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Research Papers

Solubilization of the lichen metabolite (+)-usnic acid for testing in tissue culture

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Abstract

The pharmacological testing of natural products can often be hampered by the poor solubility of such compounds in non-toxic solvents. There is thus a need for a suitable agent for solubilization of natural substances to allow testing on a variety of cell lines in-vitro. Such an agent should ideally have no direct effects on any of the commonly used cell lines from a variety of tissues and mammalian species to allow proper comparison. In this study, the lichen metabolite (+)-usnic acid, a dibenzofuran derivative, was used as a prototype for an insoluble natural product with the aim of finding a solvent that was both capable of solubilizing usnic acid and was free of direct activity against a test cell line. Solubilization was measured at different pH values in various concentrations of co-solvents (glycofurol 75, propylene glycol, polyethylene glycol 400), surfactants (polysorbate 20 and Cremophor RH40), and the complexing agent 2-hydroxypropyl-β-cyclodextrin. The solubility achieved in a 20% aqueous solution was 0.11 mg mL^{-1} for propylene glycol, 0.19 for PEG 400, 0.27 for glycofurol 75, 0.57 for Cremophor RH40, 0.68 for 2-hydroxypropyl-β-cyclodextrin and 0.84 for polysorbate 20. The direct effects of the various solvent systems were tested on the human leukaemia cell line K-562 in a standard proliferation assay. Most of the solvents proved toxic with the exception of propylene glycol, PEG 400 and 2-hydroxypropyl-β-cyclodextrin. Anti-proliferative activity of usnic acid could be demonstrated with an ED50 (amount of substance required to reduce thymidine uptake to 50% of uptake by untreated control culture) of 4.7 μg mL^{-1} using PEG 400 and 2-hydroxypropyl-β-cyclodextrin but only the latter gave satisfactory solubility. 2-Hydroxypropyl-β-cyclodextrin was thus identified as a solubilizing agent that fulfilled both set criteria of solubility and lack of toxicity against the test cells.

Introduction

Pharmacological testing of natural products can often be hampered by the poor solubility of such compounds in non-toxic solvents. We have encountered such difficulties in our studies on growth-inhibitory effects of lichen metabolites on mammalian cells in culture. For many human cell lines we have found ethanol to be a suitable solvent with little direct effect (Ögmundsdóttir et al 1998). In extending our studies to a greater variety of lichen metabolites and more cell lines, problems have arisen with inadequate solubility of substances and ethanol toxicity to the cells in the concentrations used. Dimethyl sulfoxide (DMSO) is another commonly used solvent but has often proved too toxic to human test cells at the concentrations required. There is thus a need for a more suitable agent for solubilization of natural substances to allow testing on a variety of cell lines in-vitro. Such an agent should ideally have no direct effects on any of the commonly used cell lines from a variety of tissues and mammalian species to allow proper comparison.

To increase aqueous solubility of an insoluble substance several methods can be employed; pH adjustment, a co-solvent, complexation or surfactants. These methods can be used singly or in combination. Li et al (1999a, b) have reported increased solubility of the flavonoid derivative flavopiridol by using a combination of pH control and complexation and micellization, as well as pH control and co-solvency. Use of water-miscible co-solvents is probably the most widely used technique to solubilize,

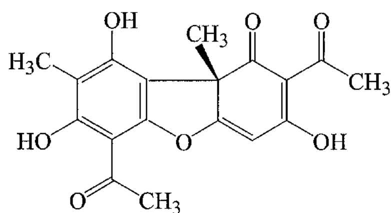

Figure 1 Chemical structure of the dibenzofuran derivative (+)-usnic acid, isolated from the lichen *Cladonia arbuscula*.

because of their relatively low toxicity (Jonkman-de Vries et al 1996). Complexation with cyclodextrin is also extensively used to increase aqueous solubility. Cyclodextrins are cyclic oligosaccharides formed from α-1,4-linked glucose units with hydroxyl groups on the outer surface and a void cavity in the centre, and are capable of forming inclusion complexes with a wide variety of hydrophobic molecules by taking up a whole molecule, or part of it, into the cavity and thereby increasing its solubility (Loftsson & Brewster 1996). Complex formation of a compound with cyclodextrin can be affected by pH and it is often assumed that the unionised species is primarily responsible for the formation of the complex and the subsequent increase in solubility.

In this study, the lichen metabolite (+)-usnic acid, a dibenzofuran derivative (Figure 1), was used as a prototype for an insoluble natural product, its aqueous solubility being less than 10 mg/100 mL at 25°C (Takai et al 1979). The pK_a value for the enolic hydroxyl group in position 3 of usnic acid is 4.4, whereas those for the phenolic hydroxyl groups in positions 9 and 7 are 8.8 and 10.7, respectively (Sharma & Jannke 1966).

Following reports of antimycobacterial activity of (−)-usnic acid (Vartia 1949) and activity of (+)-usnic acid against Gram-positive bacteria and strains of *Mycobacterium tuberculosis* (Stoll et al 1950), previous attempts were made to overcome the poor solubility of usnic acid by preparing amino derivatives (Virtanen 1954). (−)-Usnic acid was later shown to exhibit modest activity against Lewis lung carcinoma in mice (Kupchan & Kopperman 1975; Takai et al 1979) and moderate activity in the murine P388 leukaemia assay and in-vitro cytotoxic activity against cultured L1210 cells (Takai et al 1979).

The aim of this study was to find a solvent that was both capable of solubilizing usnic acid and was free of direct activity against a test cell line. To this end a variety of pharmaceutical solubilizing agents was tested for their ability to increase solubility. In choosing the solvent systems for use in the solubilization experiments emphasis was placed on their low toxicity, aqueous miscibility and their ability to increase the solubility of many non-polar drugs. Solubilization was measured at different pH values in various concentrations of co-solvents (glycofurol 75, propylene glycol, polyethylene glycol 400), surfactants (polysorbate 20, Cremophor RH40) and the complexing agent 2-hydroxypropyl-β-cyclodextrin. The direct effects of the various solvent systems were tested on the human leukaemia cell line K-562 in a standard proliferation assay.

Materials and Methods

Isolation of usnic acid

(+)-Usnic acid was isolated from *Cladonia arbuscula* (Wallr.) Rabenh. (Cladoniaceae) collected in Hveravellir, Iceland. Authentication of the plant material, isolation procedure and spectroscopic data (IR, ^{1}H and ^{13}C NMR, HMQC, HMBC) were as previously described (Ingólfsdóttir et al 1998).

Materials

Glycofurol 75, polyethylene glycol 400, propylene glycol and polysorbate 20 were purchased from Sigma Chemical Co., St Louis, USA. Cremophor RH40 was obtained from BASF, Germany and 2-hydroxypropyl-β-cyclodextrin was a gift from Cerestar, Indiana, USA. All other chemicals were of reagent grade.

Solubility determination

An excess of usnic acid was placed in a glass vial (10 mL) together with 2 mL of the solution (solvent system). The vials were kept in an orbital shaker bath (LAB-LINE Instruments, Inc., Melrose Park, IL) at 25°C. After 72 h, samples were collected, filtered through 0.45-μm membrane filters and diluted as needed. The usnic acid concentration in the samples was assayed by HPLC. Each experiment was carried out in triplicate.

HPLC assay of usnic acid

The amount of usnic acid in solution was determined using a high-performance liquid chromatography (HPLC) component system consisting of a Hewlett Packard 1050 series pump, a Hibar RP18 25 μm (3.9×250 mm) column from Merck and a Hewlett Packard 1050 series UV detector. The wavelength was 235 nm and the mobile phase consisted of methanol–water–H_3PO_4, 80:20:0.9 (Huneck & Yoshimura 1996) with the retention time being 10 min at 1.5 mL min^{-1} flow rate.

Cell proliferation assay

The human erythro-leukaemia cell line, K-562 (ATCC CLL-243) was maintained in suspension culture in RPMI medium supplemented with 10% fetal calf serum and 50 μg mL^{-1} penicillin/streptomycin (all tissue culture material from Gibco Ltd, Paisley, UK). For the proliferation assay the cells were seeded at 10^4 cells per well into 96-well tissue culture plates. Test substances were added at the concentrations indicated at the beginning of the culture period. After incubation at 37°C, 95% humidity and 5% CO_2 for 24 h, ^{3}H-thymidine (Amersham International, Amersham, Buckinghamshire, UK) was added at 10 μCi per well and incubation continued for a further 6 h. Cultures were harvested onto glass fibre filters, which were placed in MicroscintO scintillation liquid and radioactivity

counted in a TopCount scintillation counter (Packard Instruments, CT). The results were expressed as percentage of untreated control. Each experiment was carried out in triplicate.

Statistical analysis

Results for solubility of usnic acid are presented as means±s.d. and results for anti-proliferative effects of usnic acid against K-562 are presented as means±s.e.m. The differences between the means of individual treatments were compared using the Kruskal–Wallis test. In all cases, $P < 0.05$ denoted significance.

Results

Effect of solubilizing agents on the solubility of usnic acid

Figure 2 shows the effect of pH on the solubility of usnic acid in 10% aqueous solution of PEG 400, Cremophor RH40 or 2-hydroxypropyl-β-cyclodextrin. The solubility of usnic acid at pH 6 is very limited but is significantly higher at pH 8.3. In PEG 400 and Cremophor RH40 solutions the increase in usnic acid solubility is linear with increasing pH but in the case of 2-hydroxypropyl-β-cyclodextrin the increase is exponential. Despite the higher

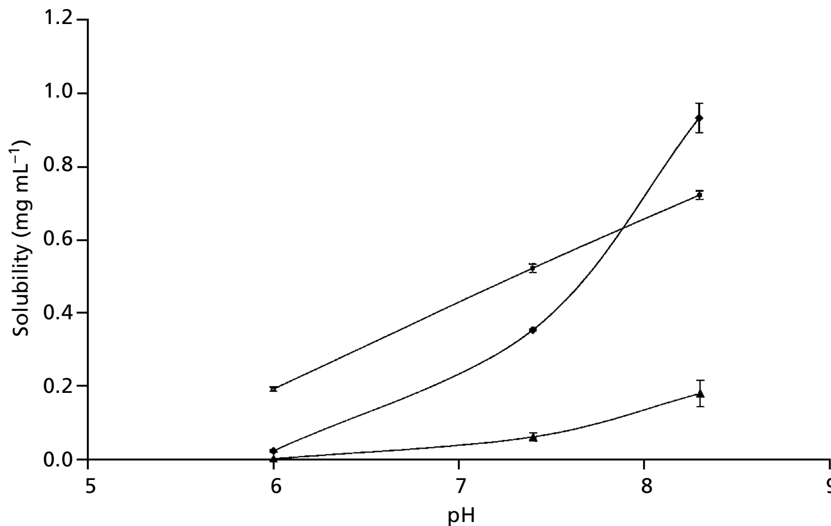

Figure 2 Effect of pH on solubility of (+)-usnic acid in 10% aqueous solutions of 2-hydroxypropyl-β-cyclodextrin (◆), Cremophor RH40 (●) or PEG 400 (▲). Each point represents the mean±s.d. of three experiments.

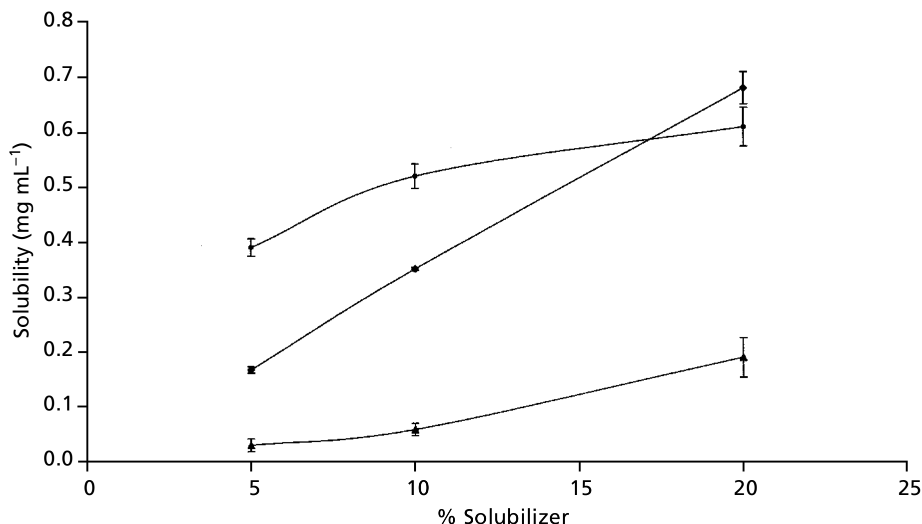

Figure 3 Effect of solubilizer concentration on (+)-usnic acid solubility at pH 7.4 in solutions of 2-hydroxypropyl-β-cyclodextrin (◆), Cremophor RH40 (●) or PEG 400 (▲). Each point represents the mean±s.d. of three experiments.

Table 1 Solubility of (+)-usnic acid in 20% of solubilizers at pH 7.4.

Solubilizer	Solubility (mg mL^{-1})
Polysorbate 20	0.840±0.036
Cremophor RH40	0.573±0.035
Propylene glycol	0.107±0.006
PEG 400	0.190±0.002
Glycofurol 75	0.271±0.009
2-Hydroxypropyl-β-cyclodextrin	0.683±0.028

Values are mean±s.d. (n = 3).

solubility of usnic acid at pH 8.3 than the physiological pH 7.4, the latter was chosen for the cell experiments. Figure 3 shows the effect of solubilizer concentration on usnic acid solubility at pH 7.4. The solubility increases as a function of solubilizer concentration and for 2-hydroxypropyl-β-cyclodextrin the increase is linear between 5 and 20%. The solubility of usnic acid in the different solvent systems at pH 7.4 is presented in Table 1. In 20% aqueous solutions the surfactants Cremophor RH40 and polysorbate 20 (critical micelle concentration values 0.039% and 0.06%, respectively) yielded greater increase in usnic acid solubility than the co-solvents but complex formation with 2-hydroxypropyl-β-cyclodextrin gave comparable results to those obtained with the surfactants.

Effects of solubilizing agents on thymidine uptake of K-562

All solubilizing agents were tested in serial dilutions starting from 20% stock solutions. Since the goal was to find a suitable solvent for testing in a proliferation assay, all test solvents were tested in this assay. As can be seen from Table 2, most of the solvents tested had strong inhibitory effects on thymidine uptake at very low doses. It should be stressed that these effects should be interpreted as toxicity rather than genuine anti-proliferative effects. From these results, three solvents appeared suitable for further testing: propylene glycol, PEG 400 and 2-hydroxypropyl-β-cyclodextrin.

Table 2 Effect of solubilizing agents on thymidine uptake in K-562 cells.

Solvent	ED50 (μL mL^{-1})
Polysorbate 20	< 1
Polysorbate 80	< 1
Cremophor RH40	1.5
Cremophor EL	< 1
Propylene glycol	> 80
PEG 400	58
2-Hydroxypropyl-β-cyclodextrin	60
Glycofurol 75	5
2-Phenoxyethanol (water/ethanol)	4
2-Phenoxyethanol(water/propylene glycol)	2

ED50 = μL mL^{-1} of solvent required to reduce thymidine uptake to 50% of uptake by untreated control culture.

Effect of usnic acid in different solvents on thymidine uptake of K-562

Usnic acid was solubilized in 10% solutions of propylene glycol, PEG 400 or 2-hydroxypropyl-β-cyclodextrin and tested on K-562. The propylene glycol solution showed no effect and the highest concentration achieved was 2.6 μg mL^{-1} at 80 μL mL^{-1} of solvent. PEG 400 gave slightly better solubility and usnic acid showed an ED50 (amount of substance required to reduce thymidine uptake to 50% of uptake by untreated control culture) of 3.0 μg mL^{-1} at a solvent concentration of 70 μL mL^{-1}. The best results in terms of solubility and effectiveness of usnic acid were obtained with 2-hydroxypropyl-β-cyclodextrin (Figure 4). The ED50 for usnic acid was 4.7 μg mL^{-1} at a solvent concentration of 10 μL mL^{-1}.

Discussion

This study has resulted in the identification of a solubilizing agent, 2-hydroxypropyl-β-cyclodextrin, which is free of toxic effects and thus suitable for use in cell culture, and at the same time capable of bringing usnic acid into solution at concentrations that give a highly significant anti-proliferative activity against a malignant human cell line.

In attempting to increase the solubility of usnic acid, the co-solvents were found to be less effective in solubilizing usnic acid than the surfactants or 2-hydroxypropyl-β-cyclodextrin. However, all the solubilizing agents were found to be more effective when usnic acid was present in the ionised form. This is in agreement with results obtained by Li et al (1998, 1999a, b) in their work on flavopiridol, but they found that the combined techniques of pH control and either co-solvents, surfactants or complexing agents increased not only the solubility of the un-ionized compound but also the solubility of the ionised solute.

The solubility of (+)-usnic acid obtained in the 20% aqueous solutions of the surfactants and the cyclodextrin is lower than the sought-after 1 mg mL^{-1}, but it is sufficient to carry out testing of the compound's activity against the human leukaemia cell line K-562. The surfactants used were, however, found to have a strong inhibitory effect on thymidine uptake and therefore not suitable for cell culture testing. Surfactants are frequently used to increase solubility in parenteral preparations but at the concentrations used in this work they could be causing disruption of cell membranes.

Three of the solubilizing agents were suitable for tissue culture use in terms of their own toxicity (i.e. propylene glycol, PEG 400 and 2-hydroxypropyl-β-cyclodextrin). The other limiting factor, solubility of usnic acid, eliminated

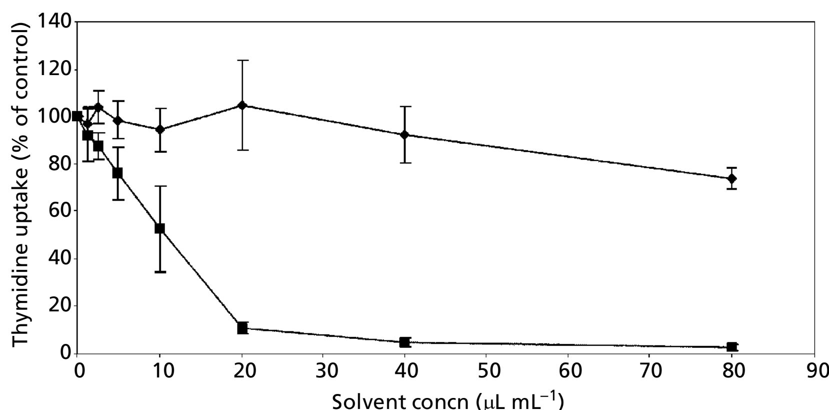

Figure 4 The effect of (+)-usnic acid on thymidine uptake of K562. Control (2-hydroxy-propyl-β-cyclodextrin only) (▲); usnic acid in 2-hydroxypropyl-β-cyclodextrin (■). Results are of three separate experiments each carried out in triplicate, bars represent s.e.m.

propylene glycol from further testing. The same ED50 for the anti-proliferative effect of usnic acid was obtained in the two remaining solvents, but the concentration of solvent required to achieve this dose was 7-fold higher for PEG 400 than for 2-hydroxypropyl-β-cyclodextrin. These results are in accord with those reported by Mottu et al (2001), who evaluated the haemolytic effect of water-miscible organic solvents used for pharmaceutical applications. They divided the solvents into four categories, and reported that DMSO was very highly haemolytic, glycofurol 75 moderately haemolytic and propylene glycol had low haemolytic activity.

Although usnic acid has been claimed in the literature to have some anti-neoplastic activity (Cardarelli et al 1997), this has not been extensively tested. The first report was from Kupchan & Kopperman (1975), who described significant inhibitory activity of (−)-usnic acid administered in Acacia gum in the Lewis lung carcinoma model in the mouse, but the effects were relatively modest. Increased survival of treated mice was defined as ⩾ 125% compared with controls, and a value of 135–152% was achieved at a dose range of 20–200 mg kg^{-1}. In a later study, several structural modifications of usnic acid were tested but no significant survival advantage could be demonstrated in two mouse tumour models, Lewis lung and P388 leukaemia (Takai et al 1979). These authors also performed in-vitro cytotoxicity tests, counting viable mouse L1210 cells following 23 and 46 h exposure to the various usnic acid derivatives in DMSO. The more lipophilic compounds, including usnic acid itself, showed marked growth inhibitory effects in this assay at the one dose tested, 1.4×10^{-7}mol mL^{-1}, but no results are shown for DMSO controls. Restricted solubility was clearly a limiting factor on these older studies. It is also possible that the two enantiomers of usnic acid differ in their potency. In the most recent study, Cardarelli et al (1997) tested commercially obtained (+)-usnic acid solubilized in DMSO against three human malignant cell lines, K-562, and cell lines from endometrial carcinoma, Ichikawa and HEC-50. No further details were given on the assay system used, but cell counts were significantly reduced following exposure for 21 h to 50 μg mL^{-1} of (+)-usnic acid, by 40, 62 and 76% for K-562, Ichikawa and HEC-50, respectively. The time factor was shown to be important, as extending exposure to 46 h increased the inhibition to 90, 70 and 85% respectively. In a recent review, Cocchietto et al (2002) concluded that the tumour-inhibitory properties of usnic acid did not appear to be very pronounced but that more studies were needed.

Conclusion

This study has identified a suitable solvent for the lichen metabolite (+)-usnic acid. This provides the opportunity for further testing of this substance for its potentially promising anti-proliferative effects against malignant cells. 2-Hydroxypropyl-β-cyclodextrin is commonly used in dosage forms and could therefore be used in dosage-form design for (+)-usnic acid if further work indicates its possible therapeutic use in the future.

References

Cardarelli, M., Serino, G., Campanella, L., Ercole, P., De Cicco Nardone, F., Alesiani, O., Rossiello, F. (1997) Antimitotic effects of usnic acid on different biological systems. *Cell. Mol. Life Sci.* **53**: 667–672

Cocchietto, M., Skert, N., Nimis, P. L., Sava, G. (2002) A review of usnic acid, an interesting natural compound. *Naturwissenschaften* **89**: 137–146

Huneck, S., Yoshimura, I. (1996) *Identification of lichen substances.* Springer Verlag, Berlin, Heidelberg, p. 493

Ingólfsdóttir, K., Chung, G. A. C., Gissurarson, S. R., Skúlason, V. G., Vilhelmsdóttir, M. (1998) *In vitro* antimycobacterial activity of lichen metabolites. *Eur. J. Pharm. Sci.* **6**: 141–144

Jonkman-de Vries, J. D., Flora, K. P., Bult, A., Beijnen, J. H. (1996) Pharmaceutical development of investigational anticancer agents for parenteral use — a review. *Drug Dev. Ind. Pharm.* **22**: 475–494

Kupchan, S. M., Kopperman, H. L. (1975) Tumor inhibitors. 105. L-Usnic acid: tumor inhibitor isolated from lichens. *Experientia* **31**: 625–626

Li, P., Tabibi, E., Yalkowsky, S. H. (1998) Combined effect of complexation and pH on solubilization. *J. Pharm. Sci.* **87**: 1535–1537

Li, P., Tabibi, E., Yalkowsky, S. H. (1999a) Solubilization of ionized and un-ionized flavopiridol by ethanol and polysorbate 20. *J. Pharm. Sci.* **88**: 507–509

Li, P., Tabibi, E., Yalkowsky, S. H. (1999b) Solubilization of flavopiridol by pH control combined with co-solvent, surfactants or complexants. *J. Pharm. Sci.* **88**: 945–947

Loftsson, T., Brewster, M. E. (1996) Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *J. Pharm. Sci.* **85**: 1017–1025

Mottu, F., Stelling, M.-J., Rüfenacht, D. A., Doelker, E. (2001) Comparative hemolytic activity of undiluted organic water-miscible solvents for intravenous and intra-arterial injection. *PDA J. Pharm. Sci. Tech.* **55**: 16–23

Ögmundsdóttir, H. M., Zoëga, G. M., Gissurarson, S. R., Ingólfsdóttir, K. (1998) Anti-proliferative effects of lichen-derived inhibitors of 5-lipoxygenase on malignant cell lines and mitogen-stimulated lymphocytes. *J. Pharm. Pharmacol.* **50**: 107–115

Sharma, R. K., Jannke, P. J. (1966) Acidity of usnic acid. *Indian J. Chem.* **4**: 16–18

Stoll, A., Brack, A., Renz, J. (1950) Die Wirkung von Flechtenstoffen auf Tuberkelbakterien und auf einige andere Mikroorganismen. *Schweiz Zeitschr. Path. Bakt.* **13**: 729–751

Takai, M., Uehara, Y., Beisler, J. A. (1979) Usnic acid derivatives as potential antineoplastic agents. *J. Med. Chem.* **22**: 1380–1384

Vartia, K. O. (1949) Antibiotics in lichens. I. *Ann. Med. Exptl. Biol. Fenn.* **27**: 46–54

Virtanen, O. E. (1954) The antibiotic activity of some amino compound derivatives of l-Usnic acid. II. *Suomen Kemistilehti* **27B**: 67–70