CHEMICAL INVESTIGATION OF INDIAN LICHENS

Part VIII. Some lichens growing on sandal trees (*Ramalina tayloriana* and *Roccella montagnei*)

BY T. R. SESHADRI AND S. SANKARA SUBRAMANIAN

(*From the Departments of Chemistry and Chemical Technology, Andhra University, Waltair*)

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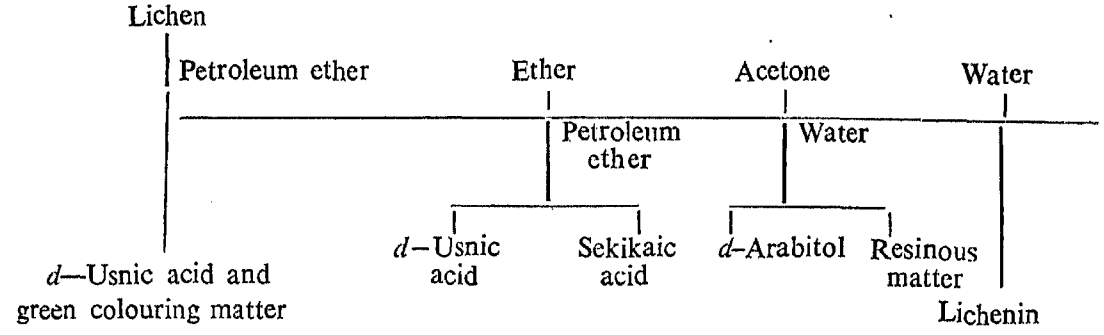
IT was reported to us recently that sandal trees grown on the Simhachalam hills which are in the neighbourhood of Waltair have been suffering from the adverse effects of the growth of lichens on them. The tender growing parts of the plants have a heavy growth of lichens and these parts soon deteriorate and die. A sample of the predominant lichen was sent to Kew for identification and it has been identified as *Ramalina tayloriana* Zahlbr. (*Ramalina canaliculata* Tayl. non-diet.). A chemical examination of the components using the general procedure adopted in the previous papers of this series has established the presence of *d*-usnic acid, sekikaic acid and *d*-arabitol. Two methods of extraction have been adopted using the sequence of solvents, petroleum ether, ether, acetone and water in the first instance and omitting petroleum ether in the second owing to lack of this solvent. Usnic acid is quite soluble in petroleum ether especially in the presence of green colouring matter and rather sparingly in ether, whereas sekikaic acid is insoluble in the former and freely soluble in the latter. Consequently usnic acid was obtained mostly from the petroleum ether extract and to a small extent from the ether extract while sekikaic acid was found only in the ether extract. The subsequent acetone extract yielded *d*-arabitol. In the second set of experiments in which petroleum ether was omitted usnic acid was obtained both from ether and acetone fractions.



In the nature of its chemical components *R. tayloriana* resembles closely *R. geniculata*[1]. Among the large number of species of the Ramalinæ so far

examined usnic acid seems to be almost invariably present; sekikaic acid which is said to be found only in the Ramalinæ[2] has been reported previously to be present in *R. dilacerata*[3], a Chinese drug known as ' Shi-hoa', *R. farinacea*[1] and *R. geniculata*[1]. Similarly *d*-arabitol[4] has been previously noted to occur in *R. scopularum* and *R. geniculata.*

In considering the possible cause of injury to the sandal trees may be mentioned here the statement of Porter[5] that the Ramalinæ are capable of causing damage to the tissues of trees by their penetrating bases. In the present case the lichen was actually found to have penetrated deep into the tissues of the sandal trees; more markedly the tender parts were attacked. This feature was also observed in other plants growing nearby and they were also adversely affected. Besides the physical injury mentioned above, it appeared to us possible that certain lichen products may have a phytocidal effect which may require consideration. Hence some experiments on the toxic properties of the components of this lichen have now been made using fish as test-animals. Usnic and sekikaic acids are found to be markedly toxic to fish and consequently some phytocidal action could be expected from them. Previously vulpinic and pinastric acids were noted to have toxic effect on living animals[6]. Usnic and sekikaic acids may now be added as poisonous lichen components. From the tentative constitution given to the former it contains the toxophore (A) and the latter has in it the grouping (B) (see Lauger *et al.*[7] and Seshadri *et al.*[8]). Here may also be mentioned the recent discovery of Stoll *et al.*[9] that usnic acid inhibits the growth of Staphylococcus aureus and various mycobacteria, but is inactive against Gram-negative bacteria.

(A) (B)

Ramalina tayloriana is found mostly at higher altitudes of the range above 1000 ft. whereas *Roccella montagnei* is more common at the lower altitudes. In some collections of the former from sandal trees small quantities of the latter were also mixed up. This admixture of lichens was clearly indicated by the chemical examination of the mixture in which the characteristic components of the Roccella, roccellic acid and erythrin, could be isolated.

Roccella montagnei has been examined earlier in detail in this laboratory[10]. As further information the study of the carotenoids present in this lichen has now been undertaken. It may be mentioned here that only stray studies of vitamin properties of lichens[11] have so far been made. Only traces of vitamin D and some ergosterol were noticed in *Cladonia rangiferina.* Short growth lichens of Alaska gave some vitamin A when fed to rats.

The presence of vitamin C was also detected by acetic acid-silver nitrate reagent. No study of the carotenoid pigments of lichens seems to have been carried out so far. We expected that lichens belonging to the foliose type (leafy and green) might contain high percentage of carotenoids just like green leaves. Further in view of (1) the contribution of algæ and fungi to the discovery of new carotenoids, (2) the presence of both these in lichens and (3) the fodder value of lichens, there appeared to be considerable interest in this particular study. Fairly high percentage of β-carotene and small amounts of kryptoxanthin are found to be present in *Roccella montagnei*. The part played by climatic conditions is also revealed in the difference between samples obtained from Waltair and from Nagarcoil in South Travancore which is located nearer to the Equator and is warmer and more humid with a higher rainfall. Samples obtained from the latter contained about 40 mg. of β-carotene per hundred grams of air-dry lichen whereas the Waltair sample contained less, about 28 mg./100 g. and both of them compare very favourably with other natural sources of β-carotene.

In the experimental portion not only is the estimation of the carotenoids described but also an improved method of separating the various components present. This lichen does not seem to exert any unfavourable effect on sandal trees probably because it does not penetrate the tissues of the plant and further has no toxic components as revealed by fish tests. It is, however, of interest in connection with the study of antibiotics. Roccellic acid derivatives have been found to be anti-tubercular[12] and more recently *l*-protolichesterinic acid and related lactone aliphatic acids have been discovered to have antibacterial proprties[13].

Experimental

Ramalina tayloriana

Extraction I.—Petroleum ether extract (d-*Usnic acid*).—The air-dried lichen (500 g.) was extracted with petroleum ether (b.p. 60-80°) in the cold by repeated maceration (three times, each time lasting for 24 hours). The solvent was then distilled and the concentrated extract allowed to stand over-night when some greenish yellow crystalline solid separated. It was filtered off, washed with a little petroleum ether and recrystallised twice from dry benzene. When finally crystallised from a mixture of chloroform and alcohol it was obtained in the form of yellow prismatic rods melting at 202-203°; yield, 1·8 g. (Found: C, 62·6; H, 5·0; $C_{18}H_{16}O_7$ requires C, 62.8; H, 4·7%). It was easily soluble in chloroform and hot benzene and sparingly in cold alcohol $[\alpha]^{30}$ in chloroform, + 469°. It dissolved in aqueous potassium hydroxide giving a yellow solution and in alcoholic solution it gave a reddish-brown colour with ferric chloride. It did not give any colour with bleaching

powder and did not undergo fission by treatment with dilute alkali (5%) like the depsides. With concentrated sulphuric acid it gave a deep yellow solution which turned orange-red on standing or addition of a drop of water. In view of all these properties the compound was identified as *d*-usnic acid. This was confirmed by preparing its acetate melting at 198-199° using acetic anhydride and pyridine.

Ether extract (*Sekikaic acid*).—The material left after extraction with petroleum ether was repeatedly extracted with ether in the cold till the last extract was almost colourless. The total extract was concentrated to a small bulk (concentration being effected in vacuo towards the end) and left over-night when a pale yellow solid separated out. It was filtered off, washed repeatedly with a good quantity of petroleum ether to remove any usnic acid and the colourless solid product crystallised from benzene when it was obtained in the form of colourless rectangular rods melting at 144°; yield, 4·5 g. (Found: C, 63·4; H, 6·3; $C_{22}H_{26}O_8$ requires C, 63·2; H, 6·2%.) It was insoluble in petroleum ether and easily soluble in alcohol and acetone. It was soluble in sodium bicarbonate solution and in alcoholic solution it was acidic to litmus and gave a violet colour with a drop of ferric chloride. It did not give any colour with bleaching powder. On acetylation with acetic anhydride and fused sodium acetate it gave a crystalline acetate melting at 162-163° (diacetyl sekikaic acid m.p. 162-163°). Therefore it was identified as sekikaic acid. This was confirmed by identifying its fission products as divaricatinic acid, m.p. 148° and hydroxy-divaricatinic acid, m.p. 157°.

Acetone extract (d-*Arabitol*).—The residue left after ether extraction was extracted with hot acetone (three times, each time lasting for 6 hours) and the combined extract after concentration was obtained as a sticky mass. It was divided into two fractions by treatment with water. The water-insoluble fraction consisting of a sticky reddish-brown resinous mass was removed by filtration and the clear aqueous solution concentrated on a water-bath. The viscous semisolid residue was left in a vacuum desiccator for some days when it gradually solidified to a sticky mass which could be obtained as a granular solid after washing with a large quantity of ether. It was very hygroscopic and when crystallised from a mixture of alcohol and acetone came out as colourless crystalline aggregates melting at 102-103°; yield, 0·5 g. It was sweet to taste and easily soluble in water, sparingly in alcohol and acetone and insoluble in ether. It gave no colour with ferric chloride and did not reduce Fehling's solution. On acetylation it gave a crystalline acetate melting at 73-74°. From these reactions it was identified as *d*-arabitol.

Water extract (*Lichenin*).—The residual lichen powder was finally boiled with water for 48 hours and the aqueous extract concentrated on a water-bath

when a brittle brown mass was obtained; yield, 15 g. It was insoluble in alcohol, sparingly soluble in cold water and easily soluble in hot water. It gave no colour with iodine or ferric chloride. It was quite soluble in ammoniacal copper sulphate solution. On hydrolysis with dilute hydrochloric acid it reduced Fehling's solution. It was therefore identified as lichenin.

Extraction II.—The lichen powder was first extracted with ether by cold percolation and then with hot acetone. The ether extract was concentrated to a small bulk and cooled when usnic acid separated out first as the less soluble component. The mother liquor was concentrated to a syrupy consistency and by judicious addition of petroleum ether sekikaic acid was precipitated and purified by crystallisation. The acetone extract was divided into two portions as before. A small quantity of usnic acid was obtained from the water-insoluble portion by treatment with ether, and *d*-arabitol from the water-soluble portion.

Mixed sample: Ramalina tayloriana + Roccella montagnei (*Usnic acid, Sekikaic acid, Roccellic acid and Erythrin*)

The powdered sample (50 grams) was extracted with petroleum ether, ether and hot acetone successively. From the petroleum ether extract *d*-usnic acid was obtained. The ether extract on concentration yielded a less soluble fraction consisting of a small quantity of *d*-usnic and mostly roccellic acids. The latter could be obtained pure by treatment with dilute sodium bicarbonate solution and subsequent acidification with dilute hydrochloric acid. When crystallised from acetone it was obtained as colourless rectangular rods melting at 132-33°. Mixed melting point with an authentic sample of roccellic acid was not depressed. From the original ether mother liquor by evaporation and treatment with a little pure chloroform, a small quantity of rocellic acid was left behind. The chloroform solution yielded sekikaic acid when treated with excess of petroleum ether.

The acetone extract on concentration to a very small bulk deposited a solid which when washed with cold water and crystallised from a mixture of alchohol and acetone came out as colourless needles melting at 154-55°. Mixed melting point with an authentic sample of erythrin was not depressed.

Roccella montagnei.—Two representative extractions were done, one with a sample obtained from Nagarcoil, South Travancore and the other with the sample collected in Waltair.

Ether extract.—The lichen in coarse powder form (300 g.) was repeatedly extracted with ether in the cold till the last extract was almost colourless.

The total extract was distilled to remove the solvent (reduced pressure was used for the last stages) and the semisolid residue was digested with petroleum ether (b.p. 40-60°, litre) and filtered (residue R). The filtrate was concentrated under reduced pressure to one-fifth of its volume and dried over anhydrous sodium sulphate. This solution was passed through a column of anhydrous alumina for chromatographic analysis. The lower-most region, broad orange red band, was eluted from the column itself by passing fresh quantities of the same solvent. This eluate was concentrated to a very small bulk and allowed to stand over-night when a beautiful crystalline solid in the form of lustrous violet-red plates separated. This was filtered off and recrystallised from benzene-methanol mixture when it was obtained as dark violet prisms in the form characteristic of β-carotene[14]; yield, 90 mg. from the Nagarcoil sample and 70 mg. from the Waltair sample. It melted at 183-184° and was optically inactive. It gave a deep blue colour with conc. sulphuric acid and a greenish blue colour with antimony trichloride in chloroform solution. Its identity as β-carotene was confirmed by its absorption spectrum in carbon disulphide solution, the absorption region being 520-485 mμ. Colorimetric estimation of the total pigment present in the eluted solution was carried out using a standard curve relating the Lovibond yellow units with pure β-carotene in micrograms per c.c. The value was 120 mg. in the case of the Nagarcoil sample and 85 mg. in the case of the Waltair sample. The other bands in the developed chromatogram were cut out and separately eluted with petroleum-ether containing ethanol (2%). The two upper most bands, olive green and bluish green were found to be due to chlorophylls and the next (lower one) orange yellow region was identified as kryptoxanthin by means of its absorption spectrum at the region 485-450 mμ (in petroleum ether b.p. 60-80°) and phase separation with methanol (95%) from petroleum ether solution. Colorimetric estimation was 2·5 mg. in the case of the Nagarcoil sample and 1·5 mg. in the case of the Waltair sample. In between the β-carotene and kryptoxanthin bands two very narrow reddish orange bands were observed which could not be identified.

The residue R was worked up according to the earlier method[10] and was found to consist of roccellic acid m.p. 133° (yield, 1·7% in Nagarcoil sample and 2·3% in Waltair sample), a small quantity of erythrin m.p. 155-56° and orcinol (yield, 0·2 and 0·6%).

Acetone extract.—The hot acetone extract was concentrated to the minimum bulk and ether was added in an equal amount when a colourless solid (1·0% in Nagarcoil sample and 1·2% in Waltair sample) was precipitated. This was filtered off, washed with ether and crystallised from alcohol when it came out as large hexagonal crystals melting at 122°. It was identified

as erythritol by preparing its acetate m.p. 91° and benzoate m.p. 185-86°. The acetone ether filtrate was evaporated off and treated with water. The water-insoluble portion was crystallised from acetone when erythrin (1·3 and 1·0%) was obtained. The aqueous filtrate was extracted three times with an equal volume of ether when orcinol was removed in the ether layer leaving montagnetol behind in water. The layers were separated and evaporated; orcinol (a small quantity) was obtained from the former and *r*-montagnetol (1·2% and 1·5%) m.p. 156° from the latter. This procedure facilitates easy isolation of montagnetol as compared with the earlier method.[15]

Toxicity tests.—Fresh water fish (*Haplochilus panchax*) were employed as experimental animals for the test which was carried out according to the method of Krishnaswami and Seshadri,[16] the 'turning time' being used as a measure of toxicity.

d-*Usnic acid.*—50 mg. of usnic acid was dissolved in 10 c.c. of hot absolute alcohol and the solution was poured into a litre of tap-water in a thin stream with continuous stirring. The average turning time for fish was found to be thirteen minutes. None of the fish recovered even after removal to fresh water and all died after some time.

Sekikaic acid.—The experiment was repeated using sekikaic acid instead of usnic acid and adding gelatin (one gram per litre of water) in order to increase the solubility of the compound in water. In a concentration of 100 mg. per litre the average turning time was found to be twenty-seven minutes and with 200 mg. the time taken was only 6 minutes. In both cases all the fish died without recovery. A peculiar slate-blue colour developed on the ventral side of the fish.

Summary

Ramalina tayloriana, a lichen growing on sandal-wood trees is considered to produce adverse effect on the trees both by physical injury caused by the hold fasts and by means of its toxic components, usnic and sekikaic acids which are found to be toxic to fish. *Roccella montagnei* appears to have no such effect. It is found to be rich in β-carotene. Convenient methods of separating the various components of these lichens are described.

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