Isolation of β-asarone, an antibacterial and anthelmintic compound, from *Acorus calamus* in South Africa

LJ McGaw, AK Jäger and J van Staden*

Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa
* Corresponding author, e-mail: vanstadenj@nu.ac.za

The aromatic rhizomes of *Acorus calamus* L. are used extensively in traditional medicine worldwide. They reportedly relieve stomach cramps, dysentery and asthma, and are used as anthelmintics, insecticides, tonics and stimulants. Alcoholic rhizome extracts of *A. calamus* growing in KwaZulu-Natal, South Africa, were previously found to have anthelmintic and antibacterial activity. Using bioassay-guided fractionation, the phenylpropanoid β-asarone was isolated from the rhizome. This compound was shown to possess anthelmintic and antibacterial activity. It has previously been isolated from *A. calamus*, and a related species, *A. gramineus*. Different varieties of *A. calamus* exhibit different levels of β-asarone, with the diploid variety containing none of the compound. Mammalian toxicity and carcinogenicity of asarones has been demonstrated by other researchers, supporting the discouragement of the medicinal use of *Acorus calamus* by traditional healers in South Africa.

Introduction

Over the last few hundred years, many introduced plants have been incorporated into traditional medicine in southern Africa. One such example is the now extensive use of *Acorus calamus*, or calamus as it is known. *Acorus calamus*, of the family Araceae, is a reed-like, semi-aquatic, perennial plant with a stout aromatic rhizome. The inconspicuous flowers are compactly arranged on a long, fleshy axis, surrounded by a large leaf-like spathe. All parts of the plant have a pungent, not unattractive smell. It is indigenous to South East Asia, but is now widely distributed in Europe, eastern North America and parts of Africa (Van Wyk *et al*. 1997). It has been cultivated in South Africa for many years and has become a popular component of Zulu medicine.

The plant is used extensively in traditional medicine worldwide. Rhizomes have been used as carminatives, stomachics and to treat dysentery in southern Africa, Europe and Asia (Watt and Breyer-Brandwijk 1962). They are used as tonics, stimulants and aphrodisiacs, and to treat rheumatism, toothache and respiratory ailments (Hutchings *et al*. 1996). In the Cape, the use of the rhizome as a carminative and diarrhoea remedy has been recorded (Watt and Breyer-Brandwijk 1962). In India, the powdered rootstock and rhizome are used as an antispasmodic, anthelmintic and insecticide (Watt and Breyer-Brandwijk 1962). The fragrant oils obtained from the rhizome are also used as flavourants in alcoholic beverages and as fragrant essences in perfumes and sacred oils (Motley 1994).

Essential oils are found in the leaves, rhizomes and roots, tannins occur in the rhizome and roots, and ascorbic acid is found in the leaves and rhizomes (Pamakstyte-Jukneviciene 1971, cited by Hutchings *et al.* 1996). Owing to its previous wide usage as a crude drug, much research has been undertaken on the constituents of the oil. The major chemical constituents of the essential oils are phenylpropanes, monoterpenes and thermolabile sesquiterpenoids (Röst and Bos 1979, cited by Motley 1994). Methyleugenol, cis-methylisoeugenol, β-asarone, geranylacetate, β-farnesene, shyobunone, epishyobunone and isoshyobunone are the most abundant chemical compounds, constituting about 20% of the essential oil (Röst and Bos 1979, cited by Motley 1994). Other chemical components include α-asarone, γ-asarone, calamenene, asaronaldehyde, acorenone, calamenone, *n*-heptanic acid, calamendiol, many sesquiterpenes, and trace amounts of other compounds (Mazza 1985). The proportion of each chemical constituent of the oil, particularly in the case of β-asarone, varies between the varieties of *A. calamus*, corresponding to the ploidy (Röst and Bos 1979, cited by Schmidt and Streloke 1994). The diploid caryotype grows in North America and in parts of Asia (Siberia), and the rhizomes contain little or no β-asarone (Röst and Bos 1979, cited by Schmidt and Streloke 1994). In the triploid caryotype, present in central Europe and Kashmir, the β-asarone content of the rhizomes varies from 9–13%, while in the tetraploid caryotype, found in India, east

Asia and Japan, the essential oil of the rhizomes is 70–96% β-asarone (Röst and Bos 1979, cited by Schmidt and Streloke 1994). The ploidy of African plants has not to our knowledge been investigated.

The compound β-asarone is known to be toxic (Abel 1987, Lander and Schreier 1990), and there is evidence that it may induce duodenal and liver cancer in rats (Haberman 1971, cited by Göggelman and Schimmer 1983). As a result, many countries have discontinued the use of *Acorus* in digestive medicines. Calamus has been banned by the FDA as a food additive and within the last few years many herbal shops have stopped recommending or dispensing it. Although calamus and its products are banned in the United States, they are authorised in Europe, where the highest acceptable concentration in foods and beverages is 0.1mg kg^{-1} and in alcoholic beverages, 1mg kg^{-1} (Lander and Schreier 1990).

Much work has been done on investigating the biological activity of *Acorus calamus*, particularly in India and Europe. Extracts from the plant show larvicidal activity (Chavan *et al*. 1976, cited by Hutchings *et al*. 1996). The antigonadal activity of β-asarone is potentially useful in insect control (Saxena *et al*. 1977). The ethanolic extract has shown anti-secretagogue, anti-ulcer and cytoprotective properties in rats (Rafatullah *et al*. 1994), supporting the use of calamus for the treatment of gastric ailments in traditional medicine. Rhizomes and roots have sedative, hypotensive, analgesic and hallucinogenic effects (Agarwal *et al*. 1956, Sharma and Dandiya 1969). The active hallucinogenic principles are presumed to be α-asarone (chemically similar to mescaline, a psychoactive alkaloid) and the cis-isomer β-asarone (chemically similar to myristicin and kava alkaloids) (Lewis and Elvin-Lewis 1977).

Fungicidal effects of *A. calamus* have been reported (Alankararao and Rajendra Prasad 1981, Vashi and Patel 1987, Saxena *et al*. 1990, Jatisatienr and Jatisienr 1999). An anti-mycotic principle was identified as β-asarone, with α-asarone displaying a similar level of activity (Ohmoto and Sung 1982). The essential oil and alcoholic extracts of the rhizomes have been shown to have antibacterial activity against many Gram-positive and Gram-negative bacteria (Kar and Jain 1971, Alankararao and Rajendra Prasad 1981, Vashi and Patel 1987). The compounds responsible for the antibacterial activity have not to our knowledge been investigated.

The dried rhizome gave positive results as an antiamoebic agent when tested on *Paramecium caudatum* (Chopra *et al*. 1957). Alcoholic extracts of the rhizomes were active against the human affecting nematode *Ascaris lumbricoides* (Kaleysa Raj 1974) and the rhizome oil was active against larvae of the root knot nematode *Meloidogyne incognita* (Singh *et al*. 1991). Both forms of asarone showed a dual effect on the larvae of the nematode *Toxocara canis* (Sugimoto *et al*. 1995). The first was a fast acting temporary paralytic effect, and the other was a slowly-emerging killing effect. Anthelmintic and pesticidal activity of a related species, *Acorus gramineus*, have been reported to be associated with the phenylphenylpropanoids α- and β-asarone (Perrett and Whitfield 1995) . Both isomers of asarone, particularly the β form, have therefore been associated with a wide range of biological activity.

Extracts of *Acorus calamus* rhizomes have previously been found to be active in antibacterial, anthelmintic and anti-amoebic tests (McGaw *et al*. 2000a). This provided impetus for a further study attempting to isolate the active compound(s) using bioassay-guided fractionation, with the antibacterial agar overlay technique (Slusarenko *et al*. 1989) and an anthelmintic assay (Rasoanaivo and Ratsimamanga-Urverg 1993) as the test bioassays.

Materials and Methods

Plant extraction

Acorus calamus L. plants were collected from a shallow (approximately 0.3m deep) pond at Silverglen Nursery in Durban, South Africa in October 1999. A voucher specimen (McGaw47NU) was deposited at the herbarium of the University of Natal, Pietermaritzburg. The leaves, roots and rhizomes were separated and dried at 50°C. The dried, powdered plant parts (4.33g, 13.27g and 17.56g for the leaves, roots and rhizomes respectively) were extracted separately with 100ml ethanol. Extraction was performed by sonication for 30min in a Julabo ultrasound bath, followed by overnight maceration. The extracts were filtered through Whatman No. 1 filter paper and the procedure was repeated twice with the same plant material. The filtrates were air-dried and stored at -20°C.

Antibacterial assays

The antibacterial activity of fractions resulting from each purification stage was tested using the bioautographic assay (Slusarenko *et al*. 1989). An inoculated layer of agar is poured over a developed TLC plate, and lack of bacterial growth in certain areas identifies the presence and location of antibacterial compounds on the TLC plate. TZC (2,3,5-triphenyltetrazolium chloride) at 0.1% (w/v) was included in the agar mixture, as an indicator of bacterial growth. The inhibition of bacterial growth by compounds separated on the TLC plate is visible as white spots against a deep red background. The test organism was *Staphylococcus aureus*.

The Minimal Inhibitory Concentration (MIC) value of the pure compound was determined against the Gram-positive *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) and the Gram-negative *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883). The microplate method of Eloff (1998) in 96-well microtiter plates was used. The pure compound was dissolved in ethanol and serially diluted twofold with water in microtiter plate wells. The antibiotic neomycin and extract-free solutions were included as standard and blank controls. An equal volume (100µl) of bacterial culture (approximately 10^6 bacteria ml^{-1}) was added to each well. Quantification of the bacterial inoculum was carried out as previously described (McGaw *et al*. 2000a). The microtiter plates were covered and incubated overnight at 37°C. As an indicator of bacterial growth, 40µl of 0.2mg ml^{-1} *p*-iodonitrotetrazolium violet (INT) solution were added to each well and incubated at 37°C for 30min. The colourless tetrazolium salt is reduced to a red-coloured product by biological activity, so inhibition of

bacterial growth is visible as a clear well. MIC values were recorded as the lowest concentration resulting in complete inhibition of bacterial growth.

Anthelmintic assay

The anthelmintic activity of extracts, fractions and the pure compound was determined by incubating the sample (at a concentration of 1mg ml^{-1}) with *Caenorhabditis elegans* var. Bristol (N2) nematodes at 25°C for 2h (Rasoanaivo and Ratsimamanga-Urverg 1993, modified by McGaw *et al.* 2000b). Solvent blanks, and levamisole as a standard anthelmintic drug, were included. The percentage and movement of live nematodes after incubation with the plant extracts was recorded and compared to that of the controls.

Isolation

Thin Layer Chromatography (TLC) analysis was used to compare the chemical composition of the crude ethanol extracts of *Acorus* leaves, roots and rhizomes. Bioassay-guided fractionation was subsequently performed using the rhizome extract. The ethanolic rhizome extract (1.14g) was separated by vacuum liquid chromatography (VLC) over silica gel (Merck 230–400 mesh) using a hexane-ethyl acetate gradient solvent system of increasing polarity. A column 13cm in length and with an internal diameter of 2cm was packed with 20g silica. The gradient solvent system was increased by 5% increments from 100% hexane to 60% hexane in ethyl acetate, and subsequently by 10% increments to 100% ethyl acetate. At each concentration, 100ml solvent was flushed through the column. Bioautography established that the most active fraction (270mg) was eluted with 10% ethyl acetate in hexane. This fraction was further separated by TLC on three silica gel plates (Merck Silica gel 60 F$_{254}$, 0.25mm thick) using toluene-ethyl acetate (93:7) to yield 15.6mg of active compound. The purity of this compound, a yellow oil, was confirmed by TLC using various solvent systems.

Identification of isolated compound

High Resolution Mass Spectrometry (MS) using a VG70-SEQ spectrometer was performed. Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR and ^{13}C NMR) was carried out to confirm the structure of the compound. A Varian Unity Inova 500MHz spectrometer was used. The MIC values of the isolated β-asarone against the four previously mentioned test bacteria were determined.

Results

The leaf, root and rhizome ethanol extracts of *Acorus calamus* exhibited antibacterial and anthelmintic activity. The rhizome extract possessed a lower Minimal Inhibitory Concentration (MIC) than the leaf and root extracts (Table 1), against *Bacillus subtilis* and *Staphylococcus aureus*. Therefore, the rhizome extract was selected to perform bioassay-guided fractionation for isolation of active compound(s). Additionally, TLC and bioautography displayed

fewer compounds, other than the target active compound, in the rhizome extract than in the leaf and root extracts. The activity-directed fractionation of 1.14g of rhizome extract yielded 15.6mg of oily yellow β-asarone, representing 1.4% of the extract and 0.09% of the rhizome. The pure compound possessed antibacterial and anthelmintic activity. The MIC values for the isolated β-asarone against the test bacteria are presented in Table 1. The anthelmintic activity of the crude rhizome extract and isolated β-asarone was very similar, both resulting in the death of 60–70% of nematodes after incubation. No other fractions resulting from the fractionation of the rhizome extract exhibited anthelmintic activity. The structure of β-asarone is presented in Figure 1.

The compound identified as β-asarone was a yellow oil, $C_{12}H_{16}O_3$ [M]$^+$ 208.11016. EIMS *m/z* (rel. int.): 208 [M]$^+$ (100), 193 (47), 177 (3), 165 (27), 162 (12), 150 (6), 134 (3), 103 (4), 91 (11), 77 (7), 69 (3), 51 (2). The ^{1}H NMR spectra in CDCl$_3$ were δ 1.84 (3H, dd, J = 7.3Hz and 1.8Hz, -CH=CH-C$\underline{H}_3$), 3.81, 3.83 and 3.89 (each 3H, s, three OCH$_3$), 5.76 (1H, dq, J = 12Hz and 6.8Hz, H-2'), 6.49 (1H, dq, J = 11.4Hz and 1.83Hz, H-1'), 6.53 (1H, s, H-3) and 6.84 (1H, s, H-6). The ^{13}C NMR spectra were recorded in CDCl$_3$, δ 151.5 s (C-4), 148.5 s (C-2), 142.3 s (C-1), 125.8 d (C-1'), 124.7 d (C-2'), 118.0 s (C-5), 114.1 d (C-6), 56.6 q, 56.4 q, 56.0 q (OC$\underline{H}_3$), 14.6 q (C-3').

Discussion

The isolated active compound β-asarone has been isolated previously by others from *Acorus calamus* growing in Europe and Asia (Baxter *et al*. 1960), but has not been evaluated for antibacterial and anthelmintic activity before. This paper demonstrates both the antibacterial and anthelmintic activity of β-asarone. The anthelmintic activity of the isolated β-asarone was very similar to that of the crude rhizome extract. The crude extract and isolated compound were both tested at the same concentration (1mg ml^{-1}). It was expected that the isolated β-asarone would display higher anthelmintic activity than the original extract as it would be present in a higher concentration. However, this was not the case, and a possible explanation may be the instability of β-asarone after isolation, as some breakdown of the compound was visible after TLC analysis. No other anthelmintic compounds were apparent in the VLC fractions.

The antibacterial activity of the isolated β-asarone was lower than that of the crude extract. This may again be partly attributed to the lability of the isolated compound. Also, several other compounds with antibacterial activity in the VLC fractions of the crude extract were visualised in the bioautography assay. They made up such a small part of the overall composition that their identification was not attempted. β-Asarone appeared to be the major component of the crude extract (from TLC analysis, and with its yield of 1.4% of the extract). However, if it were the major antibacterial compound, it would be expected to have a much lower MIC value. This was not the case, so other minor compounds may well have significantly higher antibacterial activity, contributing to a greater proportion of the overall activity even though they are not present in such high quantities as β-asarone. Unfortunately, it is not possible to quantify antibac-

Table 1: Comparison of MIC values obtained using a microplate method (Eloff 1998) for *Acorus calamus* ethanol extracts, isolated β-asarone and the antibiotic neomycin against various bacteria

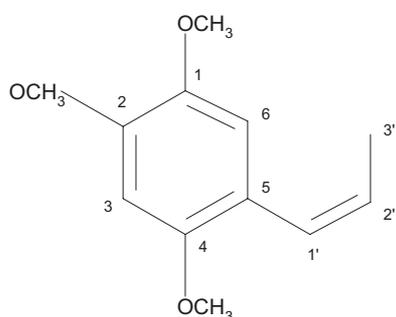| Micro-organism tested	Crude ethanol extract MIC (mg ml⁻¹)			Compound (MIC)	
	Rhizome	Root	Leaf	Isolated ß-asarone (mg ml⁻¹)	Neomycin (μg ml⁻¹)
Bacillus subtilis (ATCC 6051)	0.78	1.56	1.56	3.13	0.39
Escherichia coli (ATCC 11775)	–[1]	–	–	>12.5	0.098
Klebsiella pneumoniae (ATCC 13883)	–	–	–	>12.5	0.098
Staphylococcus aureus (ATCC 12600)	1.56	3.13	3.13	3.13	0.39

–[1] = extract not tested

Figure 1: Structure of β-asarone

terial activity from a bioautography assay, where the inhibition spot is by nature a positive/negative response, and does not reveal detail about the degree of activity of a compound.

The mass spectrometry, ^{1}H and ^{13}C NMR data were consistent with those previously reported for β-asarone (Patra and Mitra 1981, Mazza, 1985, Nigam *et al*. 1990, Oprean *et al*. 1998). Asarone has been synthesized by two different methods (Sharma and Dandiya 1969).

The evidence presented indicates that if the *Acorus calamus* tested in this study is representative of that found in southern Africa, then the South African *Acorus* is likely to be triploid or tetraploid, as these caryotypes of *A. calamus* are known to contain β-asarone (Röst and Bos 1979, cited by Schmidt and Streloke 1994). Also, its origins possibly lie in Asia or Europe. Of some concern is the evidence implicating β-asarone in causing duodenal and liver cancer in test animals. Owing to the possible carcinogenic and toxic effects, the use of *Acorus* in digestive medicines has been discontinued in most countries. In South Africa, *Acorus calamus* is widely used by Zulu traditional healers to treat a variety of ailments, including gastrointestinal disorders. The content of β-asarone substantiates the use of this plant as an anthelmintic and antibacterial remedy, but owing to the toxicity of the active compound, it is recommended that the use of the plant is restricted to external application for antibacterial use, or discontinued altogether.

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