

# Constituents of *Aristolochia manshuriensis* cell suspension culture possessing cardiotoxic activity

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**SUMMARY.** *Studies were conducted with suspension culture of A. manshuriensis (strain A2s) originated from cells transformed by Ti-plasmids of Agrobacterium tumefaciens. As shown previously, the extract from A2s biomass possessed a pronounced antihypoxic activity and decreased approximately twice a zone of myocardial necrosis in rats. Aristolactam D II, aristolactam B II, vanillic acid, dopamine and  $\beta$ -sitosterol  $\beta$ -D-glucoside were isolated from methanol extract of culture A2s.*

**Key words:** *Aristolochia manshuriensis*; cell culture; transformed culture; dopamine; aristolactams.

*Aristolochia manshuriensis* Kom. (Aristolochiaceae) is a relic of the Tertiary Manchurian flora. Stems of this plant, Kwan-Mu-Tong (in Chinese), are used as a cardiotoxic drug in China and Korea.<sup>1</sup> However, the continuous collection of stems causes depletion of natural sources of the plant. The cell culture of *A. manshuriensis* was established to investigate the possibility for creation of alternative raw source.

Earlier we have reported about isolation of *Aristolochia* callus cultures originated from 1- and 2-year stems, roots, leaves and flowers.<sup>2</sup> One-year stem culture (designated as A1 strain) was deposited at the Russian Collection of Plant Cell Cultures as a source of aristolochic acids.<sup>3</sup> Since A1 strain showed a little cardiotoxic activity, we have transformed cells of *A. manshuriensis* by plasmid DNA of *Agrobacterium tumefaciens*; this resulted in the establishment of the suspension cell culture A2s, possessing a strong cardiotoxic activity.<sup>4</sup> The extract of A2s cell culture possessed a pronounced antihypoxic effect, exceeding that of cytochrome C, and decreased approximately twice a zone of myocardial necrosis in rats.<sup>4</sup> To assess the possible role of secondary metabolites in the cardiotoxic effect of transformed *Aristolochia* cells, we investigated more closely the constituents of the methanolic extract of A2s strain.

## EXPERIMENTAL

**Culture A2s.** Cell suspension culture A2s was established after transformation of sterile plantlets of *A. manshuriensis* by *Agrobacterium tumefaciens* strain C58 as described previously.<sup>4</sup> Nutrient medium (W-0) consisted of salt components according to Murashige and Skoog.<sup>5</sup> The medium was supplemented with the following components (mg/l): thiamine·HCl (0.5), pyridoxine·HCl (0.5), nicotinic acid (0.5), myoinositol (100), peptone (50), L-cysteine·HCl (5.0), sucrose (25000) and agar (6000).

**Culture conditions and measurements of growth.** Culture A2s was cultivated in the dark at 25°C in 750-ml Erlenmeyer flasks using orbital shaker at 100 r.p.m. and amplitude 20 mm with 7-10 days intervals. The volume of medium varied within the range 140-170 ml per

flask. Cells further were transferred to bioreactors 3U (16-l), 5U (100-l) and 1T (630-l) using a step-by-step procedure.

*Fresh weight.* Disks of nylon cloth of known wet weight were used to collect the cells and the increase in weight expressed as cell fresh weight (g) per liter of medium.

*Dry weight.* The cells were separated from the culture medium by filtration and dried under flow of hot air at 60°C for 12 h. The results are expressed as cell dry weight (g) per liter.

*Growth index.* It was calculated as: [Yield (g F.W./l) - Inoculum concentration (g F.W./l)]/Inoculum concentration (g F.W./l).

*Extraction and Isolation.* Dried cell biomass of A2s strain (43.3 g) was extracted with MeOH in Zaitsev extractor for 7 h and the solvent evaporated to give a thick brown syrup (12.3 g). Si-gel CC eluting with CHCl<sub>3</sub>-MeOH mixts yielded compounds 1-5, identified as *aristolactam D II (1)*<sup>6,7</sup> (0.015%, on cell dry weight), mp 283-284 °C, *aristolactam B II (2)*<sup>6,7</sup> (0.023), mp 251-256°C, *vanillic acid (3)* (0.068), mp 204-206°C, *β-sitosterol β-D-glucoside (4)* (0.052), mp 303-305°C, *dopamine (5)* (0.052), mp 180-182°C.

## RESULTS AND DISCUSSION

### *Growth parameters of the A2s suspension culture*

Strain A2s has been adapted for laboratory cultivation in the Institute of Biology and Soil Sciences, and for industrial cultivation on Omutninsk Chemical Factory (Kirov Region, Russia). The main culture characteristics for Erlenmeyer flasks and 630-l stirred tank reactor are given in Table 1.

Parameters	750-ml flask	630-l bioreactor
Growth period [days]	10	7-13
Growth index	12-22	5-7
Inoculum concentration (g/l)	20-40	20-30
Yield [g F.W./l medium]	490-580	140-190
Yield [g D.W./l medium]	17-19	10-12
Color of aggregates	white-beige	beige

Table 1 - Growth parameters of cell suspension culture A2s.

### *Constituents of the cell suspension culture A2s and possible role of dopamine in the cardiotoxic effect*

Two aristolactams were isolated from the methanol extract of A2s cell suspension culture and identified as *aristolactam D II (1)* and *aristolactam B II (2)*.<sup>6,7</sup> They were isolated from the roots of *A. argentina*<sup>6,7</sup> and not found in *A. manshuriensis* so far.<sup>1,8</sup> Stems of *A. manshuriensis* contain aristolochic acids I, IV, D and glucoside of aristolochic acid D (aristoloside).<sup>1</sup> Aristolochic acids are tightly related to aristolactams. Little is known about the biosynthesis of these phenanthrene substances. Priestap suggested that aristolochic acids are derived from

aristolactams.<sup>6</sup> Aristolactams are supposed to originate from 3-carboxy-phenylalanine or 3-carboxy-4-hydroxyphenylalanine *via* the modified shikimate pathway.<sup>7</sup>

Callus culture A1, derived from 1-year *A. manshuriensis* stems, produces aristolochic acids in the range of 0.24-0.60%.<sup>3</sup> Cell culture A2s, derived from stem cells of *A. manshuriensis* transformed by Ti-plasmids, appears to have lost ability to synthesize aristolochic acids since these substances were not found in A2s cells during long-term cultivation. It can also be mentioned that amount of phenantrene compounds in A2s culture was one magnitude less compared with A1 culture. Described results led us to conclude that transformation of *Aristolochia* cells by plasmid DNA results in modification of the biosynthesis of phenantrene compounds.

Two derivatives of shikimate pathway were isolated from the methanolic extract of the biomass of A2s cell suspension culture: *dopamine* (5), and *vanillic acid* (3). There is only one evidence about accumulation of dopamine in plant cell cultures,<sup>9</sup> although dopamine is an important derivative of shikimate pathway of higher plants.<sup>10</sup> It may be due to high chemical activity of dopamine which rapidly converts in plants to a number of metabolites, *e.g.* 3-hydroxy-, 5-dimethoxy-phenylethylamine, benzylisoquinolinic alkaloids and others.<sup>10</sup>

Although the mechanism of protection of free dopamine in transformed *Aristolochia* cells is not yet understood, as well as pharmacodynamic properties and metabolism of dopamine absorbed in stomach, one may suppose that dopamine confers cardiotonic effect of the A2s culture.<sup>4</sup>

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