

CHAPTER IV

DISCUSSION

Usneaceae is the lichen family which accumulates various groups of compounds such as sterols, triterpenes, xanthenes, dibenzofurans, polyols, anthraquinones, depsides and depsidones. The largest number of lichen substances belong to the two groups, depsides and depsidones (Neelakantan, 1969). The survey of pertinent literature of the genus *Usnea* suggested that usnic acid is the principal constituent of members of this lichen genus (Patrocínio, 1980). This present work has led to the isolation of three compounds from the entire plant of *Usnea siamensis* Wainio. All of them are identified by using their physical properties and spectroscopic data.

Part I : Phytochemical Study

1. Structure Elucidation of the Isolated Compounds

1.1 Compound US-1

Compound US-1 was obtained as yellow needles from petroleum ether and chloroform part by successive chromatographic techniques using silica gel column (benzene : chloroform = 3:2) to yield 20.660 gm (1.247% w/w of dry weight).

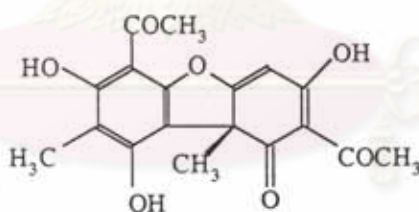
The ei mass spectrum of US-1 (Figure 17 , p.139) exhibits the molecular ion peak at m/z 344 (61.5%) corresponding to a molecular formula of $C_{18}H_{16}O_7$. The UV

spectrum (Figure 15 , p.137) shows the absorption bands at λ_{\max} 284 nm which is the characteristic of the conjugated dibenzofuran part. The ir spectrum (Figure 16 , p.138) confirms the presence of the carbonyl stretching at ν 1640 cm^{-1} and also suggests the acetyl (COOCH_3) at 1700 cm^{-1} .

US-1 can be assigned as a known dibenzofuran derivative: usnic acid by the analysis of its ^1H and ^{13}C nmr spectra. The ^1H nmr spectrum of US-1 (Figure 18 , p.140) shows the signals of 3 methyl groups, 1 acetyl group, 1 aromatic proton, and 3 hydroxy protons. The ^{13}C nmr spectrum (Figure 19 , p.141) suggests the presence of 3 carbonyl carbons, 9 sp^2 carbons, 3 methyl carbons and 1 acetyl carbon.

The assignments of protons and carbons of compound US-1 are confirmed by comparison with the data of usnic acid previously reported by Takahashi (1975) and Cooper *et al* (1992).

The assignments are summarized in Table 8 and 9 (p.78 and 79). The structure of usnic acid is shown below.



This structure is confirmed by the analysis of the mass fragmentation (Figure 17). The cleavage of the ester carbonyl and methyl provide the fragments of m/z 232(16.5%) and 217 (18.4%), respectively. Proposed mass fragmentation of compound US-1 is shown in Scheme 1.

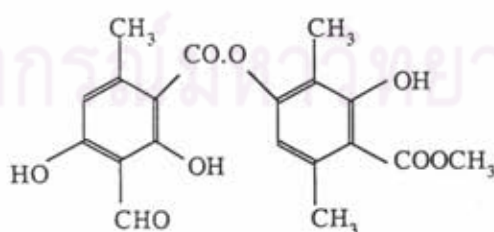
1.2 Compound US-2

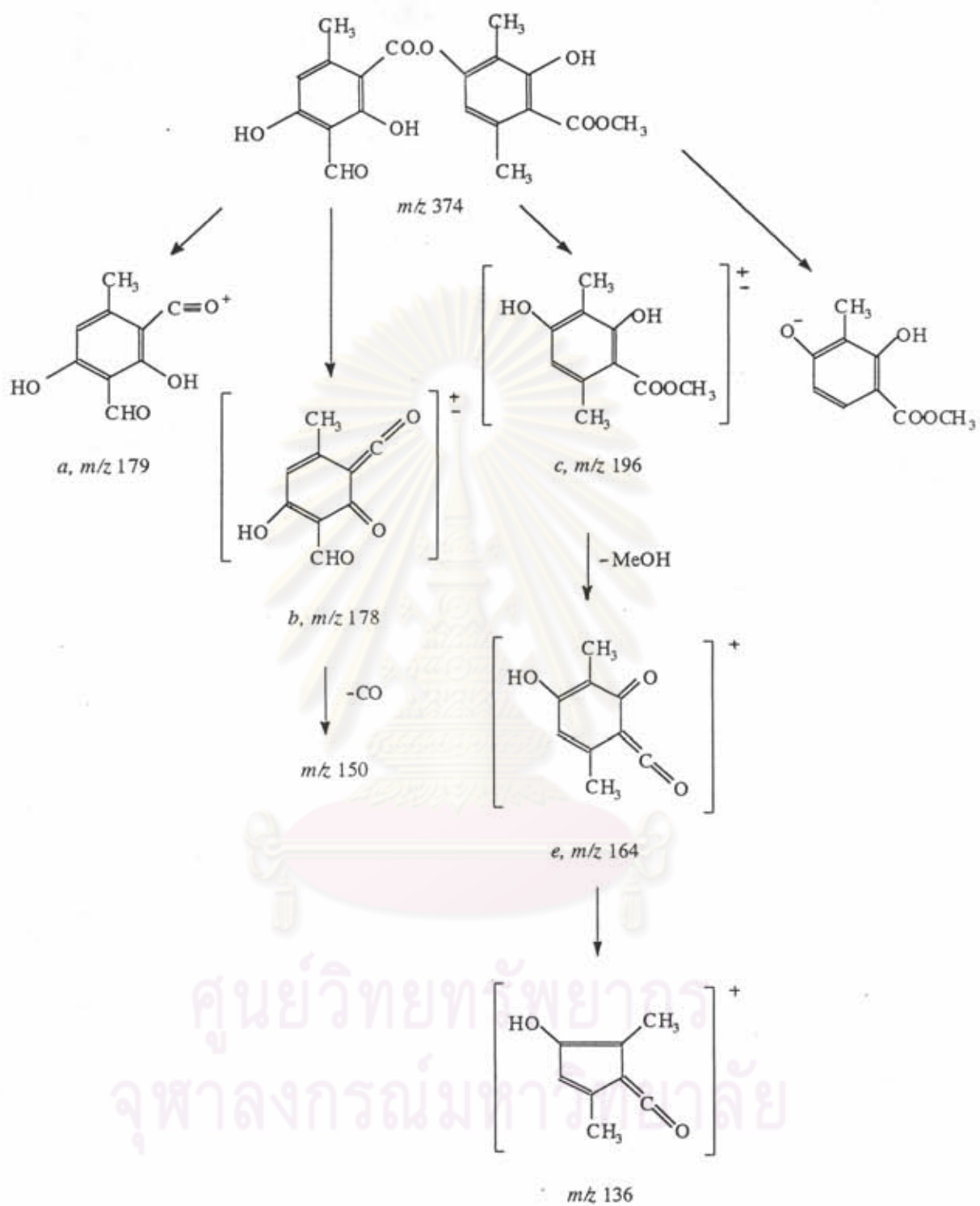
Compound US-2 was obtained as colorless needles from petroleum ether part by chromatographic techniques using silica gel column (petroleum ether : chloroform = 4:1). It yields 0.013 gm (0.009% w/w of dry weight).

The eims spectrum of US-2 (Figure 23 , p.145) exhibits the stable molecular ion peak at m/z 374 (73%) and establishes the tentative molecular formula of $C_{19}H_{18}O_8$. The uv absorption at λ_{max} 218 and 262 nm (Figure 21 , p.143) suggest the conjugated part which is confirmed by the ir absorption band ν 1660 cm^{-1} (Figure 22 , p.144).

Compound US-2 is assigned as a known depside, atranorin, by the analysis of the 1H and ^{13}C nmr spectra. The 1H nmr spectrum (Figure 24 , p.146-148) provides the signals of 3 hydroxy protons, 1 methoxy and 3 methyl groups. The ^{13}C nmr spectrum (Figure 25 , p.149) shows the signals of 3 carbonyl carbons, and 1 aldehyde carbon, 12 sp^2 carbons, 1 methoxy carbon, and 3 methyl carbons.

The assignments of carbons and protons of US-2 are supported by comparison with the reports of Hylands and Ingolfsdottir (1985). The chemical shifts of protons and carbons are summarized in Table 10 and 11 (p.82 and 83), respectively. The proposed structure of US-2 is finally confirmed by the analysis of the mass fragmentation (Scheme 2). The chemical structure of compound US-2 is shown below.





Scheme 2 The fragmentation of US-2

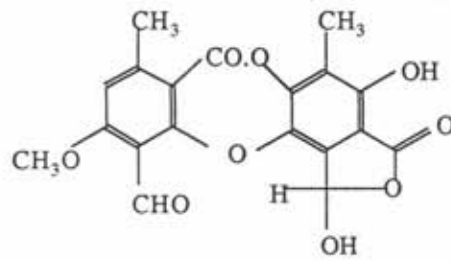
1.3 Compound US-3

Compound US-3 was obtained as white amorphous powder from acetone part by chromatographic techniques using silica gel column (10% methanol in chloroform). It yields 0.025 gm (0.017%w/w of dry weight).

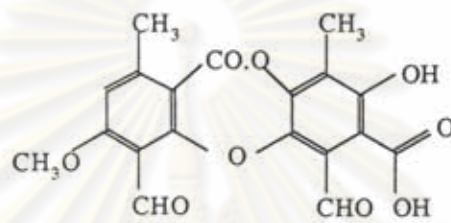
The eims spectrum (Figure 28 , p.152) shows the stable molecular ion peak at m/z 386 and establishes the tentative molecular formula of $C_{19}H_{14}O_9$. The uv absorption at λ_{max} 217, 262 and 318 nm (Figure 26 , p.150) suggests the depsidone carbonyl and carboxyl and can be confirmed by the ir absorption at ν 1745, 1730 and 1690 cm^{-1} (Figure 27 , p.151). The hydroxyl absorption at 3480 and 3250 cm^{-1} are also observed.

Compound US-3 is assigned as an isomer of the known depsidone: stictic acid. The 1H nmr (Figure 29 , p.153) and ^{13}C nmr spectra of this compound (Figure 30 , p.154) suggest the presence of 2 aldehyde groups and 1 olefinic proton (the literature indicate that the stictic acid shows 1 aldehyde group and 2 olefinic proton). The 1H nmr spectrum also provides the signals of 1 methoxy and 2 methyl groups. The ^{13}C nmr spectrum provides the signals of 16 sp^2 carbons consisting of 2 aldehyde, 1 ester carbonyl, 1 carboxylic acid group and 12 olefinic carbon connecting to the heteroatom, 1 methoxy and 3 methyl carbons.

The proton and carbon assignments of US-3 are confirmed by comparison with the data of stictic acid. The assignments are summerized in Table 12 and 13(p.86 and 87). The proposed structure of US-3 is finally confirmed by the analysis of the mass fragmentation (Scheme 3). The chemical structure of compound US-3 and stictic acid are shown below.

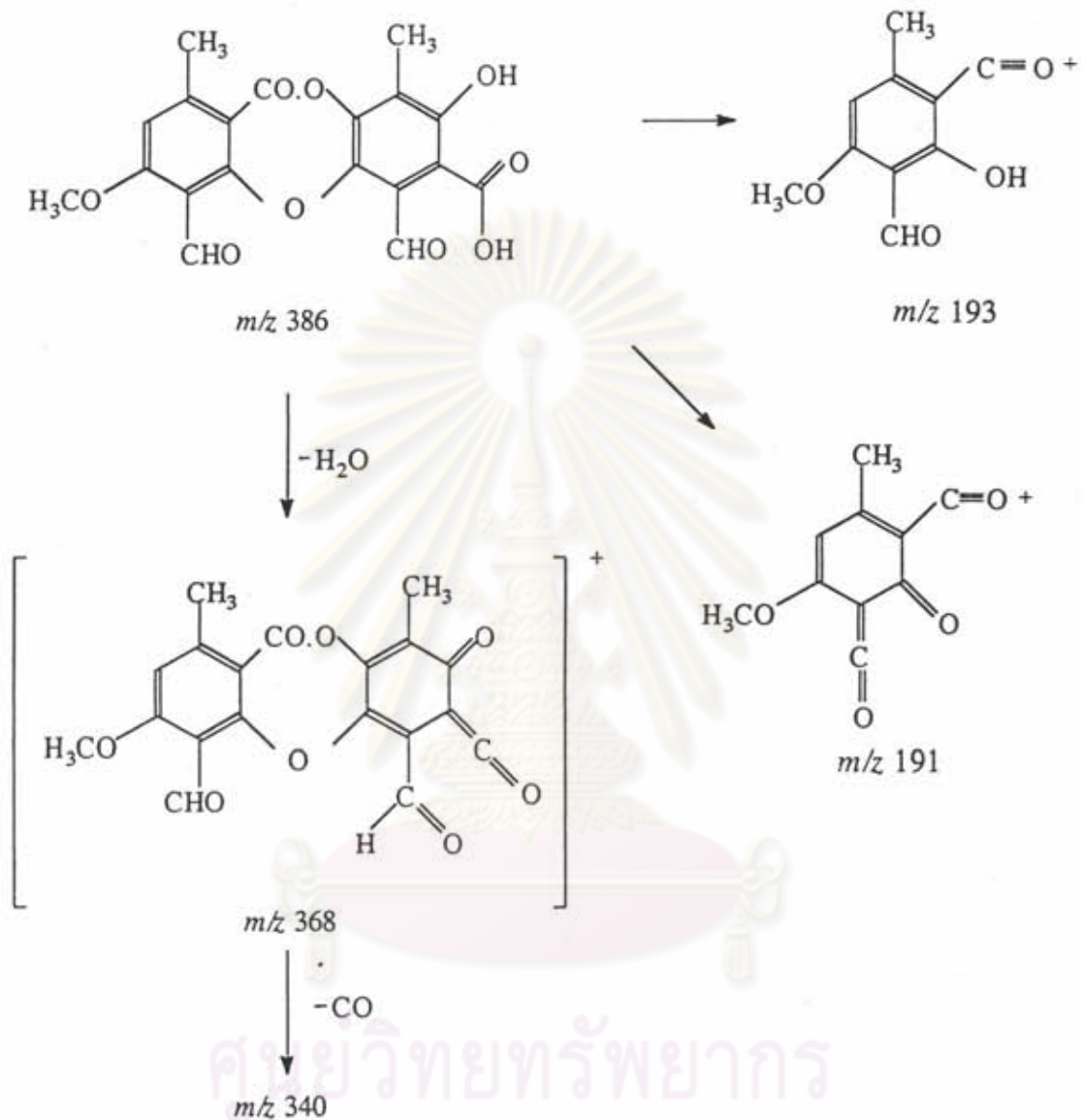


STICTIC ACID



US-3

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Scheme 3 : The fragmentation of US-3

Part II : TLC-Densitometric Analysis of Usnic Acid

Although usnic acid has been reported to be present as the common component of *Usnea* spp., there have been no information on the content of this compound in *Usnea siamensis* Wainio. In this study, we have been shown that the usnic acid content can be determined effectively by using a TLC-densitometric method. This method was developed carefully to ensure accurate quantitative analysis of usnic acid. The method is also simple and rapid since the analysis can be performed directly from the crude extracts without prior purification. It can quantitative usnic acid even the concentration is as low as 0.25 µg per spot.

In the development of this TLC-densitometry, the choosing of the solvent system for complete separation of usnic acid from other interfering substances on a TLC plate is a very important step. The spot of usnic acid on the chromatographic plate must be pure so that the absorption intensity read by TLC-densitometer is not interfered by any other substances. It appears that the composition of chloroform-hexane with the ratio 4:1 is the best solvent system for the separation. Moreover, the application volume of each assay solution must be accurate and constant in order to obtain reproducible peak area of each spot.

In addition, other factors that may affect the reliability of the method were also examined. It was found that the sample preparation by reflux extraction 60-70 °C, one hour, using ethyl acetate as extracting solvent was optimised to allow exhaustive extraction of the usnic acid in *Usnea siamensis* Wainio. Also, the choosing the wavelength 295 nm for the analysis was supported by the absorption spectrum of the compound.

Based on the development TLC-densitometric method, it appears that *Usnea siamensis* Wainio from Doi-Intanon, Chiangmai contains 4.31% of usnic acid and the one from Pu Luang, Loei contains 3.43% of dry weight.



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Part III : Antibacterial activity of usnic acid

It is well-known that intensive use of an antibiotic is often followed by the appearance of resistant strains. In view of this propensity of the resistant bacteria, the search for new antibiotic continuous unceasing. In this connection, plants continue to remain a rich source of new therapeutic compounds (Ahmad *et al*,1986)

Usnea siamensis Wainio, Thai medicinal plant which is commonly compounded in each preparation of local remedies is reasonably believed to be active against bacteria. Antibacterial properties of *Usnea siamensis* Wainio had been evaluated against nineteen representative microorganisas which were suspected to be the cause of many disease as dysentery, diarrhoea, typhoid fever and infectious skin diseases. Microorganisms used for testing are gram positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus citeus*, *Staphylococcus mirabilis*, oxacillin resistant coagulase negative staphylococcus , penicillin-ampicillin resistant staphylococcus , *Microcoocus flavus* (*Sarcina lutea*), *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumitis*, and gram negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus cereus*, *Proteus vulgaris* and *Salmonella typhi*. The selected compound tested against the microorganisms by microbiological agar diffusion method was shown inhibitory activities.

The diameters of inhibition zone of usnic acid from this study are higher than those from solvent control, 0.8% tween 80 in 95% ethanol except of inhibition zone of *Bacillus cereus*. However, there were a few different zone between usnic acid zone and solvent zone. The reason was that diffusion in inoculated agar depended on water solubility. The more water soluble, the more diffuse were there in the cup solution. Usnic acid is a non-polar compound so that it is slightly soluble in water. Tween 80, a nonionic surfactant and



95%ethanol was used in order to dissolve the compound..

The results from this study were in agreement with the results from the previous study which indicated that usnic acid showed antibacterial activity against gram-positive bacteria, for example usnic acid from *Usnea montagnei* inhibited *Bacillus subtilis*, *Micrococcus pyogenus aureus* (both penicillin sensitive and penicillin resistant strains) and *Mycobacterium tuberculosis* : MIC 1 µg/ml, 2 µg/ml and 3 µg/ml respectively (Patrocinio, 1980). Moreover usnic acid showed activities against *Sarcina lutea*, *Corynebacterium diphtheriae*, *Haemophilus pertussis* (Shaw, 1988), *Staphylococcus aureus*, *Bacillus megatherium* (Vertia, 1949) and *Streptococcus mutans* (Ghione, 1988). In Malaysia *Usnea* sp. was used as antidiarrhea and antidysentery (Burkill, 1935). Because of no activity against gram negative bacteria, so that it could be describe the mechanism of action. The difference in the structures of the gram-negative and gram-positive bacteria, in so far that the former have an outer membrane, had an interesting bearing on the resistance of these two groups to biocides. As a general rule, gram-negative bacteria often showed a greater resistance to biocides (antiseptics, disinfectants, and preservatives) than did the gram-positive cells and this was thought to be due to the protective nature of the gram-negative outer membrane, which acted as an exclusion barrier to biocidal agents. In much the same manner, the bacterial spore was considered to more resistant than its corresponding vegetable cell through the presence of its spore coats (Denyer, 1990). It indicated that the antibacterial activity of usnic acid involved the permeability of bacterial membrane.