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Pterocarpenes elicited by *Aspergillus caelatus* in peanut (*Arachis hypogaea*) seeds

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ABSTRACT

The substituted pterocarpenes named aracarpene-1 (**1**) and aracarpene-2 (**2**) were isolated from wounded peanut seeds challenged by a strain of *Aspergillus caelatus*. The structures of these putative phytoalexins were determined by interpretation of NMR and MS data. The aracarpenes were investigated for their antifungal and antibacterial activities as well as antioxidant, anti-inflammatory, and cytotoxic activities in mammalian cells. Aracarpene-2 demonstrated high antibacterial properties against tested gram-positive and gram-negative bacteria, whereas aracarpene-1 displayed low antibacterial properties against the same bacteria. Both compounds had no antifungal activity against *Aspergillus flavus*. Together with peanut stilbenoids that are also produced in the challenged seeds, these compounds may represent a class of low-molecular weight peanut metabolites with a defensive role(s) against pathogenic microorganisms.

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1. Introduction

Under favorable conditions, the leguminous peanut plant (*Arachis hypogaea* L.), when infected by a fungal pathogen, is capable of producing stilbene-derived phytoalexins (Ingham, 1976; Keen and Ingham, 1976; Aguamah et al., 1981; Cooksey et al., 1988; Sobolev et al., 2006a,b, 2009), which have been considered the backbone of the plant's inducible chemical defenses. Peanut leaves infected with the early leaf-spot fungus *Cercospora arachidicola* produced two pterocarpanoids, medicarpin and demethylmedicarpin (Edwards and Strange, 1991). However, the latter was considered a degradation product of the former (Edwards and Strange, 1991). Although accumulation of pterocarpanoid and isoflavone phytoalexins is a common reaction of several leguminous plants to challenge by host-pathogenic fungi (Al-Hazimi and Alkhathlan, 2000), medicarpin is the only induced pterocarpanoid previously detected in peanut leaves. Increased concentrations of medicarpin were found only in infected leaves, and therefore this compound has been suggested to play a defensive role as a phytoalexin.

Pterocarpanoids and isoflavones represent the most abundant class of isoflavonoid phytoalexins produced by leguminous plants (Al-Hazimi and Alkhathlan, 2000). Pterocarpans contain a tetracyclic ring system that is derived from the basic isoflavonoid skeleton; pterocarpans are isoflavans in which a furan ring is formed through generation of an ether link between the chromane and the 3-phenyl unit. Pterocarpans possess the highest antifungal activity among the phytoalexins in the flavonoid-based group of compounds (Jiménez-González et al., 2008). In addition to their defensive antifungal functions, pterocarpans display other diverse biological effects, such as antibacterial (He et al., 2006; Tanaka et al., 2004; Rukachaisirikul et al., 2007a; Jiménez-González et al., 2008; Mitscher et al., 1984), anti-inflammatory (Njamen et al., 2003; Selvam et al., 2004), antitumor (Chaudhuri et al., 1995; Maurich et al., 2006; Militao et al., 2006), antioxidant and antiallergenic (Miyase et al., 1999), and antiparasitic (Chanphen et al., 1998; Salem and Werbovetz, 2006) activities, as well as activity against *Anopheles gambiae* adult mosquitoes (Joseph et al., 2004) and common cutworm, *Spodoptera litura* (Morimoto et al., 2006).

Pterocarpenes differ from pterocarpans by incorporation of a double bond between the C-6a and C-11a atoms (Fig. 1). In contrast to hundreds of pterocarpans known from various plant sources (Al-

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Hazimi and Alkhathlan, 2000; Jiménez-González et al., 2008; Boland and Donnelly, 1998), only six members of the pterocarpene (pterocarp-6a-ene) group were known just 10 years ago (Al-Hazimi and Alkhathlan, 2000). A few more pterocarpenes have been isolated since that time, and the biological activities of some of them have been investigated (He et al., 2006; Rukachaisirikul et al., 2007a,b; Chansakaow et al., 2000; Njamen et al., 2003; Tanaka et al., 2001, 2004). Antibacterial activity similar to that of pterocarpans seems to be the most important quality of pterocarpenes. Despite their scarce distribution in plants, pterocarpenes may play an important role in the disease resistance of plants in which they are produced.

Our previous research on peanut defensive mechanisms (Sobolev et al., 2009) established the presence of unidentified compounds that were detected only in fungus-challenged seeds and therefore could be involved in peanut defense against pathogens. The compounds had complex absorption spectra similar to those of known substituted pterocarpenes, but their properties did not match those of any compounds previously reported from peanuts. The purpose of the research described here was to isolate and characterize these metabolites, as they may represent a new class of peanut phytoalexins.

2. Results and discussion

2.1. Structure elucidation of new compounds

An *A. caelatus* strain was chosen as a biotic phytoalexin elicitor because it demonstrated a higher growth rate and faster phytoalexin biosynthesis in our previous experiments (Sobolev et al., 2009). Compared to other strains, *A. caelatus* produced only a few known secondary metabolites, which were easily detected and did not interfere with peanut elicited metabolites. The compounds of interest in this study were initially detected in earlier experiments with wounded, challenged peanut seeds (Sobolev et al., 2009). Because they were observed only in inoculated seed extracts, they were suspected as possible phytoalexins. When the experimental conditions were changed from high temperature – short incubation time to low temperature – long incubation time, the production of the two major compounds of this type increased significantly. These two compounds (**1** and **2**) were targeted for further investigation and isolated from the extracts by column chromatography and HPLC.

The first of these compounds (**1**) was assigned the molecular formula $C_{16}H_{12}O_6$ (unsaturation index = 11) by analysis of NMR and HRMS data. NMR spectroscopic data (Table 1) indicated the presence of two 1,2,3,4-tetrasubstituted aromatic rings (both 1,2,3-trioxygenated according to ^{13}C NMR shift data), an isolated CH_2O unit, a tetrasubstituted double bond, and a methoxy group. ^1H NMR signals corresponding to three phenolic OH groups were also observed. The number of sp^2 carbons present in combination with the formula required a tetracyclic structure. A literature search suggested that **1** was likely to be a pterocarpene derivative (Ferreira et al., 1974; Miyase et al., 1999; He et al., 2006; Tanaka et al., 2002). The ESI-MS^2 data bore some similarities to results reported for 3,9-dihydroxypterocarp-6a-ene (Ingham and Dewick, 1978), and the UV spectrum (Fig. 2) was also consistent with such a structure. More specifically, the units present and the molecular formula were consistent with a pterocarpene system substituted with three OH groups and a methoxy group. HMQC and HMBC NMR spectroscopic data were used to independently verify this conclusion, to determine the substitution pattern, and to locate the methoxy group. The methoxy signal at δ3.94 showed a correlation to an oxygenated aromatic carbon (C-9) which was also correlated with a phenolic OH signal at δ5.70. This phenolic OH showed additional correlations to C-10 and C-10a, both of which are also oxygenated aromatic carbons of the same 1,2,3-trioxygenated aromatic ring. One of the aryl protons of this ring (H-7) showed strong correlations to two of the oxygenated carbons (C-9 and C-10a) and a weak correlation to the third (C-10), which is *para* to H-7. H-8 (*ortho*-coupled to H-7) also shows strong correlations to two of the oxygenated carbons (C-9 and C-10) and a weak correlation to the third (C-10a), which is *para* to H-8. The remaining carbon of this ring (C-7a) was located via its correlations with H-7 and H-8. C-7a was further linked to sp^2 carbon C-6a on the basis of a strong correlation of H-7 to C-6a. The isolated CH_2–O unit (CH_2-6) was connected to C-6a by virtue of its correlations to C-6a and C-7a. H_2-6 also showed strong correlations to two other oxygenated sp^2 carbons, which must correspond to C-4a and C-11a in **1**, although these two assignments could not be distinguished. Even so, these data require C-6 to be connected to the second aromatic ring via an ether linkage. The signals for the second tetrasubstituted aromatic ring could be assigned on the basis of HMBC correlations of H-1 and H-2 (Table 1). Strong correlations of H-1 to two other oxygenated carbons, aside from C-3 and C-4, required C-1a to be connected to an additional oxygenated sp^2 carbon. This was supported by weak correlations of H-2 to the same two carbons. The only ambiguity was the assignment of C-4a and C-11a. Although these two signal assignments were interchangeable, the formula requires connection of O-11 to C-11a to complete the structure of **1**. Compound **1** has not been reported in the literature, and the 3,4,9,10-oxygenation pattern shown in **1** has been described only once previously (in bryacarpene-4, a trimethoxy–monohydroxy pterocarpene derivative obtained from heartwood of *Brya ebenus*) (Ferreira et al., 1974). The common name aracarpene-1 is proposed for **1**.

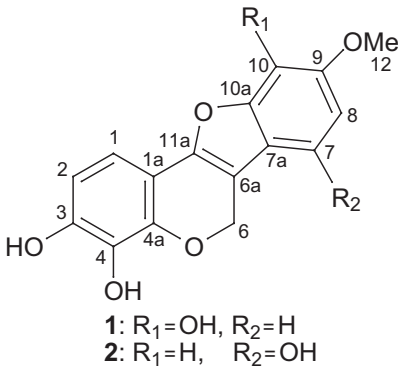

Fig. 1. Structures of aracarpene-1 (1) and aracarpene-2 (2).

Aracarpene-2 (**2**) was recognized as an isomer of **1** by analysis of NMR and HRMS data. Close similarities in the UV (Fig. 2) and NMR spectroscopic data with those of **1** indicated that **2** is also a pterocarpene derivative. The main difference relative to **1** is that the ^1H NMR data of **2** (Table 1) indicated a different substitution pattern for one of the tetrasubstituted aromatic rings. While **1** has two sets of *ortho*-coupled protons, compound **2** shows one pair that is *ortho*-coupled and one pair that is *meta*-coupled (J = 1.9 Hz). The ^{13}C NMR shifts observed also show that one of the aromatic rings in **2** is a 1,3,5-trioxygenated aromatic ring. Once again, HMBC NMR data were used to determine the substitution pattern of **2**. The methoxy group (δ3.80) showed a correlation to C-9, an oxygenated carbon of the 1,3,5-trioxygenated aromatic ring, as well as a weak correlation to C-10, a protonated aromatic carbon. H-10 was also strongly correlated with two oxygenated carbons (C-10a and C-9) and two non-oxygenated carbons (C-7a and C-8), and weakly to a third oxygenated carbon (C-7), which is *para* to H-10. H-8 was correlated with two oxygenated carbons (C-7 and C-9) and two non-oxygenated carbons (C-7a and C-10), but lacked a weak correlation to the third oxygenated carbon (C-10a). H-8 did,