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Short Communication

An arsenosugar as the major extractable arsenical in the earthworm *Lumbricus terrestris*†

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Earthworms (*Lumbricus terrestris*) were investigated for arsenic compounds by high-performance liquid chromatography inductively coupled plasma mass spectrometry MS. Total arsenic concentrations were 6.3 ± 0.4 mg kg^{-1} in the earthworms and 28.1 ± 1.9 mg kg^{-1} in the casts of the earthworms. Extraction of the samples removed ~25% of total arsenic from the earthworm tissues, but only ~0.7% from the casts. The major arsenic compound in the earthworm extracts was an arsenic-containing carbohydrate (phosphate arsenosugar, ~55%); glycerol arsenosugar, dimethylarsinic acid, methylarsonic acid, arsenate, and arsenite were also present as minor constituents. In the cast extracts, the two arsenosugars could also be detected in addition to some arsenate and arsenite. The identification of the phosphate arsenosugar was confirmed with liquid chromatography–electrospray-mass spectrometry with detection of *m/z* 75 (As^+) and *m/z* 483 $[(M + H)]^+$; the data were identical with those recorded for authentic standard material. This is the first report of an arsenosugar as the major extractable arsenical in a terrestrial animal.

KEYWORDS: *Lumbricus terrestris*; earthworm; arsenic compounds; arsenosugars; HPLC–ICP-MS; LC–ES-MS

INTRODUCTION

Arsenic-containing carbohydrates (arsenosugars) are common constituents of marine samples[1,2] and are known to be marine algal products. There have been only a few reports of their presence in freshwater and terrestrial organisms. Lai *et al.*[3] were the first to report an arsenosugar as the main arsenic constituent in an algal extract from a non-marine aquatic environment. Koch *et al.*[4] also found arsenosugars in samples from the freshwater environment. Other reports from the terrestrial environment followed, revealing a variety of green plants and lichens containing traces of the glycerol arsenosugar.[5]

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Arsenosugars are also found in animals from the marine environment,[2] but the presence of these compounds is clearly related to the algal food source of the animals. In terrestrial animals, arsenosugars (phosphate and glycerol arsenosugar) have only been reported once so far, in earthworms from Austria, and their origin is less clear.[6] The earthworm data were of interest, but their interpretation was limited by the fact that the earthworm sample comprised a mixture of different species. For example, it was not possible to say if the arsenosugars and the other arsenic compounds detected were present in the same organism. Such information may help explain the origin of the compounds in the earthworms.

In the present study we report the determination of arsenic compounds in a single species, the common earthworm *Lumbricus terrestris*. The analysis was performed using high-performance liquid chromatography–inductively coupled plasma-mass spectrometry (HPLC–ICP-MS) and liquid chromatography–electrospray-mass spectrometry (LC–ES-MS).

MATERIALS AND METHODS

Adult earthworms (*L. terrestris* L.) were collected from an agriculturally utilized field in Admont, Austria, high in arsenic due to geological reasons. Earthworms were washed free of adhering soil particles with tap water and kept for 10 days at 4 °C in order to empty their guts. Discharged earthworm casts were collected daily. All samples were freeze-dried and homogenized.

Determination of total arsenic

Approximately 0.2 g of worms or ~0.1 g of casts of freeze-dried powdered samples were digested with subboiled concentrated nitric acid and hydrogen peroxide in a microwave digestion system (MLS-1200 Mega, MWS, Leutkirch, Germany), and total arsenic concentrations were determined with an ICP–MS (VG Elemental Ltd, Winsford, UK) as previously described.[6] Pine needles (SRM 1575, National Bureau of Standards NIST, Gaithersburg, USA) served as standard reference material (certified arsenic concentration: 0.21 ± 0.04 mg kg^{-1}; measured values: 0.23 ± 0.04 mg kg^{-1}; $n = 3$).

Determination of arsenic compounds

HPLC–ICP-MS

Freeze-dried earthworm and cast samples (~0.2 g) were extracted with a methanol–water mixture (9 + 1, v/v) as previously reported.[6] The extracts were evaporated to dryness, redissolved in water, centrifuged, and filtered. Aliquots of these solutions were then directly chromatographed with an HPLC–ICP-MS system consisting of a Hewlett Packard 1050 solvent delivery unit (Hewlett Packard, Waldbronn, Germany) and a Rheodyne 9125 six-port injection valve (Rheodyne, Cotati, USA) with a 100 µl injection loop, the outlet of the HPLC column was connected to a hydraulic high-pressure nebulizer (Knauer, Berlin, Germany); the ICP–MS (VG Plasma Quad 2 Turbo Plus) served as the arsenic-specific detector. The separations were performed on an anion-exchange column (PRP-X100, Hamilton, Reno, USA) with aqueous 20 mM ammonium dihydrogen phosphate at pH 5.6 [(adjusted with aqueous ammonia (25%)] as mobile phase and on a cation-exchange column (Supelcosil LC-SCX, Supelco, Bellefonte, USA) with aqueous 5 mM pyridine at pH 2.6 (adjusted with formic acid) as mobile phase. Both columns were operated at 40 °C at a flow rate of 1.5 ml min^{-1}. Arsenic compounds were identified by comparison of the retention times with known standards, which included arsenite [As(III)], arsenate [As(V)], methylarsonic acid (MA), dimethylarsinic acid (DMA), arsenobetaine (AB), arsenocholine (AC), trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA), and two arsenosugars: glycerol (OH) arsenosugar, and phosphate (PO_4) arsenosugar.

LC–ES-MS

Aqueous worm extracts were prepared as previously reported for other worms (~0.1 mg worm powder extracted in 5 ml water with a ultrasonication probe),[7] concentrated and filtered before analysis by LC–ES-MS. A Hewlett-Packard LC-MSD system, consisting of a Series 1100 HPLC (with solvent degasser; binary pump, autosampler, and thermostatic column compartment) and a G1946A MSD single quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) LC–MS interface, was used. Chromatography was performed using a PRP-X100 anion-exchange column equilibrated at 30 °C with a mixture (1 + 9, v/v) of methanol and 20 mM NH_4HCO_3, pH 10.3 adjusted with aqueous ammonia. The flow rate was 0.4 ml min^{-1}, and the injection volume was 10 µl in all cases. Analyses were performed on standard solutions of arsenosugars or samples, using selective ion monitoring (SIM) in the positive mode with detection of m/z 75 (As) (250 V) and m/z $[M + H]^+$ (100 V) 329 for OH arsenosugar; 483 for PO_4 arsenosugar; 393 for sulfonate arsenosugar; and 409 for sulfate arsenosugar. The technique has previously been described for the detection of arsenosugars.[8,9]

RESULTS AND DISCUSSION

The arsenic concentration in *L. terrestris* was 6.3 ± 0.4 mg kg^{-1} dry mass ($n = 3$). Mixed earthworm samples from the same area have been reported to contain similar amounts of arsenic, ~5 mg kg^{-1}.[6] Meharg *et al.*[10] reported *L. terrestris* arsenic concentrations for control animals lower than 10 mg kg^{-1} dry mass and for arsenate-exposed (up to 600 mg kg^{-1} in the soil) animals up to 100 mg kg^{-1} dry mass.

The arsenic concentrations of the digested casts were 28.1 ± 1.9 mg kg^{-1} dry mass ($n = 3$), which is much higher than the earthworm tissues, but is close to that of the surrounding soil (~30 mg kg^{-1} dry mass, microwave-assisted digestion),[11] which is a main constituent of the casts.

The HPLC–ICP-MS analysis revealed that the major arsenic compound in the earthworm extracts (~25% extraction efficiency) was the PO_4 arsenosugar (~55%), and that OH arsenosugar (~8%), DMA (~5%), MA (~2%), As(V) (~8%) and As(III) (~10%) were present as minor constituents (Fig. 1). AB was not detected under these conditions, but might have been overlapped by the preceding high signals and could be present at trace levels. The most interesting aspect of the data was the presence of the PO_4 arsenosugar as the major arsenic compound. Arsenosugars have previously been reported as trace or minor constituents of terrestrial organisms. Therefore, further support for the identification of the main arsenic constituent in *L. terrestris* was sought by a technique providing structural information. The identity of the PO_4 arsenosugar in the earthworm extracts was confirmed by means of LC–ES-MS analysis (Fig. 2), revealing

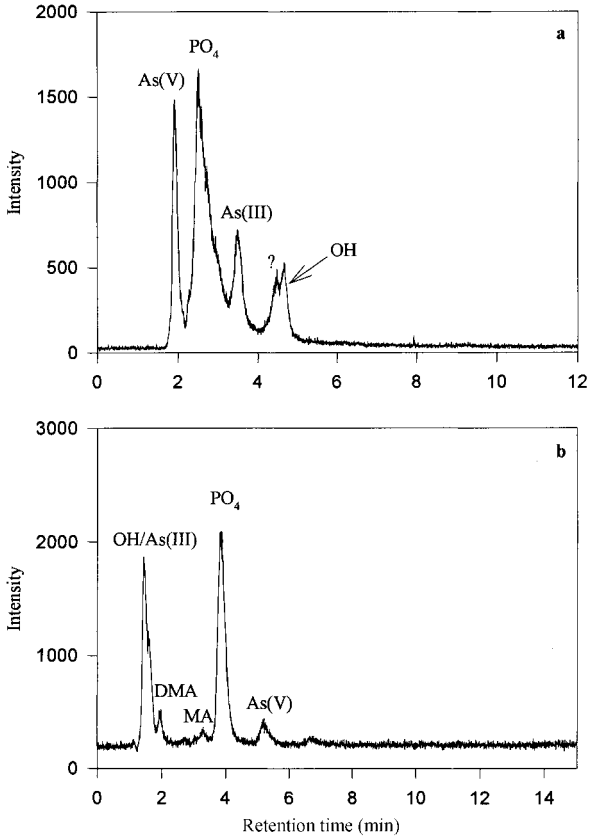

Figure 1. HPLC–ICP-MS chromatograms of an aqueous extract of *L. terrestris* (a) cation-exchange (Supelcosil LC-SCX, 5 mM pyridine, pH 2.6, 1.5 ml min^{-1}) and (b) anion-exchange (Hamilton PRP-X100, 20 mM ammonium dihydrogen phosphate, pH 5.6, 1.5 ml min^{-1}).

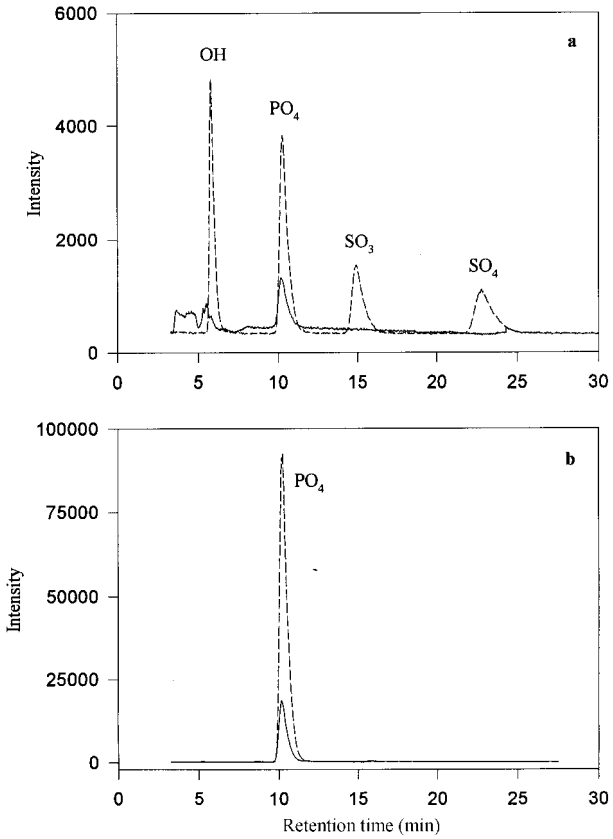

Figure 2. LC–ES-MS chromatograms of aqueous extracts of *L. terrestris* (solid line) and arsenosugar standards (0.2 ng μl^{-1}) (dotted line) at (a) *m/z* 75, 250 V and (b) *m/z* 483, 100 V. Conditions: Hamilton PRP-X100 anion-exchange column, mobile phase 20 mM NH_4HCO_3 (pH 10.3)–methanol (1 + 9, v/v) 0.4 ml min^{-1}, 30 °C, 10 µl injected.

matching retention times (10.2 min) and molecular masses (483 $[M+H]^+$) for the PO_4 arsenosugar with authentic arsenosugar standard material.

The occurrence of the arsenic compounds present in *L. terrestris* has also been reported in mixed earthworm samples from different sites in Austria;[6] however, in all those samples, arsenosugars were only minor constituents, whereas the bulk of arsenic was inorganic. Arsenosugars as minor constituents have also been detected in other (marine) worms, *Arenicola marina*[7] and *Nereis sp.*[12] (Polychaeta, Annelida). However, in *L. terrestris* extracts the PO_4 arsenosugar is the dominant arsenic compound. Small amounts of PO_4 and OH arsenosugars were recently also reported to be present in some plants in the terrestrial environment.[4,5] Thus far, only marine and freshwater algae were thought to contain arsenosugars as their main constituents.[2,3] Algae are able to biotransform arsenosugars from As(V) taken up from the water.[13] The occurrence of arsenosugars as minor constituents in marine animals is supposed to be a consequence of symbiosis with algae or feeding on them.[14,15] However, arsenosugars have also been found in mussels from a hydrothermal vent, where algal growth is unlikely.[16]

The extracts of the earthworm casts contained traces of the PO_4 arsenosugar, the OH arsenosugar, As(V) and As(III). However, only ~0.7% of the arsenic concentration in the casts was extractable with the water–methanol mixture. This low extraction efficiency is similar to the extraction efficiency of soil from this area (~0.2%).[6] This is not surprising, since it can be assumed that most of the cast consists of soil, but in soil extracts of this area only As(V) was detected. Investigations about soil at another sampling place in Austria have shown that soil can also contain TMAO and AB besides inorganic arsenic.[17] Arsenosugars have not been reported in soil. Analysis of tissue and mucus/cast samples from a marine polychaete (*A. marina*) revealed that these worms also contain the two arsenosugars and excrete the arsenosugars with their casts,[7] whereas no arsenosugars could be detected in the surrounding sediment.

The origin of the arsenosugars in the terrestrial environ-

ment in general, and in earthworms and their casts in particular, is not yet clear. The occurence of arsenosugars in worm casts (which contain soil) but not in the surrounding soil suggests that earthworms or microorganisms in contact with the worms might elaborate these compounds.

CONCLUSION

The presence of arsenosugars in earthworms, and especially the dominance of the PO_4 arsenosugar in *L. terrestris*, discloses that arsenosugars are not restricted to marine algae and marine animals feeding on them, but play an important role in the arsenic biotransformation in the terrestrial environment as well. PO_4 arsenosugar as the main arsenic compound in the earthworm extract is a novelty in the terrestrial environment. Until now, arsenosugars had only been detected in trace amounts or as minor constituents in plants and mixed earthworm samples. The results of the present investigation not only confirm previous reports on the occurrence of arsenosugars in earthworms, but, moreover, demonstrate that arsenosugars can be present as the main extractable arsenic compound in (a) an animal and (b) in the terrestrial environment.

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