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In Vitro Evaluation of the Antimicrobial Activity of Lichen Metabolites as Potential Preservatives

KRISTÍN INGÓLFSDÓTTIR,† SALLY F. BLOOMFIELD, AND PETER J. HYLANDS*

Department of Pharmacy, Chelsea College, University of London, London SW3 6LX, England

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Antimicrobial screening of several lichen species and subsequent isolation and structure elucidation of active compounds revealed that the hydrolysis products of certain lichen metabolites, i.e., depsides, were active against gram-negative bacteria and fungi as well as gram-positive bacteria. The active constituents isolated from *Stereocaulon alpinum* and *Peltigera aphthosa* were identified, respectively, as methyl β-orsellinate and a mixture of methyl and ethyl orsellinates. MIC determinations indicated that activity of these compounds was superior to that of the commonly used preservative agents methyl and propyl *p*-hydroxybenzoates and was of the same order as that of chlorocresol.

Lichens are nonflowering plants which consist of two partners living in symbiotic association, an alga (the phycobiont) and a fungus (the mycobiont). In the folklore of many European countries, lichens were used as a remedy for pulmonary tuberculosis and in the treatment of wounds and skin disorders. These medical uses to some extent have been confirmed by studies which showed that many lichen metabolites such as depsides, depsidones, and usnic acid are active against mycobacteria and gram-positive bacteria (8). With a few exceptions, however, activity of lichen metabolites against gram-negative bacteria and fungi has not been reported (2, 7).

Although the range of preservative agents investigated for use in pharmaceutical products is extensive, experience over the last few years has indicated that, within that range, the actual choice of suitable agents may be severely limited by, for example, toxicity, skin irritancy, loss of activity due to interaction with formulation ingredients, and packaging (6). In particular, whereas many currently used agents show good activity against gram-positive bacteria, activity against some gram-negative species (notably the pseudomonads) and fungi tends to be limited (6). Such problems indicate an urgent need for development of new preservatives and preservative systems.

Thus, this investigation was concerned with the antimicrobial screening of 17 lichen species indigenous to Iceland. Those species exhibiting activity against gram-negative bacteria and fungi were investigated further for the presence of potential preservatives. Several active compounds were isolated, and their chemical structures were elucidated from spectroscopic evidence. The discussion here will be restricted to compounds which represent hydrolysis products of the depsides atranorin (Fig. 1A) and tenuiorin (Fig. 1B). The spectrum of activity of each compound was established by using six test organisms.

MATERIALS AND METHODS

Microorganisms. *Staphylococcus aureus* NCTC 10788, *Bacillus subtilis* NCTC 8236, and *Pseudomonas aeruginosa* NCTC 6750 were obtained as freeze-dried cultures from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9, England. *Candida albicans* NCPF 3179 was obtained from the London School of Hygiene and Tropical Medicine, London, England, as a freeze-dried culture, and *Escherichia coli* was obtained from the Chelsea College culture collection, University of London, London, England. *Aspergillus niger* ATCC 16404 was obtained as an actively growing slope culture from Reckitt and Colman Microbiological Laboratories, Hull, England. Stock cultures were maintained on tryptone soya agar (for bacteria and yeasts) or on malt extract agar (for fungi) and subcultured once a month. All stock cultures were stored at 4°C. Before testing, fresh cultures were grown in tryptone soya broth (TSB) (18 h, 37°C) for bacteria and yeasts or on malt extract agar slopes (1 week, 25°C) for fungi. These were used to prepare washed suspensions in one-quarter-strength Ringer solution containing the required number of CFU per milliliter. All media were obtained from London Analytical and Bacteriological Media Ltd., London, England.

Isolation of antimicrobial agents. The lichens (Table 1) were collected from various locations in Iceland and cleansed of extraneous material before being ground into a fine powder. For primary screening, small samples (1 g) of powdered plant material were successively extracted in a Soxhlet apparatus by using the following solvents: light petroleum boiling range, 40 to 60°C, chloroform, acetone, ethanol, methanol, and water. Water extracts were freeze-dried, and other extracts were evaporated to dryness under reduced pressure. For preparation of test solutions, no single solvent was found capable of dissolving all extract residues; the light petroleum and chloroform extract residues were dissolved in chloroform, the acetone extracts were dissolved in acetone, the ethanol and methanol extracts were dissolved in methanol, and the water extracts were dissolved in water. The primary antimicrobial screen was orientated qualitatively rather than quantitatively, and the test solutions were prepared at maximum permissible concentrations (3.0 to 11.0 mg/ml) to ensure the maximum chance of detecting activity due to the presence of minor components (Table 2).

For quantitative secondary screening purposes, large-scale extractions were carried out. *Stereocaulon alpinum* (800 g), *Peltigera aphthosa* (512 g), and *Thamnolia subuliformis* (160 g) were extracted with solvents of increasing polarity as described above. The chloroform extract (17.7 g) of *Stereocaulon alpinum* was fractionated by passing

* Corresponding author.
† Present address: Department of Pharmacy, University of Iceland, 101 Reykjavík, Iceland.

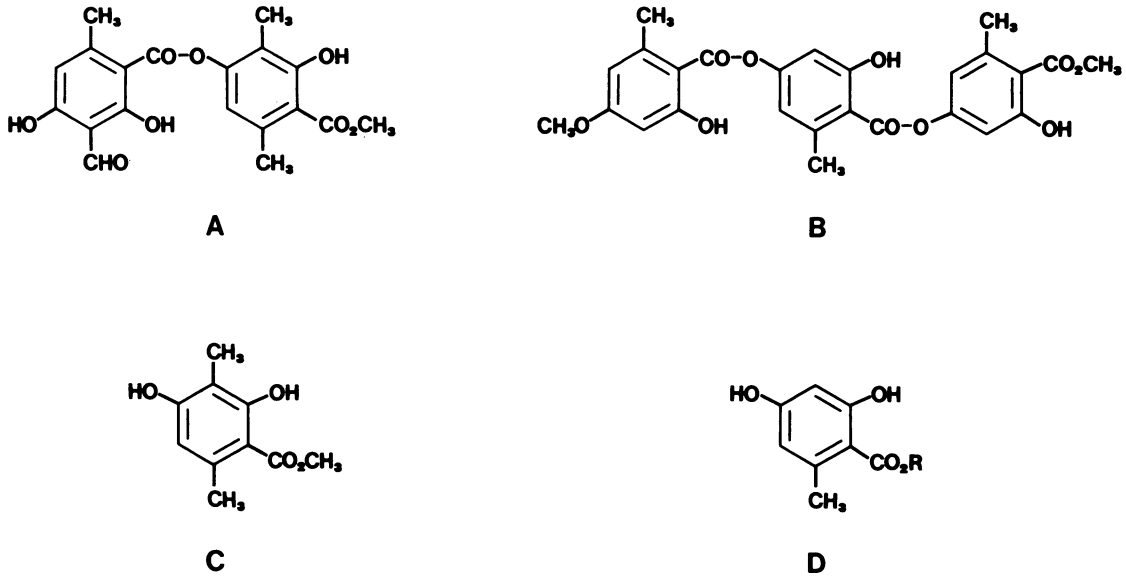

FIG. 1. Structures of atranorin (A), tenuiorin (B), methyl β-orsellinate (C), and methyl (R = CH_3) and ethyl (R = C_2H_5) orsellinates (D).

it through a column of silica gel G and eluting with hexane, then with increasing concentrations of chloroform in hexane, followed by chloroform, and finally with increasing concentrations of methanol in chloroform. Through continuous monitoring for antimicrobial activity, the fraction responsible was isolated. From this fraction, 175 mg of methyl β-orsellinate (Fig. 1C) was isolated, and its structure was elucidated from UV, infrared, nuclear magnetic resonance, and mass spectroscopic data.

The chloroform extract (33 g) of *Peltigera aphthosa* was fractionated in a similar manner to give 508 mg of a mixture of methyl and ethyl orsellinates (Fig. 1D).

Antimicrobial susceptibility testing for bacteria and yeasts. Disk diffusion and turbidimetric methods were used for the primary screening of plant extracts against bacteria and yeasts and were performed in accordance with the recommendations of the European Pharmacopoeia (3). Due to the hydrophobic nature of the majority of the extracts, which could limit agar diffusion of active constituents, the turbidimetric method was used wherever possible. However, sensitivity tests indicated that the more volatile solvents were inhibitory to *Staphylococcus aureus*, *B. subtilis*, and *C. albicans*, and the disk diffusion method was used for testing chloroform and light petroleum extracts against these species.

For the turbidimetric method, 3-ml quantities of TSB were inoculated with 50 μl of a bacterial or yeast suspension of 0.6 × 10^6 CFU/ml. Test solutions (50 μl, prepared as above) were added to the inoculated broths. (For tests with *Pseudomonas aeruginosa*, 20 μl only of test solutions in chloroform was used since the inoculum was sensitive to larger volumes.) Tubes were incubated for a period of 18 to 24 h at

TABLE 1. Summary of primary screening results

Plant species	Activity[a] against:				
	Staphylococcus aureus	*B. subtilis*	*E. coli*	*Pseudomonas aeruginosa*	*C. albicans*
Alectoria ochroleuca (Hoffm.) Mass.	PC	PCH	–	–	PCA
Cetraria islandica (L.) Ach.	P	P	–	–	PC
Cladonia arbuscula (Wallr.) Rabenh.	PC	PC	–	–	PCA
Cladonia gracilis (L.) Willd.	–	–	–	–	–
Cladonia rangiferina (L.) G. Web. ex Wigg.	P	P	–	–	P
Cladonia uncialis (L.) Wigg.	PCM	PC	–	–	PCAEM
Cornicularia aculeata (Schreb.) Ach.	PC	PC	–	–	PC
Parmelia saxatilis (L.) Ach.	–	–	–	P	–
Peltigera aphthosa (L.) Willd.	PC	A	PA	(P)(C)A	PCA
Peltigera canina (L.) Willd.	–	E	–	P	–
Peltigera leucophlebia (Nyl.) Gyeln.	C	–	P	(P)(C)	–
Pertusaria oculata (Dicks.) Th. Fr.	A	–	–	–	–
Stereocaulon alpinum Laur	CA	PA	(C)	A	PCA
Stereocaulon arcticum Lynge	C	C	–	(P)	–
Stereocaulon vanoyei Duvign.	C	–	–	(P)	–
Stereocaulon vesuvianum Pers.	C	C	–	P	W
Thamnolia subuliformis (Ehrh.) W. Culb.	P	P	P	–	P

[a] –, No activity; letters indicate activity in light petroleum b.r. 40–60°C (P), chloroform (C), acetone (A), ethanol (E), methanol (M), and water (W) extracts; ( ), variable activity.

TABLE 2. Concentration of plant extract residues in test solutions used for primary antimicrobial screening

Plant species	Concn (mg/ml) of plant extract residues					
	Light petroleum	Chloroform	Acetone	Ethanol	Methanol	Water
Alectoria ochroleuca	7.1	5.0	10.4	7.0	3.0	6.0
Cetraria islandica	4.2	4.3	7.7	5.5	4.0	10.4
Cladonia arbuscula	9.9	5.0	9.7	6.0	5.6	6.0
Cladonia gracilis	11.0	6.3	8.6	4.4	3.2	4.2
Cladonia rangiferina	6.3	7.0	11.0	4.0	3.0	4.8
Cladonia uncialis	9.0	4.5	10.2	3.5	4.2	6.3
Cornicularia aculeata	10.0	4.6	6.6	7.2	6.0	8.7
Parmelia saxatilis	8.3	7.0	6.4	5.3	3.0	7.0
Peltigera aphthosa	4.0	6.2	3.8	3.4	8.2	6.8
Peltigera canina	4.7	5.8	8.2	8.6	6.5	11.0
Peltigera leucophlebia	10.4	8.1	9.7	7.2	8.0	6.6
Pertusaria oculata	10.7	10.0	10.2	4.7	3.2	6.3
Stereocaulon alpinum	6.0	4.4	3.3	6.0	3.0	5.0
Stereocaulon arcticum	8.6	8.6	9.5	9.5	7.0	8.0
Stereocaulon vanoyei	4.0	9.3	3.7	3.1	4.5	4.3
Stereocaulon vesuvianum	9.5	6.6	4.7	5.6	6.3	4.6
Thamnolia subuliformis	4.0	8.5	3.0	4.8	4.0	6.0

37°C (bacteria) and 30°C (yeasts), after which the presence or absence of growth was recorded.

For the disk diffusion method, tryptone soya agar (4 ml) was inoculated with 66 μl of a bacteria or yeast suspension of 0.6×10^6 CFU/ml and poured onto the surface of a prepared tryptone soya agar plate. The sterile disks (diameter, 10 mm) were impregnated with 100 μl of test solution. After evaporation of the solvent, each disk contained 0.3 to 1.1 mg of crude extract. The disks were placed onto the surface of the inoculated plates and incubated for 18 to 20 h at 37°C (bacteria) or 30°C (yeasts). The diameters of inhibition zones were measured (two measurements at right angles were taken). Positive results refer to inhibition zones with a mean diameter of more than 13 mm.

MIC determinations. The broth dilution method (1) was used to determine the MICs of methyl β-orsellinate and the methyl and ethyl orsellinates against bacteria and fungi. TSB (3 ml) was inoculated with bacterial or fungal suspensions to give a final concentration of 10^4 CFU/ml. For *Pseudomonas aeruginosa* and *E. coli*, test compounds were dissolved in chloroform, and various volumes of the solution (up to 50 and 20 μl, respectively) were added to the inoculated broth. For testing against the other organisms (which were sensitive to chloroform), solutions in dimethylsulfoxide (DMSO) (up to 75 μl) were used. The MIC was determined as the lowest concentration of the test compound which prevented visible growth after incubation at 37°C for 20 h.

Preservatives. Chlorocresol and methyl and propyl *p*-hydroxybenzoates were obtained from BDH Chemicals Ltd, Poole, England. MICs were determined as described above. Test solutions were prepared in DMSO or sterile distilled water (for chlorocresol) and in DMSO or TSB (for the *p*-hydroxybenzoates). Interestingly, when the *p*-hydroxybenzoates were used as DMSO solutions, their activity was markedly reduced and therefore only MIC values for TSB solutions are quoted. By contrast, the use of DMSO or water had no effect on the MIC values for chlorocresol.

RESULTS

Preliminary investigations involved the screening of extracts from small samples of each lichen for antimicrobial activity against *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *B. subtilis*, and *C. albicans*.

The majority of lichen species exhibited activity against the gram-positive organisms. In addition, however, several extracts were active against the gram-negative organisms and *C. albicans* (Table 1). Most of the activity resided in the chloroform and acetone extracts. On the basis of these screening results, three species were chosen for further study, *Stereocaulon alpinum*, *Peltigera aphthosa*, and *T. subuliformis*, and larger quantities of these lichens were extracted and retested. For *Stereocaulon alpinum* and *Peltigera aphthosa*, activity then was found to reside in the chloroform extracts which therefore were subjected to chemical analysis after chromatographic separation.

From the chloroform extract of *Stereocaulon alpinum*, methyl β-orsellinate (methyl 2,4-dihydroxy-3,6-dimethylbenzoate; Fig. 1C) was isolated. This compound is believed to be a hydrolysis product of atranorin (Fig. 1A; 5).

From the chloroform extract of *Peltigera aphthosa*, colorless crystals were isolated which were shown by spectroscopic methods to be a mixture of methyl orsellinate (methyl 2,4-dihydroxy-6-methylbenzoate; Fig. 1D; R = Me) and ethyl orsellinate (ethyl 2,4-dihydroxy-6-methylbenzoate; Fig. 1D; R = Et). Integration of the carboxyethyl and carboxymethyl signals in the hydrogen-1 nuclear magnetic resonance spectrum revealed the ratio of ethyl or methyl esters to be 7:3. These compounds are most likely hydrolysis products of tenuiorin (Fig. 1B).

Further screening of these compounds for activity by the broth dilution method to determine MIC values (Table 3) indicated activity against a broad spectrum of bacterial and fungal species. For the gram-positive bacteria and *C. albicans* and *A. niger*, MIC values ranged from 80 to 160 μg/ml for methyl β-orsellinate and from 160 to 500 μg/ml for the methyl and ethyl orsellinate mixture. Although both compounds showed good activity against *Pseudomonas aeruginosa* (MIC values of 30 to 80 μg/ml for methyl β-orsellinate and 200 to 260 μg/ml for methyl and ethyl orsellinates), activity against *E. coli* as determined by this method was found to be variable at concentrations which represent maximal water solubility.

The light petroleum extract of *T. subuliformis* proved inhibitory against all test organisms. Unfortunately, how-

TABLE 3. MICs, determined by the broth dilution method, of isolated compounds and existing preservatives

Compound or preservative	MIC[a] (μg/ml) for:					
	B. subtilis	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*E. coli*	*C. albicans*	*A. niger*
Methyl β-orsellinate	160	80–160	30–80	(596)	80–160	80–160
Methyl and ethyl orsellinates	330	330	200–260	(833)	160–330	330–500
Chlorocresol	100	200	400	200	300	100
Methyl *p*-hydroxybenzoate	1,500	2,000	2,000	1,000	800	800
Methyl and propyl *p*-hydroxybenzoate	600	800	1,500	800	400	400

[a] ( ), Variable activity.

ever, isolation of the active principle was impeded by the lack of sufficient plant material. The lichen is extremely difficult to collect in substantial quantity as it grows mainly as tiny scattered single strands.

DISCUSSION

Antimicrobial screening of extracts from a number of Icelandic lichen species indicated that the constituent compounds methyl β-orsellinate and a mixture of methyl and ethyl orsellinates exhibit a broad spectrum of activity, being active against gram-negative bacteria and fungi as well as gram-positive bacteria. In particular, these compounds were found to be active against *Pseudomonas aeruginosa*, an organism which is quite frequently encountered in pharmaceutical products and is particularly resistant to many preservative agents currently in use (6). Structurally, methyl β-orsellinate and the orsellinic acid esters may be compared with two types of preservatives commonly used in pharmaceutical and cosmetic products, namely substituted phenols and *p*-hydroxybenzoic acid derivatives.

MIC determinations, with the exception of those for *E. coli*, indicate that both the methyl β-orsellinate and the methyl and ethyl orsellinates were noticeably more active than the *p*-hydroxybenzoates (MIC values of 30 to 500 μg/ml compared with 400 to 2,000 μg/ml for the *p*-hydroxybenzoates) and showed the same order of activity as chlorocresol (MIC values of 100 to 400 μg/ml). Despite the fact that preliminary screening of extracts showed activity against *E. coli*, MIC tests indicated that activity against this organism was relatively poor and suggested the need for further screening with other gram-negative species.

These findings are in accordance with those of Fujikawa et al. (4). In a study of the activity of orsellinic and β-orsellinic acid esters against Hiochi bacteria from Japanese sake, these workers found that methyl β-orsellinate was approximately 10 times more active than the *p*-hydroxybenzoic acid ester and 20 to 40 times more active than salicylic acid.

The broad antimicrobial spectrum and increased potency of methyl β-orsellinate and methyl and ethyl orsellinates compared with existing analogs have thus stimulated an interest both in the further development of these compounds as potentially useful preservative agents and in a more extensive examination of other lichens for this purpose.

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