IDENTIFICATION OF ARSENIC COMPOUNDS IN MUSHROOMS, AND EVIDENCE FOR MYCELIAL METHYLATION

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Since Edmonds et al. (1977) identified non-toxic 'fish arsenic' present in crustacea and fish as arsenobetaine (carboxymethyl trimethylarsonium zwitterion, $(CH_3)_3As+CH_2COO-$), and subsequently a variety of dimethyltribosylarsine oxides in marine algae, (Edmonds et al. 1981), about a dozen arsenic compounds are now known to occur in the marine environment (Cullen & Reimer 1989). In contrast, information on the compounds present in the terrestrial environment is scarce. We now wish to report progress in identification of arsenic compounds in higher fungi, and some evidence of methylation processes occurring in the mycelium. A recent study (Byrne et al. 1995) on some arsenic-accumulating mushrooms in which we found not only methylated arsenic species, but also—for the first time in the terrestrial environment—arsenobetaine (AB) prompted further investigation of the distribution of arsenic metabolites in the Fungal Kingdom. Analysis of about fifty species of basidiomycetes (Šlejkovec et al. 1997) showed AB to be the most common arsenic compound present, and also suggest its preponderance in evolutionarily more advanced fungal groups, i.e. Gastrales and Agaricaceae.

Interest in higher fungi in relation to arsenic (and other trace element) cycling in the terrestrial environment hardly needs justification in view of the dominant role of fungi in decomposition, in transfer and in transformation of nutrients and trace elements. Our earlier finding of arsenic accumulation by *Laccaria amethystina* (Byrne *et al.* 1979, Byrne & Tušek-Znidaric 1983) led to identification of the compound present as dimethylarsinic acid (DMA) (Byrne *et al.* 1991), and this stimulated us to identify the arsenic species present in a number of other arsenic-accumulating mushrooms, namely methylarsonic acid (MA) in *Sarcosphaera coronaria*, inorganic arsenic in *Entoloma lividum*, a mixture of inorganic arsenic, MA, DMA and AB in *Sarcodon imbricatus*, and AB in two *Agaricus* species (*A. placomyces* and *A. haemorrhoidarius*) (Byrne *et al.* 1995). Analyses of different collections showed the pattern to be consistently related to the mushroom species. Hence our recent survey (Šlejkovec *et al.* 1997) covering the main orders and genera of basidiomycetes was intended to identify the arsenic compounds present, and perhaps relate them to taxonomic position.

In these studies arsenic compounds were determined in methanol extracts from the mushrooms by high performance liquid chromatography (HPLC) with an inductively coupled plasma-mass spectrometer (ICP-MS) system as an arsenic specific detector. Arsenite, DMA, MA, arsenate and AB were separated on a Supelcosil LC-SAX anion exchange column, and trimethylarsine oxide (TMAO), arsenocholine (AC) and the tetraarsonium cation (TETRA) on a Supelcosil LC-SCX column, the concentrations being obtained from comparison of peak areas with the calibration responses for the authentic compounds. Radiochemical neutron activation analysis (RNAA) was used for total arsenic determination, and to lesser extent, hydride generation—atomic absorption spectroscopy (HG-AAS).

The mushrooms examined in this survey (Šljekovec *et al.* 1997) were not accumulators, but had normal arsenic concentrations. As stated above, the most frequently encountered metabolite was AB. As well as the other compounds mentioned above, some mushrooms have small amounts of unknown compounds, but these were generally insignificant. Although our survey is still very incomplete, it is already possible to report some interesting observations.

There was apparently no relation between the occurrence of AB, or we might say, the ability to metabolise it (this is discussed below) and the biology of fungi, *i.e.* saprophytic species were as apt to contain it as mycorrhizal ones. Although the number of mushrooms analysed is too small to permit firm conclusions on a relationship between arsenic metabolism and taxonomy, there is little doubt that the ability to synthesize AB was acquired only during the higher stages of fungal evolution. We find AB most expressed in the Gastrales (puffballs, and the related earth star, *Geastrum*), Table 1, which are at the top of the evolutionary ladder (Courtecuisse 1994). AB is also dominant in the more advanced gilled fungi, notably the Agaricaceae and Lepiotaceae (Table 2). The Agaricaceae have many features in common with the Lycoperdaceae, *e.g.* formation

Table 1: Arsenic compounds in some mushrooms from the order Gastrales (μg g^{-1} dry weight); individual metabolites are given as percentage of sum of species in methanol extract.

Mushroom	Total	Sum of species	As(III)	DMA	AB	MA	As(V)
Calvatia excipuliformis	0.72±0.06	0.8	4%	20%	72%	–	4%
Calvatia utriformis	0.79±0.07	0.5	trace	9%	85%	–	6%
Lycoperdon echinatum	1.23±0.10	0.3	trace	12%	78%	–	10%
Lycoperdon perlatum	2.81±0.24	3.6	trace	5%	88%	7%	trace
Lycoperdon piriforme	0.46±0.09	0.5	8%	trace	62%	trace	30%
Geastrum sp.	3.12±0.20	2.9	2%	2%	94%	trace	2%

(Total arsenic in Tables 1 and 2 was determined in 2 or 3 replicate determination using RNAA.)

of urea (Stijve & Diserens 1988), accumulation of silver (Schmitt *et al.* 1978, Byrne *et al.* 1979) of selenium (Stijve 1977) and mercury, and biosynthesis of methylmercury (Stegnar *et al.* 1973, Stijve & Roschnik 1974).

Other evolutionarily advanced gilled fungi are the Cortinariaceae, a few genera of which are clearly linked to Gasteromycetes. Two arsenic-rich *Cortinarius* species, *Telamonia bivela* and *Phlegmacium melleolens*, collected in a former mining area, were found to contain virtually only AB (Gössler *et al.* unpublished). Such links are less evident in the Amanitaceae, but at least in the Fly Agaric (*A. muscaria*), AB is the predominant metabolite, with the unusual precursor arsenocholine (AC). This was also the finding of Kühnelt *et al.* (1997) in some Austrian specimens.

The Aphyllophorales are a most heterogeneous group, especially from the morphological point of view. *Sarcodon imbricatus*, a representative analysed earlier (Byrne *et al.* 1995) contains AB, but apparently biosynthesis is not very efficient, since many intermediates are present at significant levels. The genus *Albatrellus* (which are terrestrial polypores), *Ramaria pallida* and *Sparassis crispa*, contain AB and several other organic and inorganic arsenic compounds. In *Sparassis crispa* arsenocholine is the predominant metabolite. In *Gomphus clavatus*, an intermediary species between the Clavaricales and the Cantharellaceae (the latter having rudimentary gills), AB predominates to the extent of 90 per cent, while in *Thelephora terrestris*, belonging to the more primitive crust and parchment fungi, AB is absent.

In the Tricholomataceae, another diverse group, AB occurred less frequently (only in 3 of 6 genera investigated). But in the more developed genus *Collybia*, in two species collected near an old smelter site in southern Austria and containing much arsenic, AB was the main compound (Kühnelt *et al.* 1997). We also analysed a few reddish spored fungi belonging to the Plutaceae and the Entolomataceae, which are rather difficult to classify. It is generally recognized that Plutaceae are the more evolved ones and indeed in *Volvariella volvacea* arsenic is largely present as DMA with a trace of AB, whereas in *Entoloma rhodopolium* there is mainly inorganic arsenic, just like in *Entoloma lividum* analysed earlier (Byrne *et al.* 1995).

The question of course arises whether biosynthesis, *i.e.* methylation, is involved, and this presumably in the mycelium which is very long-lived, rather than in the ephemeral fruitbodies themselves (where the compounds were detected). The aternative hypothesis is selective uptake and transport of compounds from soil or pore water, synthesised by other means, *e.g.* microorganisms (Cullen *et al.* 1995). The fact that mushroom species from different sites and countries display a consistent arsenic compound pattern supports the idea of direct

Table 2: Arsenic compounds in some mushrooms belonging to Agaricaceae and Lepiotaceae (µg g^{-1} dry weight); Individual metabolites are given as percentage of sum of species in methanol extract.

Mushroom	Total	Sum of species	As(III)	DMA	AB	MA	As(V)	TETRA	Unknown[1]
Macrolepiota procera	0.42±0.04	0.1	trace	trace	100%	trace	trace		
Leucocoprinus badhamii	2.9±0.7	2.6	trace	47%	49%	trace	2%	trace	2%(460)
Agaricus abruptibulbus	3.49±0.45	3.0	1%	3%	88%	5%	3%		
Agraricus bisporus	1.00±0.03	0.8	-	27%	55%	6%	12%		
Agaricus campester	1.32±0.17	1.3	trace	trace	96%	trace	-	4%	
Agaricus elvensis	2.43±0.29	2.6	2%	4%	84%	2%	4%		4%(335)
Agaricus fuscofibrillosus	2.54, 2.81	2.7	trace	4%	95%	-	1%		
Agaricus lilaceps	1.78±0.08	2.3	6%	24%	66%	-	3%		1%(460)
Agaricus macrosporus	3.32±0.36	2.4	-	8%	85%	4%	3%		
Agaricus silvicola	6.2±0.5	7.2	1%	4%	90%	-	3%		2%(460)
Agaricus subrutilescens	10.8±0.4	10.4	0.5%	1.5%	96%	1%	1%		

[1] For unknown compounds retention times are given in parentheses.

Table 3: Distribution of arsenic compounds in mycelia grown for 39 days on potato dextrose agar spiked with arsenic compounds and in growth media (PDA) that had been in contact with the mycelia for 39 days, expressed as % of the sum of species for each sample.

Compound added	Sample	As(III)	AB	DMA	As(V)	MA	TETRA	TMAO
As(III)	Mycelium	78.3	b.d.[1]	b.d.	21.7	b.d.	b.d.	b.d.
	PDA	80.2	b.d.	b.d.	19.8	b.d.	b.d.	b.d.
As(V)	Mycelium	75.7	b.d.	b.d.	24.3	b.d.	b.d.	b.d.
	PDA	79.2	b.d.	b.d.	20.8	b.d.	b.d.	b.d.
MA	Mycelium	b.d.	b.d.	16.8	b.d.	83.2	b.d.	b.d.
	PDA	b.d.	b.d.	6.8	b.d.	93.2	b.d.	b.d.
DMA	Mycelium	1.0	b.d.	99.0	b.d.	b.d.	b.d.	b.d.
	PDA	b.d.	b.d.	100	b.d.	b.d.	b.d.	b.d.
AB	Mycelium	b.d.	100	b.d.	b.d.	b.d.	b.d.	b.d.
	PDA	b.d.	100	b.d.	b.d.	b.d.	b.d.	b.d.
TETRA	Mycelium	b.d.	b.d.	b.d.	b.d.	b.d.	100	b.d.
	PDA	b.d.	b.d.	b.d.	b.d.	b.d.	100	b.d.
TMAO	Mycelium	b.d.	trace	trace	b.d.	b.d.	b.d.	100
	PDA	b.d.	b.d.	4.0	b.d.	b.d.	b.d.	96.0

[1] b.d.: below the limit of detection (representing about <0.1–0.3%)

biosynthesis. Selective uptake could also account for it, but the major arsenic compounds in soils are arsenite and arsenate (Woolson 1977, Masscheleyn *et al.* 1991) while DMA is only a minor component (<0.05% of total arsenic (Pohl & Bächmann 1986)). In pore waters MA (10% of total arsenic) but no DMA was found (Haswell *et al.* 1985). AB has not yet been identified in soil or pore water. Our failure to detect TMAO in any mushroom when this compound is a common metabolite of methylation by microorganisms also supports the hypothesis of mycelial methylation of the other compounds.

To investigate this question, experiments on the cultivation of the Oyster mushroom, *Pleurotus sp.*, with added arsenate, and mycelium cultures of *Agaricus placomyces* on potato dextrose agar plates, spiked with various arsenic compounds, were performed (Šlejkovec *et al.* 1996). *Agaricus placomyces* was chosen since it was found to contain only AB in nature (Byrne *et al.* 1995).

In the experiments with *Pleurotus* grown on straw, the major form of arsenic found in the fruitbody was arsenite, and to lesser extent, arsenate. After more than 55 days, about 1 per cent of the arsenic present was in the form of MA (1.4 $mg.kg^{-1}$; arsenite ca. 100, arsenate ca. 50). However, the observed reduction and partial methylation could have been carried out by microorganisms present in the incompletely sterile substrate.

In the case of agar cultures of *Agaricus placomyces*, it was found that arsenobetaine and tetramethylarsonium iodide were taken up efficiently by the mycelium from the medium (concentration factors of about 4.4 and 6, respectively), whereas the more toxic compounds were much less accumulated. (The growth medium and mycelium were separated by a membrane filter, allowing compounds to pass through, but preventing physical contamination of the harvested mycelium). Perhaps the most important finding, in addition to reduction of arsenate to arsenite in the presence of mycelium, was the presence of DMA in the mycelium growing on medium to which MA had been added (about 83% MA, and 17% DMA in the mycelium after 39 days) (Table 3). This provides direct proof of methylation by *Agaricus placomyces* mycelium, even in the short period of the experiment.

The more general question can be posed as to the potential importance of fungi in the terrestrial cycle of arsenic. The occurrence of methylated arsenic compounds in fruitbodies is evidently the expression of a considerable arsenic pool in the fungal biomass, whose magnitude in relation to the total arsenic in the upper soil horizons at present cannot even be guessed at. It is of interest to note that in the case of radiocaesium (one of whose manifestations has been high levels in certain mushrooms) some recent estimates suggest that as much as about 30 per cent (Olsen & Bakken 1990) or 40 per cent (Guillite *et al.* 1994) can be contained in the fungal biomass, and that its upwards transport in fruitbodies is an important process in the dynamics of radiocaesium movement in soil. We have observed evidence for the 'pumping' of arsenic through transfer to arsenic-accumulating fruitbodies of *Laccaria amethystina*, in areas where these grew persistently, as increased levels of arsenic in surface soil. Thus the role of fungi in relation to the input (atmospheric deposition, plant decay), output (leaching, volatilisation) and transformation (reduction, methylation) of arsenic in soils, and its overall terrestrial cycle, could turn out to be of some importance, and certainly worthy of further study.

References:

Byrne, A.R., Dermelj, M. & Vakselj, A. (1979). Silver accumulation by fungi. *Chemosphere* **10,** 815–821.

Byrne, A.R., Šlejkovec, Z., Stijve, T., Fay, L., Goessler, W., Gailer, J. & Irgolic, K.J. (1995). Arsenobetaine and other arsenic species in mushrooms. *Appl. Organomet. Chem.* **9,** 305–313.

Byrne, A.R. & Tušek-Znidaric, M. (1983). Arsenic accumulation in the mushroom *Laccaria amethystina*. *Chemosphere* **12,** 1113–1117.

Byrne, A.R., Tušek-Znidaric, M., Puri, B.K. & Irgolic, K.J. (1991). Studies of the uptake and binding of trace metals in fungi. Part II Arsenic compounds in *Laccaria amethystina*. *Appl. Organomet. Chem.* **5,** 25–32.

Courtecuisse, R. (1994). *Guide des Champignons de France et d'Europe.* (Delachaux et Niestle: Lausanne.)

Cullen, W.R. & Reimer, K.J. (1989). Arsenic speciation in the environment. *Chem. Rev.* **89,** 713–764.

Cullen, W.R., Li, H., Pergantis, S.A., Eigendorf , G.K. & Mosi, A.A. (1995). Arsenic biomethylation by the microorganism *Apiotrichum humicola* in the presence of L-methionine-methyl-d3. *Appl. Organomet. Chem.* **9,** 507–515.

Edmonds, J.S. & Francesconi, K.A. (1981). Arseno-sugars from brown kelp (*Ecklonia radiata*) as intermediates in cycling arsenic in marine ecosystems. *Nature* **289,** 602–604.

Edmonds, J.S., Francesconi, K.A., Canon, J.R., Raston, C.L., Skelton, B.W. & White, A.H. (1977). Isolation, crystal structure and synthesis of arsenobetaine, the arsenical constituent of the western rock lobster *Panulirus Longipes cygnus* George. *Tetrahedron Lett.* **18,** 1543–1546.

Guillite, O., Melin, J. & Wallberg, L. (1994). Biological pathways of radionuclides originating from Chernobyl fallout in a boreal forest ecosystem. *Sci. Total Environ* **157,** 207–215.

Haswell, S.J., O'Neill, P. & Bancroft, K.C.C. (1985). Arsenic speciation in soil pore-waters from mineralized and unmineralized areas of south-west England. *Talanta* **32,** 69–72.

Kühnelt, D., Goessler, W. & Irgolic, K.J. (1977). Arsenic in terrestrial organisms. 1: *Collybia maculata, Collybia butyracea* and *Amanita muscaria* from arsenic smelter sites in Austria. *Appl. Organomet. Chem.* **11,** 289–299.

Masscheleyn, P.H., Delaune, R.D. & Patrick Jr., W.H. (1991). Effect of redox potential and pH on arsenic speciation and solubility in contaminated soil. *Environ. Sci. Technol.* **25,** 1414–1419.

Olsen, R.A. & Bakken, L.R. (1990). Soil fungi and fate of radiocaesium in the soil ecosystem—a discussion of possible mechanisms involved in the radiocaesium accumulation in fungi, and the role of fungi as a Cs sink in the soil, *in* G. Desmet, P. Nassimbeni & M. Belli (eds), Transfer of radionuclides in natural and semi-natural environments, *Proc. Workshop on Transfer of Radionuclides in Natural and Semi-natural Environments* 657–663. (Elsevier Applied Science, Barking, U.K.)

Pohl, B. & Bächmann, K. (1986). Vergleichende Untersuchungen der Konzentrationen von Dimethylarsonsäure, As(III) und As(V) in Boden und Luftstaubproben. *Fresenius* Z. *Anal. Chem.* **323,** 859–964.

Schmitt, J.A., Meisch, H.U. & Reinle, W. (1978). Schwermetalle in höheren Pilzen, IV, Silber. *Z. Naturforsch.* **33c,** 608–615.

Šlejkovec, Z., Byrne, A.R., Goessler, W., Kuehnelt, D., Irgolic, K.J. & Pohleven, F. (1996). Methylation of arsenic in *Pleurotus* sp. and *Agaricus placomyces*. *Acta Chim. Slovenica* **43,** 269–283.

Šlejkovec, Z., Byrne, A.R., Stijve T., Goessler, W. & Irgolic, K.J. (1997, in press). Arsenic compounds in higher fungi. *App. Organomet. Chem.* **11.**

Stegnar, P., Byrne, A.R., Kosta, L. & Ravnik, V. (1973). The accumulation of mercury by, and the occurrence of methyl mercury in some fungi. *Chemosphere* **2,** 57–63.

Stijve, T. (1977). Selenium Content of Mushrooms. *Z. Lebensm. Unters.—Forsch.* **164,** 201–203.

Stijve, T. & Diserens, H. (1988). Harnstoff in Speisepilzen. *Deutsche Lebensm. Rundschau* **84,** 248–252.

Stijve, T & Roschnik, R. (1974). Mercury and Methyl Mercury Content of Different Species of Fungi. *Trav. Chim. Aliment. Hyg.* **65,** 209–220.

Woolson, E.A. (1977). Generation of alkylarsines from soil. *Weed Sci.* **25,** 412–416.