ANTIBIOTIC COMPOUNDS FROM NEW ZEALAND PLANTS: METHYL HAEMATOMMATE, AN ANTI-FUNGAL AGENT FROM *STEREOCAULON RAMULOSUM*

B.J. HICKEY, A.J. LUMSDEN, A.L.J. COLE, & J.R.L. WALKER[1]

Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand.

(Received 24 April, 1990; revised and accepted 27 May, 1990)

ABSTRACT

Hickey, B.J., Lumsden, A.J., Cole, A.L.J & Walker, J.R.L. (1990). Antibiotic compounds from New Zealand plants: Methyl haematommate, an anti-fungal agent from *Stereocaulon ramulosum*. *New Zealand Natural Sciences* 17: 49-53.

Methyl haematommate (methyl, 3-aldo-2:4-dihydroxy-6-methylbenzoate) was isolated from the lichen *Stereocaulon ramulosum*. This compound showed marked antibiotic activity against dermatophytic fungi: treated cells showed gross morphological changes and marked differences in their pattern of sterol metabolism.

KEYWORDS: *Stereocaulon ramulosum* - lichen - anti-mycotic - dermatophytes - methyl haematommate - steroid biosynthesis.

INTRODUCTION

Earlier studies in this laboratory (Calder *et al.* 1986) found that extracts from a number of New Zealand's unique plants exhibited different types of antibiotic activity. Here we wish to report the isolation and identification of an anti-dermatophyte compound from the lichen *Stereocaulon ramulosum* (SW.) Rausch.

MATERIALS AND METHODS

Lichen was collected from various sites on the Port Hills, near Christchurch, N.Z. and a voucher specimen was deposited in the Department's herbarium. Samples, cleaned of extraneous moss and soil, were preserved by freeze-drying, and stored in sealed containers at 4°C.

EXTRACTION PROCEDURES

Freeze-dried lichen was ground to a coarse powder in a small hammer mill, and 1 g samples extracted with a range of different solvents to determine the optimum extractant for maximum antibiotic activity: methanol was found to be the best extractant. Subsequently 25 g samples were extracted with three successive 150 ml portions of methanol for 12 h in a soxhlet apparatus. These were then concentrated in a rotary vacuum evaporator care being taken to avoid total drying of the extract (Fraction I).

CHROMATOGRAPHIC SEPARATIONS

Thin-layer chromatography (TLC) was carried out on silica gel coated sheets developed with Culberson's solvent system C (toluene, acetic acid, 85:45) (Culberson & Kristinson 1970). Lichen constituents were visualised by examination under UV light (254 and 365 nm), exposure to iodine vapour or spraying with 10% (v/v) sulphuric acid followed by heating at 90°C to bring about colour development.

Fungal steroids were extracted from freeze-dried mycelium with dimethyl sulphoxide-hexane or chloroform-methanol and then analysed by TLC on silver nitrate-impregnated silica gel plates (Parkes *et al.* 1985) developed with benzene-ethyl acetate (1:2 v/v) (Lisboa 1969). Cholesterol, ergosterol and squalene were included as standards. Steroids were visualised by spray-

[1] To whom correspondence should be addressed

ing the TLC plates with Liebermann-Burchard reagent (Lisboa 1969) or concentrated sulphuric acid -methanol (1:1) followed by heating at 90°C for 10 min (Stahl 1969).

ANTIBIOTIC ASSAY

For the routine bioassay of antibiotic activity, test samples were dried onto 6 mm antibiotic assay (AA) discs and placed on agar plates previously seeded with a suspension of the test organism. Dermatophyte fungi were all recent isolates from local clinical laboratories.

Whole TLC plates (50 x 200 mm) were bioassayed to locate active zones by placing them in sterile polypropylene food trays and covering them with molten agar previously seeded with the test organism. All bioassay plates were incubated at 25° or 30°C for 2 - 3 days, and the diameter of any zones of inhibition recorded.

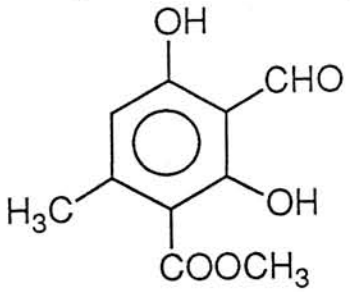
The effect of methyl haematommate on spore germination and mycelial growth in liquid culture was assessed as described previously (Muir *et al.* 1982).

RESULTS

Preliminary bioassays of crude methanolic extracts (Fraction I) revealed a broad spectrum of activity against dermatophyte fungi, whilst direct bioassay of TLC plates indicated two zones of antibiotic activity at Rf (0.82 and 0.65), with maximum activity in the former (= Compound I).

In order to isolate Compound I a preliminary partitioning was carried out by washing the methanolic extract (Fraction I) with hexane. The pooled hexane extracts were evaporated to dryness *in vacuo* and then extracted with three successive aliquots of acetone which removed the active principle. Following repeated recrystallisation from acetone-hexane (3:1), 50 g of dried lichen finally yielded 23 mg of pale-yellow, long, needle-shaped crystals, M.Pt 147°.

Identification of the structure of Compound I was achieved by means of UV, ^{13}C and ^{1}H-NMR spectroscopy. The UV spectrum in methanol showed peaks at 207, 241, 262 and 336 nm. Addition of sodium acetate caused bathochromic shifts to 213, 272, and 378 nm, whilst in the presence of sodium methoxide peaks were located at 221, 252, 292 and 382 nm. These observations suggested the presence of a phenolic compound. This was confirmed by NMR spectroscopy which suggested that Compound I was a small, 10C, multiple-substituted, phenolic compound. The NMR chemical shifts indicated the presence of only one aromatic proton (δH 6.28), two phenolic groups (δH 12.41 and 12.88), a formyl proton (δH 10.33) with associated carbonyl (δC 193.67) plus a further ester linkage carbonyl (δC 171.83), a methoxyl group (δH 3.96, δC 52.33) and a methyl group (δH 2.53). The ^{13}C-NMR spectrum also showed signals corresponding to Ar-OH (δ 168.10, 166.46), Ar-H (δ 152.18, 112.04, 108.34, 103.78) and Ar-Me (δ 25.27). Consideration of the above data suggested a molecular formula of $C_{10}H_{10}O_5$ and a structural formula corresponding to methyl,3-aldo-2:4-dihydroxy-6-methyl benzoate which has the trivial name of methyl haematommate (Asahina & Shibata 1954a).



SPECTRUM OF ANTIBIOTIC ACTIVITY

The antibiotic activity of methyl haematommate was assayed against a range of bacteria and fungi. It appeared to show preferential antibiotic activity against dermatophytes (Table 1). Repetition of these bioassays by liquid culture assays and slide germination tests (Muir *et al.* 1982) confirmed these results, and showed that methyl haematommate was active against both spores and growing mycelium.

MICROSCOPY

Examination of methyl haematommate-treated macroconidia of *M. gypseum* revealed marked disruption of the cytoplasmic organisation and thickening of the cell wall and membrane. Methyl haematommate-treated spores failed to germinate after 14 days.

Further examination of treated spores using

Table 1. Antimicrobial activity of methyl haematommate (MH) as determined by AA disc bioassay. Numbers in brackets = number of strains tested.

ORGANISM	DIAMETER OF ZONE OF INHIBITION (mm)						
	Discs applied before germination				Discs applied after germination		
MH (μg/disc)	340	34	3.4	0.34	340	34	3.4
Dermatophytes							
Epidermophyton floccosum (3)	11-12	5-8	2	-	5-6	3-4	1
Microsporum canis (2)	17-18	7-9	2	-	5-6	1	-
M. nanum (1)	15	10	3	-	5	2	-
M. gypseum (1)	19	14	4	1	9	5	1
Trichophyton rubrum (3)	14-17	7-8	-	-	5	2	-
T. mentagrophytes (2)	13-16	8-11	3-4	-	7-9	2-6	1
T. mentagrophytes var *interdigitale* (2)	14-15	8-11	-	-	6-7	2	-
Non dermatophytes							
Aspergillus fumigatus	-	-	-	-	-	-	-
A. niger	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-
Candida albicans	-	-	-	-	3	3	1
Cladosporium resinae	4	3	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-
Fusarium oxysporum	4	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-
Verticillium dahliae	15	11	3	-	3	-	-

transmission electron microscopy showed considerable loss of internal organisation, and disruption of the cell membrane. Control (untreated) spores had intact cytoplasm and membranes, and many had produced germ tubes.

STEROL ANALYSIS

A number of potent anti-fungal agents are known to affect ergosterol biosynthesis in fungi (Nozawa & Morita 1986). In preliminary tests *E. floccosum, T. mentagrophytes* and *M. canis* were grown on Sabouraud agar containing a sub-lethal concentration (17mg ml^{-1}) of methyl haematommate. Their sterols were then extracted and compared by TLC to those extracted from control mycelium grown in the absence of methyl haematommate. Chromatograms of the untreated dermatophytes exhibited spots possessing the same R_f and colour reactions as ergosterol, whereas the methyl haematommate-treated cells showed loss of the ergosterol spot, and gained a new spot with the same R_f as squalene.

DISCUSSION

Lichens produce an extraordinarily diverse range of secondary metabolites whose exact role in nature is still unclear. Many have antibiotic properties. Lichen extracts have been used as a component of the folk medicines of a number of peoples (Asahina & Shibata 1954b, Richardson 1988). Lichen acids have been shown to retard the growth of Gram-positive bacteria (Vartia 1973) whilst more recently Ingolfsdottir *et al.* (1985) screened Icelandic lichens for antibiotic activity and showed that the products of hydroly-

sis of certain depsides were active against bacteria and fungi. They identified the active compounds as methyl β-orsellinate and the methyl and ethyl esters of orsellinic acid, and suggested that these esters were hydrolysis products of the depsides atranorin and tenuiorin.

In this paper we have shown that methyl haematommate from *S. ramulosum* exhibits antibiotic activity against dermatophytic fungi, probably by affecting steroid biosynthesis and the organisation of membrane structure. Haematommic acid, or its esters, does not normally occur as such in lichens but it is found as a component of common lichen depsides such as atranorin and norstictic acid and from which it may be obtained by alcoholysis (Asahina & Shibata 1954a). Both of these depsides occur in *S. ramulosum* (Lamb 1951) and it is possible that our isolation of methyl haematommate could be an artefact of the isolation procedure (Hylands & Ingolfsdottir 1985). However when small portions of intact thallus were subjected to agar plate bioassay, zones of inhibition were observed around them; thus it is probable that free methyl haematommate was present in *S. ramulosum*. Culberson (1969) suggested that these depsides are formed by the phycobiont from aromatic precursors synthesised by the mycobiont. By analogy with glycoside formation in higher plants this synthesis could serve as an internal protective mechanism for the algal partner. Lichens also secrete enzymes that can hydrolyse depsides to release free phenolic compounds which might act in chemical defence mechanisms (Mosbach & Ehrensvaard 1966).

ACKNOWLEDGEMENTS

The authors wish to thank Dr J.W. Blunt and Dr M.H.G. Munro for assistance with the NMR analyses, Mr Ron Bridger (Godfrey Pathology Lab) and Messrs G. Paltridge and B. Harris (Christchurch Public Hospital) for clinical isolates of microorganisms, the University Grants Committee and the University of Canterbury for the provision of research equipment and facilities. Mrs Margaret Stevens provided competent technical assistance.

REFERENCES

Asahina, Y. and Shibata, S. (1954a). *Chemistry of Lichen Substances*, p95. Japan Society for Promotion of Science. Tokyo.

Asahina, Y. & Shibata, S. (1954b). *Chemistry of Lichen Substances*. Japan Society for Promotion of Science. Tokyo, Chapter 3, pp216-224.

Calder, V.L., Cole, A.L.J. & Walker, J.R.L. (1986). Antibiotic compounds from N.Z. plants: III, a survey of N.Z. plants for antibiotic substances. *Journal Royal Society of New Zealand* 16: 169-181.

Culberson, C.F. & Kristinsson, H. (1970). A standardised method for identification of lichen products. *Journal of Chromatography* 46: 85-93

Culberson, C.F. (1969). *Chemical and Botanical Guide to Lichen Products* p23. University of North Carolina Press, Chapel Hill, U.S.A.

Hylands, P.J. & Ingolfsdottir, K. (1985). The isolation of methyl β-orsellinate from *Stereocaulon alpinum* and comments on the isolation of 4:6-dihydroxy-2-methoxy-7-methylacetophenone from *Stereocaulon* species. *Phytochemistry* 24: 127-129 .

Ingolfsdottir, K., Bloomfield, S.F. & Hylands, P.J. (1985). *In vitro* evaluation of the antimicrobial activity of lichen metabolites as potential preservatives. *Antimicrobial Agents and Chemotherapy* 28: 289-292.

Lamb, I.M. (1951). On the morphology, phylogeny and taxonomy of the lichen genus *Stereocaulon*. *Canadian Journal of Botany* 29: 522-584 .

Lisboa, B.P. (1969). In *Methods in Enzymology* (ed. R.B. Clayton) Vol 15, pp3-157. Academic Press, New York.

Mosbach, K. and Ehrensvaard, U. (1966). Studies on lichen enzymes. I. Preparation and properties of a depside hydrolysing esterase and of orsellinic acid decarboxylase. *Biochemical and Biophysical Research Communications* 22: 145-150..

Muir, A.D., Cole, A.L.J. & Walker, J.R.L. (1982). Antibiotic compounds from New Zealand plants. I; Falcarindiol, an anti-dermatophyte agent from *Schefflera digitata*. *Journal of Medicinal Plant Research* 44: 129-133.

Nozawa, Y. & Morita, T. (1986). In *In vitro and In vivo Evaluation of Antifungal Agents.* (ed. K. Iwata & H. Vanden Bossche), pp111-122, Elsevier Science Publishers B.V. Amsterdam.

Parkes, L.W., Botlema, C.D.K., Rodriguez, R.J. & Lewis, T.A. (1985). In *Methods in Enzymology* (ed. J.H. Law & H.C. Rilling), Vol 111. pp333-345. Academic Press Ltd. New York.

Richardson, D.H.S. (1988). In *CRC Handbook of Lichenology.* (ed. M. Galun), Vol III. pp103-105. CRC Press Inc. Boca Raton. Florida, U.S.A.

Stahl, E. (Ed.) (1969). *Thin Layer Chromatography*, 2nd edition. George Allen & Unwin Ltd. London.

Vartia, K.O. (1973). In *The Lichens*, (ed. V. Ahmadjian and M.E. Hale), pp145-150. Academic Press, New York.