Known and Novel Terpenes from *Buddleja globosa* Displaying Selective Antifungal Activity Against Dermatophytes

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Lipophilic extracts of the stembark of *Buddleja globosa* were found to have antifungal activity at 125 μg/mL against three dermatophytic fungal species but had no activity at 1000 μg/mL against four other fungal species or two yeast species. Bioassay-guided fractionation of Si gel column eluates using the sensitive fungal species resulted in active fractions from which were isolated five compounds that were characterized by spectroscopic methods as one novel and four known compounds. The known compounds were the diterpene buddlejone (**1**), the bisditerpene maytenone, and the two sesquiterpenes buddledin A and buddledin B, while the novel compound was characterized as the diterpene deoxybuddlejone (**2**). The minimum inhibitory concentration of all the compounds was determined against all the microorganisms under test, and buddledins A and B were shown to exhibit the greatest antifungal activity, with values of 43 μM and 51 μM, respectively, against the sensitive fungi *Trichophyton rubrum*, *Tricophyton interdigitale*, and *Epidermophyton floccosum*.

Ethnopharmacological and chemotaxonomic considerations have led to several investigations of the chemical constituents of *Buddleja* species in recent years. Although the majority of uses of this genus in traditional medicine are concerned with the leaves and flowers, some interesting caryophyllane sesquiterpenes[1−3] and one diterpene[4] have been reported from the roots and stembark of a few species. The caryophyllane buddledins A, B, and C have been shown to have piscicidal activity[1,2] and also to inhibit eicosanoid synthesis in vitro.[3] The presence of a reasonably strong odor when the roots of *B. globosa* were dug out of the ground led us to speculate on the ecological role that the volatile substances might play against soil organisms. Preliminary studies showed that the stembark of this species contained similar compounds and, because this stembark was easier to collect, it was used for the current investigation. Fungal species are often found in the soil, and so it was decided to test lipophilic extracts of *B. globosa* bark and roots, which would most likely contain the volatiles, against a range of fungal species, some of which, for example, *Trichophyton* and *Epidermophyum* spp., are of clinical importance as the agents of fungal skin infections. In the past these have been of interest only as the cause of relatively harmless cosmetic disfigurements, but in recent years they have assumed a more serious role as agents of serious opportunistic infections in immunocompromised patients. This has occasioned more effort in the search for novel antifungal agents, and the work reported here is a contribution in this area.

Results and Discussion

Compound **1** was identified as the known compound buddlejone and the others as buddledin A, buddledin B, and maytenone. Buddlejone (**1**) was previously reported from the roots of *B. globosa*,[4] but this is the first report of its presence in the stem bark. The two sesquiterpene buddledins (A and B) have been previously reported from *B. globosa* roots.[3] The presence of maytenone has not been previously reported from members of the Buddlejaceae, but, because its structure corresponds to two fused units similar in structure to buddlejone (**1**), it is very likely to share a common biogenetic pathway.

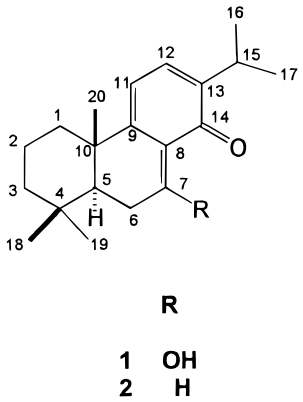

Figure 1. Structures of buddlejone **1** and compound **2**.

The spectral characteristics of compound **2** were similar to buddlejone (**1**).[4] The HRFABMS of **2** gave a $[M + H]^+$ peak at *m*/*z* 285 consistent with the molecular formula $C_{20}H_{28}O$ (D. B. E. = 7), which indicated that it might be a diterpene. Analysis of the ^{13}C NMR spectra for the hydrogen-bonded carbons obtained from the DEPT experiment showed the presence of five methyl, four methylene, five methine, and six quaternary carbons. Examination of the ^1H NMR and 2D COSY spectra indicated the presence of an isopropyl group evident from two methyl doublets at δ 1.13 and 1.14 coupled to a methine group at δ 3.25. The spectrum also showed two geminal methyl groups at δ 0.90 and 0.98 and a tertiary methyl group at δ 1.12. This combination of signals suggested an abietane skeleton similar to **1** but with loss of an O atom. The UV spectrum showed absorption maxima at 397, 288, and 248 nm and no shift on the addition of NaOH, implying the absence of a free phenolic group. The downfield signal in the ^{13}C NMR spectrum at 201.7 confirmed that the oxygen formed part

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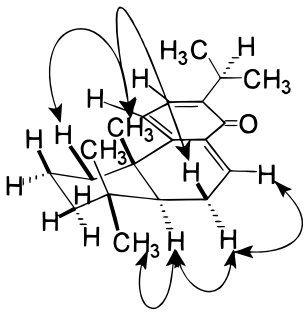

Figure 2. Significant NOE relationships observed in compound **2**.

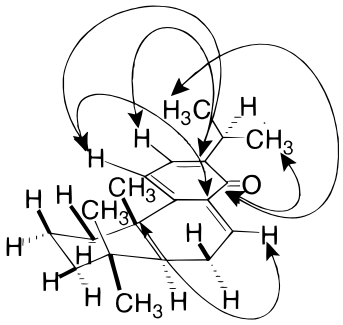

Figure 3. Significant HMBC couplings observed in compound **2**.

of a ketone carbonyl group in the molecule and thus established the absence of an OH group. The yellow color of the compound and its UV spectrum indicated that it possessed an extended conjugated π electron system. The 2D COSY spectrum also showed that the two methine signals at δ 5.96 (H-9) and 7.30 (H-12) were coupled to each other and showed close proximity to each other in the NOESY spectrum. The position of the angular methyl group at C-20 was assigned by COLOC, which showed long-range coupling of δ 1.12 with δ_C 157.5 (C-9). The assignment was fully supported by the one-bond $^{13}C-^{1}H$-correlated spectrum. The close proximity of the protons represented by the methine signal at δ 5.96 (H-11) to the doublets at δ 2.01 (H_a-1) and multiplet at δ 1.49 (H_a-3) was established by NOESY. Similarly, the proximity of the isopropyl group to the aromatic methine at δ 7.30 (H-12) was observed. The NOESY spectrum also indicated that the methylene group at C-6, showing signals at δ 2.63 and 2.61, was spatially close to the methine group at δ 7.85 (H-7). The relative stereochemistry of the C-20-methyl group and 5-H were found to be trans, as the NOE correlation spectrum showed that they were spatially close to the opposite methyl groups attached to C-4 and the protons at C-6. By analogy with buddlejone, the 20-CH_3 was deemed to be β, that is, above the plane of the ring system, thus making the 5-Hα (see Figure 2). All nonquaternary carbons were assigned by the $^{13}C-^{1}H$ correlation spectrum. The quaternary carbons were assigned by $^{13}C-^{1}H$ COLOC (see Table 1, Figure 3) and by comparing the chemical shifts of the similar abietane compound buddlejone (**1**). Because the compound showed spectral characteristics similar to **1** and the major difference is the absence of the C-7 OH, the compound was named deoxybuddlejone and assigned the structure **2**, as shown, which is 7,9,12-abietrien-14-one. This novel compound is the second diterpene to be isolated from the genus *Buddleja*.

The chloroform extract of stem bark showed no activity, even at 1000 μg/mL against six of the fungal species tested: *Aspergillus niger*, *Candida albicans*, *Penicillium notatum*, *Saccharamyces cerevisiae*, *Scopulariopsis brevicaulis*, and *Scytalidium dimidiatum*. In contrast, extracts at 250 μg/mL inhibited the growth of the three dermatophytes *Trichophyton rubrum*, *Trichophyton interdigitale*, and *Epidermophyton floccosum*. It is interesting, as these dermatophytes are responsible for some skin infections, that extracts of some *Buddleja* species are reportedly used to treat unspecified skin diseases,[5] and the antifungal activity may be connected with these uses.

The results (Table 2) show that most of the isolated compounds inhibited the growth of only the dermatophytes (*T. rubrum*, *T. interdigitale*, and *E. floccosum*) at concentrations less than 1000 μg/mL.

The MIC values obtained for the isolated compounds show that the most active of the isolated compounds compared to miconazole were buddledin A (**3**) and buddledin B (**4**), which are caryophyllane sesquiterpenes. They

Table 1. NMR Spectral Data for Compound **2** in $CDCl_3$

C/H no.	^{1}H (δ, J in Hz)	^{13}C[a]	$^{1}H-^{1}H$ COSY	COLOC
1	ax 2.01, d (11.1)	37.1, t	H-2 ax, H-11, Me-20	H-3, H-5, Me-20
	eq 1.55, m		H-2 eq	
2	ax 1.72, m	19.0, t	H-1 ax, H-3 ax	
	eq 1.55, m		H-1 eq, H-3 eq	
3	ax 1.49, m	42.4, t	H-2 ax, Me-19	H-1, H-5, Me-18, Me-19
	eq 1.46, m		H-2 eq	
4		33.6, s		H-2, H-6
5	1.63, m	52.9, t	H-6 eq, Me-19	H-3, H-7, Me-18, Me-19, Me-20
6	ax 2.61, m[b] (6.8)	27.6, t	Me-20 H-5, H-7, Me-19	
	eq 2.63, m[b] (6.8)			
7	7.85, t (5.8)	148.3, d	H-6 eq	5-H
8		140.6, s		6-H, 11-H
9		157.5, s		1-H, 5-H, 12-H, Me-20
10		36.7, s		2-H, 6-H, 11-H
11	5.96, s	117.9, d	H-12	
12	7.30, d (1.6)	143.2, d	H-11, H-15, Me-16, Me-17	15-H
13		128.4, s		11-H
14		201.7, s		12-H, Me-16, Me-17
15	3.25, dq (4.3, 1.6)	36.9, d	H-12, Me-16, Me-17	12-H
16	1.14, d (4.3)	20.4, q	H-12, H-15	Me-17
17	1.13, d (4.3)	20.3, q	H-12, H-15	Me-16
18	0.98, s	33.4, q	Me-20	3-H, 5-H, Me-19
19	0.93, s	22.4, q	H-3 ax, H-5, H-6	3-H, 5-H, Me-18
20	1.12, s	22.0, q	H-6 ax, H-11, Me-18	1-H, 5-H

[a] Multiplicity deduced from DEPT spectroscopy. [b] Signal appeared as a 'split' multiplet, J value given is that due to coupling between 6-H geminal protons.

Table 2. Minimum Inhibitory Concentration μg/mL (μM) for Isolated Compounds Against Fungal Species[a]

	compound					
fungal species	miconazole 2.4 μg/mL (ca. 5 μM)	buddlejone (**1**)	deoxybuddlejone (**2**)	buddledin A (**3**)	buddledin B (**4**)	maytenone (**5**)
E. floccosum	NGO[b]	750 (2500 μM)	750 (2640 μM)	12 (43 μM)	12 (51 μM)	375 (625 μM)
T. rubrum	NGO	750 (2500 μM)	750 (2640 μM)	12 (43 μM)	12 (51 μM)	375 (625 μM)
T. interdigitale	NGO	750 (2500 μM)	750 (2640 μM)	12 (43 μM)	12 (51 μM)	375 (625 μM)

[a] No inhibition of growth was observed at 1000 μg/mL for any of the test compounds against *A. niger*, *C. albicans*, *P. notatum*, *S. cerevisiae*, *S. brevicaulis*, and *S. dimidiatum*. [b] NGO = no growth observed.

both inhibited the dermatophytes at a concentration of 12 μg/mL (equivalent to 43 μM for **3** and 51 μM for **4**). The bisditerpene maytenone (**5**), for which the MIC value was 375 μg/mL (620 μM), displayed only weak activity, while the diterpenes buddlejone (**1**) and deoxybuddlejone (**2**) were comparatively inactive. The activities of the most active isolated compounds were less than that of the control broad-spectrum antifungal agent miconazole by at least 1 order of magnitude. The antifungal activity of caryophyllane sesquiterpenes has not been previously reported, so the activity for such compounds reported here is of interest from an ecological perspective, as they might protect the plant from fungal attack. The novelty of this class of compounds may also form the basis of further investigations into their potential as lead molecules for clinically useful antifungals.

Experimental Section

General Experimental Procedures. UV spectra were acquired on an AVIV 17DS spectrometer using MeOH as solvent. The IR spectra were measured on a Perkin-Elmer 1600 series FTIR spectrometer (Nujol, KBr disks). ^{1}H and ^{13}C NMR spectra were recorded at 400/100 MHz on a Bruker AMX 400 NMR spectrometer (ppm, *J* in Hz, using TMS as internal standard). DEPT experiments were carried out with the polarization pulse $\theta = 45°$, 90°, and 135°. Standard programs from the library $^{n=3}J_{CH} = 10$ Hz were used for the COLOC experiment. The optical rotation was recorded on a Perkin-Elmer 141 polarimeter, calibrated with sucrose solution in water (10 mg/mL, $[\alpha]_D = 66.8°$). FABMS and EIMS data were recorded on a high-resolution spectrometer (KRATOS MS890MS and JEOL JMS-AX505W spectrometers). Melting points (uncorrected) were determined on a Townson and Mercer melting point apparatus.

Plant Material. Stem bark of *B. globosa* was obtained in May 1997, from a garden specimen in southwest London. The plant was authenticated by one of us (PJH), and a voucher specimen (Bg 004) is deposited in the herbarium of the Department of Pharmacy, King's College, London. A sample of the bark is also deposited as sample Bud 21M1 in the museum of the Department of Pharmacy, King's College, London.

Extraction and Isolation. The freshly peeled stem bark of *B. globosa* (250 g) was dried in the shade, coarsely powdered, and extracted with $CHCl_3$ (1.0 L) at 50 °C in a Soxhlet apparatus for 48 h. The extract was concentrated under reduced pressure to a viscous syrupy residue (2.8 g). The extract was then subjected to flash column chromatography on Si gel (40−60 Å) using the solvent system: light petroleum ether (40−60 °C), $CHCl_3$, and MeOH in order of increasing polarity. Fractions were monitored by TLC, and similar fractions were combined to give 20 fractions MM1−MM20. Each column fraction was tested at 125 μg/mL for antifungal activity against the three sensitive species, and only fractions MM4−MM6 inhibited growth.

The active fractions MM4−MM6 were combined and five compounds isolated by preparative Si gel TLC (light petroleum ether−EtOAc, 15:1) as **1** (20 mg) [hR_f 70], **2** (15 mg) [hR_f 62], **3** (12 mg) [hR_f 49], **4** (10 mg) [hR_f 48], and **5** (20 mg) [hR_f 46].

Compound **1** was identified as buddlejone by comparing its spectral and chromatographic characteristics with an authentic sample previously isolated in our laboratories,[4] while **3** and **4** were identified as buddledin A and buddledin B, and **5** was identified as the bisditerpene maytenone by comparing their spectroscopic data with the literature.[1,6,7] Attempts to procure an authentic sample of maytenone for chromatographic comparison were unsuccessful.

Deoxybuddlejone (2): obtained as a yellow oil, $[\alpha]_D$ −13.82°, ($CHCl_3$; *c* 1.302 mg/mL); UV (EtOH) λ_{max} nm (log ϵ) 397 (2.80), 288 (3.39), 248 (3.46); IR (KBr) ν_{max} cm^{-1}: 2936, 1662, 1634, 1386 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 400 MHz), see Table 1; HRFABMS $[M + H]^+$ measured 285.2230 (calcd for $C_{20}H_{29}O$ 285.2218); EIMS (probe) 70 eV *m/z* (rel int) 284 (100) $[M]^+$, 241 (60), 152 (38), 128 (25).

Test Organisms for Antifungal Assay. *Saccharomyces cerevisiae* NCIMB 080178, *Candida albicans* ATTC 10234, *Aspergillus niger* ATCC 16404, and *Penicillium notatum* ATCC 11654 were obtained as freeze-dried cultures from the Department of Pharmacy culture collection. *Trichophyton rubrum* EL 5095, *Trichophyton interdigitale* EL 5171, *Epidermophyton floccosum* SJH, *Scopulariopsis brevicaulis* EL 3839, and *Scytalidium dimidiatum* EL 936 were obtained from the Dermatology Department of St. Thomas's Hospital, London. The yeasts were grown on Sabouraud's dextrose agar at 30 °C for 24 h before use in the assay. The molds were also cultured on Sabouraud's dextrose agar, incubated at 26 °C, and grown for 10 days before being used for the assay. The cultured microorganisms were harvested by adding sterile phosphate-buffered saline (PBS) to the agar and gently scraping the surface of the agar to release the organism, which was then transferred to a sterile test tube. The molds were filtered through sterile glass wool to remove the mycelium but allowing the spores to pass through. Freshly prepared spore and yeast suspensions were prepared for each assay.

Standardization of Test Inocula. A series of suspensions with a nephelometer reading of between 4 and 6 was prepared and viable counts determined as described briefly below. The suspension of the organism was diluted 1 in 10^6, and 1 mL of this dilution was added to a sterile Petri dish. Next, 20 mL of molten agar was added to the Petri dish, and the agar allowed to set. Then it was incubated for 24 h. The colony-forming units (CFU) were counted and the number of viable organisms in the original yeast suspension estimated by extrapolation, taking the dilution factor into consideration.[8] The yeast suspension was standardized such that 100 μL would contain 10^5 CFU. In the case of the molds, a similar viable count method was used to standardize the mold suspension so that 100 μL would contain 10^4 CFU.

Antifungal Assay Procedure. The agar dilution method was chosen as the appropriate assay to determine the minimum inhibitory concentration (MIC) of the extract on the growth of the fungi, in as much as the hydrophobic nature of the $CHCl_3$ extract of *B. globosa* may prevent its diffusion into the aqueous matrix of the agar or broth medium. DMSO was chosen as the appropriate solvent to dissolve the extract, and, in order not to miss any antifungal activity of the extract, a very high initial concentration (1000 μg/mL) of the test solution was prepared.

A stock solution of the extract was prepared by dissolving 4 mg of the extract in 50 μL of DMSO and sonicating to ensure thorough mixing. Sterile water was added to make the volume up to 2 mL with sonication. Six steps of a 1:2 serial dilution of 1 mL of the test solution were then carried out in a microtiter well plate, and 1 mL molten double-strength Sabouraud's

dextrose agar was added to each dilution of the extract. The dilutions were thoroughly mixed, and 200 μL of the test extract were introduced into 96-well plates in order of increasing concentration. Miconazole was used as a positive antifungal control. For the *C. albicans*, 100 μL of the suspension was added to each well and the plate was incubated at 30 °C for 24 h. A similar procedure was repeated for the other test organisms, and the molds were maintained at 26 °C for 10 days. The plates were examined daily throughout the incubation period for growth of mycelia, and activity was determined by the minimum concentration showing absence of growth. The experiment was repeated three times. Results are presented in Table 1.

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