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Isolation and Identification of Usnic Acid and Atranorin from Some Saudi-Arabian Lichens

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ABSTRACT. (+) Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H, 9bH)-dibenzofurandione), an antibiotic, was found in large amount (about 1% based on dry weight) in the Saudi-Arabia lichen *Usnea articulata* which grows in abundance on the Asir mountains of the South-Western region. This finding was confirmed by various chemical means including mass spectra, NMR, IR, UV spectrometry and polarimetry. Atranorin (3-formyl-2,4-dihydroxy-6-methylbenzoic acid 3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl ester), of a less known medical importance, was found in a lesser quantity (about 0.1%) in the edible lichen *Parmelia tinctorum* which is consumed as a food spice in some Arab countries (called in Arabic- Al-Sheba). The presence of atranorin, which is known to be a food deterrent for insects and other herbivores, was also confirmed by the preceeding methods of chemical analysis.

Chemistry of lichens is one of the more thoroughly explored and active area in lichenology. Many chemists have long been attracted to lichens and their unique products (Huneck 1974, Bruno *et al.* 1983, Elix *et al.* 1984, Venkataramana and Krishna 1992). Some of these products, namely lichen acids, are unique to the lichen symbiosis (Lawrey 1986) and are known to possess a wide spectrum of biological activities such as antibiotic (Asahina and Shibata 1954, Vartia 1973, Kupchan and Kopperman 1975), antitumor (Kupchan and Kopperman 1975, Takai *et al.* 1979), anti-inflammatory (Skidmore and Whitehouse 1965, Handa *et al.* 1992), inhibitors of prostaglandin biosynthesis (Sankawa *et al.* 1982) and invertebrate deterrent properties (Slansky 1979, Lawrey 1983, Seyd and Seaward 1984). In fact, lichens and their chemical products have appeared in the herbal literature of many countries

(Kirtikar and Basu 1984, Huovinen 1988, Hobbs 1990).

Yet, the chemical and biological properties of Saudi-Arabian lichens and their products have not been explored. This study deals with the chemical identification of two lichen acids; (+)usnic acid and atranorin isolated from two local lichen species *viz: Usnea articulata* and *Parmelia tinctorum* respectively. The former is a fruticose type found at high altitudes of the Asir mountains in the South-Western region, while the latter lichen species is a foliose type (called in Arabic Al-Sheba) which is consumed heavily as a food spice, in the Western region of Saudi-Arabia as well as in many other Arab countries.

Materials and Methods

Collection of lichens:

Two lichen species were collected from different localities as follows:

- *Usnea articulata* was collected during the winter season of 1986 as festons loosely hanging from Juniper trees (*Juniperus procera*) in Al-Sawdah at Asir region in the South Western part of Saudi Arabia.
- *Parmelia tinctorum* (Al-Sheba) was obtained from a local market in Jeddah.

Extraction of lichen acids:

Thalli of *U. articulata* were air-dried, coarsely sheared and soaked (100 gm) for 5 days in one liter of chloroform. By filtering through several layers of clean cheese-cloth, the residue was discarded and the crude chloroform extract was refiltered twice using filter paper. The solvent was then removed by evaporation using a rotary evaporator. Long yellowish crystalline prisms were precipitated when ethanol (3 vol.) was added to the concentrated crude chloroform extract followed by overnight cooling at 0-4 °C. The obtained substance was purified by recrystallization in ethanol, weighed and kept in a stoppered vial at room temperature. Likewise, thalli of *P. tinctorum* (Al-Sheba) (100 gm) were soaked in one liter of benzene. By filtering through several layers of clean cheese-cloth, the residue was discarded and the crude benzene extract was refiltered twice with filter paper. The solvent was removed by evaporation. A light yellowish amorphous granules precipitated when ethanol (3 vol.) was added to the concentrated crude benzene extract followed by overnight cooling at 0-4 °C. The precipitated substance was purified by recrystallization in ethanol, weighed and kept in a stoppered vial at room temperature.

Identification of lichen acids:

Colour tests were made by applying a drop of the reagent directly on the anonymous lichen substances. If the test was positive, there would be a rapid colour change, usually red or yellow, if negative, nothing happens. A drop of 10% potassium hydroxide solution (KOH, abbreviated K) was applied directly on both compounds isolated from *U. articulata* and *P. tinctorum* using a fine medical dropper and followed immediately by a drop of sodium hypochlorite (liquid bleache, Chlorox, abbreviated C). Concentrated sulfuric acid was also used separately for further identification. Lichen acids were identified by comparing any colour change as the reagent was being applied, with standard colour keys (Dahl and Krog 1973).

Elemental analysis for %C and %H was carried out using a Perkin-Elmer 240-B.

Melting points were determined and compared with the melting points of authentic samples of (+)usnic acid and atranorin.

Optical rotation of (+)usnic acid isolated from *U. articulata* (recrystallized in chloroform) was conducted at room temperature using a Carl Zeiss polarimeter (length of column = 20.2 cm).

TLC was carried out as described by Culberson and Kristinsson (1970). Samples of compounds isolated from *U. articulata* and *P. tinctorum* were spotted on 40 x 80 mm precoated silica gel F254 plastic sheets, and developed with three solvent systems as follows: solvent A: benzene - dioxane - acetic acid (90 : 25 : 4 v/v); solvent B: hexane - diethyl ether - formic acid (5 : 4 : 1 v/v); solvent C: toluene - acetic acid (85 : 15 v/v). After developing, sheets were examined under UV (both at long and short wavelengths of 254 and 300 nm). The location of colourless spots on the sheet was also visualized by spraying with a 10% solution of sulfuric acid and heating for 10 min. at 110 °C.

Mass spectra of (+)usnic acid and atranorin isolated from *U. articulata* and *P. tinctorum* respectively, were recorded with El HX-100 mass spectrometer, using the direct inlet system (ion source temperature 270 °C, ionizing current 60 μA, electron energy 70 eV). IR spectra of (+)usnic acid and atranorin (2 mg/0.1 ml chloroform each) were measured on a Perkin Elmer 1310 infrared spectrometer.

NMR spectra were recorded with Varian EM-390 NMR spectrometer at 60 MC using $CDCl_3$ as a solvent and 1% TMS as an internal standard.

UV spectra of benzene solutions (1 mg/ml) of (+)usnic acid, atranorin and the authentic samples were recorded with Hitachi 100-80 spectrophotometer.

Results

The chloroform extract of *U. articulata* yielded long yellow prisms (yield 0.7-1% based on dry weight) which were identified by various chemical means as (+)usnic acid (Fig. 1) according to the following results: colour test gave a deep yellow coloration with KC reagent. Elemental analysis C (62.83%), H (4.71%) and O (32.46%) which are close to the expected values (62.79, 4.68 and 32.53% respectively) corresponding to the molecular formula ($C_{18}H_{16}O_7$) and molecular weight (344.31) of usnic acid. The recrystallzied substance (ethanol) melted at 200 °C and has $[\alpha]_D^{25}$ = (+)495. R_f values (TLC, using different solvents) of the isolated and authentic usnic acid were identical: solvents A (0.69), B (0.56) and C (0.63). The extract of *U. articulata* shows no presence of atranorin or any other lichen acids.

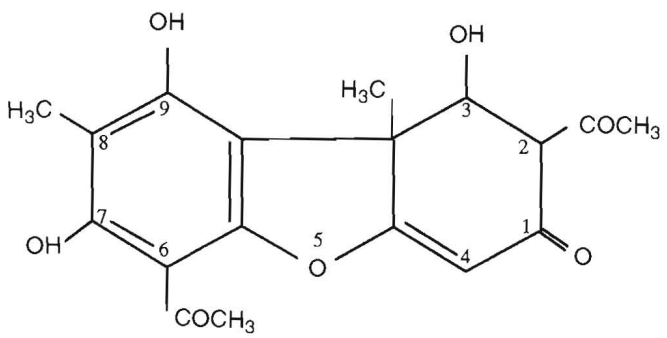


Fig. 1. Chemical structure of (+)usnic acid (2.6-diacetyl-7,9-dihydroxy-8,9b-di-methyl-1,3 (2H,9bH)-dibenzofurandione).

UV absorption spectrum of the substance isolated from *U. articulata* in benzene had two maxima at 310 and 325 nm that were identical with authentic (+)usnic acid.

Mass spectrum of (+)usnic acid (Fig. 2) showed four prominent peaks at m/e 260 (relative abundance 64.7%), 233 (100%), 217 (17.6%) and 43 (25.7%) in addition to the molecular ion peak (M^+) 344 (64.6%). To account for such peaks, it could be assumed that fragmentation occurs in two principal ways (Scheme-1).

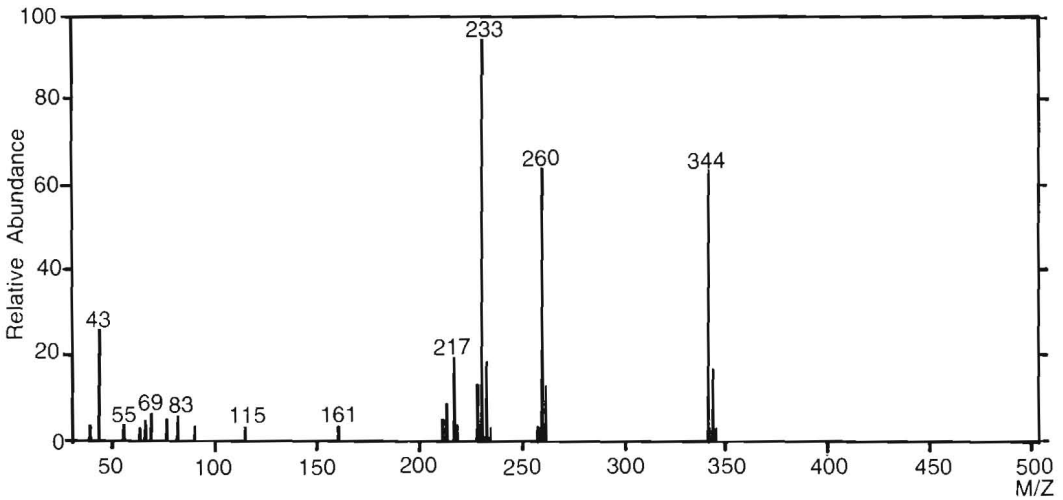


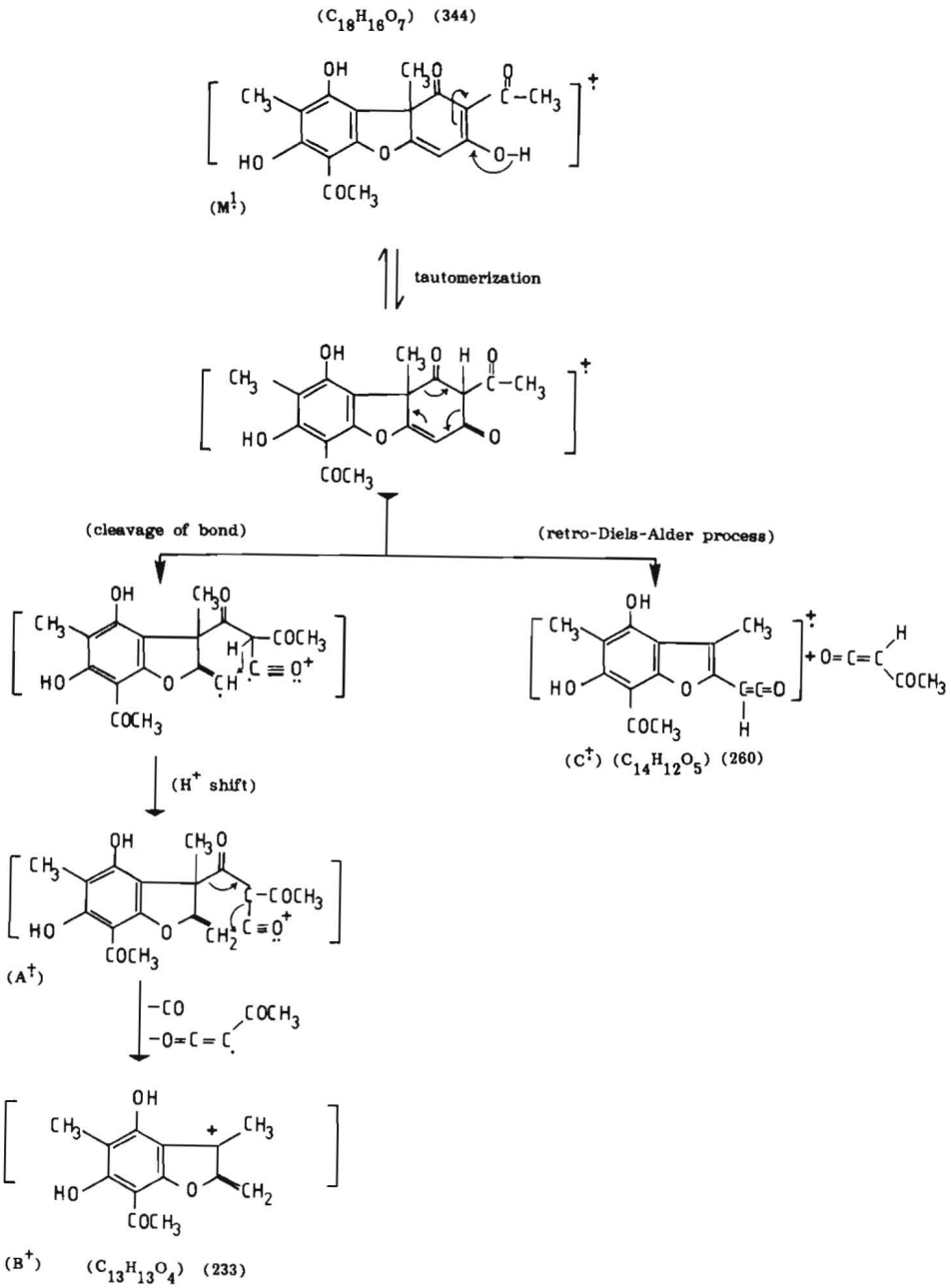
Fig. 2. Mass spectrum of (+)usnic acid isolated from *Usnea articulata.*

The primary cleavage of the keto tautomer of usnic acid, as with aliphatic cyclic ketones, occurs adjacent to the CO group, followed by a hydrogen shift. The rearranged radical cation (A^+) thus formed eliminates CO and acetyl ketene radical yielding the base peak at m/e 233 which corresponds to the resonance stabilized cation (B^+).

The other unique mode of cleavage of the keto form of (+)usnic acid, is a type of homolytic retro-Diels-Alder reaction (Scheme-1). This mode of fragmentation yields acetyl ketene molecule and the radical cation (C^+) of m/e 260. Elimination of an acetyl radical from the latter radical cation would form the peak at m/e 217. The peak at m/e 43 is undoubtedly due to an acetyl cation CH_3CO^+.

The infrared (IR) spectrum of (+)usnic acid showed characteristic peaks maxima in the regions 1685-1373 cm^{-1}. There is no absorption in the region 3500-3100 cm^{-1}, where simple hydrogen bonded hydroxyl bands normally appear. It also showed strong bands in the region 1710-1670 cm^{-1}, which are close to the range 1685-1660 cm^{-1} assigned to α, ß– unsaturated ketones and may reasonably be attributed to conjugated carbonyl groups.

The proton NMR spectrum of (+)usnic acid (Fig. 3) showed no douplets or multiplets with five major peaks as follows; 1.9 (a) methyl group of C_{9b}, 2.2 (b) methyl group of C_8, 2.7 (c, d) methyl groups of acetyl groups at both C_2 and C_6, 6.0 for the enolic hydrogen at C_4 and 7.2 of chloroform as an impurity with $CDCl_3$.



Scheme 1. A proposed MS-fragmentation pattern of (+)usnic acid isolated from *Usnea articulata.*

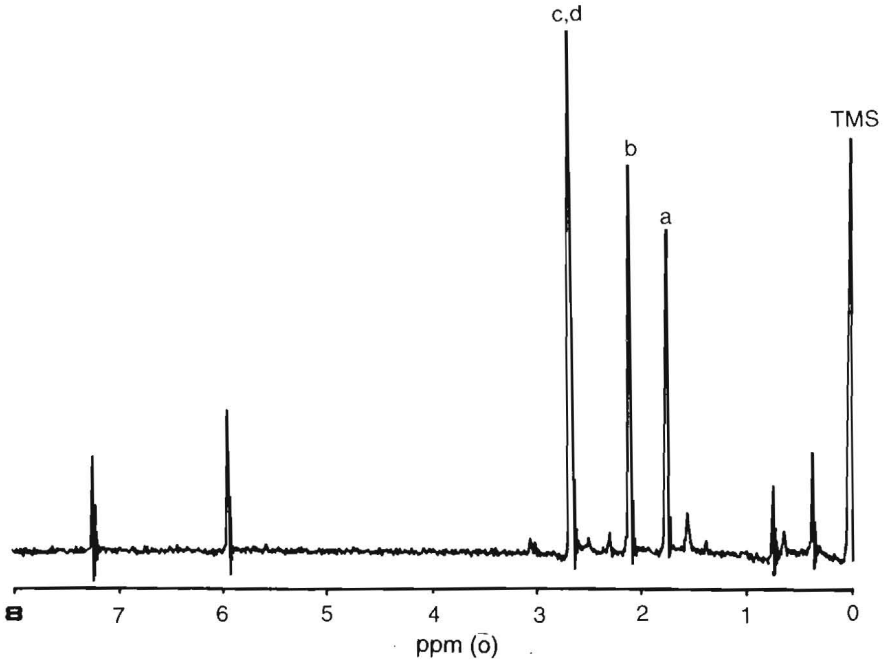


Fig. 3. NMR spectrum of (+)usnic acid isolated from *Usnea articulata.*

The benzene extract of *P. tinctorum* yielded a light yellowish amorphous substance (yield: 0.08-0.1%) which was identified as atranorin (Fig. 4) by comparing to the following results: colour tests gave intense yellowish reaction with K reagent and a dark yellow colour with conc. H_2SO_4. Elemental analysis showed C (62.37%), H (6.54%) and O (31.09%) which are near the expected values (60.96, 4.85 and 34.19% respectively) corresponding to the molecular formula ($C_{19}H_{18}O_8$) and the molecular weight (374.33) of atranorin (the amorphous nature of atranorin and the presence of an impurity, possibly lichenan, could be the causes of this deviation from the expected theoretical values).

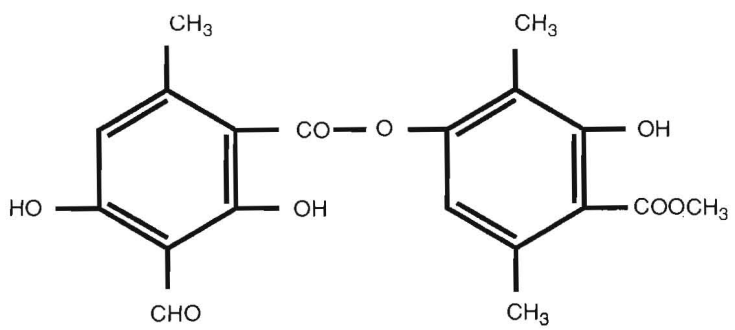


Fig. 4. Chemical structure of atranorin (3-formyl-2,4-dihydroxy-6, methylbenzoic acid, 3,hydroxy-4-(methyoxycarbonyl)-2,5-dimethyl-phenyl ester).

The melting point (recrystallized in acetone) was 192.7 - 193.8 °C. R_f values of TLC were: solvent A (0.85); B (0.70) and C (0.65). These values corresponded to an authentic sample of atranorin. Benzene extract of *P. tinctorum* shows no presence of usnic or any other lichen acids.

UV absorption spectrum of atranorin in benzene had two maxima at 252 and 323 nm which were identical with those of an authentic sample of atranorin.

Mass spectrum of atranorin (Fig. 5) showed five prominent peaks at m/e 196 (relative abandance 77%), 179 (85.8%), 165 (23.7%), 164 (100%) and 136 (46.2%) in addition to the molecular ion peak (M^+) 374 (18.9%). It could be assumed that fragmentation pattern of the molecular ion of atranorin is of typical aryl o-hydroxybenzoate esters. As shown in Scheme-2, the radical cation of atranorin eliminates a neutral ketene derivative through the so-called "ortho" effect, and forms the radical cation of methyl 2,4-dihydroxy-3,6-dimethyl benzoate (m/e 196). The largest peak at m/e 164 resutls from the elimination of a methanol molecule from the latter ester radical cation. Elimination of CO from the radical cation of m/e 164 leads to the peak at m/e 136. The peak of m/e 179 corresponds to the cationic fragment left after elimination of 3-hydroxy-4-methoxy carbonyl-5-methyl-phenoxy radical via homolytic cleavage of the acyl-oxygen bond.

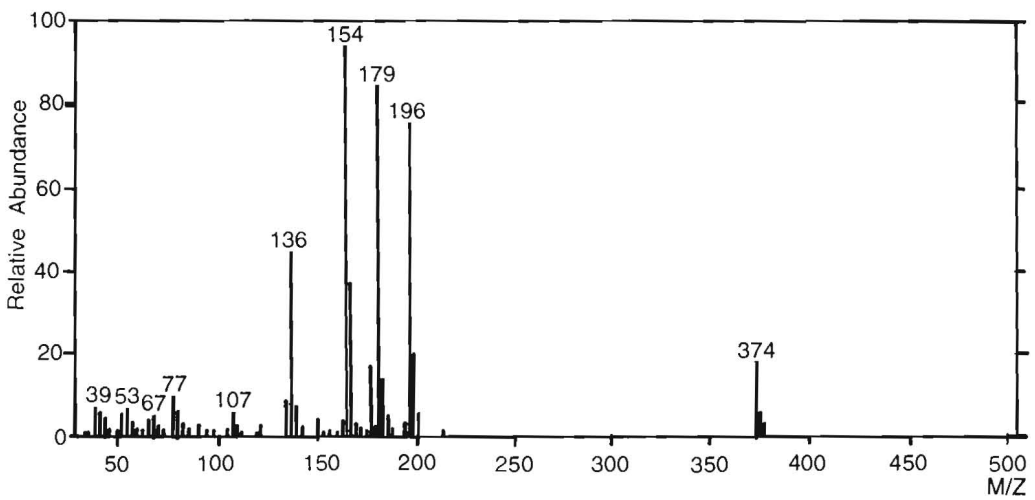
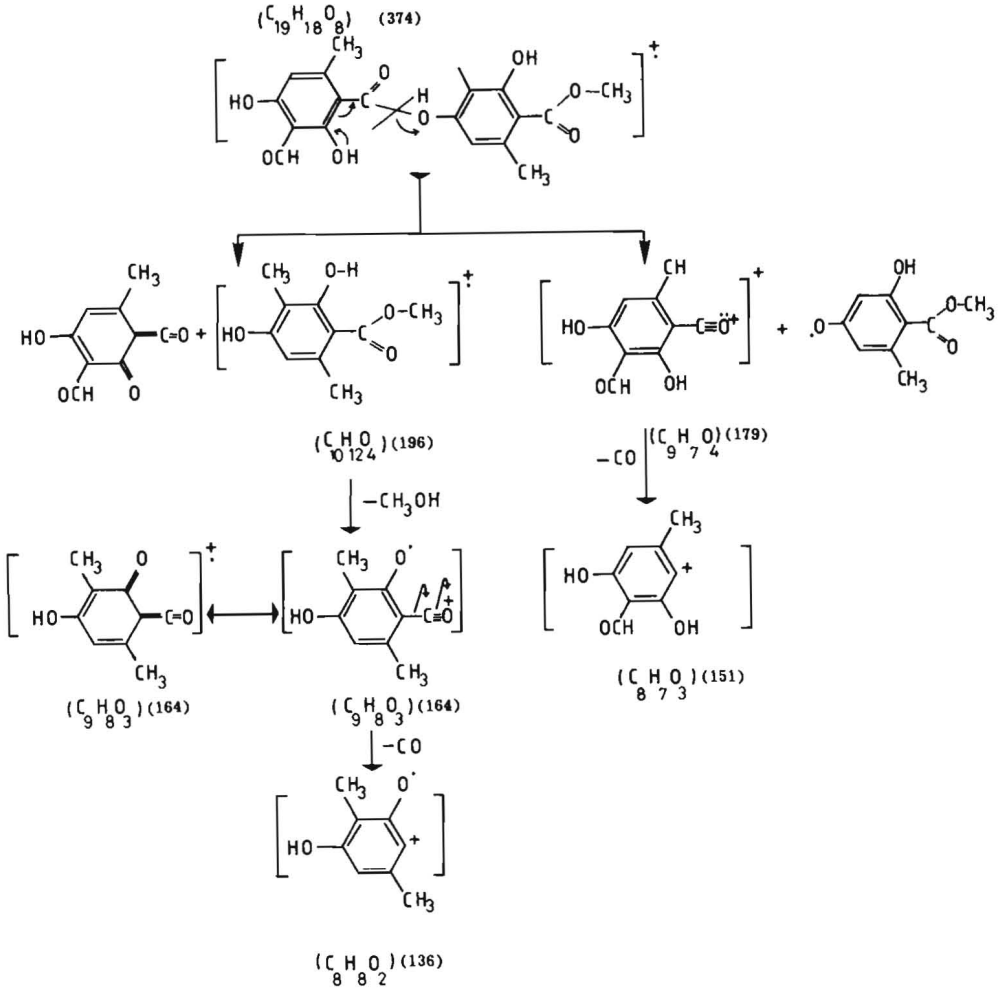


Fig. 5. Mass spectrum of atranorin isolated from *Parmelia tinctorum*.



Scheme 2. A proposed MS-fragmentation pattern of atranorin isolated from *Parmelia tinctorum*.

The IR spectrum of atranorin exhibited five characteristic bands at 1270 (Ph-OH); 1390 (–CH_3); 1450 (CH_3COO–); 1650 (–CO linked) and 2900-2950 (–CHO). These values were identical with those given by an authentic sample of atranorin.

NMR spectrum of atranorin showed major peaks at 1.27, 2.09, 2.55, 2.70, 3.98 (strong), 6.40 (douplets), 7.23, 10.35, 11.90 and 12.55 (douplets).

Discussion

Usnic acid, a widespread secondary metabolite of lichens (Shibamota and Wei 1984) was found in large quantities (0.7-1.0%) in *U. articulata* which grows and flourishes at high altitudes (~7,000 feet above sea level) on the mountains of Asir in the South-Western region of Saudi-Arabia. Chemical identification of (+)Usnic acid was made using mass spectrometry, NMR, IR and other chemical and physical means. All the analysis performed confirmed the presence of (+)usnic acid as a major constituent present in the thalli of *U. articulata*.

The mass spectra of a large number of secondary lichen compounds have been published (Huneck *et al.* 1968, Huneck 1974). The mass spectrum of (+)usnic acid obtained in this study was identical with that found in other lichen species from Europe (Shibata and Taguchi 1967, Santesson 1969, Nourish and Oliver 1976). Similarly, ^{1}HNMR and IR spectra were identical with those obtained for (+)usnic acid found in other lichen species (Shibata and Taguchi 1967, Blanco *et al.* 1984).

It is well documented that usnic acid is the most active antibiotic produced by lichens (Brightman 1960, Bustinza 1960, Shibamoto and Wei 1984, Rowe *et al.* 1991). This fact together with its presence in abundance in the local lichen *U. articulata*, put special emphasis on its clinical usefullness.

Some Arab countries such as Saudi-Arabia, Kuwait, and Oman use the lichen *P. tinctorum* (called locally Al-Sheba) as a spice or food additive. Our results indicated that this lichen species contains a fairly large amount of atranorin (0.1% based on dry weight). The chemical identity of atranorin was confirmed on the basis of spectroscopic methods and other means. Previous identification of atranorin from other lichen species was done by several workers (Shibata and Taguchi 1967, Nourish and Oliver 1976, Blanco *et al.* 1984) and our results confirm theirs.

It has been suggested that atranorin is an accessory photosynthetic pigment in the lichen thallus which acts as a light-screening agent to protect the light-sensitive

underlying algal partner (phycobiont) of the lichen (Scott 1964, Hill and Woolhouse 1966, Richardson 1967, Rundel 1969). Atranorin has also a growth-retarding effect on some herbivores (Lawrey 1986).

No doubt that the chemical identification of lichen acids isolated from Saudi-Arabian lichens would constitute an important step towards the understanding of their biochemical modes of action, and to their potential use in chemotherapy.

Acknowledgements

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عزل والتعرف على حمض الأوزنيك والأترانورين من بعض أشنات المملكة العربية السعودية

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المضاد الحيوي الطبيعي حمض الأوزنيك ، يوجد بكمية كبيرة (نحو واحد بالمئة من المحتوى الجاف) في أشنة *Usnea articulata* التي تنمو بغزارة فوق مرتفعات عسير في المنطقة الجنوبية – الغربية للمملكة العربية السعودية . ولقد تأكدت هذه النتيجة بالتحليل الكيميائي الدقيق بإستخدام طيف الكتلة ، والرنين النووي المغناطيسي ، والأشعة تحت الحمراء ، والأشعة فوق البنفسجية ، وبالطريقة البولارمترية . أما الأترانورين ، وهي مادة أشنية حمضية أقل أهمية من الناحية الطبية ، فقد وجدت بكميات أقل (نحو عُشْر بالمئة من المحتوى الجاف) في الأشنة الصالحة للأكل *Parmelia tinctorum* التي تستهلكها بعض البلدان العربية كنوع من التوابل . كذلك ، فإن لهذه المادة خصائص مانعة لتغذية الحشرات . ولقد تأكد وجود الأترانورين في هذه الأشنة بإستخدام طرق التحليل الكيميائي الدقيق سالفة الذكر .