
360 derived from biotic flux in the sub-vent biosphere. This implication becomes even

stronger when considering previous reports that the racemization rate constant is higher under hot geothermal conditions (Bada, 1972). The D/L ratio of amino acids in the present core samples therefore reflect microbiological activity, as well as hydrothermal water samples (Horiuchi *et al.*, 2003).

365 In addition to hydrothermal racemization, the D-amino acids may also originate from peptidoglycans in bacterial cell walls (Nyberg *et al.*, 2001). Peptidoglycans are a product of bacterial metabolism and the principal biochemical sources of D-amino acids. D-Ala and D-Glu are among the most common D-amino acids found in bacterial cell walls (Friedman, 1999). The low D/L ratio for aspartic acid, glutamic acid and alanine
370 in the vertical profile may contain two origins of D-form amino acids: i) racemized D-amino acids from protein L-form analogs, ii) minor composition, however, D-amino acids derived from microbial constituents such as bacterial cell walls.

Temperature limit and microbial habitat

375 The currently accepted thermal limit of life is 113 °C (Blochl *et al.*, 1997), and although some proteins from hyperthermophiles are more active at high pressure (Bernhardt, 1984), high pressure does not increase the thermal stability of micromolecules. Many microbiologists seem willing to speculate that the maximum may be closer to 150 °C (Deming and Baross, 1993). The recent discovery of a
380 microbe living at 121 °C has broken the established temperature limit and extended the zone of microbial habitable temperature (Kashefi and Lovley, 2003). It is interesting to note that approximately 10^4 – 10^5 cell/ml of microbes were found in 308 °C hydrothermal fluid from the drill hole in the Suiyo hydrothermal area when the drill hole was cased with metal to block the infiltration of interstitial water (Maruyama *et al.*,
385 2001). The hydrothermal gradient zone may be such that optimum fluid temperatures for microbial life occur in the sub-vent habitable regions.

Microbial diversity and populations in a hydrothermal plume that was present inside the caldera of the Suiyo Seamount were investigated by performing a phylogenetic analysis of the 16S rRNA gene and by using fluorescence in situ hybridization (FISH)
390 (Sunamura *et al.*, 2004). An indicator of turbidity, the vertical total cell count varied from 5.6×10^4 to 1.1×10^5 cells/ml. Not only the sub-vent environment but also the hydrothermal plume are habitable spaces for microbes. Thus, Suiyo Seamount caldera

has functioned as a natural continuous incubator for microbes in the deep-sea environment (Sunamura *et al.*, 2004; Nakagawa *et al.*, 2004).

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Conclusions

This report presents three significant kinds of evidence of a deep-sea hydrothermal sub-vent biosphere. The first evidence is that two sensitive biochemical indicators, aspartic acid versus β -alanine and glutamic acid versus γ -aminobutyric acid, are
400 consistent with a large microbial population. Low β -alanine and γ -aminobutyric acid contents and β -ala/Asp and γ -ABA/Glu ratios are indicative of relatively fresh organic matter and partial hydrothermal alteration. The lack of abiotically synthesized amino acids such as ω -amino acid specimens supports the biological origins of the amino acids. The difference between deep-sea biogenic sediment and sub-vent core samples was
405 clarified based on the trend of aspartic acid to β -alanine and glutamic acid to γ -aminobutyric acid.

The second line of evidence is that the D/L ratios of chiral amino acids were quite low on average, indicating a biotic origin of the detected amino acids. An unexpected vertical distribution with large enantiomeric excesses of L-form amino acids supports
410 the existence of subterranean microbial activity.

Finally, the relation between the concentration of aliphatic amino acids and their carbon number was clearly different from experiments involving the abiotic formation of amino acids. This is strong evidence that the sampled sub-vent amino acids are derived from subterranean biogenic processes. The present findings reveal that
415 hydrothermal sub-vent regions are a previously unknown extreme-environment biosphere.

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Figure and Table

Figure 1 (a) Geological location of the Izu-Bonin Arc on the eastern edge of the Philippine Sea plate, western Pacific ocean. (b) Topographic map of the Suiyo seamount in the Shichiyo seamount chain (Cited from Tsunogai *et al.*, 1994). *Si* = Suiyo Seamount; *OR* = Ogasawara Ridge; *OT* = Ogasawara Trough; *S* = Sofugan Island; *N* = Nichiyo Seamount; *G* = Getsuyo Smt.; *K* = Kayo Smt.; *M* = Mokuyo Smt.; *Kn* = Kinyo Smt.; *D* = Doyo Smt.; *Ns* = Nishinoshima Island. (c) Distribution of chimneys, mounds, and BMS drilling sites in the bottom of the caldera at Suiyo Seamount (Cited from Marumo *et al.*, 2004)

Figure 2 The vertical variations in the mineral assemblages of the core samples. Cited and modified from Marumo *et al.*, 2004.

Figure 3 Typical ion-exchanged chromatogram of hydrolyzed amino acids in deep-sea hydrothermal sub-vent core samples from APSK 10. Major constituents were protein amino acid analogs, with minor non-protein analogs. Asp = aspartic acid; Thr = Threonine; Ser = serine; Glu = glutamic acid; α -AAA = α -aminoadipic acid; Gly = glycine; Ala = alanine; α -ABA = α -aminobutyric acid; Val = valine; Met = Methionine; Ile = isoleucine; Leu = leucine; Tyr = tyrosine; Phe = phenylalanine; β -Ala = β -alanine; γ -ABA = γ -aminobutyric acid.

Figure 4 Molar ratio of hydrolyzed amino acid in deep-sea hydrothermal sub-vent core samples at sites APSK 08, 09 and 10. Each marker indicates the individual amino acid component.

Figure 5 Vertical distribution of THAA and specific biogeochemical indicators in sub-vent core samples (a) APSK 08 (b) APSK 09 and (c) APSK 10.

Table 1 Vertical concentration of amino acids in submarine hydrothermal sub-vent core samples of APSK 08 at Suiyo seamount, Izu-bonin arc, Pacific ocean. The

maximum depth of the coring was 3,807 mm below sea floor where the hydrothermal fluid temperature was measured at 62.5 °C.

675 Table 2 Vertical concentration of amino acids in submarine hydrothermal sub-vent core samples of APSK 09 at Suiyo seamount, Izu-bonin arc, Pacific ocean. The maximum depth of the coring was 8,992 mm below sea floor where the hydrothermal fluid temperature could not measured by weather condition.

680 Table 3 Vertical concentration of amino acids in submarine hydrothermal sub-vent core samples of APSK 10 at Suiyo seamount, Izu-bonin arc, Pacific ocean. The maximum depth of the coring was 7,035 mm below sea floor where the hydrothermal fluid temperature was measured at 89.3 °C.

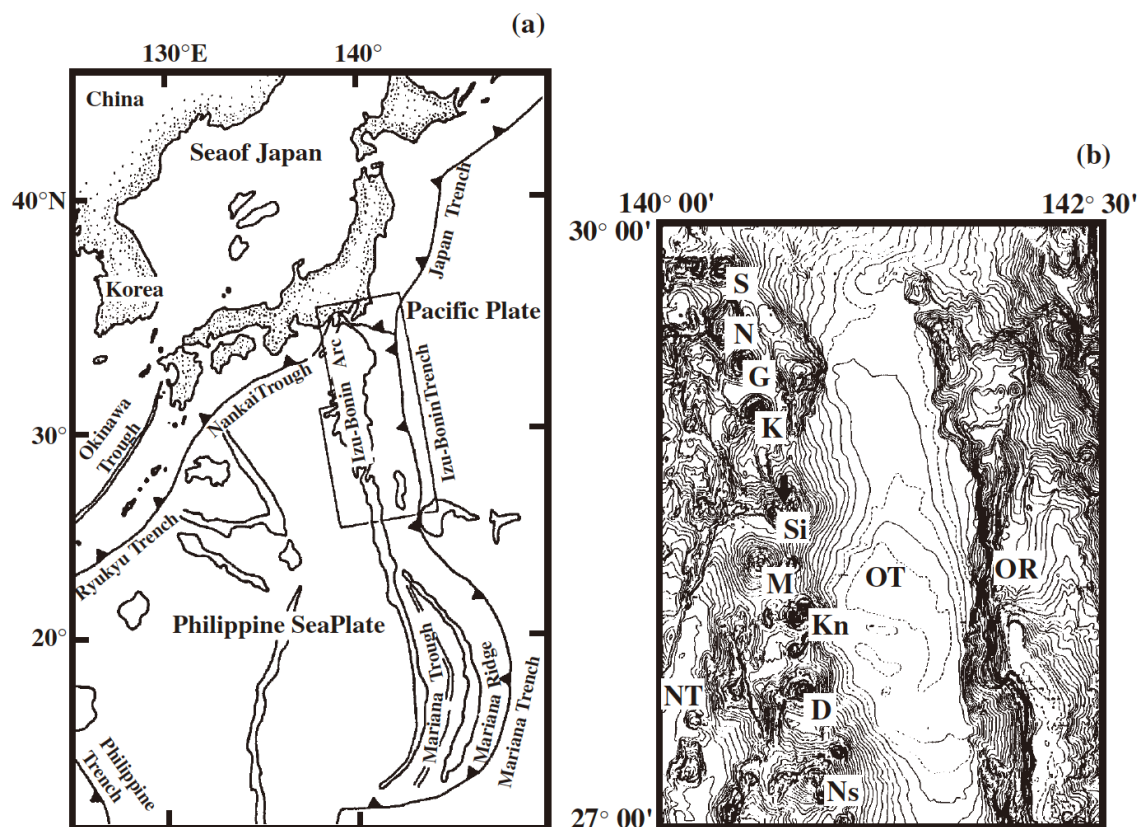
685 Table 4 D/L ratio of aspartic acid (Asp), glutamic acid (Glu) and alanine (Ala) in the site of APSK 08 at Suiyo seamount, Izu-bonin arc, Pacific ocean.

Table 5 D/L ratio of aspartic acid (Asp), glutamic acid (Glu) and alanine (Ala) in the site of APSK 09 at Suiyo seamount, Izu-bonin arc, Pacific ocean.

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Table 6 D/L ratio of aspartic acid (Asp), glutamic acid (Glu) and alanine (Ala) in the site of APSK 10 at Suiyo seamount, Izu-bonin arc, Pacific ocean.

Figure 1



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Figure 1

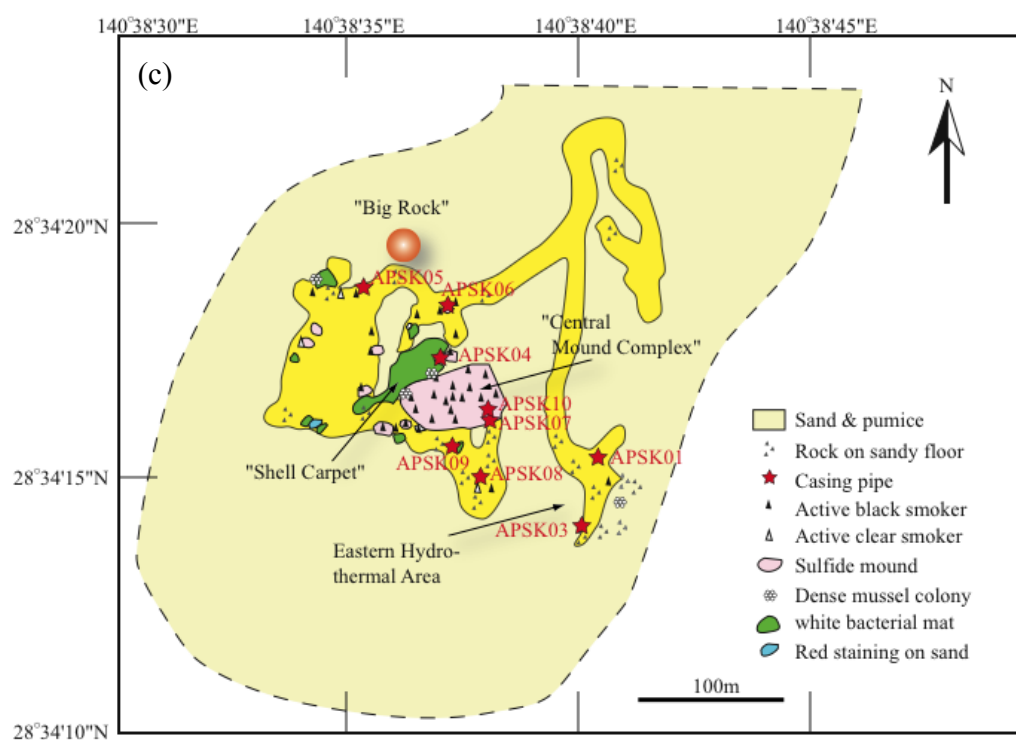


Figure 2

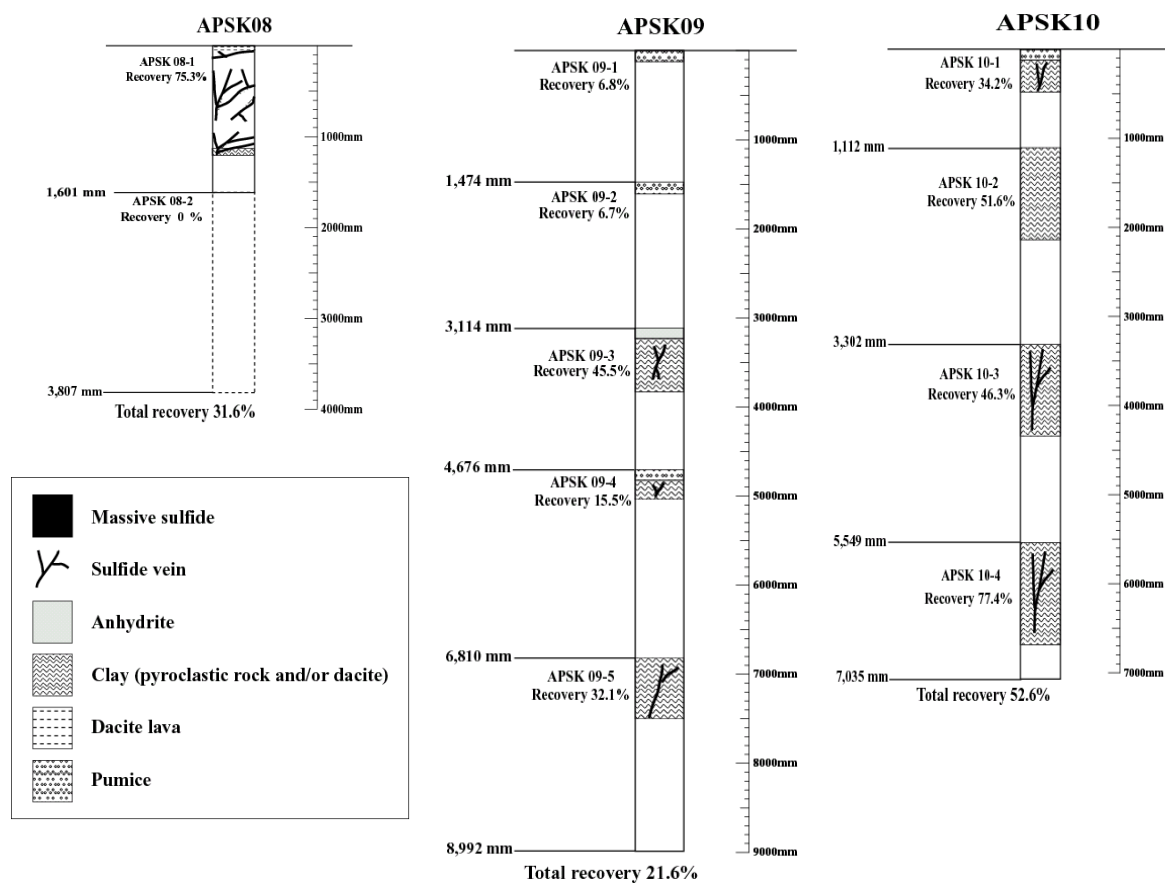


Figure 3

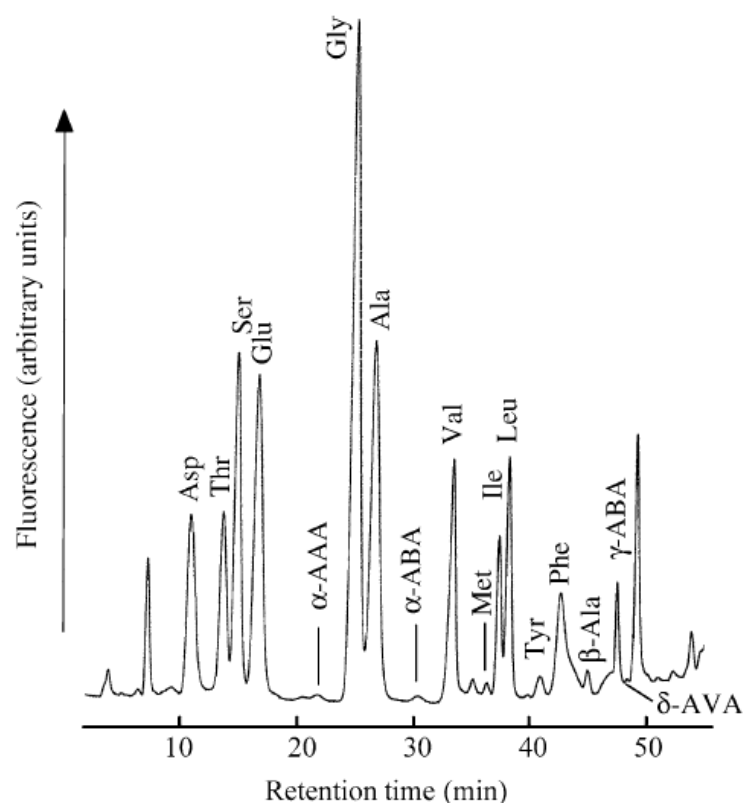


Figure 4

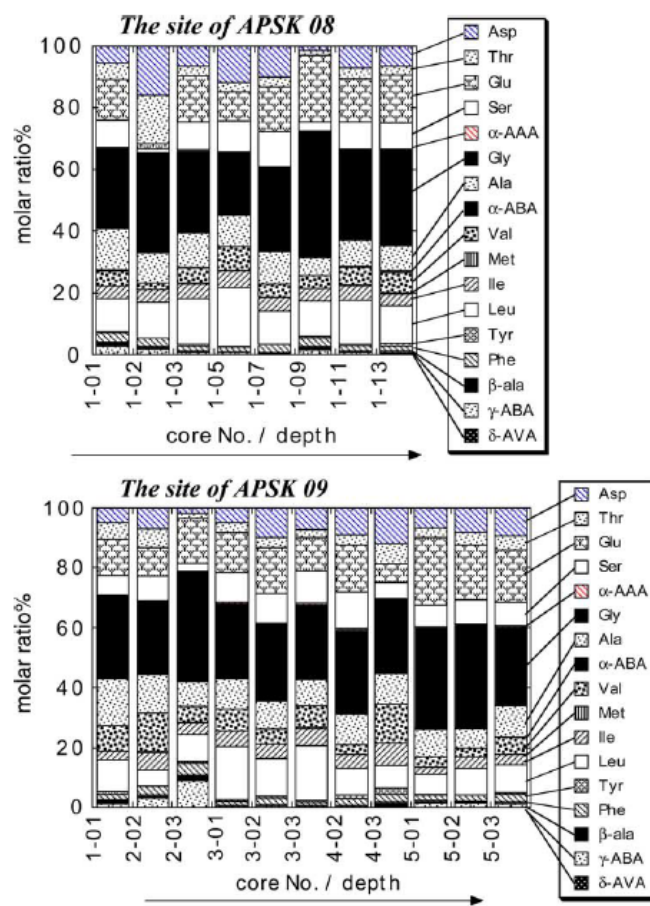


Figure 4

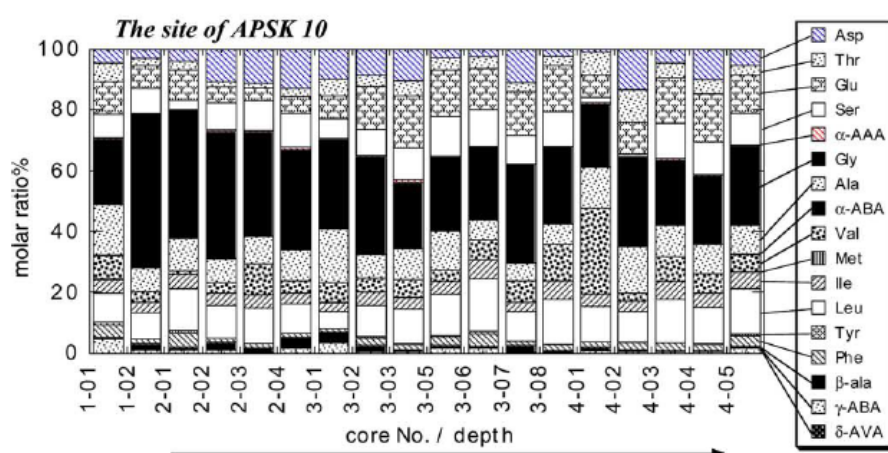
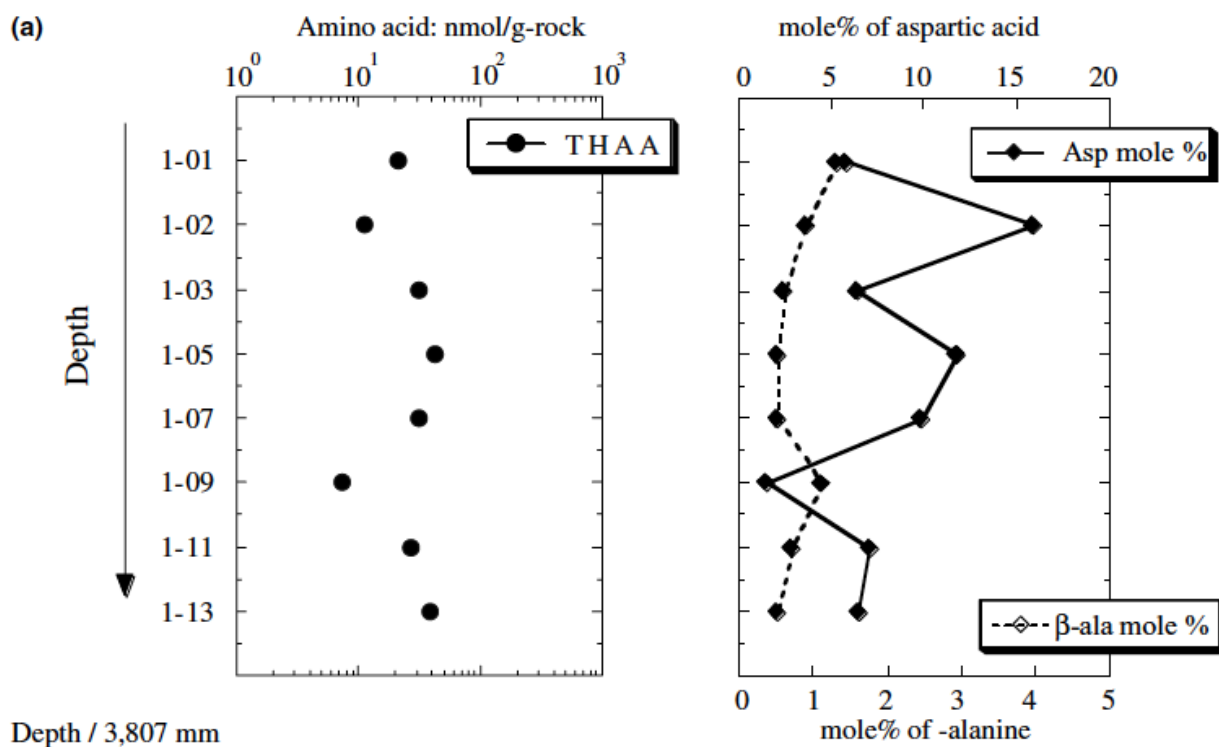
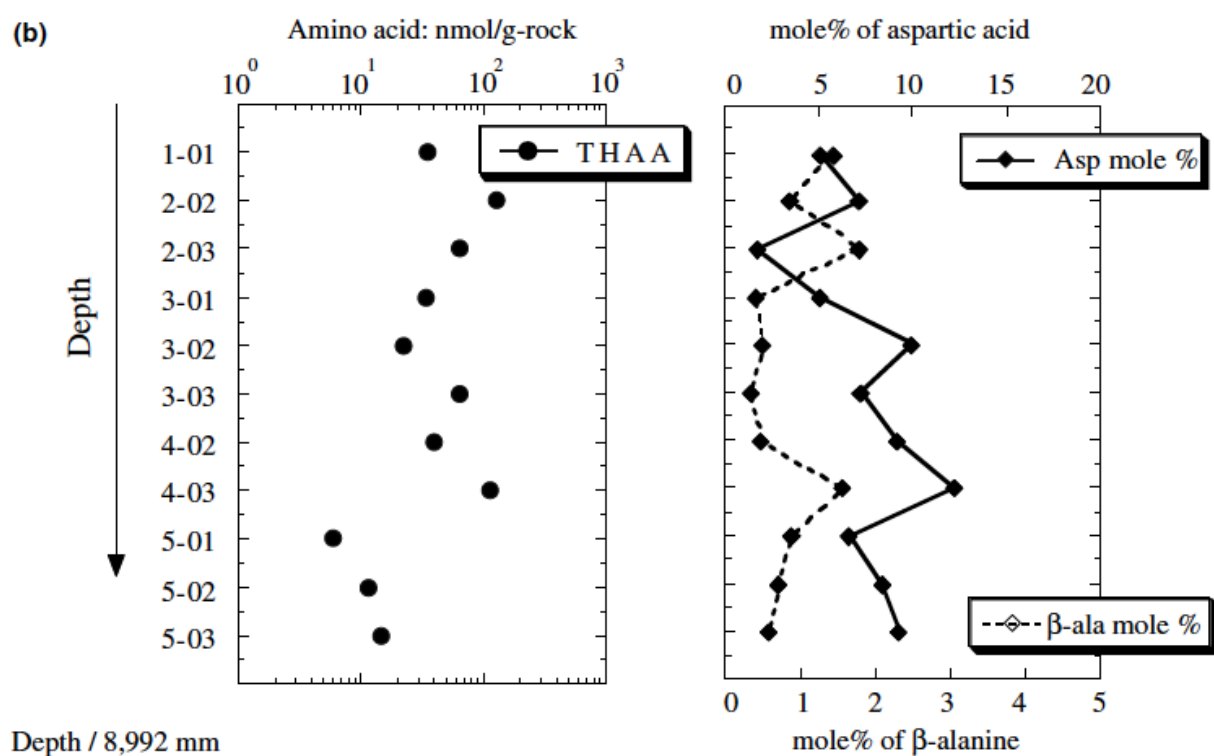


Figure 5



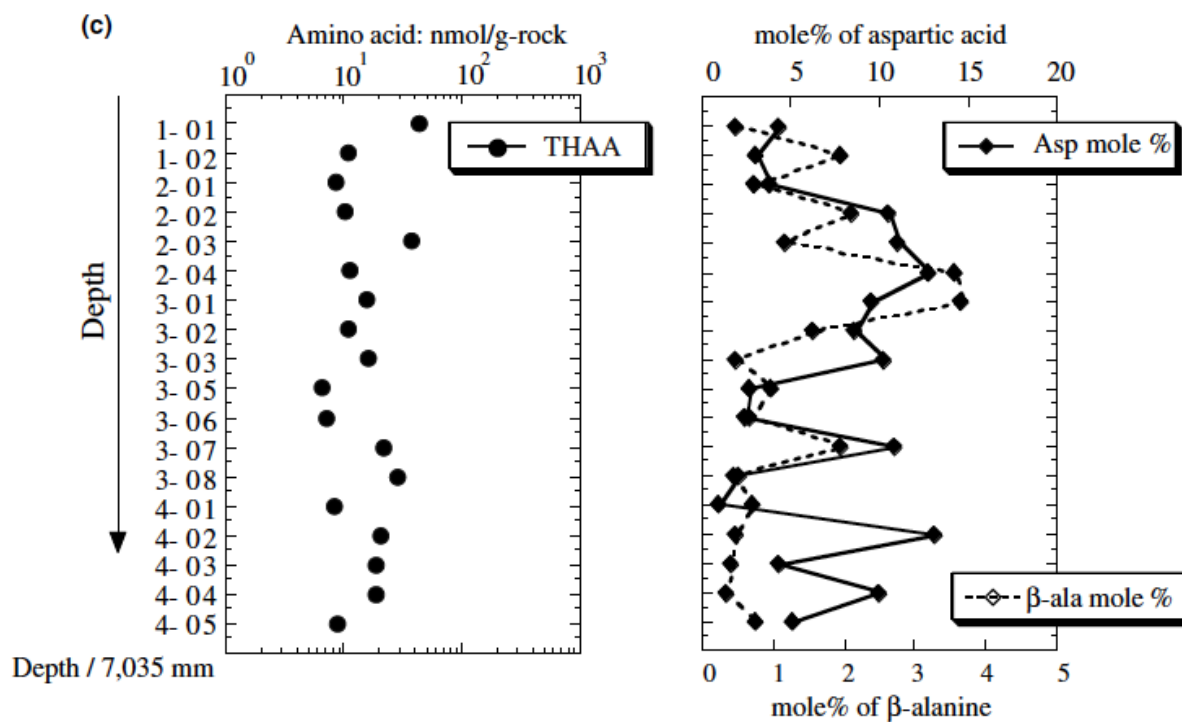
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Figure 5



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Figure 5



APSK08

Table 1

sample No.	Asp	Thr	Ser	Glu	α -AAA	Gly	Ala	α -ABA	Val	Met	Ile	Leu	Tyr	Phe	β -Ala	γ -ABA	δ -AVA	Total
1-01	1.19	1.07	2.80	1.81	n.d.	5.51	2.82	0.08	1.05	n.d.	0.86	2.28	0.06	0.60	0.27	0.58	tr.	20.99
1-02	1.78	1.79	0.19	0.12	n.d.	3.66	1.13	n.d.	0.21	n.d.	0.48	1.30	0.02	0.27	0.10	0.21	tr.	11.25
1-03	1.94	1.06	4.58	2.76	0.06	8.22	3.45	0.09	1.48	0.10	1.44	4.64	0.15	0.47	0.19	0.17	tr.	30.79
1-05	4.93	1.35	3.95	4.21	0.09	8.59	4.37	n.d.	3.26	0.06	2.35	8.02	0.15	0.57	0.20	0.21	tr.	42.3
1-07	3.08	1.08	4.55	3.67	n.d.	8.66	3.26	n.d.	1.39	n.d.	1.43	3.40	n.d.	0.76	0.15	0.08	tr.	31.51
1-09	0.10	0.11	1.57	0.21	n.d.	2.97	0.44	n.d.	0.31	n.d.	0.28	0.83	0.05	0.19	0.08	0.12	tr.	7.25
1-11	1.91	0.90	3.85	2.40	n.d.	7.98	2.24	0.09	1.60	0.06	1.33	3.81	0.12	0.44	0.18	0.16	tr.	27.08
1-13	2.45	1.15	5.85	3.32	n.d.	11.89	3.19	0.17	2.60	0.13	1.39	4.78	0.25	0.59	0.17	0.14	0.15	38.23

n.d.: not detected, tr.: trace amount (detected but not quantified)

sample No.	Asp	Thr	Ser	Glu	α -AAA	Gly	Ala	α -ABA	Val	Met	Ile	Leu	Tyr	Phe	β -Ala	γ -ABA	δ -AVA	Total
1-01	1.74	1.90	4.21	2.40	n.d.	9.67	5.59	n.d.	2.85	n.d.	1.08	3.78	0.25	0.60	0.50	0.46	tr.	35.04
2-02	9.26	8.39	12.17	10.56	0.33	31.54	16.80	0.12	16.77	0.16	7.51	6.88	0.67	3.64	1.12	4.24	tr.	130.15
2-03	1.03	1.12	9.76	1.45	0.17	23.38	5.46	n.d.	3.43	0.10	2.46	5.58	0.40	2.63	1.14	5.77	tr.	63.89
3-01	1.69	1.11	4.57	3.34	0.17	8.48	3.52	n.d.	2.40	n.d.	1.71	6.12	0.16	0.44	0.14	0.11	tr.	33.95
3-02	2.21	0.81	3.42	2.18	0.07	5.77	2.09	n.d.	1.05	n.d.	1.15	2.83	0.10	0.51	0.11	0.11	tr.	22.42
3-03	4.67	1.83	7.11	7.07	0.37	16.06	5.76	n.d.	4.77	0.06	3.75	11.96	0.26	0.80	0.22	0.17	tr.	64.84
4-02	3.63	1.46	6.18	4.90	0.22	11.25	3.94	n.d.	1.39	n.d.	1.77	3.70	0.46	0.90	0.18	0.09	tr.	40.07
4-03	13.80	8.03	6.81	6.39	0.17	28.13	11.50	0.15	14.52	0.09	8.75	8.66	2.33	3.01	1.77	0.12	0.07	114.23
5-01	0.39	0.19	1.32	0.43	n.d.	2.00	0.53	n.d.	0.21	n.d.	0.14	0.39	0.02	0.10	0.05	0.09	tr.	5.87
5-02	0.96	0.49	2.12	0.92	n.d.	4.02	0.76	n.d.	0.33	n.d.	0.46	1.02	tr.	0.24	0.08	0.17	tr.	11.55
5-03	1.37	0.76	2.57	1.15	0.06	3.90	1.55	n.d.	0.85	n.d.	0.51	1.40	0.07	0.39	0.08	0.19	tr.	14.84

n.d.: not detected, tr.: trace amount (detected but not quantified)

sample No.	Asp	Thr	Ser	Glu	α -AAA	Gly	Ala	α -ABA	Val	Met	Ile	Leu	Tyr	Phe	β -Ala	γ -ABA	δ -AVA	Total
1-01	1.86	2.69	4.67	3.26	0.11	9.45	7.15	0.14	3.31	0.09	1.91	4.16	0.27	1.86	0.20	1.86	0.04	43.0
1-02	0.32	0.24	0.83	0.87	0.03	5.43	0.86	n.d.	0.37	n.d.	0.36	0.94	n.d.	0.16	0.21	0.12	tr.	10.7
2-01	0.32	0.24	0.83	0.29	n.d.	3.56	0.89	n.d.	0.11	n.d.	0.40	1.14	0.08	0.39	0.06	0.10	tr.	8.4
2-02	1.07	0.18	0.56	0.90	0.06	4.30	0.83	n.d.	0.34	n.d.	0.43	1.11	n.d.	0.13	0.21	0.14	tr.	10.3
2-03	4.18	0.58	1.62	3.69	0.18	13.18	3.40	n.d.	3.86	n.d.	1.73	4.42	n.d.	0.52	0.43	0.16	tr.	37.9
2-04	1.44	0.30	0.65	1.24	0.11	3.73	1.18	n.d.	0.46	n.d.	0.38	1.08	0.02	0.17	0.40	0.15	tr.	11.3
3-01	1.46	0.87	1.15	1.01	0.04	4.56	2.73	0.02	1.00	0.02	0.45	0.86	n.d.	0.17	0.56	0.50	tr.	15.4
3-02	0.92	0.37	1.56	0.92	0.04	3.49	0.87	n.d.	0.51	n.d.	0.44	1.13	0.06	0.26	0.17	0.08	tr.	10.8
3-03	1.65	0.79	2.77	1.74	0.12	3.54	1.63	n.d.	0.94	n.d.	0.63	1.81	0.06	0.26	0.07	0.10	tr.	16.1
3-05	0.17	0.26	0.98	0.85	n.d.	1.61	0.84	n.d.	0.23	n.d.	0.27	0.90	0.02	0.19	0.06	0.09	tr.	6.5
3-06	0.17	0.26	0.98	0.84	n.d.	1.70	0.47	n.d.	0.47	n.d.	0.45	1.22	0.05	0.29	0.05	0.10	tr.	7.1
3-07	2.36	0.61	3.22	2.04	n.d.	7.06	1.32	n.d.	1.50	n.d.	0.71	2.10	n.d.	0.33	0.42	0.06	tr.	21.7
3-08	0.56	0.93	4.36	3.19	n.d.	7.40	1.85	n.d.	3.46	n.d.	1.71	4.25	n.d.	0.56	0.12	0.09	tr.	28.5
4-01	0.07	0.62	0.60	0.15	0.05	1.70	1.11	n.d.	2.30	n.d.	0.35	0.94	n.d.	0.16	0.06	0.08	tr.	8.2
4-02	2.65	2.20	2.12	0.16	n.d.	6.00	3.11	n.d.	0.51	n.d.	0.70	2.02	0.07	0.41	0.09	0.14	tr.	20.2
4-03	0.79	0.93	2.78	2.14	0.09	3.95	1.93	n.d.	1.53	0.02	1.05	2.65	0.01	0.40	0.07	0.10	tr.	18.5
4-04	1.89	0.91	2.96	2.04	0.07	4.33	1.84	n.d.	1.23	n.d.	0.85	2.31	0.03	0.34	0.06	0.11	tr.	19.0
4-05	0.45	0.30	1.10	0.93	n.d.	2.33	0.85	n.d.	0.53	n.d.	0.50	1.32	0.04	0.31	0.07	0.12	tr.	8.8

n.d.: not detected, tr.: trace amount (detected but not quantified)

core	D/L ratio		
	Asp	Glu	Ala
1-01	0.02	0.02	0.12
1-02	0.02	0.06	0.05
1-03	0.02	0.06	0.05
1-05	0.11	0.05	0.08
1-07	0.00	0.00	0.00
1-09	0.02	0.06	0.05
1-11	0.09	0.05	0.08
1-13	0.01	0.05	0.03

core	D/L ratio		
	Asp	Glu	Ala
1-01	0.00	0.01	0.02
2-02	0.08	0.02	0.02
2-03	0.04	0.04	0.02
3-01	0.00	0.00	0.03
3-02	0.04	0.04	0.05
3-03	0.07	0.03	0.05
4-02	0.02	0.00	0.11
4-03	0.01	0.00	0.02
5-01	0.01	0.01	0.00
5-02	0.05	0.01	0.11
5-03	0.00	0.01	0.03

core	D/L ratio		
	Asp	Glu	Ala
1-01	0.04	0.07	0.16
1-02	0.00	0.00	0.00
2-01	0.00	0.00	0.00
2-02	0.12	0.00	0.00
2-03	0.07	0.02	0.00
2-04	0.02	0.00	0.00
3-01	0.10	0.06	0.05
3-02	0.05	0.01	0.00
3-03	0.01	0.00	0.03
3-05	0.03	0.06	0.05
3-06	0.01	0.00	0.03
3-07	0.01	0.00	0.03
3-08	0.03	0.06	0.05
4-01	0.02	0.00	0.00
4-02	0.01	0.00	0.21
4-03	0.11	0.02	0.16
4-04	0.02	0.00	0.14
4-05	0.02	0.00	0.00