THE CHEMICAL CONSTITUENTS OF KNOWLTONIA CAPENSIS

by

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SUMMARY

Many members of the Ranunculaceae have antibacterial and antifungal activity due to the liberation of protoanemonin from ranunculin or from some other, as yet unidentified, precursor. Also many members of the family contain alkaloids and, in particular the genus thalictrum of the subfamily Anemoneae has given rise to much interest due to the wide variety of bisbenzylisoquinoline and other related alkaloids which have been isolated from it, especially since a number of these have shown antitumour activity. In connection with this it was noted that the genus knowltonia which is also a member of this subfamily had not been subjected to chemical study. Thus a typical member of this genus, Knowltonia capensis, was examined for its chemical constituents. The plant was tested and was found to have antibiotic activity and possibly also antitumour activity, but this has still to be confirmed. Extraction of the dried plant yielded anemonin, choline and glucose, and revealed that the plant was completely lacking in alkaloids. Extraction of the fresh plant yielded ranunculin and protoanemonin. Thus it was shown that the genus knowltonia, as typified by Knowltonia capensis, is chemotaxonomically related to the other genera of its subfamily, the Anemoneae, with the exception of the genus thalictrum which appears to be anomalous.

INTRODUCTION

1.0 KNOWLTONIA

The plant Knowltonia capensis is a member of the family Ranunculaceae. This family comprises some 40 genera containing about 1100 species, most of which are North Temperate. The Ranunculaceae are mostly poisonous due to their alkaloid content, though some have been reported to have medicinal properties. The classification and chief genera (after Prantl) of this family, where the numbers in brackets refer to the approximate number of species in each genus, are as follows:-

A: Ovules many; follicle, berry or capsule.

1. Paeonieae Paeonia(15).

2. Helleboreae Caltha(20), Actaea(15), Aquilegia(75), Trollius(12), Helleborus(15), Nigella(16), Eranthis(7), Delphinium(175), Aconitum(110).

B: Ovule one; acheme.

3. Anemoneae Anemone(120), Clematis(220), Ranunculus(300), Thalictrum(10), Knowltonia(6).

The genus Knowltonia was originally classified by Engler and Prantl as part of the genus Anemone but was later established by Salisbury to be an independent genus.[1] It is endemic to South Africa and the only chemical studies that have been conducted on it previously were done by Brandwijk and Watt[2] on the species Knowltonia transvaalensis in 1925. They report that this plant contains a white crystalline compound which they call anemonol. They claim that this anemonol readily decomposes to anemonin and isoanemonic acid and they attribute the strongly irritant and vesicant properties of the fresh leaves to it. These findings however are not at all in keeping with later studies on anemonin (see section 2.0).

The above study was carried out as a result of the large number of reported uses of the members of this genus in folk medicine. Thus in their book "Medicinal and Poisonous Plants of Southern and Eastern Africa" Brandwijk and Watt[3] record the following. "In the Transvaal the crushed fresh leaf of Knowltonia transvaalensis is sniffed to relieve headache and a decoction of the leaf is used as a lotion for festering wounds and to relieve poisonous bites. The fresh crushed leaf is found to blister the skin, but the leaf loses this property when dried. The early colonists at the Cape are reported as having used Knowltonia capensis as a Cantharides substitute and as a remedy for sciatica and rheumatism probably by local application. Knowltonia bracheata is acrid and is said to have been one of the factors causing the death of an African and to have been used in South Africa for homicidal purposes. The plant contains an inactive resin and anemonin. An infusion of the leaf of Knowltonia gracilis is used by the Zulu for syphilis. It is either taken by the mouth or injected as an enema. For headaches they inhale the smoke from burning the leaf. Knowltonia rigida is acrid and has been used in the treatment of rheumatism and lumbago, usually as a vesicant. Also the leaf of Knowltonia vesicatoria has very generally been used as a vesicant especially in rheumatism and lumbago. The plant is acrid and even the juice of the ripe drupe is pungent. The root is also irritant and the sore resulting from its application heals very slowly. The coloured people in the Belville district drink a hot infusion of the root and that of Pelargonium grossularoides in large doses for colds and influenza. The treatment produces free perspiration. The plant apparently contains a cardioactive substance and the FD of an alcoholic extract is 33." No other studies of chemical

or pharmacollogical interest have been carried out on this plant genus.

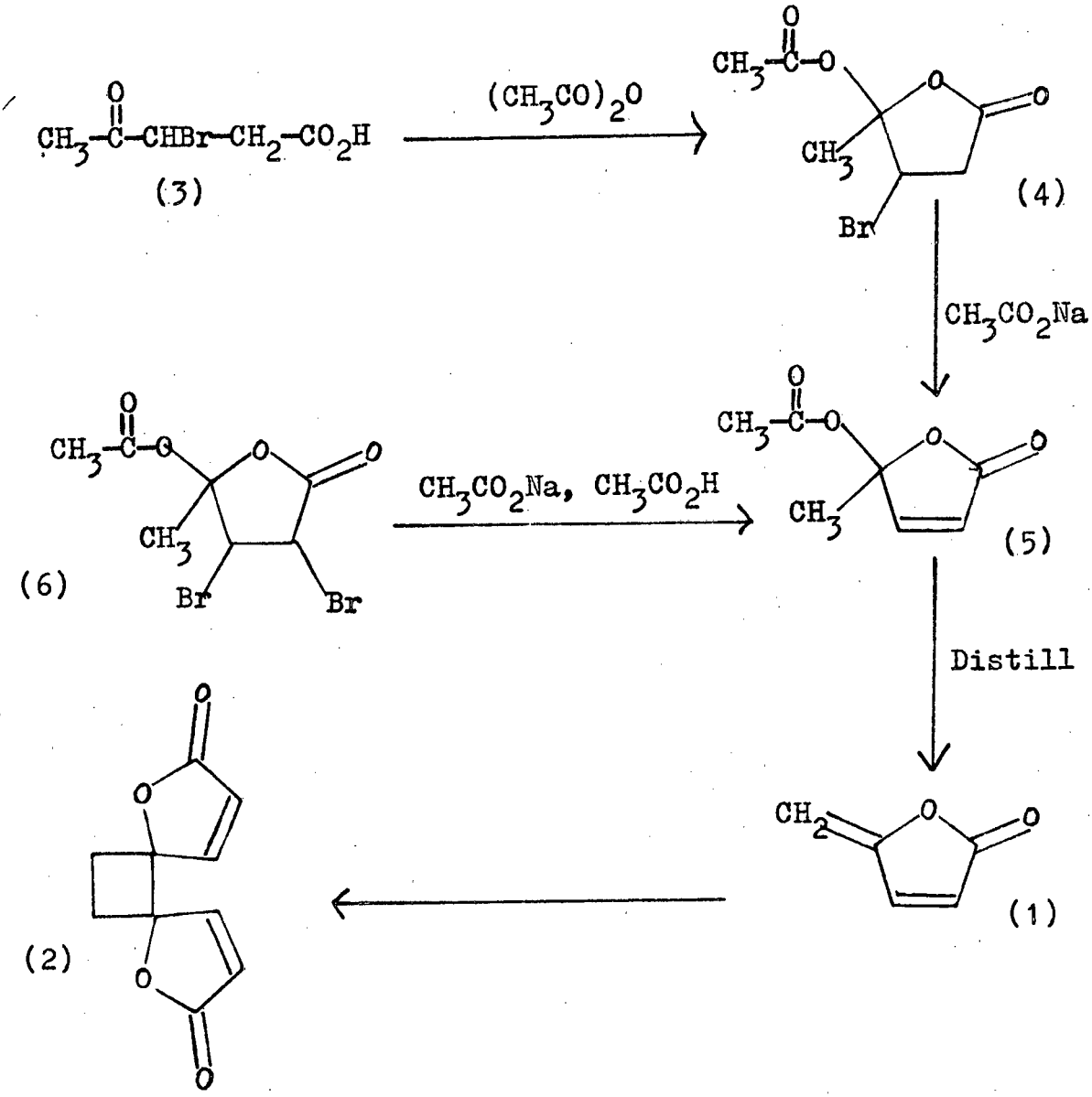
2.0 ANEMONIN, PROTOANEMONIN AND RANUNCULIN.

The presence of a vesicant lachrymogenic compound in certain members of the Ranunculaceae was noted as long ago as 1792 by Heyer[4]. Subsequent study has shown that this compound which has been given the name protoanemonin occurs exclusively in the subfamily of the Ranunculaceae, the Anemoneae, and in the genus helleborus. Moreover, it is present in all members of this subfamily and of the genus helleborus, with the exception of the genus thalictrum. There does, however, seem to be considerable variation in the amount present.[5]

Protoanemonin(1) was first isolated by Asahina and Fujita in 1922[6] by steam distillation of Ranunculus japonicus. It is obtained as a pale yellow oil which rapidly undergoes spontaneous dimerization to yield crystalline anemonin(2), a compound which Asahina and Fujita had already noted in extracts of these plants in 1920[7]. They determined the structure of protoanemonin and anemonin and confirmed these by the following synthesis.

β-bromolevulinic acid(3) reacts with acetic anhydride to give a syrup, acetylbromolevulinic acid(4). Adding dry sodium acetate to this gives acetylacetoacrylic acid(5) and this, when distilled, gives the acrid volatile oil protoanemonin. Angelica lactone dibromide(6) can be used in the place of β-bromolevulinic acid since,with sodium acetate in acetic acid,it also gives acetylacetoacryllic acid. Anemonin is then readily obtained from the protoanemonin thus formed by allowing it to dimerize. (see Scheme I)

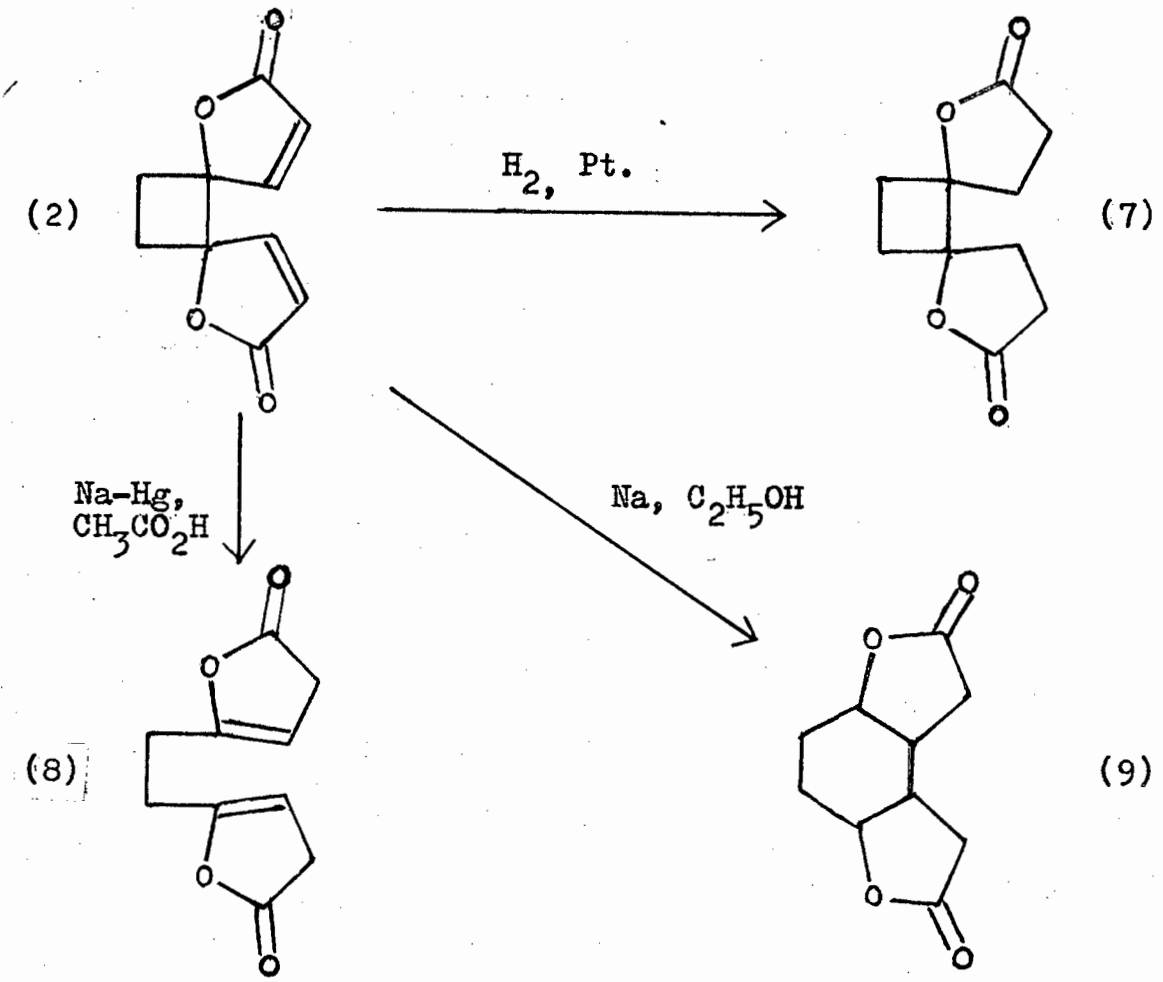
SCHEME I



2.1 Chemistry of Anemonin.

Asahina and Fujita then continued to make a fairly detailed study of the chemistry of anemonin(2)[6, 8]. Catalytic reduction of anemonin with platinum yields tetrahydroanemonin(7), but reduction with sodium amalgam and acetic acid ruptures the cyclobutane ring, leaving the double bonds unattacked, to form the straight chain derivative, dihydroanemonin(8). Reduction with sodium and alcohol gives isotetrahydroanemonin(9), a derivative of cyclohexane. (Scheme II)

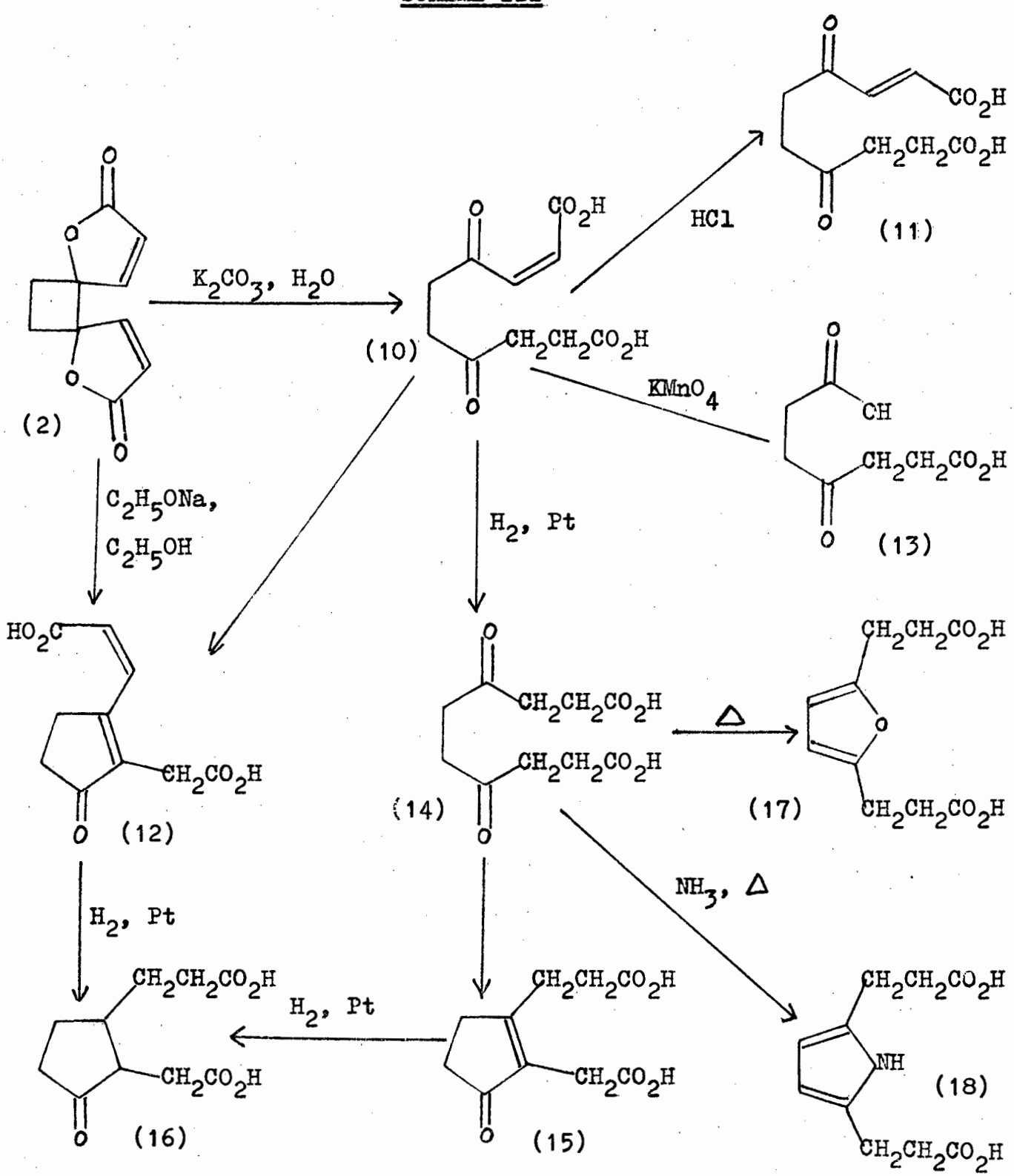
SCHEME II



Aqueous potassium carbonate hydrolyses the lactone groupings of anemonin and opens the cyclobutane ring to give α-anemoninic acid(10), which rearranges with hydrochloric acid to the β-form(11). However if sodium ethoxide is used for the hydrolysis of anemonin the α-anemoninic acid formed undergoes an internal aldol condensation to produce the cyclopentenone derivative anemonic acid(12), which also rearranges on treatment with hydrochloric acid to the β-form. Oxidation of anemoninic acid gives acetonediacetic acid(13), and its reduction gives anemonolic acid(14), which under alkaline conditions also undergoes an internal aldol condensation, in the same way as α-anemoninic acid, to form anhydroanemonolic acid(15). Both anemonic acid and anhydro-

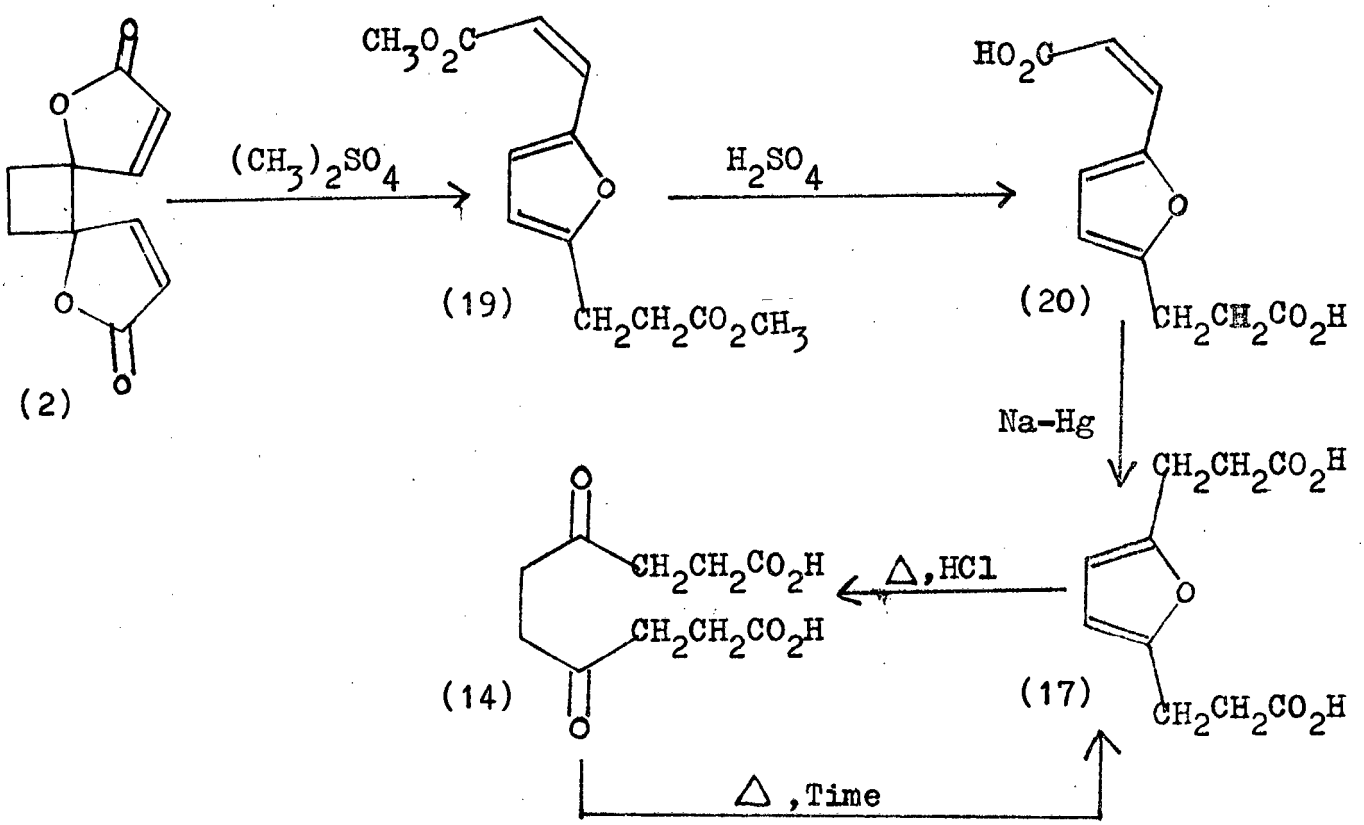
anemonolic acid undergo catalytic hydrogenation to form tetrahydro-anemonic acid(16). As a 1,4-diketone anemonolic acid(14) on heating is converted into furan-2,5-dipropionic acid(17), and when it is heated with ammonia, it gives pyrrole-2,5-dipropionic acid(18). Likewise anemoninic acid(10) is converted into furan-2,5-acrylic-propionic acid and pyrrole-2,5-acrylicpropionic acid. (Scheme III)

SCHEME III



When warmed with methyl sulphate anemonin(2) gives furan-2,5-acrylic-propionic acid dimethyl ester(19) and this on acidification gives the corresponding free acid(20), which is easily reduced to furan-2,5-dipropionic acid(17). Furan-2,5-dipropionic acid on heating with hydrochloric acid gives anemonolic acid(14), which on prolonged heating regenerates (17). (Scheme IV)

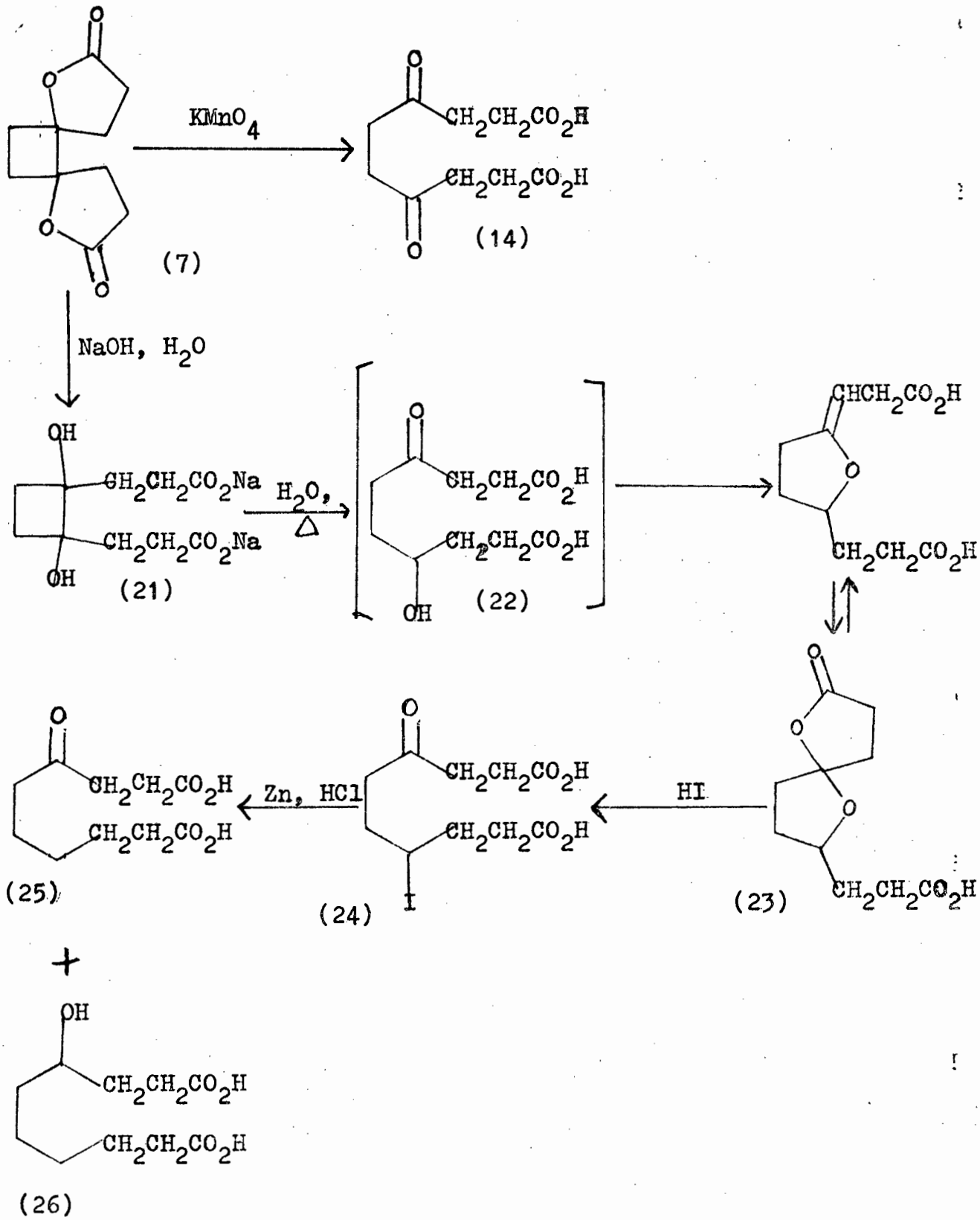
SCHEME IV



Oxidation of tetrahydroanemonin(7) with potassium permanganate gives anemonolic acid(14) and alkaline hydrolysis gives the disodium salt(21). When this sodium salt is heated with water the intermediate compound(22) first formed cyclises to the tetrahydrofuran derivative, ψ-tetrahydroanemonic acid(23). This is converted by hydrogen iodide into 3-iodo-octane-6-one-1,8-dicarboxylic acid(24), which on reduction gives acetone-acetic-valeric acid(25)

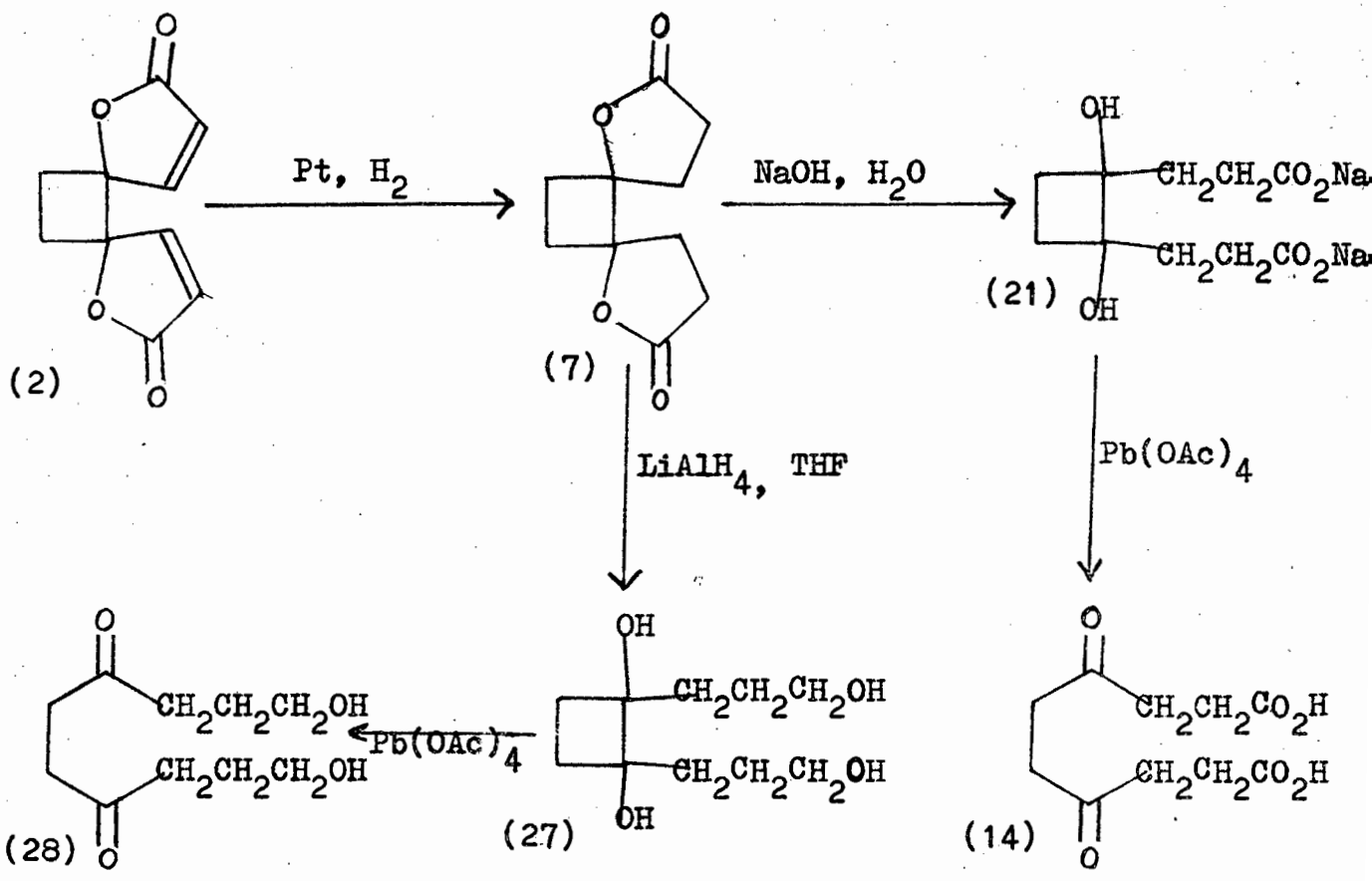
and γ-hydroxysebacic acid(26). (Scheme V)

SCHEME V



These chemical studies all served to establish firmly the structural formula of anemonin but there was still doubt about the stereochemical configuration of the lactone rings. Then in 1959 Harris[9] reduced anemonin(2) catalytically to tetrahydroanemonin(7) which was then further reduced by lithium aluminium hydride in tetrahydrofuran to 1,2-di(3-hydroxypropyl)-1,2-cyclobutanediol, tetrol(27). This compound was cleaved by lead tetra-acetate to decane-1,10-diol-4,7-dione(28). The sodium salt of tetrahydroanemonin(21) was cleaved in the same way to anemonolic acid(14). Periodic acid also cleaved the tetrol to give the same product as was obtained by the lead tetra-acetate cleavage. (Scheme VI)

SCHEME VI



On the basis of the above evidence Harris concluded that the dilactone rings were cis.

However, in 1963 Moriarty, Romain, Karle and Karle[10] investigated a crystal of anemonin by X-ray diffraction and found that the molecule was in the trans configuration. In addition the crystal structure determination showed that the cyclobutane ring is not planar but assumes a bent configuration with a dihedral angle of 152°, and that the carbon-carbon bond lengths in the cyclobutane ring have normal single bond values near 1,54Å. (Anemonin crystallizes in the orthorhombic system, space group Pbca with eight molecules in the unit cell and cell parameters a=11,65; b=13,86 and c=11,07 Å. The structure was solved by obtaining the phases directly from the structure factor magnitudes by means of the symbolic addition procedure.) Independent confirmation of the dihedral angle was obtained from nmr studies of the molecule.[11]

Further evidence for the configuration being trans was obtained by Romain[12] from studies of the dipole moment. Theoretical calculation of the dipole moment of tetrahydroanemonin gives a value of 6,7D for the cis- and 4,6D for the trans- isomer, and practical determinations of this constant through measurements of the dielectric constants and densities of dilute solutions of the solute in benzene gave a value of 4,5D. Then in 1967 Sugiyama, Kataoka and Yamada[13] reported that they had reduced tetrahydroanemonin(7) to tetrol(27) by the same method as that used by Harris and they found that their product was not oxidized by lead tetraacetate. Thus Harris's work was shown to be in error and the conformation was confirmed as trans.

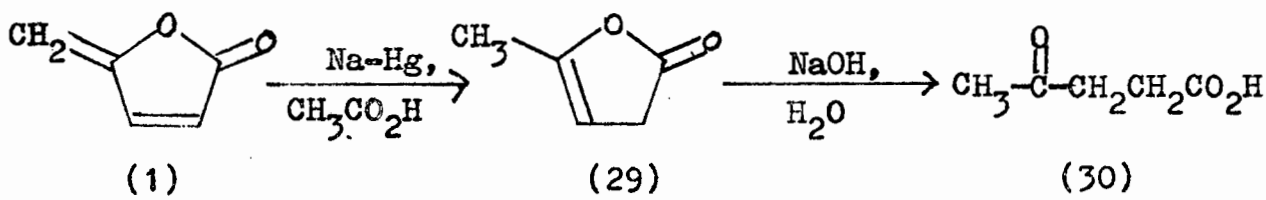
2.2 Synthesis of Anemonin.

The only syntheses of anemonin that have been reported have been merely dimerizations of protoanemonin - a spontaneous process. Thus anemonin is obtained as a by-product in nearly all the protoanemonin syntheses that have been reported. However, although

anemonin is the only one of the more than twelve theoretical dimerization products of protoanemonin, which is actually formed in practice, the reaction is not a clean one and a large amount of uncharacterizable polymer is formed as well, causing the yield of pure anemonin to be low. Consequently in recent years various methods have been studied for controlling the dimerization to give a higher yield of anemonin. Thus in 1966 Font and Pascual[14] obtained a 30% yield of pure anemonin by dissolving protoanemonin in acetone in the presence of catalytic amounts of hydroquinone. Better results were obtained by Kataoka, Yamada and Sugiyama[13, 15] by irradiating a methanol solution of protoanemonin at -50°C with uv light from a 250W mercury lamp. They report yields of anemonin varying between 40 and 75%.

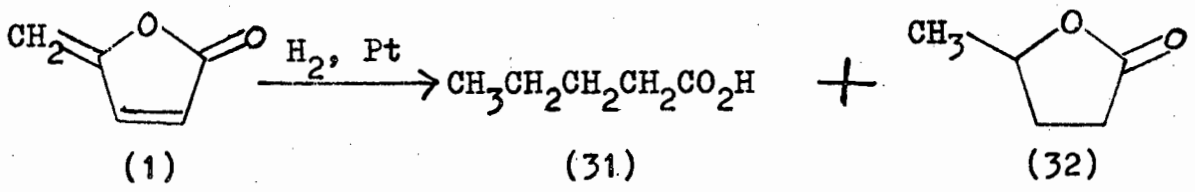
2.3 Chemistry of Protoanemonin.

The chemistry of protoanemonin itself has not been nearly as extensively studied as that of anemonin since the rapidity with which it polymerizes to form anemonin makes it nearly impossible to obtain pure compound for chemical studies; these studies have consequently been carried out on the crude product. However Asahina and Fujita[6] have shown that when protoanemonin(1) in methanolic solution is reduced with sodium amalgam and acetic acid it gives a neutral liquid dihydroprotoanemonin(29), which on treatment with alkali yields levulinic acid(30).



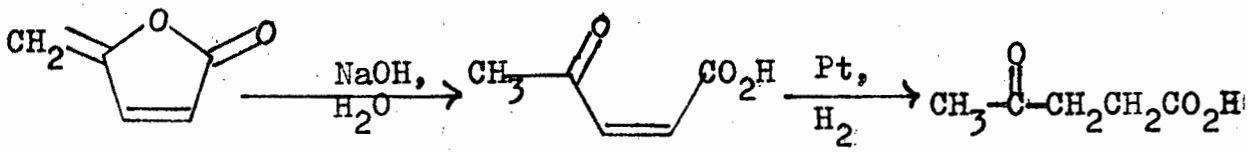
(1) (29) (30)

Further work by Kipping[16] showed that protoanemonin(1) is easily reduced by hydrogen in the presence of platinum, yielding mainly n-valeric acid(31) with some γ-valerolactone(32).



(1) (31) (32)

Also protoanemonin(1) was hydrolyzed by alkali giving rise to β-acetylacrylic acid(33) which was then reduced catalytically to levulinic acid(30). However it is comparatively stable to acid hydrolysis.



(1) (33) (30)

Studies on the stability of protoanemonin have shown that it polymerizes most rapidly when in the pure form and that it is more stable in dilute solution (0,2 - 1,0%) in ether, absolute alcohol or acetone than in aqueous solution in similar concentrations. However it can be stabilized in aqueous solution by the addition of catalytic amounts of hydroquinone.

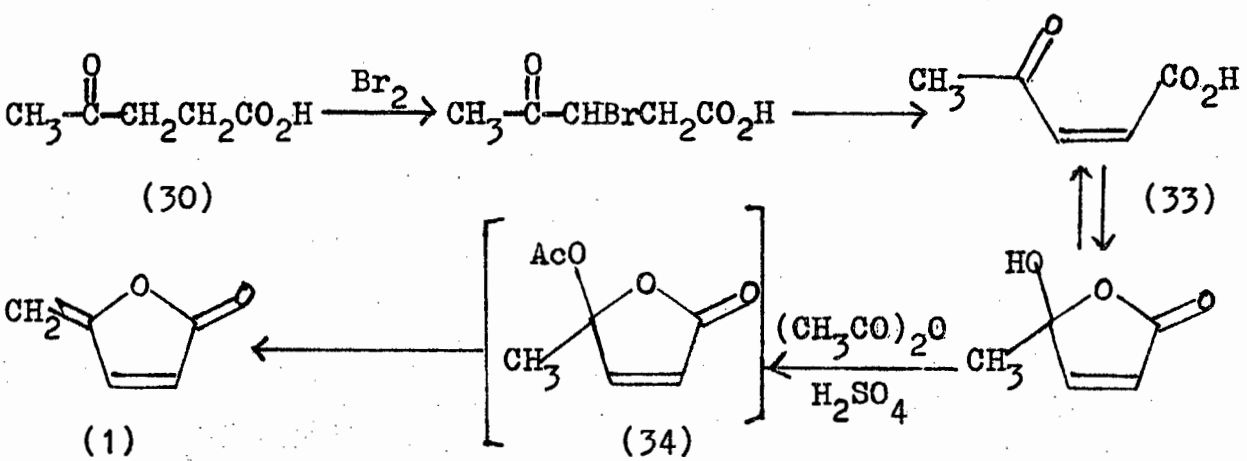
2.4 Synthesis of Protoanemonin.

Numerous syntheses of protoanemonin have been developed. The first was the one previously mentioned which was devised by Asahina and Fujita[6, 8] (see section 2.0) to confirm their proposed structure for protoanemonin. Then in 1930 Muskat, Becker and Lowenstein[17] reported a synthesis, by the pyrolysis of 4,5-dibro-

movinylacrylic acid, of a lactone to which they assigned the protoanemonin structure, but which was shown by Kipping[16] to be in fact anemonin, though protoanemonin was presumably formed as an intermediate. Both the above syntheses gave minute yields.

The first reasonably efficient synthesis was that proposed by Shaw in 1946[18]. In this synthesis β-acetylacrylic acid(33) was cyclized by the acid catalysed action of acetic anhydride. On the basis of spectroscopic evidence Shaw considers that β-acetylacrylic acid exists as the lactone and suggests that the apparent cyclization is merely a straight forward dehydration proceeding via an acetyl intermediate(34). The β-acetylacrylic acid is obtained from levulinic acid(30) by bromination followed by dehydrobromination. Protoanemonin(1) was obtained in 30% yield from the β-acetylacrylic acid.(Scheme VII)

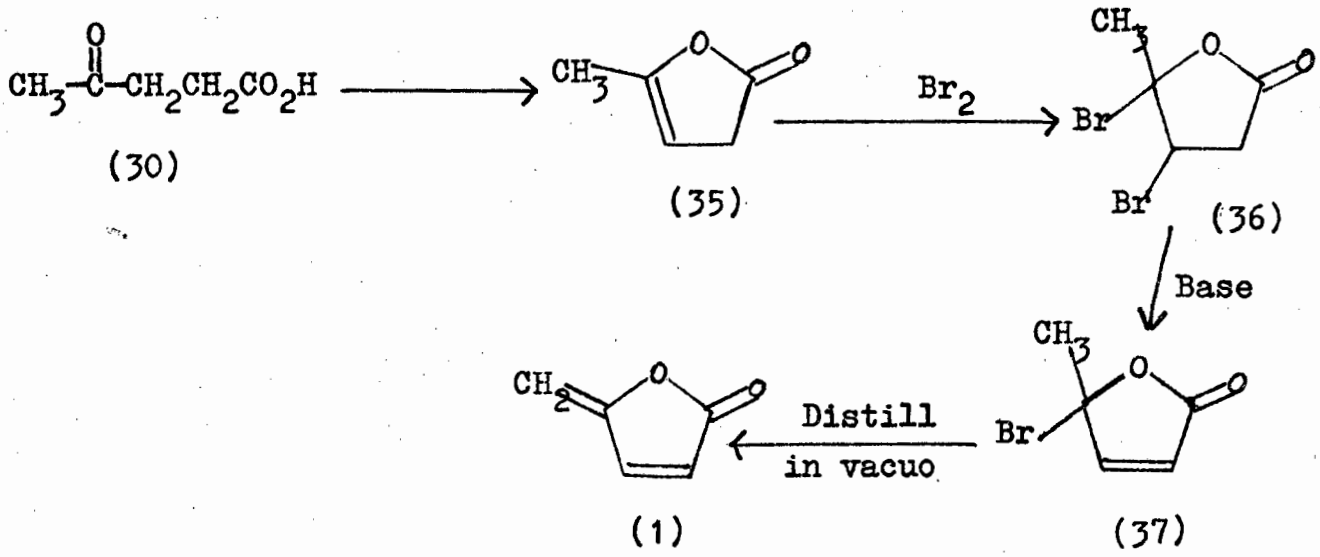
SCHEME VII



A modification of this synthesis proceeding in vastly improved yields was reported by Grundmann and Kober in 1955[19]. In this synthesis levulinic acid(30) is first converted to α-angelica lactone(35) and then brominated to form the 3,4-dibromo-4-valerolactone(36), which is then easily dehydrobrominated, without further purification using a tertiary base in an inert solvent to

give protoanemonin(1). This reaction occurs stepwise, the first molecule of hydrogen bromide is eliminated easily at room temperature to give an unsaturated monobromolactone(37) which, when distilled in vacuo, loses the second molecule of hydrogen bromide to give protoanemonin. The yields depend on the solvent and the base but average at about 70%. This synthesis was patented in 1956 by Olin Mathieson Chemical Corporation. (Scheme VIII)

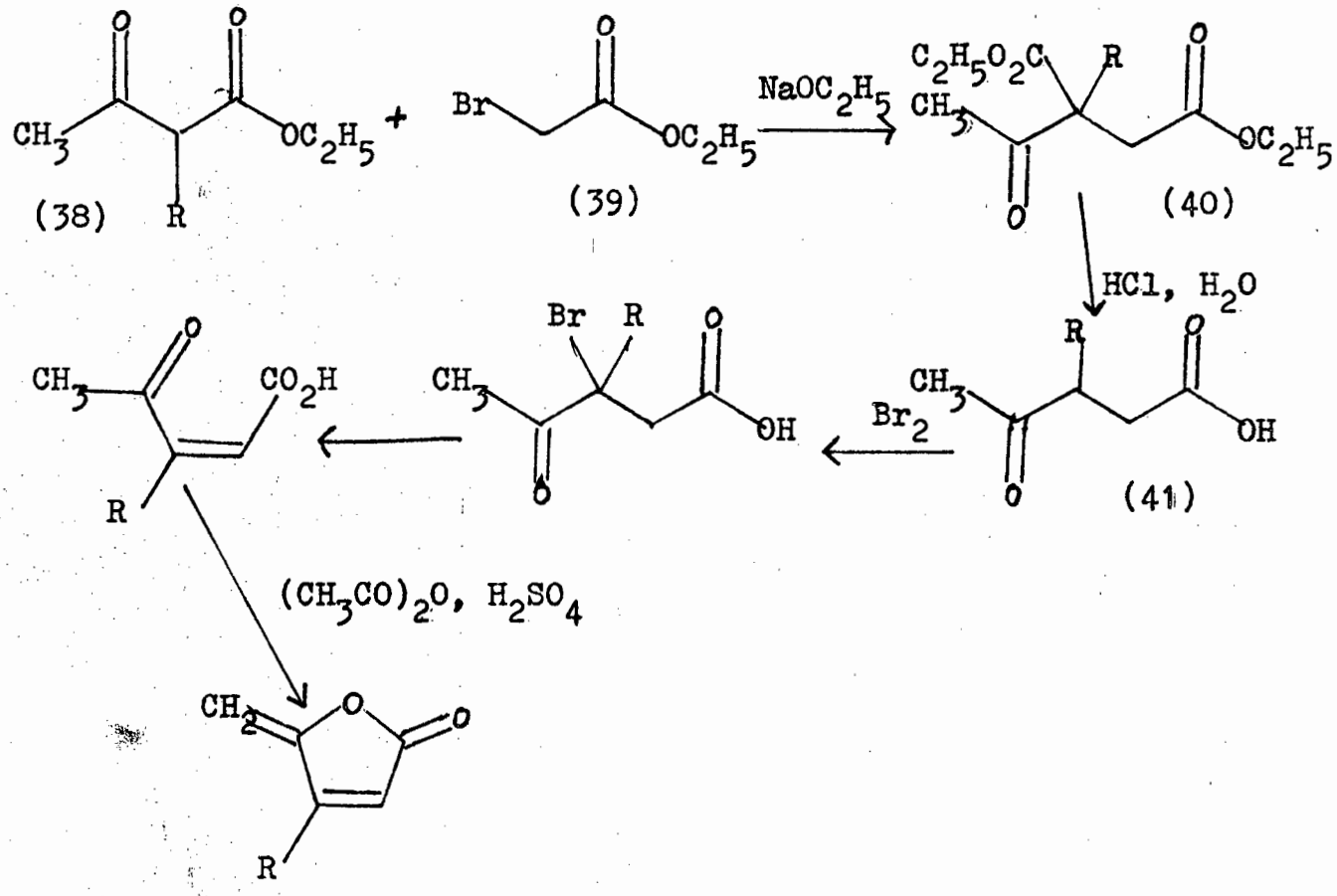
SCHEME VIII



About the same time Stocklmayer and Meinhard[20] devised a general synthesis that could be used for preparing not only protoanemonin itself but a number of its derivatives. The sodium derivative of an ethyl 2-alkylacetoacetate(38) is condensed with ethyl bromoacetate(39) to give a diethyl 3-alkyl-3-carboxylevulinate(40) which,after hydrolysis with aqueous hydrochloric acid, undergoes spontaneous decarboxylation to give 3-alkyllevulinic acid(41). This is then brominated, dehydrobrominated and cyclized by acetic anhydride containing a trace of sulphuric acid as in Shaw's synthesis. (Scheme IX). Ethylacetoacetate gives protoanemonin itself and the analogs with R=CH_3, R=C_2H_5, R=iso-C_3H_7 and

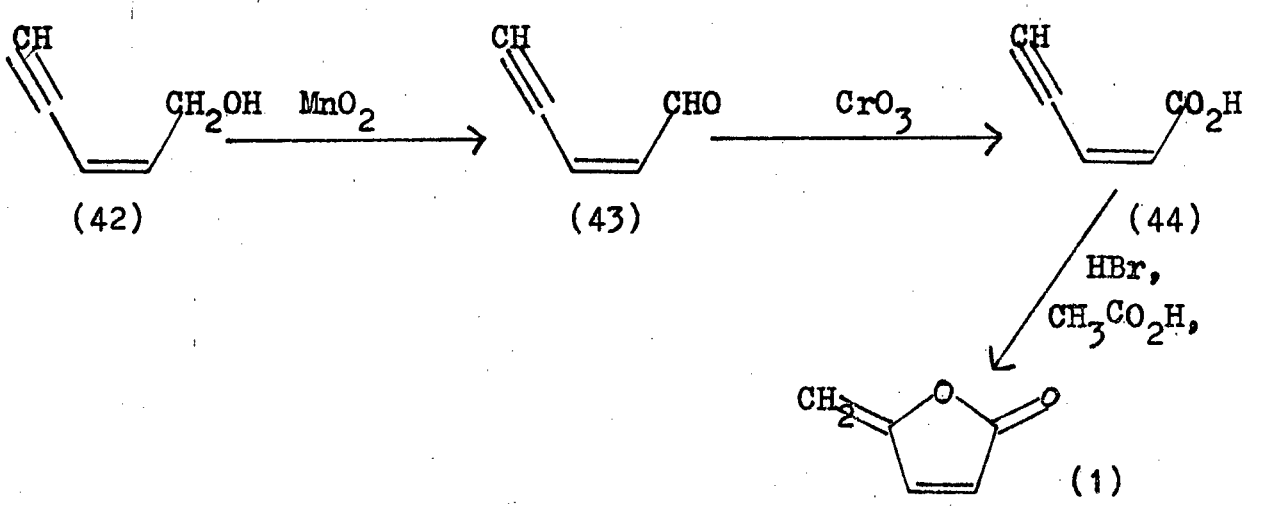
R=C_6H_5–CH_2 were also prepared. These derivatives show similar uv spectra to protoanemonin and are labile compounds losing formaldehyde and polymerizing. The yields of all these derivatives were low.

SCHEME IX



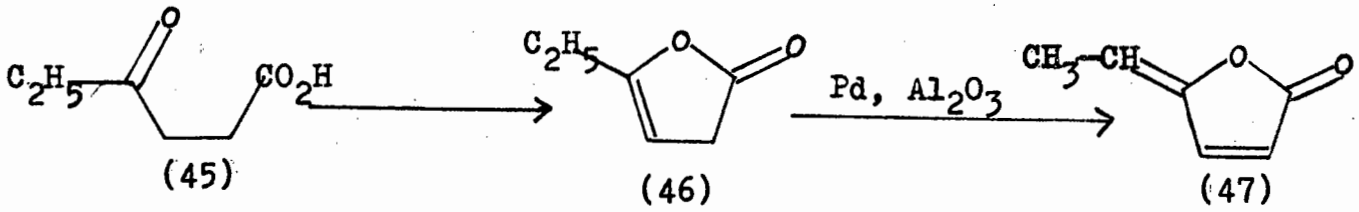
As part of their studies on acetylene carboxylic acids Schulte and Baranowsky[21] achieved a synthesis of protoanemonin from 2-pentene-4-yne-1-ol(42). This was first oxidized with manganese oxide to 2-pentene-4-yne-1-al(43) and then further oxidized with chromic acid to 2-penten-4-yne-1-oic acid (44). This was cyclized to protoanemonin by treating with hydrogen bromide in acetic acid and distilling. The overall yield,however,was only 7,0%. (Scheme X)

SCHEME X



Another industrial synthesis of protoanemonin and its homologs, which involves passing aliphatic γ-oxo-carboxylic acids or their esters in the liquid or gaseous state at elevated temperatures over dehydrogenation and dehydration catalysts, was patented by the Badische Anilin an Soda Fabriek[22] in 1957. Thus β-propionylpropionic acid(45) and 3% aqueous boric acid were refluxed and then distilled over a column to obtain water and crude 3-hexen-1,4-olide(46), which was first purified and then passed through a column containing palladium on aluminium trioxide as catalyst at elevated temperature and reduced pressure. The resulting product mixture yielded methyl protoanemonin(47) after fractional distillation. In a similar manner levulinic acid gave 3-penten-1,4-olide, which in turn gave protoanemonin and anemonin. The yields depend on the temperature, pressure, reaction time and composition of the catalyst. (Scheme XI)

SCHEME XI



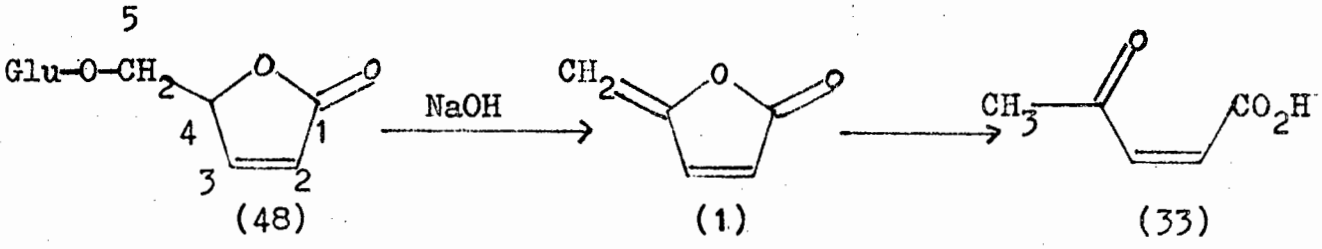
(45) (46) (47)

In 1963, Osipenko and Dovoshkevich[23] reported an attempt to synthesize protoanemonin and its 2,4-heptadiene homolog from the β-lactones of the corresponding γ-oxo acids by the use of N-bromosuccinimide and the direct dehydrobromination of the obtained bromolactone, as an alternative to the use of molecular bromine but the results were far less satisfactory. The most effective of the many syntheses of protoanemonin which have been reported and which are reviewed above is the one due to Grundmann and Kober (Scheme VIII) which proceeds in the highest yield and is the most convenient to carry out in a laboratory.

2.5 Chemistry of Ranunculin.

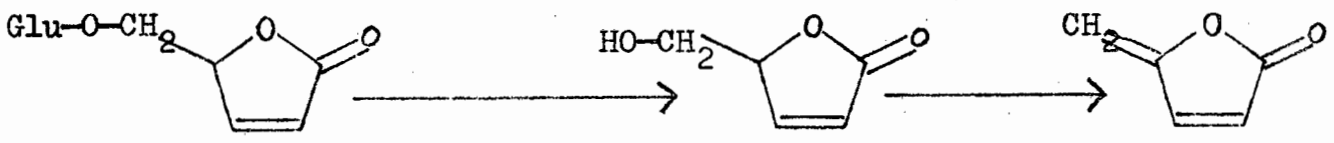
The extreme instability of protoanemonin both when pure and in aqueous solution makes it highly unlikely that it appears in this form in the plant and in 1951 Hill and van Heyningen[24] undertook a study to find out how the protoanemonin was both stable and harmless in the plant itself. They observed that when fresh leaves of Ranunculus species were crushed a strong smell of protoanemonin developed after 3-5 mins and was not observed immediately, and thus they deduced that when the tissues are crushed protoanemonin is released enzymatically from some precursor. Thus by destroying the enzymes by crushing the plant with dilute hydrochloric acid they were able to isolate a crystalline glucoside precursor which they called ranunculin. Ranunculin was found to be stable both as the solid and in aqueous solution. It is

also stable in acid solution but under alkaline conditions it readily breaks down to glucose and protoanemonin(1) which is converted into β-acetylacrylic acid(33). Ranunculin, on distillation with aqueous sodium acetate, gives a nearly quantitative yield of protoanemonin. On the basis of the above facts and the observation that neither the glucoside nor its tetra-acetate show any sign of having a free carboxyl group it was deduced that the glucoside has the structure (48).



(48) (1) (33)

Hellstrom[25] after studying the optical rotation, ir spectra, and the action of β-glucosidases and other enzymes on ranunculin (48), concluded that the glucosidic linkage was in the β-configuration. Also by carefully controlled hydrolysis experiments in buffered solutions he obtained an optically active aglucone(49) and showed that it had a greater power to reduce ammoniacal silver nitrate than ranunculin or β-acetylacrylic acid.

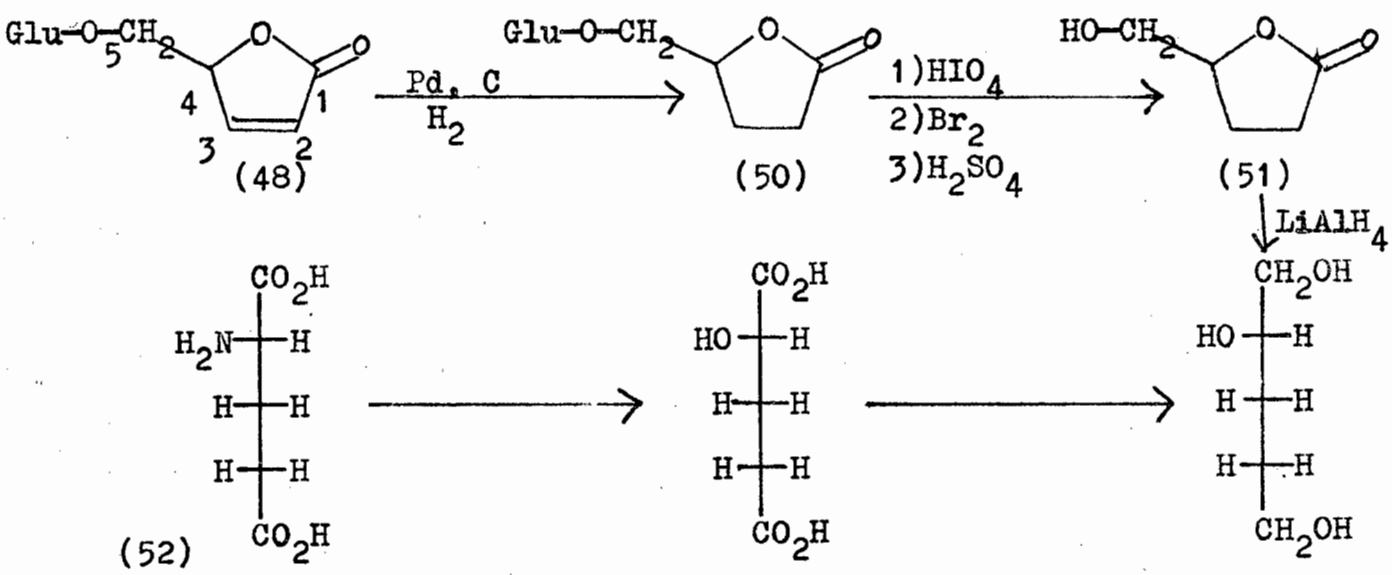


(48) (49) (1)

Further studies by Bredenberg[26] showed that ranunculin did decompose slowly in ethanolic or aqueous solution to give protoanemonin and that when it was warmed in dry methanol D-glucose was formed. The nmr spectrum of ranunculin was studied by

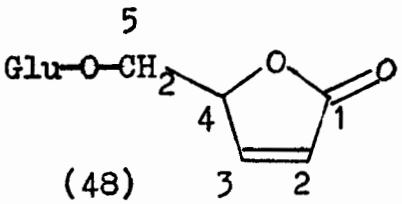
Benn and Yelland[27] and found to be consistent with the structure proposed by Hill and van Heyningen[24]. They then determined the configuration at the C4 position of the lactone ring as follows. They hydrogenated ranunculin over palladium-charcoal to form dihydroranunculin(50) which was not obtained crystalline but which yielded a crystalline tetra-acetate which gave ir and nmr spectra that were consistent with the expected product. The glucose ring of the dihydroranunculin was then cleaved by periodate oxidation and the initial product further oxidized with bromine. Acid hydrolysis then yielded the dihydroaglucone(51) of ranunculin, which was obtained as a syrup and reduced with lithium aluminium hydride to give an oily triol, which was shown to be identical with L-1,2,5-pentanetriol prepared from L-glutamic acid(52). Thus the configuration at the C4 position of the lactone moiety of ranunculin was shown to be S. (Scheme XII)

SCHEME XII



Circular dichroism measurements by Boll[28] also showed the configuration at the C4 position of the aglucone to be S.

Ruijgrok[5] reported finding ranunculin in Ranuculus repens and Helleborus foetidus in 0,06 and 1,4% yield respectively. So when Tscheshe, Welmar, Wulff and Snatzke[29] extracted these plants with aqueous isopropyl alcohol and with methanol they were surprised to find no ranunculin at all but rather the alkylglucosides of the appropriate alcohols ie. isopropyl-β-D-glucopyranoside and methyl-β-D-glucopyranoside. Since ranunculin itself is stable in alcohols they deduced from this that the plants did not genuinely contain ranunculin but a reactive precursor which was responsible for the formation of the alkylglucoside. To resolve this question they extracted these same plants using the same method as Hill and van Heyningen[24] and found not only ranunculin(48) but also an isomeric compound, isoranunculin(53).



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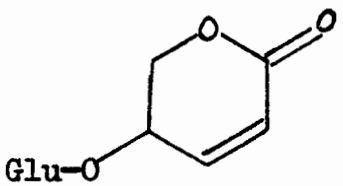
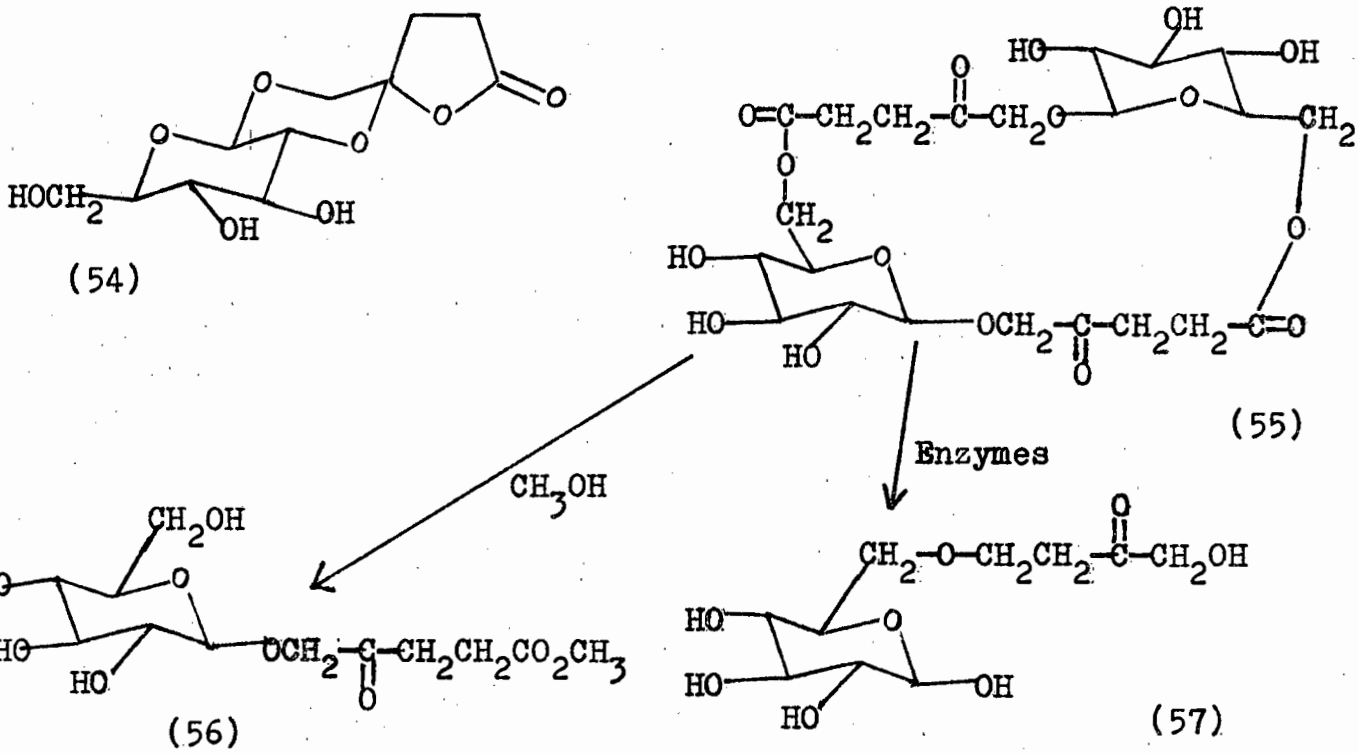


(53)

Since neither of these compounds had been found after the alcohol extraction of the plant materials Tscheshe and co-workers concluded that they must be formed under the acid extraction conditions. So they devised an alternative extraction procedure that would involve the use of neither acid nor alcohol. Thus the leaves of Helleborus foetidus were freeze dried and then extracted with acetone-water. This extract was examined by thin layer and paper chromatography and was found to contain neither ranunculin nor isoranunculin. However they found along with glucose and fructose two other non-reduceable glucose derivatives which they separated by column chromatography. These two new compounds were named ranuncoside(54) and ranunculoside(55). The structures

of all three of these new compounds were determined from spectroscopic data, chiefly nmr. Ranunculoside was also subjected to methanolysis and enzymic hydrolysis to give compounds (56) and (57) respectively, whose structures were also determined spectroscopically.



Since both ranuncoside and ranunculoside are very stable compounds and neither of them breaks down to give protoanemonin, these clearly can not be genuine precursor substances either. Thus Tschesche and co-workers conclude that all these compounds are artifacts generated by the extraction procedures and that the genuine precursor of protoanemonin has yet to be isolated. No syntheses of ranunculin or any of these other glucosides have been reported.

2.6 Antibiotic and Pharmacological Activity of Anemonin and Protoanemonin.

The presence of protoanemonin causes the fresh leaves and

sap of ranunculin containing plants to produce an irritating and blistering effect on the skin[2]. In dilute aqueous solution protoanemonin shows antibacterial activity against a wide variety of gram-positive, gram-negative and acid-fast bacteria[30]. It is most active against the erbethellas and in descending order against the shigellas, salmonellas and eschericias and least active against the staphylococci and streptococci[31]. It has been suggested that the inactivating effect against bacteria is due in part to the blocking of sulfhydral groups in the bacterial enzyme systems, but other factors also appear to be operative[32]. Anemonin on the other hand is only weakly antibacterial. Baer, Holden and Seegal[33] report that it exhibited practically no antibacterial activity towards Eschericia coli, Candida albicans and Staphylococcus aureus. However Boas and Steude[34] using different methods of testing apparently found a high order of activity for anemonin against these organisms. Toshkov et al.[35] report that both protoanemonin and anemonin not only show antibacterial activity but also antiviral activity and deactivated in vitro diphtheria toxin and were cytopathaic to both normal and tumor tissue cultures. This is not surprising since protoanemonin has been shown to manifest mitostatic action in the metaphase due to a combination of protoanemonin with the sulfhydral groups of the polypeptide chains of the spindle fibres[36]. Miyaki, Mizuno, Narita, Takeuchi, Ukita and Yamamoto[37] have also demonstrated that protoanemonin inhibits tumor cells in vitro. On the other hand protoanemonin in common with other 5 membered lactones has been reported to be a slow acting carcinogen by Dickens[38]. Protoanemonin has also been found to be toxic to many fungi[39] and its effect on higher plants was tested on barley seedlings. Protoanemonin inhibited the growth of barley seedlings but increased the chlorophyll and vitamin C contents, the catalase

activity and the intensity of respiration[40].

A study of the pharmacodynamic properties of anemonin showed that an injection of a sufficient dose of the active principle of anemone in the saphic vein caused a hypotension which varies with the size of the dose. This hypotension is not of vagal origin nor with small doses is it a result of cardiac depression. It is probably of vascular origin. In a chloralized dog of 10Kg in weight the intravenous injection of 200mg of anemonin quickly arrests respiration and is soon followed by arrest of the heart[41]. Protoanemonin and anemonin containing preparations obtained from various members of the Ranunculaceae have been widely used in folk remedies against a large variety of ailments. However its chief uses have been as a vesicant and as a cure for rheumatism and a variety of skin diseases by local application[42].

3.0 OTHER CONSTITUENTS OF THE SUBFAMILY, THE ANEMONEAE.

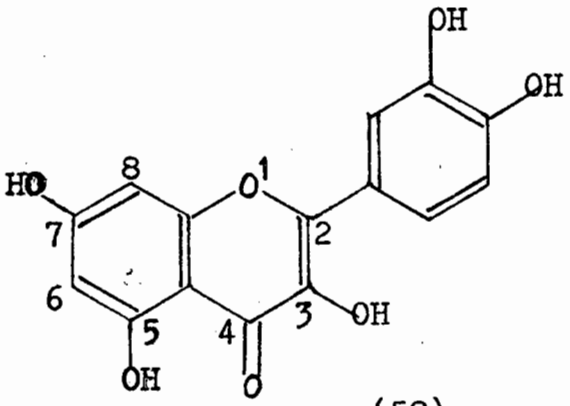
3.1 The Genus Anemone.

Not much systematic study on the chemical constituents of the genus anemone has been carried out, but what work has been done shows that the two main classes of compound that have been found to be of interest in this genus are the saponins and flavenoid glycosides. Most of the species of this genus that have been tested for saponins have been found to contain them. Luft[43] discusses the distribution of tannins and saponins in Anemone hepatica and Anemone pulsatilla. Both tannins and saponins are found in high yield in the rhizomes of these plants but the concentration in other parts of the plant is low. Gilg and Schurhoff[44] tested a number of Anemone species and found them all to contain saponins. Further work on Anemone pulsatilla by Balansard[45, 46] led to the isolation of a saponoside by salting the aqueous extract with

saturated ammonium sulphate solutions, and recrystallizing from isopropanol. This saponin had a melting point of 199-203°C and hydrolysis of it yielded glucose. Huang, Chen, Chou and Chu[47] isolated the active principle of a Chinese drug obtained from Anemone chinensis, which was a saponin, and attempted to determine the structure of the sapogenin, anemosapogenin, $C_{30}H_{48}O_4$, melting point 300-302°C. The results of their studies indicated that it was possibly a pentacyclic triterpenoid compound with two secondary hydroxyl groups and one hindered carboxyl group, and a non-hydrogenable double bond. The sugar portion contained glucose, rhamnose and one unidentified sugar. Bienfait[48] obtained a saponin from Anemone nemorosa by precipitating it from the alcoholic extract with ether. After prolonged hydrolysis of this product he obtained a genin, $C_{30}H_{38}O_4$, which after recrystallization from alcohol had a melting point of 317,5°C. Arabinose, rhamnose and glucose were also identified in the hydrolysate by chromatography and ionophoresis.

As in the case of the saponins most species of anemone seem to contain flavenoid glycosides but not much work seems to have been done on isolating and identifying them. Egger[49, 50] has, however, considered the differences in the flavonol glycosides of fifty-one species of Ranunculaceae and concludes that, in spite of all the variability, the glycoside contents in comparable organs show a relation to taxonomic groups and, therefore, the flavonol glycosides are valuable tools for the chemotaxonomy of the Ranunculaceae. A flavenoid glycoside, $C_{22}H_{25}O_9$, was isolated but not identified by Fil[51] from Anemone pratensis in 1962, and another from Anemone nigricans in 1961[52]. Raynaud and Lebreton[53] in 1970 identified the following glycosides in Anemone hepatica by chromatography, uv spectrophotometry and acid and enzyme hydrolysis.

The flavones of the leaves were quercetin-3-glucoside, quercetin-7-glucoside and quercetin-3-glucuronide. The flavones of the flowers were kaempferol-3-glucoside, kaempferol-7-glucoside, kaempferol-3,7-diglucoside and kaempferol-3-glucuronide. The structures of quercetin(58) and kaempferol(59) are as follows:-



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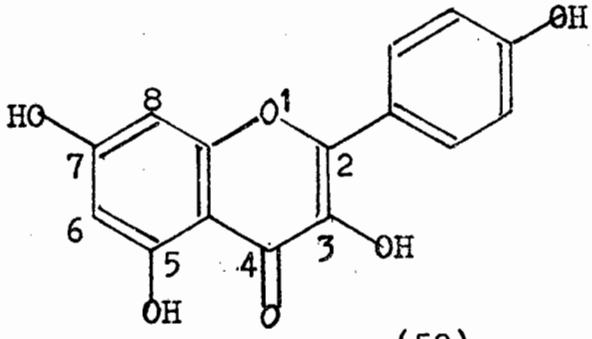
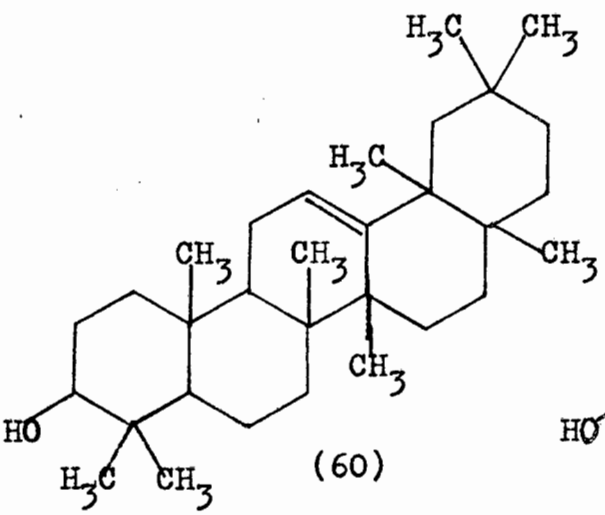


(59)

Fil[52] also reports a trace of alkaloids in Anemone nigricans but, it would appear from the general survey of the occurrence of alkaloids in the Ranunculaceae carried out by Frencel[54] in 1965, that most anemone species are lacking in alkaloids. Rolski and Przyborowski[55] in 1961 reported the isolation of a triterpene from Anemone pratensis which they conclude is probably the acetate of β-amyrin(60) or β-sitosterol(61).



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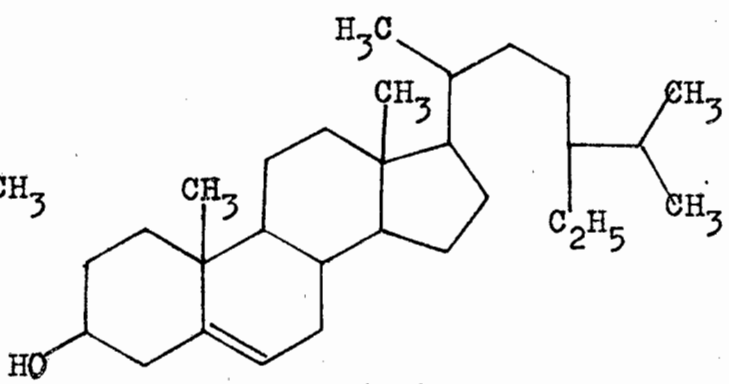
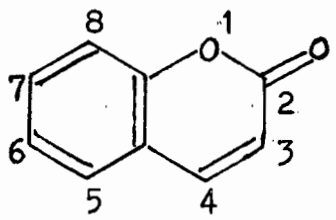


(61)

Drozd, Komissarenko and Litvinenko[56] report that, of eighteen species of Ranunculaceae tested, all contained coumarins; it is thus probable that anemone species contain coumarins(62), though no references have been found reporting the isolation of these compounds from this genus.

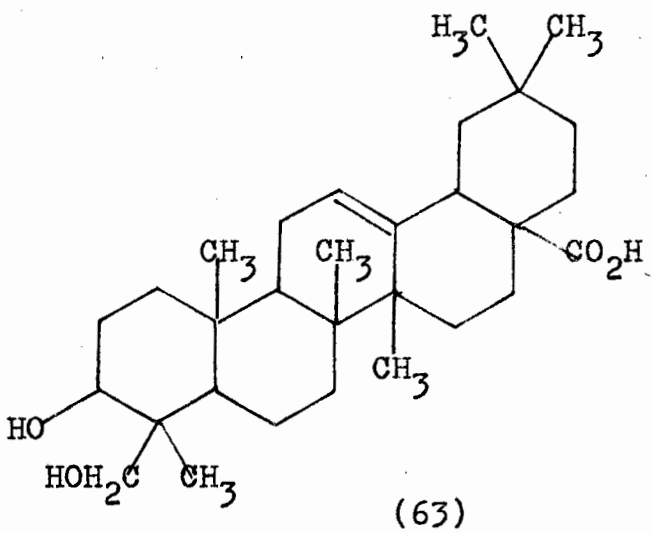


(62)

All species of the genus anemone seem to contain protoanemonin and, thus, pharmacollogically they all show the vesicant properties due to anemonin (see section 2.6). In addition Leclerc[57] discusses more fully the pharmaceutical and pharmacological properties of Anemone pulsatilla in relation to its chemical constituents. Batrak, Furs and Khrustalev[58] studied the effect of a 20% tincture of Anemone montana (1ml/Kg) on the cerebrocortical and cardiac bioelectric activity, blood pressure and respiration of dogs. The first effect was sedation, followed by a drop in arterial tension and strengthening of cardiac contraction. The pulse rate decreased and respiration was first faster and then slower with greater amplitude. Finally bioelectric activity was affected. Changes then traversed the reverse course restoring normality in 2-3 hours. Fil[52], in connection with his study of the chemical composition of Anemone nigricans, also observed that the plant had sedative and hypotensive properties. The Societe Cortial[59] patented a preparation from Anemone hepatica which functions as a cholagogue and muscle relaxant and which they report is rich in anthocyanins, flavones and flavonic acids.

3.2 The Genus Clematis

Most work on the constituents of clematis has been done on the saponins which occur in most of the clematis species as well as in the other Ranunculaceae; however, Clematis hexaseptala has been reported as containing no saponins, tannins or cardiogluco-sides[60] so these compounds do not occur in all members of this genus. Bernard and Sice[61] obtained, by salting out with ammonium sulphate, a saponoside from Clematis flammula which they do not identify but which they consider to be the same as the saponoside they obtained from Anemone pulsatilla (see section 3.1). Ishiwatori, Nakano and Shinkawa[62] identified the genin of the saponin in the drug "Radix Clematidis" obtained from the root of Clematis panic-ulata as hederagenin. A study of the saponins of Clematis vitalba by Chirva, Kintya and Melnikow[63] led to the isolation of free hed-eragenin(63), free oleanolic acid(64) and eight glycosides con-taining these triterpenes as aglycones, from the roots. The car-bohydrate portion in three of the glycosides was ribose which is unusual in saponins.



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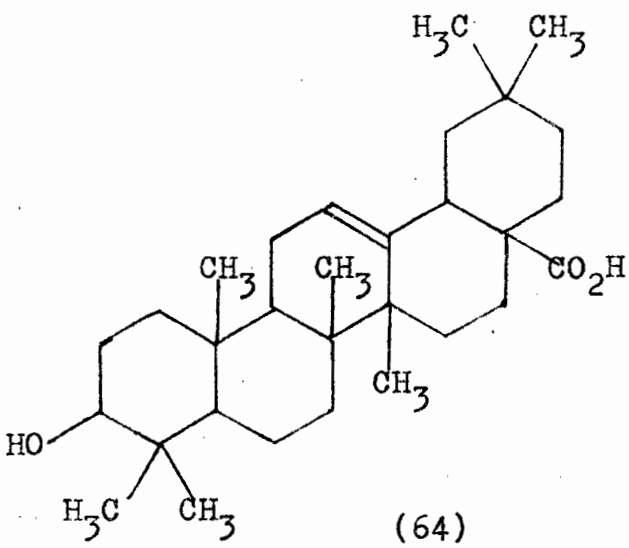
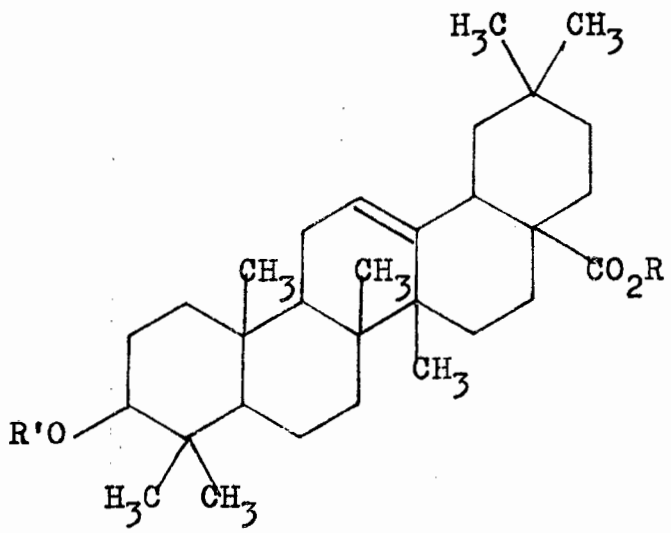


(64)

Also Clematis songorica of Tien Shan was investigated by Zakharov and Boryaev[64] and was shown to contain saponins and other glycosides. Work on Clematis mandschurica and other plants by Kochetkov and Khorlin[65] in 1963 led to the isolation of the oligoside, clematoside C(68), as well as a mixture of saponins. Further work on this compound led to the elucidation of the complete structure which was reported in 1965[66]. Later these same workers [67, 68] obtained from Clematis mandschurica three new oligosides, Clematoside A(65), Clematoside A'(66) and Clematoside B(67) which were isolated and separated by chromatography on silica gel and identified by chemical and spectroscopic means.



(65) R: L-Rha-(1→4)-D-Glc-(1→4)-D-Glc-(1→6)-D-Glc
R': D-Glc-(1→4)-D-Xyl-(1→2)-L-Ara-(1→2)-L-Ara-(1→4)-L-Rha

(66) R: H R': D-Glc-(1→4)-D-Xyl-(1→2)-L-Ara-(1→4)-L-Rha

(67) R: L-Rha-(1→4)-D-Glc-(1→4)-D-Glc-(1→6)-D-Glc
R': D-Glc-(1→4)-D-Glc-(1→4)-D-Xyl-(1→2)-L-Ara-(1→2)-L-Ara-(1→4)-L-Rha

(68) R: L-Rha-(1→4)-D-Glc-(1→4)-D-Glc-(1→6)-D-Glc
R': L-Rha-(1→6)-D-Glc-(1→4)-D-Glc-(1→4)-D-Xyl-(1→2)-L-Ara-(1→2)-L-Ara-(1→4)-L-Rha

Again it is clear from the survey by Egger[49, 50] that most of the clematis species contain flavones, but not many have been identified. A study conducted by Haw and Ko[69] disclosed that Clematis brachyura contained a flavone which they called clematisin (structure uncertain), and that the content of this flavone showed considerable seasonal variation. Clematis mandschurica, which was studied at the same time by these workers was found to contain no flavone at any stage during the experimental period. 0,23% flavenoids were detected in Clematis hexaseptala by Udaltsova, Minina and Chernysheva[60] and were resolved into four compounds, one of which was identified as kaempferol(59).

Clematis species also seem to characteristically contain coumarins; thus Drozd et al. detected the presence of five coumarins in Clematis vitalba. A medicinal preparation derived from from clematis roots is used in trichomoniasis treatment and contains coumarin derivatives as the active principle[70]. Also Udals'tsova, Minina and Chernsheva[60] have shown that the foliage of Clematis hexaseptala contains 0,82% coumarins.

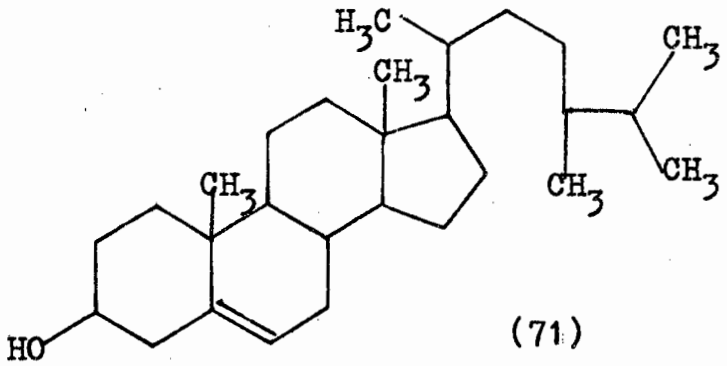
In his survey of the occurrence of alkaloids in the Ranunculaceae Frencel[54] tested three clematis species for alkaloids and found that one, Clematis tangutica, contained alkaloids, but the other two species, Clematis alpina and Clematis vitalba, were lacking these compounds. Also Tang and Chao[71] report the presence of an unidentified alkaloid in Clematis angustifolia which functions as an anesthetic, but it would seem that most clematis species are lacking in alkaloids.

The following six compounds were isolated from the leaves and stems of Clematis vitalba by Uleben[72] and identified by melting points, optical rotation, elemental analysis and uv and ir spectroscopy:- n-triacontane ($C_{30}H_{62}$), n-nonacosane ($C_{29}H_{60}$),

ginnone(69), ginnol(70), β-sitosterol(61) and campesterol(71).

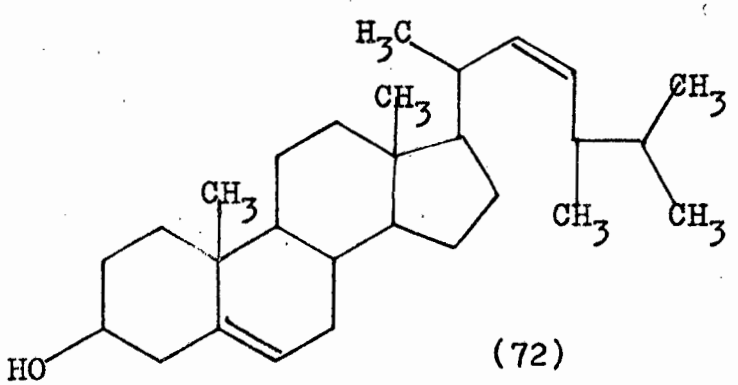
$CH_3(CH_2)_8\overset{O}{\overset{\|}{C}}(CH_2)_{18}CH_3$ (69)

$CH_3(CH_2)_8CHOH(CH_2)_{18}CH_3$ (70)



(71)

Clematis hookerina was examined by Cambie and Parnell[73] as part of a general phytochemical survey of dicotyledonous plants of New Zealand and they reported β-sitosterol(61) as the only compound readily identifiable in extracts of this plant. Dominguez, Davila and Merijanian[74] isolated and identified stigmasterol(72) and the hydrocarbons octacosane ($C_{28}H_{58}$) and dotriacontane ($C_{32}H_{66}$).



(72)

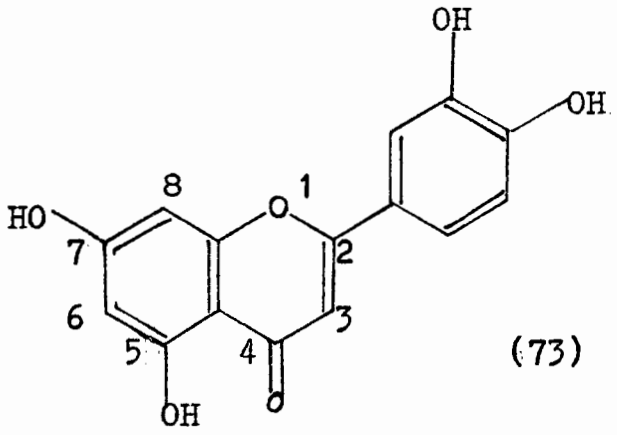
Like anemone, all species of clematis contain protoanemonin and thus show the pharmacological properties associated with protoanemonin which have been discussed earlier (section 2.7). In

addition, the pharmacological action of clematis species on some of the following isolated organs; stomach, intestine, bladder, uterus and heart, and also on blood pressure and respiration, has been studied by Boyd[75]. Also, the general physiological action and therapeutic use of Clematis recta has been studied by Dewey[76], while Tang and Chao[71] report that Clematis angustifolia, which is used as a Chinese drug, functions as an anesthetic and contains an unidentified alkaloid. Clematis flammula is used as an aphrogenic and contains an incompletely characterized saponin as the active principle[61], and a medicinal preparation for trichomoniasis treatment containing coumarins is obtained from clematis roots[70].

3.3 The Genus Ranunculus.

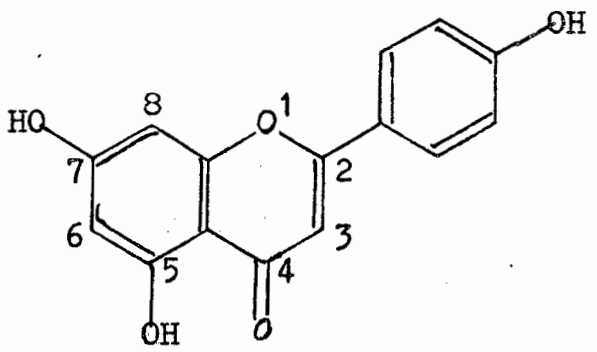
Like anemone and clematis, the majority of ranunculus species contain saponins. As part of a survey of drug plants in 1937[77], Ranunculus ficaria was found to contain saponin. Later work by Bergman in 1944[78] showed that the saponin content of Ranunculus ficaria varies with the weather conditions and the time of day. As part of a preliminiary phytochemical study of certain ranunculus species Kolesnik[79] noted a high saponin content in Ranunculus repens and Ranunculus ficaria. A method for isolating pure saponins from the roots of Ranunculus ficaria was patented in 1964[80]. This method yields 18gm of saponins per kilogram of dried root, and these saponins are used in the preparation of antihemorrhoidal ointments. Further work on Ranunculus ficaria by Pourrat and Pourrat[82] showed that the saponins of this plant give mainly hederagenin(63) and oleanolic acid(64) after saponification. A phytochemical investigation of Ranunculus sceleratus by Saber, Mahran and El'Alfy[81] showed that this plant contains tannins of the pyrogallol group.

Most ranunculus species also contain flavones. Flavenoids were detected in Ranunculus baidarae by means of paper chromatography by Bandyukova and Shinkarenko[83]. Flavenoids were also detected in Ranunculus scleratus by means of chromatography by Saber, Mahran and El'Alfy[81]. A more complete study on the glycoflavenoids of Ranunculus lingua by Drozd, Koreschuk and Litvinenko[84] led to the isolation, by column chromatography, and the identification of orientin (luteolin-8-C-glucopyranoside), homo-orientin (luteolin-6-C-glucopyranoside) and luteolin-8-C-(β-D-glucopyranosyl-6-O- β -D-xyloside). The structure of luteolin(73) is as follows.



(73)

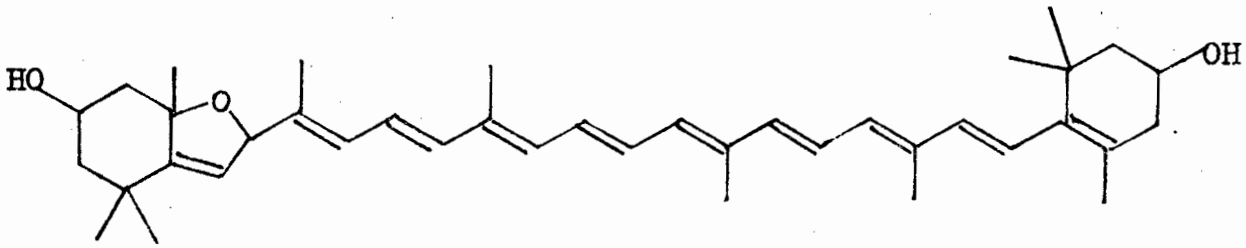
Drozd and Litvinenko[85] also made a careful study of the flavenoids of Ranunculus illyricus. These same workers then turned their attention to Ranunculus repens and, in 1969[86], they isolated, by repeated chromatography on polyamide columns, two of the six flavones that had been noted in the plant. These two flavenoids were identified as apigenin-8-C- β -D-glucopyranoside (vitexin) and its rotational isomer apigenin-8-C- α -D-glucopyranoside (saponaretin). Continuation of this work led to the isolation of a flavenoid[87] which was characterized as apigenin-6-C- β -D-glucopyranoside (isovitexin). The structure of apigenin(74) is as follows.



(74)

Drozd, Koreschuk and Litvinenko[88] have also studied the glycoflavenoids of Ranunculus polyanthemos. By means of paper chromatography several substances with flavenoid characteristics were found. Repeated chromatography on polyamide and cellulose columns yielded two substances A and B. Substance A was identified as vitexin and substance B behaved similarly to A and was probably the rotational isomer of vitexin, saponaretin.

A study of Ranunculus acer by Karrer, Jucher, Rutschmann and Steinlin[89] led to the isolation and identification of the following carotenoid compounds after hydrolysis; flavoxanthin(75), xanthophyll(76), chrysanthemaxanthin (stereo-isomer of 75), taraxanthin(77) and a compound which was probably also a xanthophyll epoxide, as well as α-carotene-5,6-epoxide(78), flavochrome(79) and some α-carotene(80) and β-carotene(81)



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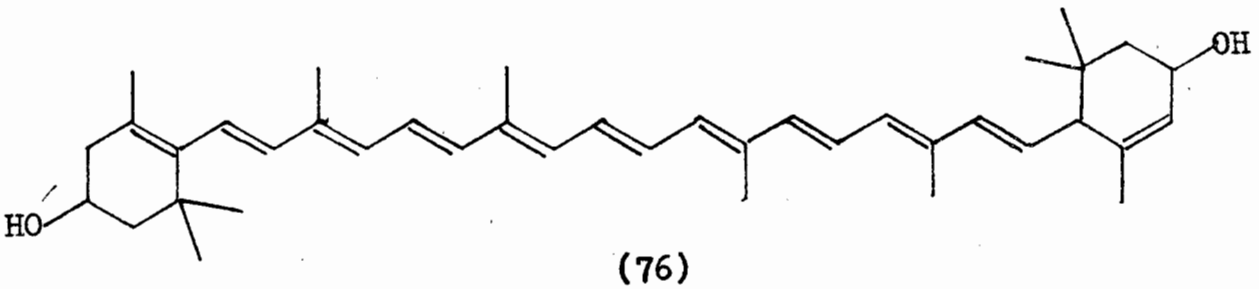

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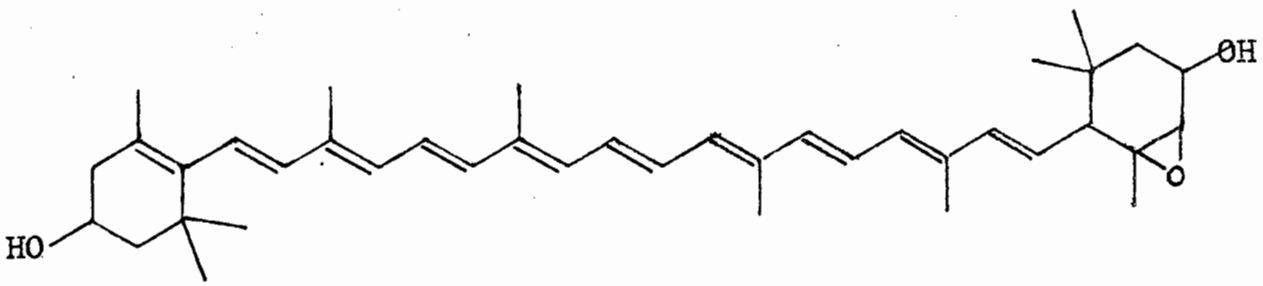

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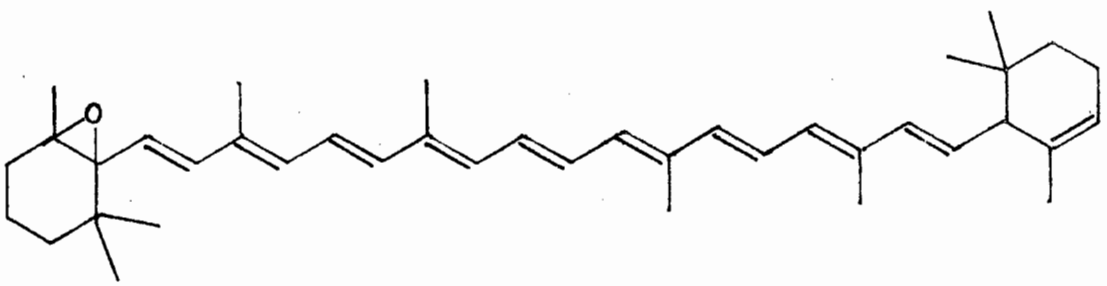

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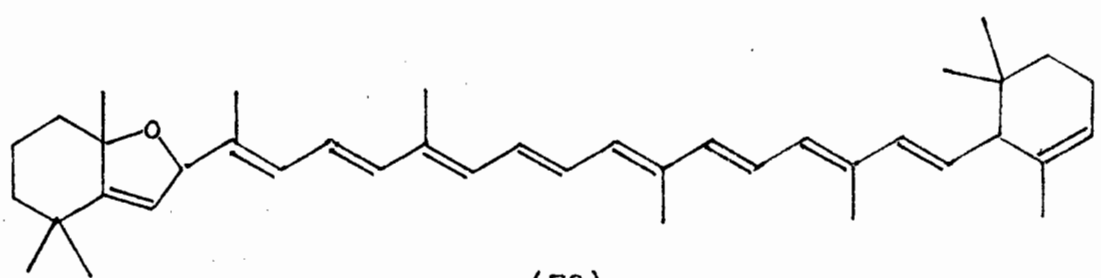

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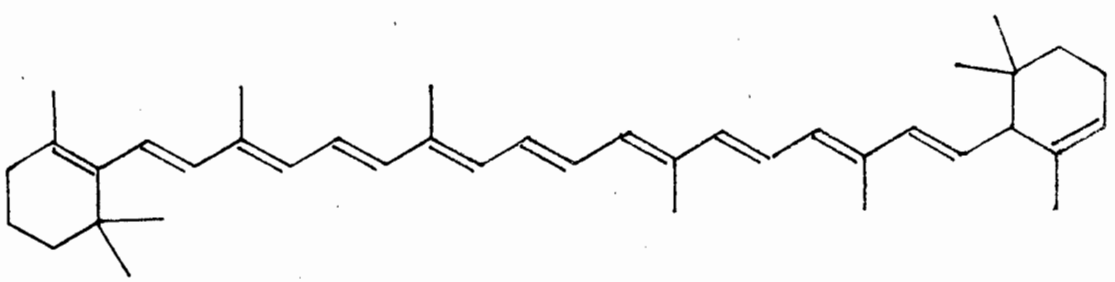

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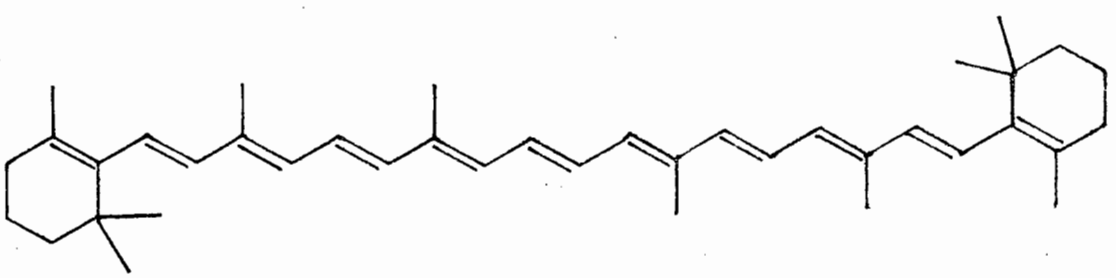
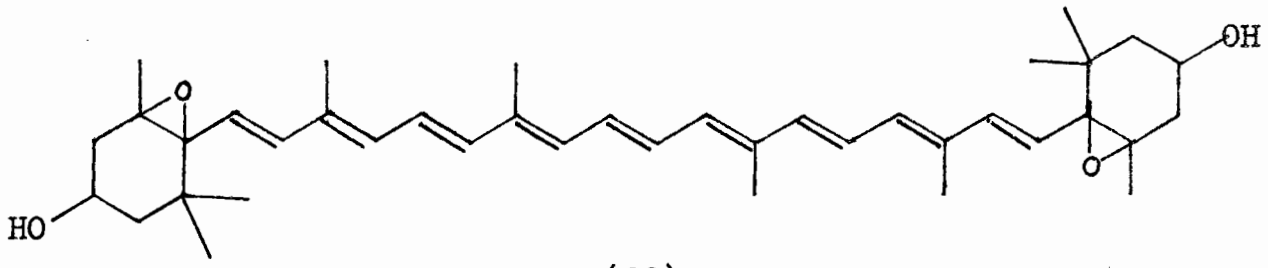


(81)

The carotenoids of Ranunculus carpaticus were studied by Neamtu, Tamas and Bodea[90]. The pigments isolated from this plant were predominantly α-carotenoids and were found to be taraxanthin (77), violaxanthin(82), flavoxanthin(75), antheraxanthin(83), a flavoxanthin ester, an eloxanthin ester(84), a xanthophyll ester and physoxanthin(85), as well as α-carotene-5,6-epoxide(78) and α-carotene(80) and β-carotene(81).



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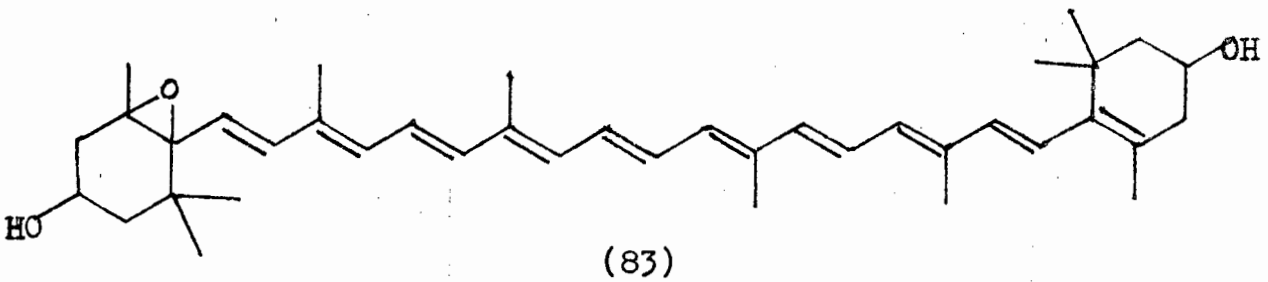

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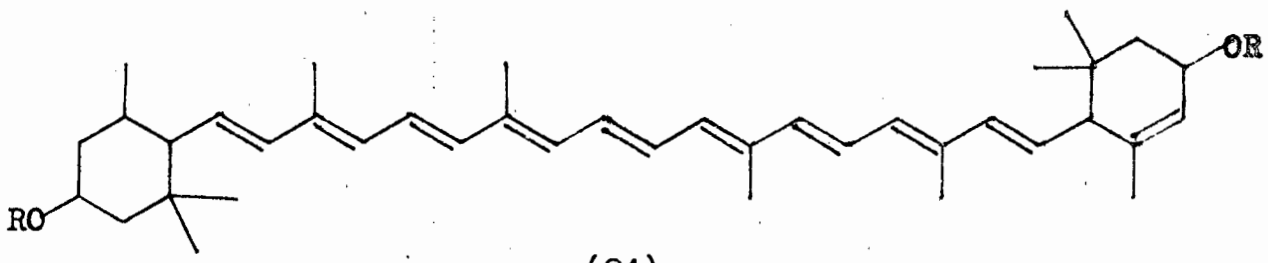

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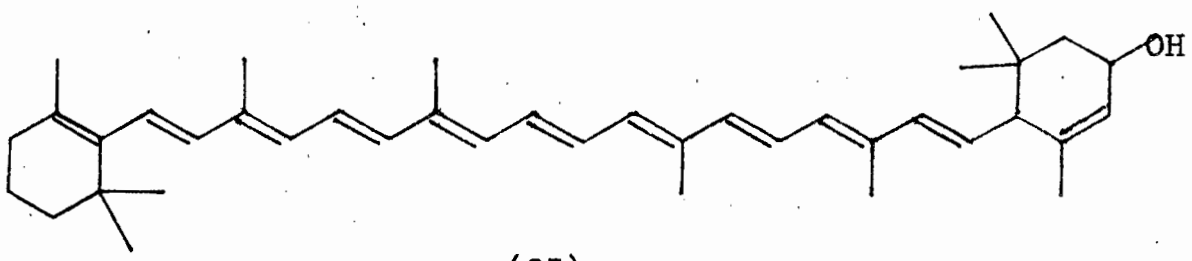
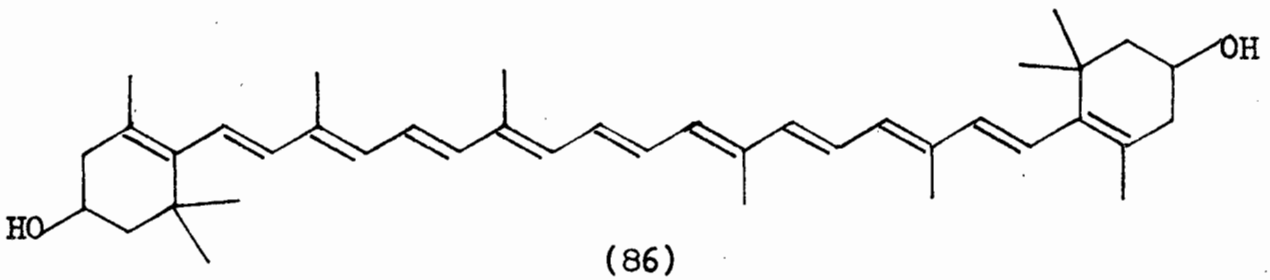


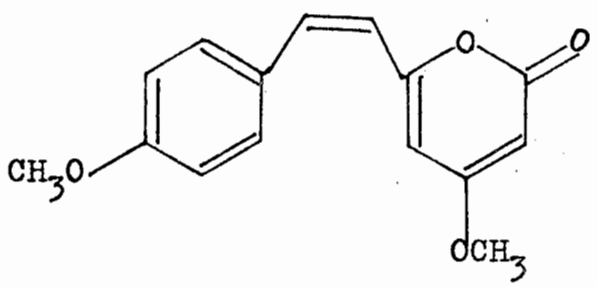
(85)

Kuhn and Brockman[91] found that the sepals of Ranunculus acer contain at least five xanthophylls, about 50% esterified. Xanthophyll(76) itself predominates, then follows β-xanthophyll(86), and finally in smaller amounts taraxanthin(77) accompanied by some violaxanthin(82) and flavoxanthin(75).



(86)

A phytochemical study of Ranunculus quelpaertensis conducted by Shibata, Shibuya and Doj[92] yielded amongst other compounds, palmitic acid ($C_{15}H_{31}CO_2H$), stearic acid ($C_{17}H_{35}CO_2H$), stigmasterol(72), β-sitosterol(61), hexacontanol ($C_{26}H_{53}OH$) and yangonin(87).



(87)

Zhuravlega[93] has isolated the antibiotic, lutidin (structure uncertain), from Ranunculus acris. Saber, Mahran and El'Alfy[81] noted the presence of an unidentified alkaloid in Ranunculus sceleratus, but it would seem that, like anemone and clematis, the majority of ranunculus species are lacking in alkaloids. Drozd, Komissarenko and Litvinenko[56] examined twelve species of ranunculus; Ranunculus lingua, R. flanmula, R. auricomus, R. cassubicus,

R. scleratus, R. repens, R. polyanthemus, R. bulbosus, R. acer, R. japonicus, R. illyricus and R. pedatus and found that all of them contained coumarin(62) derivatives. Scopolethin (6-methoxy-7-oxycoumarin) was found in all twelve species and umbelliferone (7-oxycoumarin) was found in two of the species.

Like anemone and clematis, all members of the genus ranunculus contain protoanemonin and most of the pharmacological and physiological affects of this plant are due to this substance. However, a preparation using a fat extract of Ranunculus ficaria has been patented by Yves Roche[80] as a vesicant, rubefacient and sclerosis ointment used in skin disorder treatment. Also another preparation containing saponins, from the roots of Ranunculus ficaria, has been patented by Cuenca[94] as an antihemorrhoidal ointment.

3.4 The Genus Thalictrum

Nuralieva[95] reports the presence of saponins and heart glycosides as well as 5,45% tannin substances in Thalictrum foetidum. No other work has, however, been reported on saponins in thalictrum and thus it would seem that the genus thalictrum differs from anemone, clematis and ranunculus in that most species of this genus do not contain saponins or tannins.

Sekiguchi[96] obtained a flavenoid from the leaves of Thalictrum minus by extraction with methanol, which he identified as apigenin-7-galactoside. Kowalewski, Frencel and Schumacher[97] carried out a comparative analysis of the flavenoid aglycons in the fruit of thirteen thalictrum species. They found that kaempferol(59) was the principle component of the aglucon fraction and that quercetin(58) occurred less abundantly, while apigenin(74) was seldom detected. Thalictrum foetidum is reported by Nuralieva[95] to contain 1,04% flavenoids. Further work by Nuralieva, Litvinenko

and Alimbaeva[98] led to the isolation, by chromatography, and identification of rutin (quercetin-3-rutinoside) and 7-methylquercetin-3-β-D-glucopyranoside, while Mollov, Georgiev, Panov and Kotsev[99] isolated the flavenols rhamnetin (7-methylquercetin) and quercetin(58). Wagner, Iyengar and Beal[100], working on the aerial parts of three thalictrum species, obtained the flavone-C-glycosides, vitexin (apigenin-8-C-β-D-glucopyranoside), saponaretin (apigenin-8-C-α-D-glucopyranoside), orientin (luteolin-8-C-β-D-glucopyranoside) and homo-orientin (luteolin-6-C-β-D-glucopyranoside), and the flavone-O-glycosides, rutin (quercetin-3-rutinoside) and kaempferol-3-rhamnoglucoside.

Kuczynski and Chyczewski[101] found, by paper chromatography, that various thalictrum species contained from six to sixteen flavenoids. Column chromatography of the aglycon fractions of some species revealed the presence of the flavenoids quercetin(58) and kaempferol(59) and of the flavones apigenin(74) and luteolin(73). Further work by Kuczynski and Chyczewski[102] led to the detection of six derivatives of luteolin(73) and apigenin(74) in Thalictrum rugosum. Three of these were present in amounts permitting isolation and identification and these were isovitexin-5-rhamnoside (isovitexin is apigenin-6-C-glucoside), luteolin-7-glucoside and apigenin-7-diglucoside.

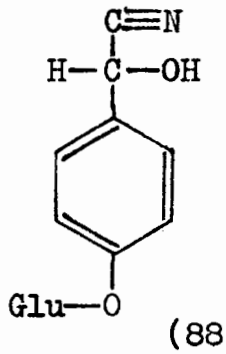
The seed oil of thalictrum species has been found to contain a number of unusual fatty acids. Thus, in 1962 Bagby, Smith, Mikolajczak and Wolff[103] reported that the principle fatty acid of Thalictrum polycarpum seed oil is the previously unknown trans-5,cis-9,cis-12-octadecatrienoic acid (35%). The oil also contains cis-9-octadecenoic (oleic) acid, and trans-5-octadecenoic acid which has not previously been demonstrated in plant materials, as well as two other major components which were not characterized.

Bhatty and Craig[104] determined the complete fatty acid composition of the seed oil of Thalictrum venulosum. They found the previously unknown trans-5-hexadecenoic and trans-5,cis-9-octadecadienoic acids. The complete analysis showed two predominant and one minor system of fatty acids. The first system is; trans-5-hexadecenoic acid, trans-5-octadecenoic acid, trans-5,cis-9-octadecadienoic acid and trans-5,cis-9,cis-12-octadecatrienoic acid. The second system comprises cis-9-hexadecenoic acid, cis-9-octadecenoic acid, cis-9,cis-12-octadecadienoic acid and cis-9,cis-12,cis-15-octadecatrienoic acid, and the third system comprises cis-5-hexadecenoic acid, cis-5-octadecenoic acid and cis-5-eicosenoic acid.

Markman and Freiman[105] identified hexadecanoic acid, octadecanoic acid, eicosanoic acid, docosanoic acid ($C_{21}H_{43}CO_2H$), tetracosanoic acid ($C_{23}H_{47}CO_2H$), cis-9-octadecenoic acid, trans-5,cis-9-octadecadienoic acid, cis-9,cis-12-octadecadienoic acid, cis-9,cis-12,cis-15-octadecatrienoic acid and trans-5,cis-9,cis-12-octadecatrienoic acid, in the seed oil of Thalictrum flavum by paper chromatography, uv spectroscopy and chemical methods. Freiman and Markman[106] also demonstrated the presence of trans-5-octadecenoic acid in the seed oil of Thalictrum minus, Thalictrum flavum, Thalictrum foetidum and Thalictrum simplex. Likewise they showed that trans-5,cis-9,cis-12-octadecatrienoic acid occurs generally in all the above species of thalictrum[107]. Rankov, Panov and Daleva[108] studying the fatty acid composition of five thalictrum species, confirmed that the major component is trans-5,cis-9,cis-12-octadecatrienoic acid, and that the fatty acids include mainly acids with trans double bonds in the 5-position.

Thalictrum also differs from the other genera of this subfamily in that it contains cyanogenic glycosides. Thus, Hegnauer[109] has reported that Thalictrum polycarpum releases hydrogen cy-

anide and Abrol[110] in a more detailed study, has measured the amount of hydrogen cyanide produced by young shoots of Thalictrum polycarpum and Thalictrum aquilegifolium. Sharples and Stoker[111] report the presence of three cyanogenic constituents in Thalictrum aquilegifolium and the identification of two of them as p-glucosyloxymandelonitrile(88) and p-glucosyloxymandelonitrile β-glucoside(89). Feeding with labelled precursors showed that these compounds were biosynthetically derived from tyrosine. They later reported[112] the isolation and structural elucidation of the third and major cyanogenetic glycoside of Thalictrum aquilegifolium which has structure (90).



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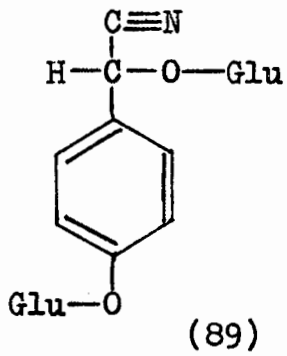

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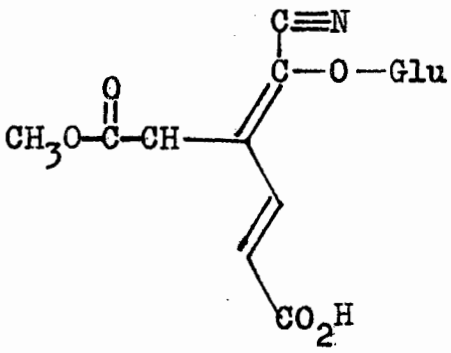


(90)

The most distinctive differences between the genus thalictrum and the other genera of this subfamily are that it contains no protoanemonin or its precursor ranunculin and that all its species contain a large number and variety of aporphine and benzylisoquinoline alkaloids and their dimers. All the reported pharmacological effects of thalictrum species are due to their alkaloid content and thus will be discussed at the end of the next section.

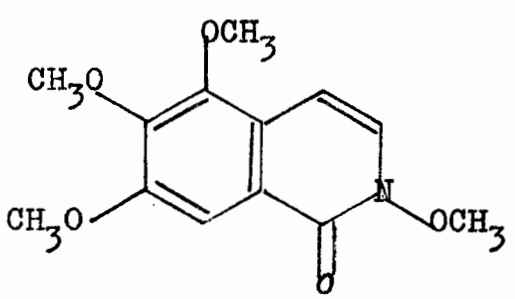
3.5 The Alkaloids of Thalictrum.[113, 114, 115, 116]

The first reports concerning phytochemical studies of the thalictrum plants date from the end of the 19th century. In the

early 1930's in the USSR, the study of the alkaloid composition of various species of thalictrum was begun. The school of Orekhov showed the presence of alkaloids in Thalictrum minus, T. petaloideum, T. angustifolium, T. flavum and T. simplex, distributed on the territory of the Soviet Union. The last 10-15 years is a period typical for the detailed investigation of the alkaloid composition of these plants. The most intensive work is being carried out in Japan by Tomita, Furukawa and Tomimatsu; in the USA by Shamma, Kupchan, Doskotch and Beal; in the USSR by Yunusov, and in Bulgaria by Mollov.

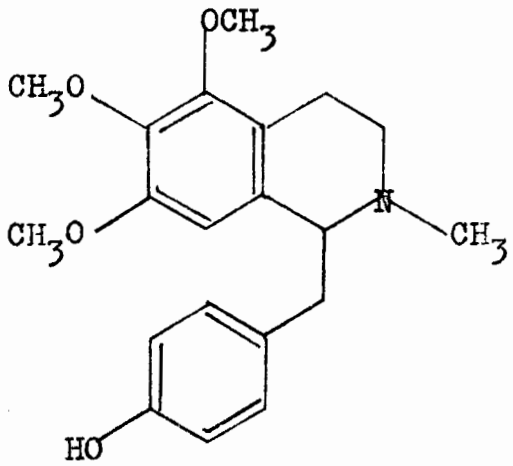
The alkaloids found in the various plants of the genus thalictrum belong to the isoquinoline type. Monomolecular isoquinoline type alkaloids belonging to the aporphine, benzylisoquinoline, isoquinoline, protoberberine and protopine series have been isolated. The majority of the compounds isolated are bismolecular isoquinoline alkaloids, which can be divided into two groups, the bisbenzylisoquinoline alkaloids and the aporphine-isoquinoline alkaloids. Pavine and isopavine alkaloids have also been found in these plants.

It was 1969 when for the first time some simple isoquinoline alkaloils were found in thalictrum species. Since then nine of these alkaloids have been isolated and identified. These are; thalactamine isolated from T. minus, corypalline from T. dasycarpum, noroxyhydrastinine and thalifoline from T. minus var. adiantifolium, thalflavine from T. flavum[117], N-methylthalidaldine and N-methylcorydaldine from T. fendleri[118], and pallidine and corydine from T. dioicum. A typical structure is that of thalactamine (91).



(91)

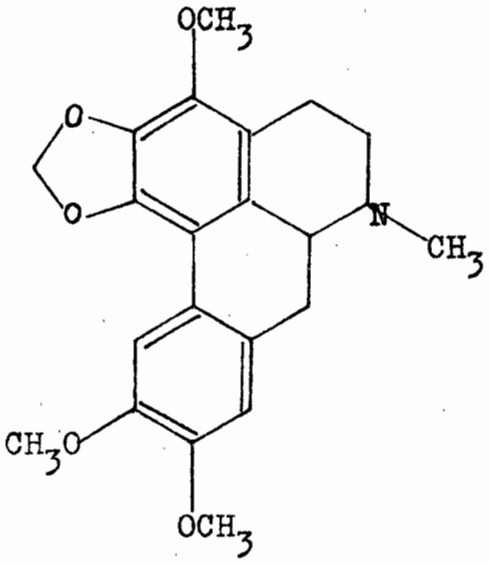
The benzylisoquinoline alkaloids are not widely distributed in plants of the genus thalictrum and so far only five of these alkaloids have been discovered. These are takatonine from T. thunbergii, thalifendlerine from T. fendleri, laudanidine from T. dasycarpum, veronamine from T. fendleri[118] and N-methylpalaudinium chloride from T. polygamum[120]. A typical structure is that of thalifendlerine(92).



(92)

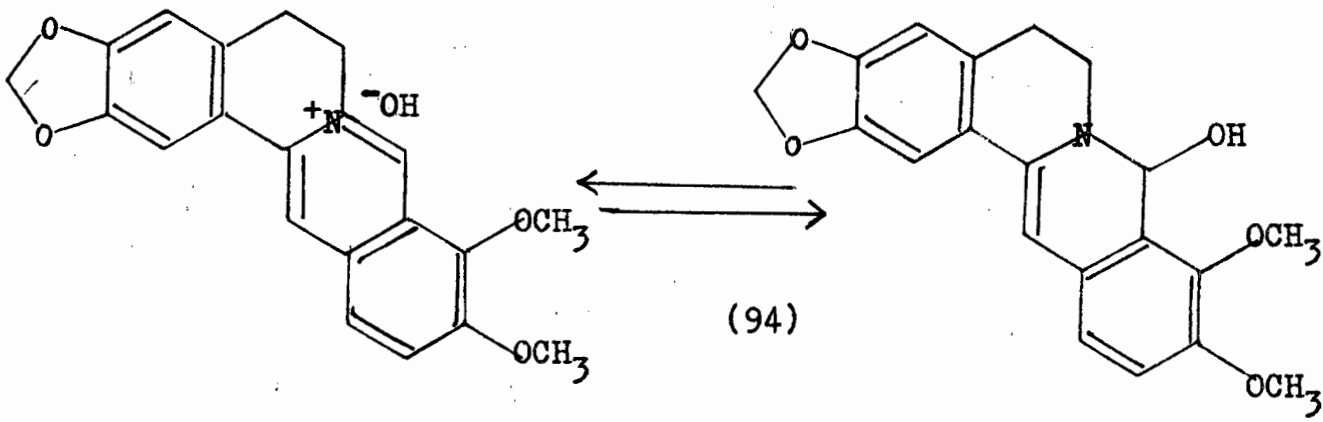
Among the great number of aporphine alkaloids wide spread in nature, sixteen are so far known from the various representatives of the genus thalictrum. These are thalicmine (ocoteine) from T. minus, T. isopyroides and T. fendleri; dehydrothalicmine from T. isopyroides[121]; thalicmidine and thalicmidine N-oxide from T. minus[122]; glaucine from T. minus and T. fendleri; magnoflorine from T. dasycarpum, T. minus, T.flavum, T. foliolsum, T. foetidum, T. fendleri, T. isopyroides, T. thunbergii, T. simplex, T. rugosum and T. rochebrunianum; isocorydine from T. aquilegifolium; thalicsimine from T. simplex; thalicminine from T. minus, T. simplex, and T. isopyroides; thaliporphine from T. fendleri; preocoteine from T. fendleri; preocoteine N-oxide from T. minus[122]; thalglucine and thalglucinone from T. rugosum[123]; thalphenine chloride and thalphenine methine from T. polygamum[124]; and thal-

icsin from T. longipedunculatum[125]. A typical structure is that of thalicmine(93).



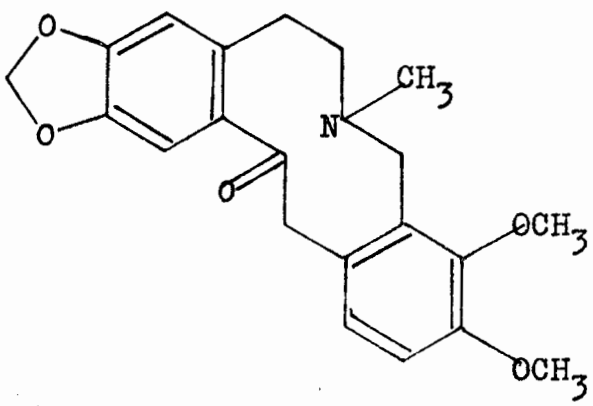
(93)

The protoberberine alkaloids are widely distributed in the Berberidaceae, Ranunculaceae, Anonaceae, Menispermaceae, Papaveraceae and Rutaceae. Those that have been found in members of the genus thalictrum are berberine from T. dasycarpum, T. flavum, T. fendleri, T. foliolosum, T. foetidum, T. minus, T. longipedunculatum, T. rugosum, T. rochebrunianum, T. tubiferum, T. thunbergii and T. simplex; tetrahydroberberine from T. actaefolium; canadine from T. minus; jatrorrhizine from T. minus, T. fendleri, T. foliolosum, T. rugosum and T. rochebrunianum; columbamine from T. rugosum; palmatine from T. foliolosum and T. minus; thalifendine from T. fendleri and T. minus; tetrahydrothalifendine from T. fendleri[118]; and thalidastine from T. fendleri. A typical example of these alkaloids is berberine(94) itself.



(94)

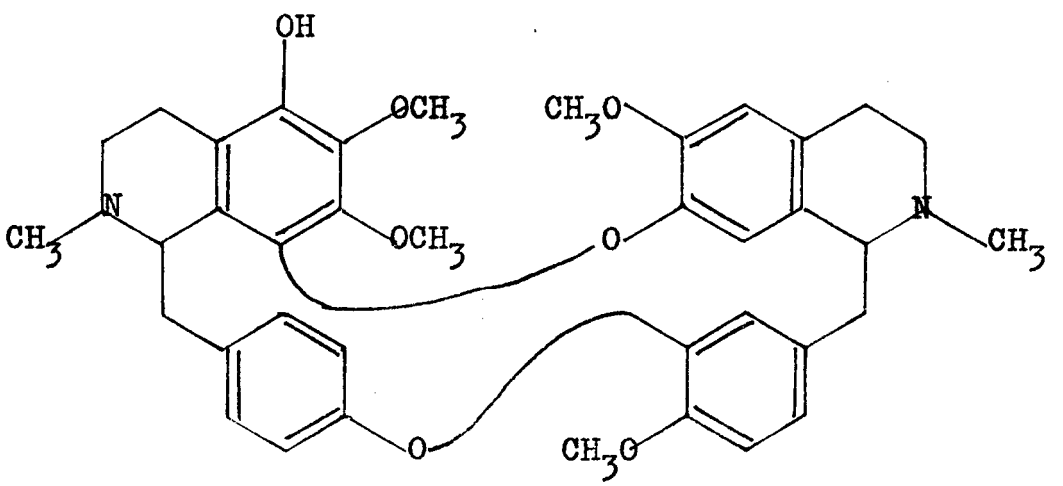
In recent years it has been found that thalictrum species contain some protopine alkaloids. So far three have been isolated and identified and these are; thalisopyrine from T. isopyroides and T. flavum; thalictrimine (β-allocryptopine) from T. minus and T. simplex; and thalictrisine from T. simplex. A typical structure is that of thalictrimine(95).



(95)

The bisbenzylisoquinoline alkaloids constitute the group of alkaloids which is most widely distributed in thalictrum species. In these alkaloids two benzylisoquinoline units are joined by ether bridges in different ways. The alkaloids that have so far been found in thalictrum species are; thalisopine and O-methylthalisopine from T. isopyroides; berbamine from T. foetidum; obamegine (stepholine) from T. rugosum; isotetrandine from T.

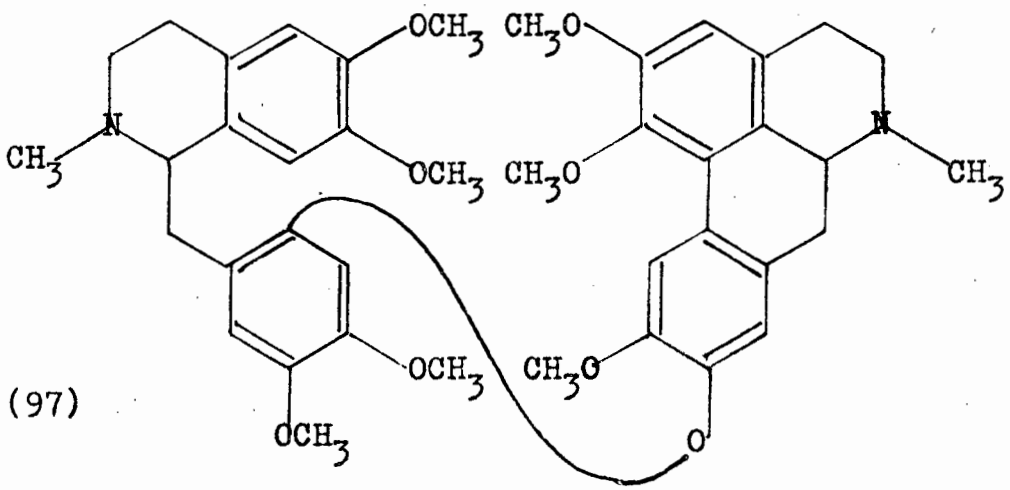
foetidum; thalidezine from T. simplex, T. rugosum and T. fendleri; hernandezine (thalicsimine) from T. hernandezii, T. rochebrunianum, T. flavum, T. alpinum, T. simplex and T. fendleri; thalisamine from T. simplex; thalidasine from T. dasycarpum and T. rugosum; thalicberine from T. thunbergii and T. minus; O-methylthalicberine (thalmidine) from T. thunbergii, T. minus and T. isopyroides; thalfoetidine (thalictrinine) from T. foetidum and T. longipedunculatum; thalmine from T. simplex and T. minus; thalicrine (aromoline) and homothalicrine (homoaromoline) from T. thunbergii; thalmetine and O-methylthalmetine from T. minus; thalsimine and thalsimidine from T. simplex; thalrugosamine from T. rugosum[126] thalphinine and thalphine from T. foetidum[127]; and thalgine from T. polygamum[128]. A typical structure is that of thalisopine(96).



(96)

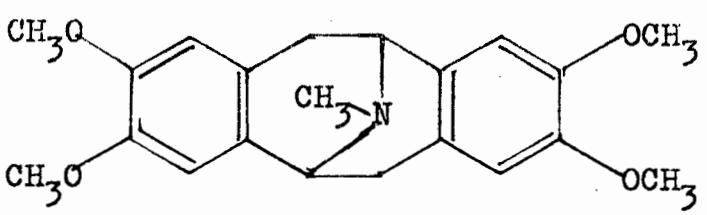
The aporphine-benzylisoquinoline alkaloids constitute a new group of alkaloids in which both parts of the molecule, the aporphine and the benzylisoquinoline are joined by an ether bridge between C-10 of the benzylisoquinoline and C-9 of the aporphine. The following alkaloids have been isolated from thalictrum; thalicarpine from T. dasycarpum, T. minus, T. revolutum and T. fendleri; dehydrothalicarpine (thalictrucarpine) from T. minus and T. dasycarpum; thalmelatine and dehydrothalmelatine from T. minus; adian-

tifoline from T. minus var. adiantifolium; foetidine from T. foetidum; thalictropine and thalictrogamine from T. polygamum[129]; thalmelatidine from T. minus var. elatum[130]; thalmineline from T. minus[131]; pennsylvanine and pennsylvanamine from T. polygamum[132]; and thalidoxine from T. dioicum[133]. A typical structure is that of thalicarpine(97).



(97)

The following four pavine and isopavine alkaloids have been obtained from thalictrum species; thalisopavine, argemonine norargemonine and bisnorargemonine all from T. dasycarpum. A typical structure is that of argemonine(98).



(98)

The various thalictrum species have been known for a long time as folk remedies. On this question Kupchan[134] writes: "Many medicinal uses of thalictrum species in folk remedies have been recorded. Thalictrum foliolosum is found throughout the Himalayas and taken as a tonic, aperient, purgative, diuretic, febri-

fuge, a remedy for atonic dyspepsia, and used as an application for ophthalmia. Thalictrum thunbergii is used in Japan as a home remedy against stomach ache and diarrhea. Thalictrum collinum and Thalictrum silvaticum have been used in Ukrainian folk medicine as diuretics. Thalictrum fendleri was prepared by the Indians of Nevada as a tea to cure gonorrhea; a decoction of the root was used against colds. Thalictrum minus is used in South Africa to treat fevers. Intravenous injection of the hydrochlorides of the extract of the total alkaloids from Thalictrum minus has recently been shown to exert an effect on the blood pressure and pulse of frogs, cats and dogs." Bulgarian folk medicine uses Thalictrum aquilegifolium against diphtheria, jaundice and for reducing nervous tension.

Alkaloid mixtures or individual alkaloids isolated from various thalictrum species have been the object of pharmacological screening since only quite recently. In 1956 appeared a report dealing with the pharmacological activity of a preparation obtained from Thalictrum minus found in the Armenian SSR. The Thalictrum minus distributed in the Byelorussian SSR contains phytoncides and exhibits bactericidal properties against gram-positive and gram-negative bacteria. The pharmacological examination of the alkaloids of Thalictrum minus carried out in Bulgaria, shows that they cause a significant, but transient hypotensive effect connected to a certain extent, with the excitation of the M-cholinoreactive systems. A more detailed investigation of their hypotensive action indicates that it is associated with the noradrenaline metabolism. These alkaloids also possess spasmolytic and diuretic properties.

The alkaloid thalsimine isolated from Thalictrum simplex has hypotensive, tranquillizing, cholinomimetic and adrenolytic

properties. Another alkaloid from the same plant, thalsine, also shows hypotensive and adrenolytic properties; the alkaloid hernandezine exhibits a two phase effect on the blood pressure, the latter increases or decreases depending on the administered dose.

A thorough pharmacological evaluation of Thalictrum foetidum has been carried out in the Soviet Union. It is reported that its alkaloid mixture lowers the blood pressure, produces slight spasmolytic effects in the intestinal muscle and also, depending on the dose and time of its administration, initially depresses the conditioned reflexes and subsequently heightens the excitation of the central nervous system. Clear spasmolytic action caused by the thalisopine alkaloid isolated from Thalictrum isopyroides is reported for the first time for thalictrum alkaloids by a group of Soviet chemists.

The alkaloid thalicarpine isolated from Thalictrum dasycarpum, Thalictrum revolutum and Thalictrum minus has been evaluated pharmacologically in the United States. It is reported to lower, but not significantly, the blood pressure and not to exhibit anti-inflammatory, anticoagulant, hypoglycemic and diuretic properties. Pharmacological studies of the alkaloid mixture obtained from Thalictrum rochebrunianum have indicated that doses of 2mg/Kg introduced intravenously lower the blood pressure.

In addition Svendsen and Anders[137] review the medicinal uses of the different species of thalictrum. They report that thalictrum is a natural source of antitumoural products. Of particular interest are the bisbenzylisoquinoline alkaloid thalidasine and the aporphine-isoquinoline alkaloid thalicarpine, which are proposed as models for further research in antitumoural synthetic products. Kupchan[138] reports that thalidasine shows inhibitory activity against Walker intramuscular carcinosarcoma 256 in rats

at 200mg/Kg. Likewise he[139] reports that thalicarpine exhibits a significant inhibitory activity against Walker intramuscular carcinosarcoma in rats over a wide dosage range. This alkaloid has undergone extensive preclinical toxicological studies and is now in clinical trial under the auspices of the National Cancer institute. It can be said in conclusion that the alkaloids obtained from thalictrum plants possess a variety of clearly exhibited pharmacological properties which make them of considerable promise for medicinal use.

3.6 General distribution of classes of compound in the Ranunculaceae[5]

We have already noted (section 2.0) that protoanemonin or, more accurately, its precursor ranunculin, occurs exclusively in the subfamily, Anemoneae, and in the genus helleborus of the subfamily Helleboreae. We also noted that the genus thalictrum was exceptional in that it is the only genus of this subfamily which does not contain ranunculin. Ruijgrok[5] reports that, in the detailed survey on which this analysis is based, he noted that a few species of the plants he tested contained a ranunculin-like main constituent whose R_f value deviated from that of pure ranunculin; since one of these plants was Ranunculus repens it is probable that this compound was the isoranunculin found in this plant by Tschesche, Welmar, Wulff and Snatzke[29](see section 2.5).

The genus thalictrum is also distinct from the other genera of this subfamily in that all the other genera of this subfamily seem to be largely lacking in alkaloids while thalictrum species all seem to contain a great variety of isoquinoline-type alkaloids. Other genera in which isoquinoline-type alkaloids are common are, aquilegia, coptis, hydrastis and xanthorrhiza, which are all members of the Helleboreae. Diterpene derived alkaloids are found in the genera aconitum and delphinium, and damascenine

is characteristic of nigella. In addition magnoflorine is found in most of these genera and in many other genera of the Helleboreae.

Flavenoids, particularly kaempferol and quercetin, are very widespread in the Ranunculaceae. In particular quercetin-7-glucoside and kaempferol-7-glucoside occur consistently in extracts of the leaves of representatives of the genera, paeonia, helleborus, caltha, anemone and ranunculus, but they are rare or totally lacking in the genera, cimicifuga, aquilegia, trollius, nigella, aconitum, delphinium, adonis and callianthemum.

Saponins are widespread in the genera anemone, clematis and ranunculus, and they have also been detected in some species of thalictrum and trollius but seem to be absent in adonis and myosurus. Tannins seem to be rare or absent in all genera of the Ranunculaceae. Drozd[56] has tested representatives of the genera caltha, nigella, clematis, batrachium, ranunculus and adonis and has found them all to contain coumarins. All species of aquilegia and isopyrum so far investigated contain cyanogenetic compounds and in addition some species of thalictrum and a few species of ranunculus and clematis release hydrogen cyanide on bruising. Whether thalictrum generates hydrogen cyanide in the same way as aquilegia and isopyrum is still unknown.

DISCUSSION

In the preceeding review it has been shown that many members of the Ranunculaceae have antibacterial and antifungal activity due to the liberation of protoanemonin(1) from ranunculin (48) or from some other, as yet, unidentified precursor. Also many members of the family contain alkaloids and, in particular, the genus thalictrum of the subfamily Anemoneae has given rise to much interest due to the wide variety of bisbenzylisoquinoline and other related alkaloids which it contains, especially since a number of these have shown antitumour activity. In connection with this it was noted that the genus Knowltonia which is endemic to South Africa and is also a member of the subfamily Anemoneae had not been subjected to any chemical study at all apart from one very confused report on one member of the genus, Knowltonia transvaalensis, by Brandwyjk and Watt[2] in 1925. However, Brandwyjk and Watt[3] (section 1.0) report that virtually all species of this genus are used as African folk remedies for a variety of ailments. Consequently it seemed worthwhile to investigate the chemical constituents of a botanically typical member of this genus.

Such a species is Knowltonia capensis which was convenient for this study since it grows wild on the lower slopes of Table mountain and was thus readily available in large quantities. It was observed that when the plant was crushed it gave a sharp acrid odour and the crushed fresh plant also caused a burning on the skin. These properties were later attributed to protoanemonin. As a preliminary study crude aqueous and alcohol extracts were prepared by grinding two portions of the fresh plant with water and alcohol respectively in a Waring blender. These extracts were tested against Staphylococcus aureus for antibiotic activity

and were both found to be active, which was to be expected if the plant contained protoanemonin(1), as already deduced from the acrid property of the fresh leaves. The alcohol extract was also tested with Dragendorf's reagent for the presence of alkaloids and surprisingly a thick yellow precipitate was formed which was identical in appearance with the precipitate obtained when testing a very dilute solution of strychnine with Dragendorf's reagent. Thus it appeared that Knowltonia capensis contained alkaloids as well as protoanemonin, which would have been most unusual in the light of the earlier studies on the Ranunculaceae reported at the end of this section. However, since all subsequent studies failed to indicate the presence of alkaloids it must be deduced that what was precipitated by the Dragendorf's reagent was not alkaloids, but the choline ester that was isolated or possibly also proteins.

A further alcohol extract was obtained by soxhlet extraction of 100 g of the dried ground plant for 24 hrs. This extract was then fractionated between aqueous methanol, petroleum ether, chloroform, ether and water as shown in Table 1. Each of these fractions, as well as the original extract, was tested for antibiotic activity against Staphylococcus aureus and most of the activity was found to be concentrated in the chloroform fraction. This fraction was obtained as a pale pungent yellow oil, which solidified overnight and was, therefore, probably mainly protoanemonin(1), It would appear from this that protoanemonin is more readily extracted from aqueous solution with chloroform than with ether and also that it is more stable to the above extraction procedure than would be expected from the rapidity with which it polymerizes when pure.

Thin layer chromatography studies of the freshly obtained

chloroform fraction showed the presence of four components, one of which was not evident when the plate was visualized with ultraviolet light but only when it was sprayed with Dragendorf's reagent, and one of which showed very marked fluorescence under ultraviolet light. Two of these compounds could be expected to be protoanemonin(1) and its dimer anemonin(2), but there remained a strong possibility that the other two could be alkaloidal. After the fraction had already solidified an attempt was made to isolate anemonin from it by extraction with absolute alcohol, but this extract was again obtained as a gum which underwent further polymerization on standing.

To enable a more detailed investigation of this fraction to be undertaken more plant material was collected and extracted in bulk. Two alternative fractionation procedures were investigated. The first was the same as that used in the preliminary investigation (Table 1) and involved extracting the plant material with ethanol, taking the ethanol extract to a paste, dissolving this paste in dilute hydrochloric acid and extracting thoroughly with ether, taking the ether extract to a paste, dissolving this in aqueous methanol and extracting thoroughly with petroleum ether, and finally basifying the acid aqueous fraction with sodium hydroxide and extracting thoroughly with chloroform. The second procedure involved extracting the plant material with petroleum ether and subsequently with ethanol, and then taking the ethanol extract to a paste, dissolving it in dilute hydrochloric acid, extracting with ether, basifying the aqueous fraction with sodium hydroxide and extracting thoroughly with chloroform. A thin layer chromatogram comparison of the fractions obtained by these two methods showed that, while the second avoided the problem of having to evaporate down the aqueous methanol

fraction which was accompanied by extensive frothing, which is probably indicative of saponins in this fraction , it had the disadvantage that the petroleum ether extraction was not complete and there was an overlap of the components found in this fraction and in the ether fraction. Thus the first method was deemed to be the more satisfactory.

During the course of the large scale extraction by the second method, a crystalline compound separated out from the hot ethanol extract as it was allowed to cool and it was isolated by decantation, and examined separately. Paper chromatography of this compound and its hydrolysate both indicated that it was glucose, and gas-liquid chromatographic studies of the trimethylsilyl ether derivative of the compound and the hexa-acetate of the reduced compound both confirmed this identification. Moreover, the phenylosazone derivative and the β-penta-acetate derivative of the compound were both found to be identical with the corresponding derivatives of glucose. The optical rotation, melting point and microanalysis, however, were not consistent with the corresponding constants of glucose, and repeated recrystallizations merely served to confirm the difference, although a comparison of the nuclear magnetic resonance spectrum of the compound and that of an authentic sample of glucose showed that the two were identical. This question has not been resolved but there seems to be no doubt that the compound isolated from the plant is in fact glucose.

The chloroform fractions obtained from the large scale extractions described above were combined and were kept in the refrigerator in dilute chloroform solution to prevent polymerization. When this extract was examined by thin layer chromatography only two spots,in addition to the spot on the base line,

were observed, one of which was strongly fluorescent under ultraviolet light. Since separation by thin layer chromatography was good, an attempt was made to separate 2 g of this chloroform fraction on a silica column. The first component eluted from the column was the one corresponding to the strongly flourescent spot on the thin layer plate and was obtained as an oil which polymerized overnight and was, therefore, probably protoanemonin(1). The second component was crystalline and had a melting point, composition figures, and mass,infrared and nuclear magnetic resonance spectra which were consistent with its identification as anemonin(2). Prominent peaks in the mass spectrum corresponded to the molecular ion (m/e 192), the loss of one (m/e 164) and two (m/e 136) units of carbon monoxide and the monomer protoanemonin (m/e 96), which was the base peak. The loss of first one and then a second unit of carbon monoxide was confirmed by metastable peaks at m/e 140 and m/e 113. The infrared spectrum, which is reported and interpreted in Table 3, confirmed the presence of the α,β-unsaturated γ-lactone system. In the nuclear magnetic resonance spectrum (Table 4) the corresponding protons in the lactone rings showed up as two sharp doublets at low field integrating for two protons each (J 6 Hz). The four protons on the cyclobutane ring showed up as a complex multiplet at τ 7,35.

No other pure compounds were obtained from this column. The more polar compounds were not separated and were obtained by stripping the column with methanol. However, when these fractions were evaporated down more anemonin separated out. This was filtered off and identified by its nuclear magnetic resonance spectrum. The remaining dark filtrate was fractionated on a sephadex LH 20 column, eluted with methanol. Examination of the fractions by thin layer chromatography showed approximately four components. Two of

the fractions gave amounts of impure crystalline material, which were considered too small for further purification and identification. The first fraction eluted from this column was found to comprise most of the material and, since thin layer chromatography of this fraction run in a more polar solvent showed three components and a base spot, a further attempt was made separate this by chromatography on silica, but the only crystalline compound that was obtained was shown to be anemonin. It was decided to repeat this chromatographic work with the remainder of the chloroform fraction in the hope of obtaining the two crystalline compounds in sufficiently large yield to permit identification. However, the only pure crystalline compound that was obtained was anemonin, all the other components being obtained as intractable oils or gums many of which seemed to be unstable. In retrospect, it seems probable that the only true component of the chloroform fraction was protoanemonin(1) which then dimerized to anemonin(2), and also yielded a mixture of polymers and possibly also breakdown products of anemonin, such as anemoninic acid(10), during the course of the attempts at chromatographic separation.

The alkaloids that had appeared to be present when the crude extract was tested with Dragendorf's reagent might possibly have been quaternary alkaloids, and might be present in solution in the aqueous fraction, remaining after the chloroform extraction. Thus ammonium reineckate was prepared and used to precitate reineckate salts from the aqueous fraction obtained from the large scale extractions and fractionations described above. Trial precipitations showed that the reineckate was precipitated best at pH 4. None the less the precipitate appeared very slowly and the yield was low. Microanalysis gave composition figures corresponding to choline reineckate and, since choline is a very simple

and extremely common natural compound, it did not seem worthwhile to pursue this further. It was shown, however, that choline occurs in the plant as an ester only and not in the free form by treating the aqueous fraction obtained after a similar fractionation which had been done entirely without heating. In this case no precipitate was obtained, but when another portion of this aqueous fraction was acidified and heated on a steam bath for three hours, and then treated with ammonium reineckate a precipitate of choline reineckate was obtained as before.

To finally confirm that there were no alkaloids in this plant after all, fresh plant material was carefully extracted and fractionated in the cold. The fresh plant material was ground up with ethanol in a Waring blender and the ethanol extract then evaporated to a paste, which was dissolved in dilute hydrochloric acid and thoroughly extracted first with ether then with chloroform. When these chloroform extracts were evaporated down, they yielded an acrid yellow oil which polymerized on standing and which was probably protoanemonin(1). The aqueous fraction was then basified and thoroughly extracted with chloroform again. When the chloroform fractions were evaporated they yielded no alkaloid fraction. This finding was, in fact, in keeping with other chemotaxonomic studies on this plant family[5] which have shown that those genera which contain alkaloids do not contain protoanemonin and vice-versa.

The ranunculin(48) content of the plant was then examined. Firstly protoanemonin was extracted by the usual method of grinding fresh plant material with water and then steam distilling. The ultraviolet absorbtion of the distillate was measured after each 100 ml had been collected, and it was observed that from 100 g of fresh plant almost all the protoanemonin had been

obtained after 300 ml of distillate had been collected. This first 300 ml of distillate was then thoroughly extracted with chloroform which was evaporated under reduced pressure yielding an acrid volatile oil which polymerized in a couple of hours and showed the characteristic ultraviolet absorbtion of protoanemonin(1). To confirm the identification of this compound as protoanemonin, a further 100 g of fresh plant material was steam distilled and a slight excess of dilute sodium hydroxide was added to the first 300 ml of steam distillate to open the lactone ring of the protoanemonin and yield the sodium salt of β-acetylacrylic acid(33). This solution was then evaporated to dryness on a steam bath leaving the sodium salt as a white crystalline deposit. The sodium salt was then taken up in a small volume of water, acidified with dilute hydrochloric acid, and the free β-acetylacrylic acid extracted with ether, which was then evaporated off to give the crystalline product. This was identified from the melting point, composition figures and ultraviolet, infrared and nuclear magnetic resonance spectra. Shaw[18] has pointed out that the ultraviolet spectrum of β-acetylacrylic acid in aqueous solution has a single absorbtion maximum at 225 nm, which is the value expected for the lactone rather than the straight chain form, and has proposed that in aqueous solution this compound is only found in the cyclic form. The nuclear magnetic resonance spectrum in D_2O provided a clear confirmation of this. One sharp doublet was observed for each of the protons attached to the double bond showing that there was no equilibrium between the straight chain and cyclic forms, unless this equilibration is extremely fast which is highly unlikely for this type of isomerization and would not be consistent with the evidence of the ultraviolet spectrum, The coupling between the protons attached to the double bond is 16 Hz

(Table 6). On the other hand the infrared spectrum of the crystalline form (nujol mull) shows a peak due to alkene C–H stretching, a broad peak due to intramolecularly bonded O–H stretching, a peak due to an intramolecularly hydrogen bonded α,β-unsaturated carbonyl group, a peak due to C=C stretching in a α,β-unsaturated carbonyl system and peaks due to C–O stretching. This indicates that the crystalline compound is in the straight chain form. (Table 5)

Having thus conclusively demonstrated the presence of protoanemonin, an attempt was made to extract ranunculin(48) from the plant by the method of Hill and van Heyningen[24]. This involves grinding the fresh plant with acid to destroy the enzymes which are responsible for the breakdown of ranunculin to protoanemonin, followed by filtering off the plant material and centrifuging the filtrate. The filtrate was stirred with activated charcoal to absorb the ranunculin which was finally eluted off the column of activated charcoal with 50% aqueous ethanol. The product was obtained as a gum which could not be induced to crystallize. It was therefore placed on a silica column and eluted with 30% methanol in chloroform. The strong fluorescence of ranunculin(48) under ultraviolet light was used to follow its elution from the column, and it was obtained as a gum which crystallized on being pumped out under high vacuum. This gave the expected melting point, composition figures and infrared and nuclear magnetic resonance spectra for pure ranunculin. The infrared spectrum (Table 7) showed the O–H and C–O stretching absorptions of the glucose moiety, and an alkene C–H stretching absorption, a carbonyl stretching absorption for an α,β-unsaturated γ-lactone, and a stretching absorption for C=C conjugated with C=O. The nuclear magnetic resonance spectrum (Table 8) showed clearly that the

product obtained was pure ranunculin and that there was no admixture with isoranunculin(53) as found by Tschesche, Welmar, Wulff and Snatzke[29] (section 2.5) in Ranunculus repens. The spectrum showed a low field doublet corresponding to the proton in position 2, a low field doublet of doublets corresponding to the proton in position 3, a broad absorption corresponding to the proton in position 4, the doublet due to the anomeric proton in the glucose moiety and a complex band in the vicinity of $\tau 6$ corresponding to the remainder of the protons. With regard to the work of Tschesche et al.[29] it seems possible that ranunculin is not the true precursor of protoanemonin, since Tschesche could not obtain it by alcohol extraction. However, since both Tschesche et al. and Ruijgrok[5] report the presence of more than one glucoside of the ranunculin type in Ranunculus repens and Helleborus foetidus when they are extracted according to the method of Hill and van Heyningen whereas Knowltonia capensis clearly only contains one, it also seems possible that the former two plants genuinely contain a variety of ranunculin-like glucosides. Whether or not Knowltonia capensis would yield different products if it were extracted by the method devised by Tschesche was not investigated.

To provide further confirmation of the identity of ranunculin it was acetylated with acetic anhydride in pyridine to yield the tetra-acetate. It was found that the tetra-acetate could not be precipitated by adding water to the reaction mixture itself, and it was necessary to evaporate off nearly all the pyridine, acetic acid and acetic anhydride before the addition of water gave a crystalline product. This compound was shown to be ranunculin tetra-acetate by the melting point, composition figures and infrared and nuclear magnetic resonance spectra. The infrared spectrum (Table 9) again showed a peak due to alkene C–H stretch-

ing, a peak due to C=O stretching in α,β-unsaturated γ-lactones, a peak due to C=C stretching conjugated with C=O and peaks due to C—O stretching. The nuclear magnetic resonance spectrum (Table 10) was also consistent with that expected for ranunculin tetra-acetate. The low field resonances of the protons in positions 1 and 2 were now both observed as doublets of doublets and the protons in positions 4 and 5 occurred as a broad multiplet at τ 6. The methyl protons of the acetate groups formed a group of singlets just below τ 8.

Finally, a portion of the dried ground plant material was extracted and partitioned according to the standard procedure laid down by the National Cancer Institute of America. This involved soxhlet extracting the plant material with petroleum ether, discarding the petroleum ether extract, and re-extracting the plant material with ethanol. The ethanol extract was then taken to a paste and a small portion of this was taken as sample A. The remainder of the ethanol extract was then partitioned between chloroform and water, and the chloroform fraction was also taken to a paste and was taken as sample B. Samples A and B were sent to the National Cancer Institute for testing for antitumour activity. Sample B has been reported to be active in the mouse leukemia P-388 system, but this is still being confirmed. The source of the activity is possibly anemonin, since protoanemonin is known to show anticancer activity[37]. The activity could not be due to ranunculin or protoanemonin itself since these are too unstable to survive the extraction procedure, and the long delay before tests can be carried out. However, it is possible that the source of the activity is some compound not yet isolated.

Thus we have shown that the genus knowltonia as represent-sented by the species Knowltonia capensis is in line with the

other genera of the subfamily Anemoneae in that it contains ranunculin and it does not contain alkaloids. No plant genera are known that contain both ranunculin and alkaloids. The genus thalictrum is anomalous in that it is the only genus of this subfamily which does not contain ranunculin and does contain alkaloids. Likewise, the genus helleborus is also anomalous in that it is the only genus outside of the subfamily Anemoneae which does contain ranunculin. Thus chemical studies have called into question the traditional subdivision of the family on the basis of botanical characteristics, since thalictrum is chemically more closely linked with hydrastis, coptis, xanthoriza and aquilegia, all of the Helleboreae, than with the other genera of the Anemoneae. Also helleborus itself is chemically more closely related to the genera of the Anemoneae, than to the other genera of its own subfamily. Also chromosome studies of the Ranunculaceae[5] have shown that the chromosomes fall into two groups on the basis of shape and size, namely the Ranunculus-type (R-type) and the Thalictrum-type (T-type). Very interestingly the subdivision of the family on the basis of these chromosome differences, which is shown below, is more in line with the results of chemical studies than is the traditional subdivision of Prantl (see section 1.0)

R-type chromosomes: Caltha, Eranthis, Nigella, Acteae, Delphinium, Aconitum, Helleborus, Anemoneae, Clematis, and Ranunculus.

T-type chromosomes: Aquilegia, Coptis, Xanthoriza, and Thalictrum.

EXPERIMENTAL

NMR spectra were measured on a Varian XL-100 spectrometer with tetramethylsilane as internal standard. Infrared spectra were determined on a Perkin-Elmer model 237 spectrometer, and uv spectra on a Perkin-Elmer spectrophotometer. In the ir spectra w stands for weak, m for medium, s for strong and br for broad. The mass spectrum was recorded on an AEI MS9 double focussing spectrometer. Melting points were determined on a Fischer-Johns hot stage apparatus and are uncorrected. Petroleum ether refers to the fraction, boiling point 60-80°C. TLC experiments were performed using Merck aluminium backed silica plates containing fluorescent indicator.

1.0 PRELIMINARY INVESTIGATION

1.1 Test of Knowltonia for antibiotic activity.

20 g of fresh stems and leaves of Knowltonia capensis collected in February on the lower slopes of Table mountain above Kirstenbosch gardens, Cape Town, was macerated in a Waring blender with 100 ml ethanol. The resulting slurry was allowed to stand overnight to ensure complete extraction and then filtered through fluted filter paper. The ethanol was evaporated under vacuum to give a dark gum. A portion of this was partially dissolved and partially suspended in distilled water and tested for bacteriological activity by placing a drop in the middle of a plate containing a culture of Staphylococcus aureus in agar. The extract showed marked bacteriocidal activity.

A further 20 g of fresh stems and leaves was macerated in a Waring blender with 100 ml water. The resulting slurry was again allowed to stand overnight and then filtered through fluted filter paper. This filtrate was tested for bacteriological activity against Staphylococcus aureus as before and was found to show

marked bacteriocidal activity.

1.2 Test for the presence of alkaloids.

Dragendorf's reagent was prepared as follows[135]: 5 ml of solution A was mixed with 5 ml of solution B and 20 ml of glacial acetic acid and made up to 100 ml with distilled water. Solution A was prepared by dissolving 0,85 g of bismuth subnitrate in a mixture of 10 ml glacial acetic acid and 40 ml of water. Solution B consisted of 8,0 g of potassium iodide in 20 ml of water.

About 1 g of the black gum obtained from the alcohol extraction described in section 1.1 was dissolved in about 2 ml of distilled water and about four drops of this solution was added to about 2 ml of Dragendorf's solution. A yellow suspension was formed which settled slowly. A blank test was performed by adding four drops of distilled water to 2 ml of Dragendorf's solution. When four drops of a very dilute aqueous solution of strychnine was added to 2 ml of Dragendorf's solution a yellow precipitate was formed which was similar in appearance to that obtained in the test with the plant extract.

1.3 Trial Alkaloid Extraction and Preliminary Fractionation.

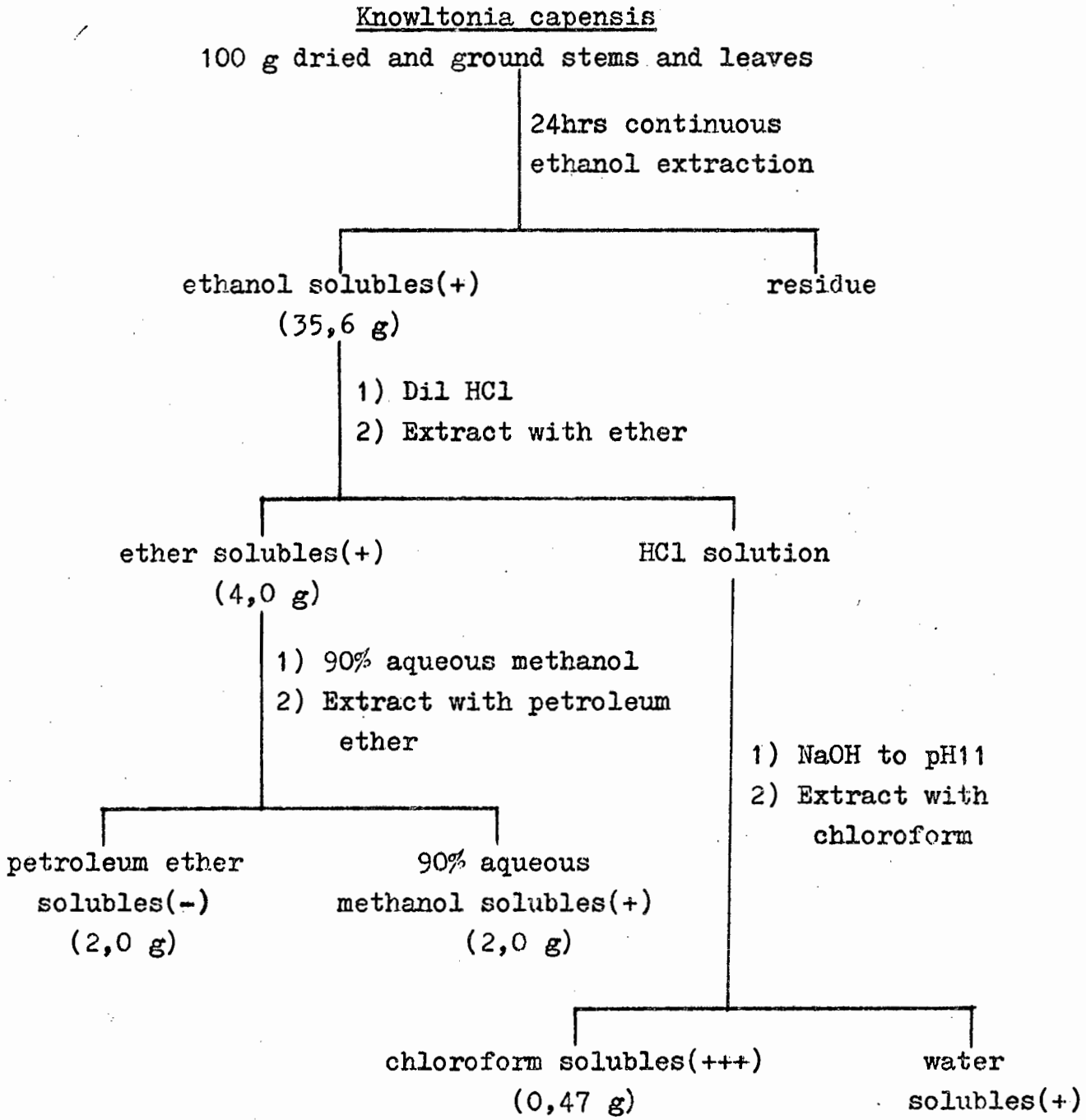
100 g of plant material, collected at the same time as in section 1.1, which had been thoroughly dried in an oven at 35-40°C and then ground to a fine powder in a Wiley hammer mill, was extracted for 24 hrs in a soxhlet apparatus with 1,2 l of ethanol. The resulting solution was evaporated under reduced pressure to give 35,6 g of a dark gum. A small portion of this was submitted for bacteriological testing. The remainder was dissolved in 40 ml of water and acidified to pH 1 with concentrated hydrochloric acid. This solution was then thoroughly extracted with 50 ml aliquots of ether until the ether extracts were nearly colourless. The ether extract was then taken to dryness under vacuum, yielding 4,0 g of

dark gum. A small portion of this was also submitted for bacteriological testing. The remainder was dissolved in 40 ml of 90% aqueous methanol and thoroughly extracted with 50 ml aliquots of petroleum ether until the petroleum ether extracts were nearly colourless. The petroleum ether extracts were then taken to dryness under vacuum, yielding 2,0 g of a dark gum. The aqueous methanol fraction was evaporated down to about 5 ml and then taken to a thick paste by azeotroping with benzene. It was finally dried completely by placing under high vacuum overnight. This yielded 2,0 g of a dark gum. Portions of both the petroleum ether and the aqueous methanol fractions were submitted for bacteriological testing. The dilute hydrochloric acid fraction was then basified with sodium hydroxide to about pH 11 and thoroughly extracted with 50 ml aliquots of chloroform until the chloroform was quite colourless. The chloroform was then taken to dryness under reduced pressure to yield 0,47 g of a pale yellow oil which solidified overnight. Again a small portion of this oil was tested for bacteriological activity, and finally the aqueous fraction remaining was neutralized with hydrochloric acid and also tested for bacteriological activity. These bacteriological tests were carried out against Staphylococcus aureus in the same manner as before, and the results of these tests indicated that the chloroform extract possessed a high degree of bacteriocidal activity, while the ethanol, ether, aqeous methanol and aqueous fractions showed slight bacteriocidal activity and the petroleum ether fraction was completely inactive against Staphyloccus aureus. (See Table 1)

1.4 TLC Studies on the Fractions obtained.

Two TLC's of the petroleum ether, aqueous methanol, chloroform and aqueous fractions were run. The two plates were developed in chloroform and ethyl acetate respectively, and visualized

TABLE 1



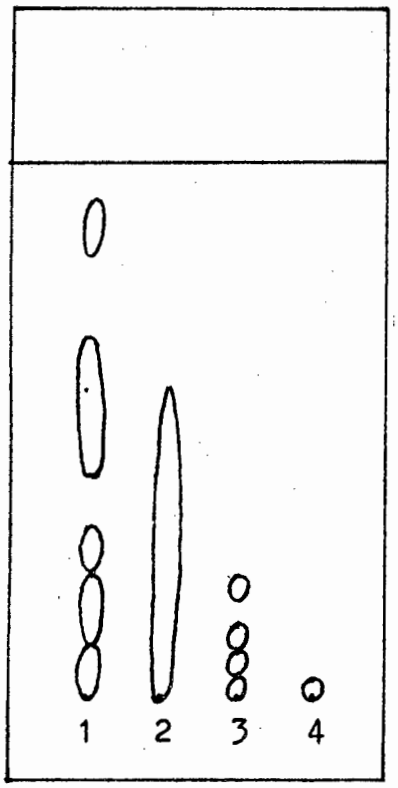
(+++): Highly active against Staphylococcus aureus

(+) : Slightly active against Staphylococcus aureus

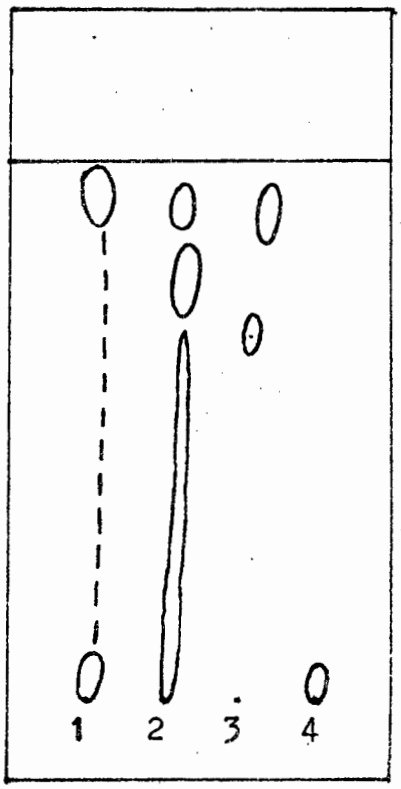
(-) : Inactive against Staphylococcus aureus

under uv light. The appearance of these TLC's was as follows.

Developing solvent: $CHCl_3$

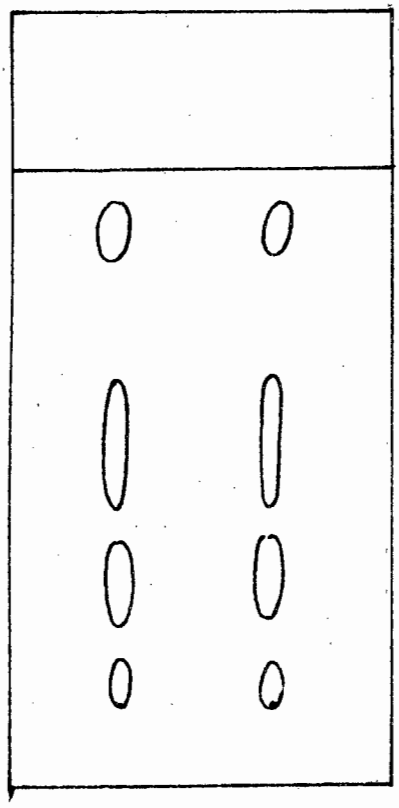


Developing solvent: EtOAc

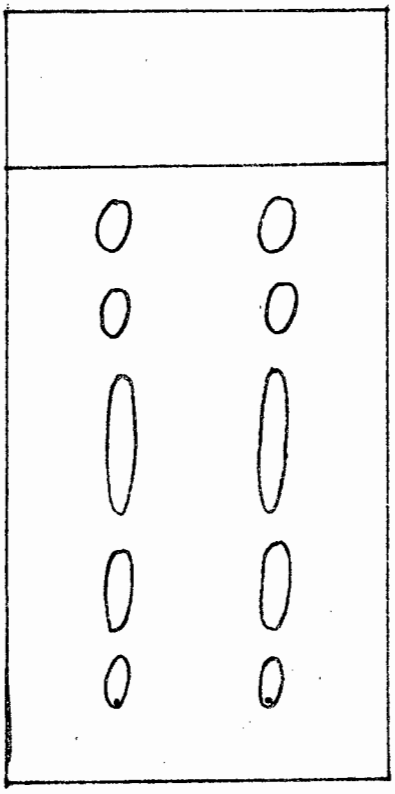


1) Petroleum ether fraction
2) Aqueous methanol fraction
3) Chloroform fraction
4) Aqueous fraction

A further TLC of the chloroform fraction was developed in ethyl acetate, chloroform and methanol in the ratio 4:4:1. When it was examined under uv light it showed four spots as in the plate developed in chloroform above, but when it was subsequently sprayed with Dragendorff's reagent (see section 1.2) an additional spot was observed as shown on the next page.



Visualized under uv light



Visualized with Dragendorff's reagent

1.5 Preliminary Study of the Chloroform fraction.

The chloroform fraction, after it had solidified, was refluxed with 70 ml of absolute alcohol for 3 hrs and was found to separate into 0,38 g alcohol solubles and 0,09 g insoluble material. This insoluble material was found to be insoluble in all other solvents tested and it did not melt below 300°C; thus it was clearly polymeric. Attempts were made to crystallize the soluble portion from alcohol, ethylacetate and other common organic solvents, but the material only came out as a gum, which, on standing, yielded more polymeric material.

2.0 FURTHER EXTRACTIONS AND STUDIES ON GLUCOSE

2.1 Extraction of Knowltonia capensis

More plant material was collected above Kirstenbosch Gardens in June, air dried and ground in a Wiley Hammer mill. 3 Kg of the dried, ground plant material was extracted, in two batches of 1,5 Kg each, in a soxhlet with ethanol for 10-12 days. The solvent was completely changed after six days. The total volume of ethanol used was about 25 l and this extract was then concentrated to about 5 l. Finally the remainder was evaporated under reduced pressure at 40-60°C to a paste. The yield was 1,067 Kg of crude extract. This was taken up in 1500ml of water, acidified with concentrated hydrochloric acid to pH1 and extracted with 6 l of ether. Emulsions were broken by filtration through cotton wool. The ether extract was evaporated to dryness under vacuum to yield 120 g of the ether soluble fraction. This ether soluble fraction was taken up in 96% aqueous methanol and extracted with 4 l of petroleum ether. Again emulsions were broken by filtration. The petroleum ether extract was evaporated to dryness under vacuum and gave 60 g of extract. The aqueous methanol fraction was concentrated under reduced pressure; extensive frothing occurred and it was finally taken to dryness in a evaporating basin under an infrared lamp. This yielded 50 g of extract.

2.2 Alternative extraction procedure.

As an alternative procedure another 3 Kg of the dried ground plant material was extracted in two batches of 1,5 Kg in the soxhlet apparatus for six days with a total of 25 l of petroleum ether. The petroleum ether extract was distilled down to 5 l and then concentrated to a paste under vacuum at 40-60°C, to give 35 g of extract. The same plant material was then extracted in the soxhlet for a further six days with 25 l of ethanol. This was then concen-

trated to 4 l. On standing crystals separated out and were isolated by decantation. These were washed three times with absolute alcohol and then filtered to yield 12 g of white crystalline material (see section 2.3). The remaining ethanol extract was taken to a paste under vacuum to yield 900 g of residue. This was taken up in 1,5 l of water, acidified with concentrated hydrochloric acid and extracted with 4 l of ether. The ether extract was taken to dryness under reduced pressure to give 45 g of the ether soluble fraction.

The dilute hydrochloric acid fractions from this extraction procedure and that described in section 2.1 were combined and basified with dilute sodium hydroxide. These basified aqueous fractions were then extracted with 5 l of chloroform over a pH range of 8-12. The chloroform fraction was evaporated under reduced pressure at not more than 40°C and yielded 22 g of extract.

2.3 Identification of the Crystalline Fraction

The crystalline compound obtained as described in section 2.2 was recrystallized twice from ethanol to give white crystals; m. pt. 168-170°C; $[\alpha]_D^{20}$ +84° mutarotating to +39° on standing overnight or on the addition of base. (Found: %C 33,9; %H 5,7) $C_6H_{12}O_6 \cdot H_2O$ requires %C 33,6; %H 6,6. For details of the nmr spectrum see table 3. A paper chromatogram of the compound and a mixture of glucose, galactose and mannose as standards was run on Whatman no. 1 paper using ethyl acetate, pyridine and water in the ratio 8:2:1 as developing solvent. The chromatogram was visualized by spraying with p-anisidine hydrochloride in butanol, ethanol and water 4:2:1 and heating at 100°C for 10 minutes. R_f of compound is 0,60 (R_f values of glucose, galactose and mannose are 0,60; 0,50 and 0,69 respectively) Treatment of the compound with refluxing 0,1M sulphuric acid for 3 hrs followed by paper chromatography of the resulting product as above gave identical results.

TABLE 2

NMR Spectrum of Glucose. (D_6DMSO with D_2O wash)

τ	Multiplicity	Integration	Assignment
3.77	doublet	1 proton	anomeric –OH, J = 4 Hz disappears after D_2O wash
4.98	doublet	1 proton	anomeric C–H, J = 4 Hz
5.0-5.6	multiplet	4 protons	other –OH's, disappears after D_2O wash
6.4-7.0	multiplet	5 protons	other C–H's

The trimethyl silyl ether (TMS) derivative was prepared by treating the compound (10 mg) in pyridine (1,0 ml) with hexamethyldisilasane (0,1 ml) and trimethylsilyl chloride (0,2 ml). GLC of the derivative on a 3% SE-52 column at 160°C gave a retention time of 10,3 min (retention time of the TMS derivative of glucose is 10,3 min) GLC of a mixture of the TMS derivatives of the compound and glucose gave a single enhanced peak.

Reduction of the compound (20 mg) with lithium aluminium hydride (20 mg) in ether (2,0 ml) followed by acetylation with acetic anhydride (1,0 ml) in pyridine (1,0 ml) gave an acetate derivative. GLC of the derivative under the same conditions as above gave a retention time of 13,2 min. (retention time of glucitol hexa-acetate is 13,2 min)

A phenylosazone derivative of the compound was prepared in parallel with a similar preparation using an authentic sample of glucose. Both osazones formed after 4,5 minutes and the crystal formations were similar. The phenylosazones were recrystallized from 60% ethanol and both melted at 188-192°C and the mixed melting point showed no depression. (Found: %C 59,9; %H 6,2 and %N 14,9. $C_{18}H_{22}N_4O_4$ requires %C 60,3; %H 6,2 and %N 15,6)

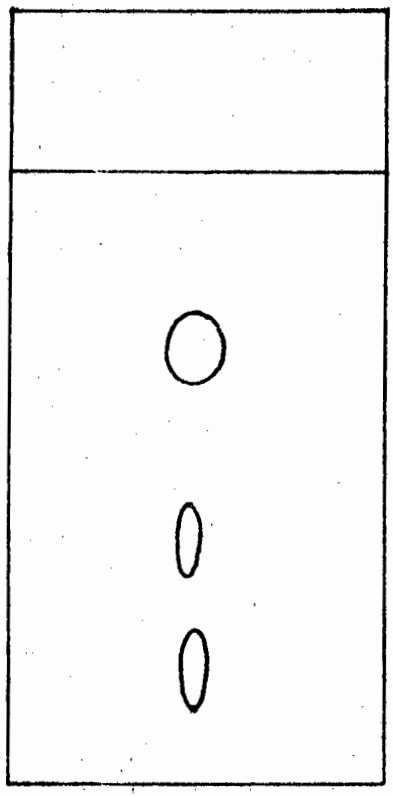
The β-penta-acetate derivative was prepared by refluxing the compound (0,5 g) with acetic anhydride (2,5 ml) and anhydrous sodium acetate (0,4 g). Two recrystallizations from methanol gave a crystalline product, m. pt. 131-132°C; mixed melting point with authentic glucose β-penta-acetate, 131-132°C.

3.0 FURTHER STUDIES ON THE CHLOROFORM FRACTION.

3.1 Small Scale Silica Column

A TLC of the freshly obtained chloroform fraction run in 60% ethyl acetate in petroleum ether and visualized under uv light

appeared as below. The spot at R_f0,7 was strongly fluorescent under uv light.



Consequently 2,0 g of the chloroform fraction was absorbed onto 3 g of silica. A column was dry packed with 50 g of silica with the 5 g of compound and silica on top and topped with sand. The column was eluted with 200 ml 20% ethyl acetate in petroleum ether; 100 ml 40% ethyl acetate in petroleum ether; 100 ml 60% ethyl acetate in petroleum ether; 100 ml ethyl acetate; 100 ml 10% methanol in ethyl acetate; and finally 200 ml 50% methanol in ethyl acetate. 20 ml fractions were collected and those that looked the same on TLC were recombined. Fractions 8-12 corresponding to the very strongly fluorescent compound, were obtained as an oil which gave a polymeric solid on standing overnight; yield 60 mg. Fractions 14-18 yielded 140 mg of crystalline material. This was recrystallized from methanol and identified as anemonin(2); m. pt. 147-149°C.

(Lit. value 151-152°C[16]) (Found %C 61,7; %H 4,2: $C_{10}H_8O_4$ requires %C 62,5; %H 4,2). Molecular ion at m/e 192(4,3%), other prominent fragments at m/e 164(15,2%), 136(6,3%), 110(8,7%), 96(100%), 82 (16,7%), 68(56,5%), 54(58,7%), 42(43,5%), and 26(32,6%). Metastable peaks at m/e 140 and 113. Details of the ir and nmr spectra are given in tables 3 and 4 respectively.

All subsequent fractions showed streaking all the way up the TLC plate and were consequently merely recombined and evaporated down to yield 1,6 g of dark gum. This was taken up in a small quantity of methanol and on being left standing more anemonin crystallized out. This was filtered off, recrystallized as before and identified by its nmr spectrum.

3.2 Small Scale Sephadex Column.

The remaining dark filtrate from section 3.1 was placed on a Sephadex LH20 column (100 g) and eluted with methanol, dripping rate about 1 drop every 5 sec. The fractions were collected on an automatic fraction collector at intervals of 75 mins, giving fractions of approximately 15-20 ml. The fractions were again examined by TLC, run in 10% methanol in ethyl acetate and visualized by uv light. These appeared as follows.

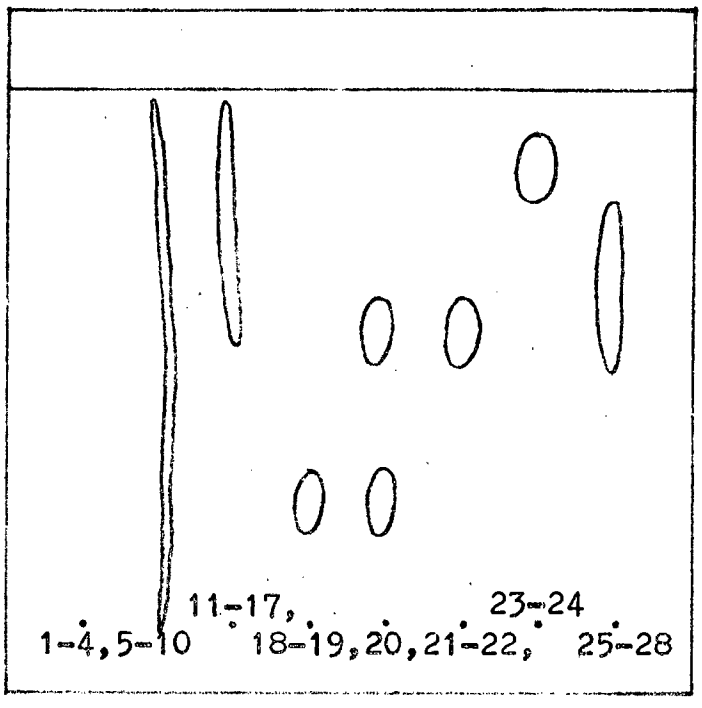


TABLE 3

Infra red spectrum of Anemonin. (nujol mul)

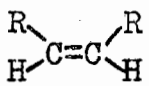
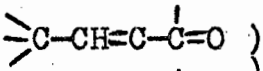
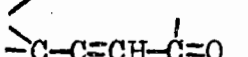
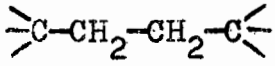
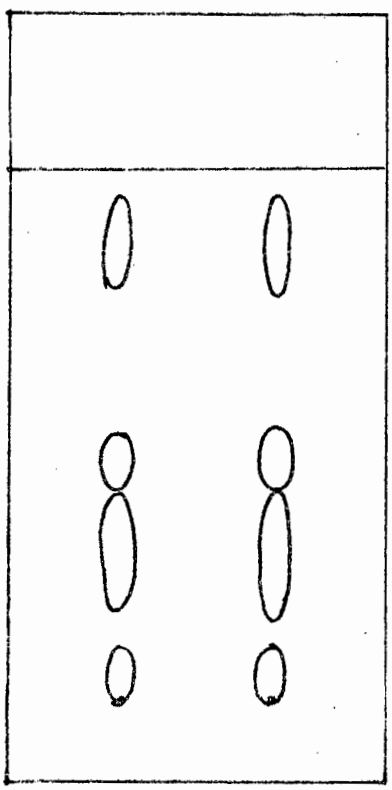
ν	Possible assignment
3200w 3110m 3095m	C=C–H stretch
2900vs	nujol
1745s	C=O stretch in α,β-unsaturated γ-lactones
1599m	C=C stretch in α,β-unsaturated γ-lactones
1249m 1230m	C–O stretching
1180w	
1137s 1115s	C–O stretching
1055m	
1018s	
937s	
910s	
839m	
810s	
737m 700m	

TABLE 4

NMR Spectrum of Anemonin. ($CDCl_3$)

τ	Multiplicity	Integration	Assignment	
2.25	doublet	2 protons		J = 6 Hz
3.87	doublet	2 protons		
7.35	multiplet	4 protons		

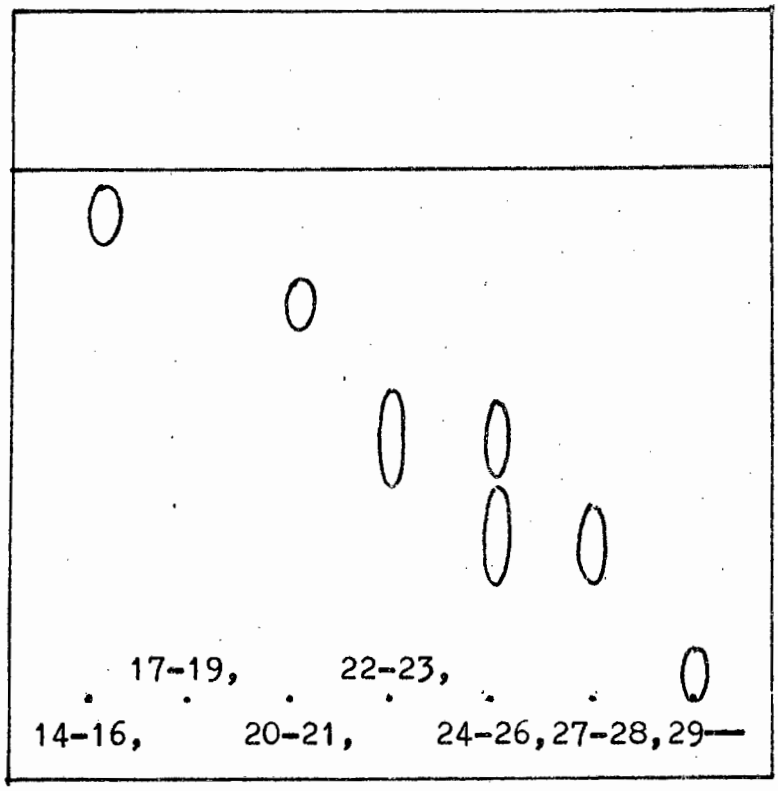
Fractions (21-22) and (23-24) gave very small yields of impure crystalline material, about 10 mg, but the quantities were too small for further purification and identification. The other fractions were all gummy. Fractions (11-17) comprised about 1,0 g of dark gum. When this was examined on a silica TLC plate run in chloroform and diethylamine in the ratio 9:1 and visualized with iodoplatinate spray (prepared[135] by mixing 3 ml of 5% platinic chloride solution made up to 50 ml with distilled water, with 50 ml aqueous potassium iodide solution) the plate appeared as follows.



3.3 Second Small Scale Silica Column.

Consequently fractions (11-17) were adsorbed on 2,0 g of silica and placed on a dry packed column comprising 50 g of silica. The column was eluted with 300 ml of chloroform; 200 ml of 5% methanol in chloroform; and 500 ml of 50% methanol in chloroform. 20 ml fractions were collected and successive fractions were examined

by TLC run in chloroform and diethylamine in the ratio 9:1 and visualized by uv light and iodoplatinate spray. Those fractions which were the same were recombined. The resulting separation was as follows.

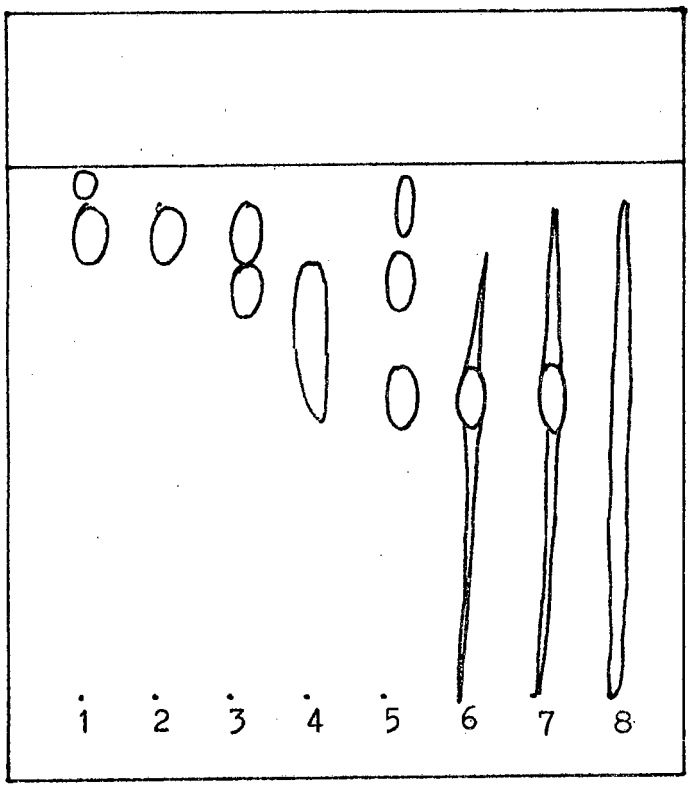


Fractions (14-16) yielded 110 mg of crystals which were shown by nmr to be anemonin. Fractions (24-26) yielded 460 mg of gum. An attempt was made to separate the two components by preparative TLC on silica plates (about 150 mg of mixture applied per plate, 3 plates) but nothing was regained from the silica except the gummy base spot which seemed to comprise the majority of the mixture. The other fractions all yielded only very small quantities of gum.

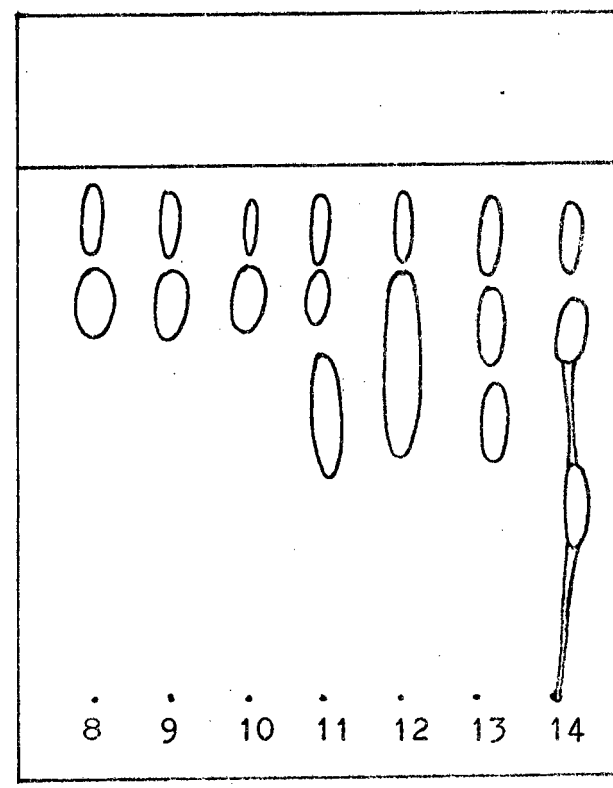
3.4 Large scale Silica and Sephadex Columns.

The remaining 10 g of the chloroform fraction (section 2.2), which had been stored meanwhile in the fridge in dilute chloroform solution to prevent polymerization, was adsorbed onto 15 g of silica. A column was dry packed with 600 g of silica and topped with the

chloroform fraction on silica. The column was eluted successively with chloroform ; chloroform - ethyl acetate mixtures; ethyl acetate; ethyl acetate - methanol mixtures; and finally stripped with methanol. 9 g of material was recovered. 20 ml fractions were collected and were examined by TLC using ethyl acetate and 50% ethyl acetate in methanol as developing solvents, and examined under uv light. Those fractions which appeared the same were recombined, giving the following set of fractions as observed on TLC.



Developing solvent: EtOAc



Developing solvent: 50% EtOAc/ MeOH

Fraction 4 gave 3,7 g of crystalline material which was recrystallized first from water then from acetone-water to give white needles m.pt. 149-150°C, shown by nmr and microanalysis to be anemonin. None of the other fractions yielded identifiable crystalline material but only intractable gums. Fractions 8,9 and 10 when combined yielded 1,9 g of gum. This was placed on a Sephadex LH20

column and eluted with methanol but no improvement in the separation was obtained. Likewise fractions 11,12 and 13 were also recombined to yield 1,5 g of gum, which was placed on a sephadex LH20 column and eluted with methanol, but again no improvement in separation was obtained and no identifiable compounds could be isolated.

4.0 STUDIES ON THE AQUEOUS FRACTION.

4.1 Preparation of Ammonium Reineckate[136] $\overset{+}{N}H_4\overset{-}{[}Cr(NH_3)_2(SCN)_4]\cdot H_2O$

400 g of ammonium thiocyanate was gently heated by means of a rose bunsen in an enamel basin. The mass was stirred with a thermometer enclosed in a glass tube until the solid had partially melted and the temperature had reached 145-150°C. At this point an intimate mixture of 185 g of finely powdered ammonium dichromate and 100 g of ammonium thiocyanate was added in portions of 10-12 g with constant stirring. After about five such portions had been added a fairly vigorous reaction took place with the evolution of ammonia and the temperature rose to about 160°C. The flame was extinguished, and the remainder of the mixture was added at such a rate that the heat of reaction maintained the temperature at 160°C. Stirring was continued while the mass cooled and the lumps of solid which formed round the sides of the vessel were broken loose. The product, while still warm, was finely powdered with pestle and mortar and stirred into 325 ml of ice water in a beaker. After 15 minutes the insoluble portion was filtered under suction, freed as completely as possible from mother liquor without washing, and stirred into 1250 ml of water previously warmed to 65°C. The temperature was then rapidly raised to 60°C and the solution filtered at once under suction. The filtrate was placed in a refrigerator overnight and the resulting crystals were col-

lected and the mother liquor was employed for a second similar extraction of the residue at 60°C. The total yield of ammonium reineckate was 50 g.

4.2 Precipitation of the Aqueous Fraction with Ammonium Reineckate

The aqueous fraction remaining after the large scale extraction described in section 2.2 was reacidified with dilute hydrochloric acid and an excess of saturated ammonium reineckate solution was added to a small portion. A red crystalline reineckate settled out very slowly at pH4. At lower pH a black amorphous precipitate was obtained. The remainder of the aqueous fraction was similarly treated at pH4. The crystals obtained were filtered off under suction and then recrystallized twice by dissolving them in a minimum volume of acetone and pouring the acetone solution into water warmed to about 60°C. The solution was then allowed to cool and the fine plate like crystals were filtered off under suction. This yielded about 3,0 g of crystals. (Found %C 26,5; %H 4,6; %N 22,1 and residue 17,8%. $HOCH_2CH_2\overset{+}{N}(CH_3)_3^-[Cr(NH_3)_2(SCN)_4]$ requires %C 25,6; %H 4,7; %N 23,2; and residue 17,9%.)

4.3 Indication that Choline occurs as an ester.

The aqueous fraction remaining after the extraction described in section 5.1 which was similar to the large scale extraction in section 2.2 but which was done without any heating of the extracts at any stage, when acidified to pH4 with dilute hydrochloric acid and treated with an excess of saturated ammonium reineckate solution as before, did not yield any reineckate salt. Therefore a second portion of the same aqueous fraction was taken and after it had been acidified with hydrochloric acid to pH4 it was heated on a steam bath for 3hrs. It was then allowed to cool and an excess of saturated ammonium reineckate was added and in this case a precipitate of choline reineckate was obtained.

5.0 STUDIES ON FRESH PLANT MATERIAL

5.1 Trial alkaloid extraction.

50 g of fresh plant material collected in October 1974 was macerated with 100 ml of ethanol in a Waring blender in two lots of 25 g. The mixture was then filtered under suction and the ethanol extract was evaporated to a paste under reduced pressure at 40°C. The residual paste was suspended in 50 ml of water, acidified with hydrochloric acid and thoroughly extracted with 20 ml aliquots of ether until the ether was quite colourless. The ether extracts were discarded. The acid solution was then further extracted with five 20 ml aliquots of chloroform. The chloroform was evaporated under reduced pressure and an acrid yellow oil was obtained which polymerized on standing. The acid solution was then basified with ammonium hydroxide to pH 11 and extracted with a further five 20 ml aliquots of chloroform. When this chloroform extract was evaporated to to dryness no residue remained.

5.2 Extraction of Protoanemonin(1).

100 g of fresh plant material was macerated in a Waring blender with water and the resulting slurry was immediately transferred to a flask and steam distilled. The uv absorbtion of the distillate at 260 nm was measured after each 100 ml of distillate had been collected, and the concentration of protoanemonin was seen to decrease in each successive fraction and to be almost zero after the third 100 ml fraction. Thus the first 300 ml of distillate were taken and thoroughly extracted with 50 ml aliquots of chloroform. The chloroform was then evaporated under reduced pressure at 40°C to yield an acrid volatile yellow oil which rapidly polymerized. This showed the characteristic uv absorption at 260 nm of protoanemonin[18].

5.3 Extraction of β-acetylacrylic acid(33).

100 g of fresh plant material was steam distilled as before (section 5.2) yielding 300 ml of steam distillate. 15 ml of 0,1M sodium hydroxide was added to the distillate and then it was evaporated to dryness on a steam bath leaving a white crystalline material. This white crystalline sodium salt was taken up in 15 ml of water, acidified with dilute hydrochloric acid and then extracted thoroughly with ether. The ether extracts were then evaporated under reduced pressure to yield a crystalline product. Recrystallization from benzene gave β-acetylacrylic acid, m. pt. 121-123°C (literature value 122-125°C[18]) (Found %C 51,8; %H 5,0; $C_5H_6O_3$ requires %C 52,5; %H 5,2) λ_{max} 225 nm. Details of the ir and nmr spectra are given in tables 5 and 6 respectively.

5.4 Extraction of Ranunculin(48)[24].

300 g of fresh plant material was ground up in a Waring blender in 50 g lots with 150 ml of dilute hydrochloric acid. The slurry was then filtered through cloth and the filtrate returned to the blender to extract the next 50 g lot, until 150 g of plant material had been ground. The process was repeated on the second 150 g of plant material. The 300 ml of filtrate obtained in this way was then centrifuged and the slightly turbid supernatant liquid was decanted off and stirred with 10 g of degassed activated charcoal for 20 minutes. The charcoal was then filtered off and the filtrate stirred with a further 36 g of degassed activated charcoal. This charcoal was then filtered off as before and washed with water until the washings were no longer acid. The charcoal was then placed in a column and eluted slowly with 600 ml of 50% aqueous ethanol. This eluent was evaporated under reduced pressure to yield 3 g of gum. This was taken up in 10 ml of methanol and filtered and then allowed to evaporate slowly in an angled

TABLE 5

Infra red spectrum of β acetylacrylic acid. (nujol mull)

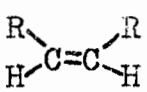
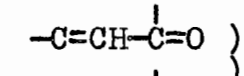
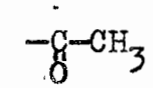
ν	Possible assignment
3055m	C=C–H stretching
3000s	nujol
3000br	O–H stretch intramolecularly H-bonded
2720w	O–H stretch combination band in carboxylic acids
1665s,br	C=O stretch intramolecularly H-bonded α,β-unsaturated ketone
1620m	C=C stretch in α,β-unsaturated carbonyl system
1300s, 1237s, 1235m, 1212m	C–O stretching
1167w	
1022m, 1000s	C–O stretching
927m	
890m	
720s, 675m	

TABLE 6

NMR Spectrum of β-acetylacrylic acid. (D_2O)

τ	Multiplicity	Integration	Assignment	
2.97	doublet	1 proton		J = 16 Hz
3.24	doublet	1 proton		
7.54	singlet	3 protons		

test tube but the material did not crystallize. Thus it was adsorbed onto 5 g of silica and placed on a column, dry packed with 100 g of silica, which was then eluted slowly with 500 ml of 30% methanol in chloroform. 20 ml fractions were collected and these were monitored by TLC run in 30% methanol in chloroform and visualized under uv light. The ranunculin showed up clearly as a strongly fluorescent spot in fractions 10-12. When these fractions were evaporated down under reduced pressure, ranunculin was obtained as a gum which crystallized on being pumped out under high vacuum, yield 1,3 g. It was recrystallized by dissolving in 10 ml of methanol and filtering and then evaporating down to a gum and pumping out under high vacuum. m. pt. 135-140°C (literature value 140-142°C[24]) (Found %C 46,8; %H 6,1; $C_{11}H_{16}O_8$ requires %C 47,8; %H 5,8) $[\alpha]_D^{20}$ -77,3° (literature value $[\alpha]_D^{20}$ -80,7°) Details of ir and nmr spectra are given in tables 7 and 8 respectively.

5.5 Acetylation of ranunculin(48)[24].

500 mg of this ranunculin was acetylated by suspending it in 12 ml of acetic anhydride containing 1 ml of glacial acetic acid and slowly adding 12 ml of dry pyridine via an equilibrating funnel, keeping the apparatus sealed against moisture and cooling in ice. The reaction mixture was then allowed to stand for 3 hrs. After this time it was poured into 50 ml of ice water and shaken but no product separated. Thus the solution was taken down to about 3 ml under reduced pressure and then precipitated by adding water. The crystals thus obtained were filtered off by suction and recrystallized from absolute ethanol, giving ranunculin m.pt. 133- 135°C (literature value 136-137°C[24]) (Found %C 51,1; %H 5,5; $C_{19}H_{24}O_{12}$ requires %C 51,4; %H 5,4) Details of ir and nmr spectra are given in tables 9 and 10 respectively. $[\alpha]_D^{20}$ -74,4°

TABLE 7

Infra red spectrum of Ranunculin. ($CHCl_3$ solution)

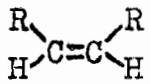
ν	Possible assignment
3380br	O–H stretch H-bonded
3010s	C=C–H stretch
2950s, 2920s, 2855s	–C–H stretch
1712s	C=O stretch in α,β-unsaturated γ-lactone
1595w, 1575w	C=C stretch conjugated with C=O
1460s, 1372m, 1360m	C–C stretch
1280s	O–H bend
1200s, 1125m, 1070m	C–O stretch
1033w	
950w	
720s	 bend
600m	

TABLE 8

NMR Spectrum of Ranunculin. (D_2O)

τ	Multiplicity	Integration	Assignment	
2.25	doublet	1 proton	H in position 2	$J = 6$ Hz
3.69	double-doublet	1 proton	H in position 3	$J = 6$ Hz
4,52	broad singlet	1 proton	H in position 4	
5.50	doublet	1 proton	Anomeric H in glucose	$J = 8$ Hz
5.6-6.8	complex band	8 protons	C-H's on glucose and H's in position 5	

TABLE 9

Infra red spectrum of Ranunculin tetra-acetate. (Nujol)

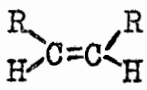
ν	Possible assignment
3100w	C=C–H stretch
2940s	nujol
1752s	C=O stretch in α,β-unsaturated γ-lactone and acetate groups
1590w	C=C stretch conjugated with C=O.
1320m	C–C stretch
1210s, 1175m, 1080s, 1060s, 1025s	C–O stretch
950s	
910m	
890m	
825s	
800m	
725m, 695m	

TABLE 10

NMR Spectrum of ranunculin tetra-acetate. ($CDCl_3$)

τ	Multiplicity	Integration	Assignment	
2,49	double-doublet	1 proton	H in position 2	J = 6 Hz
2,78	double-doublet	1 proton	H in position 3	J = 6 Hz
4,6–5,1	multiplet	3 protons	H's in positions 4 and 5	
5,36	doublet	1 proton	anomeric H in glucose, J=8 Hz	
5,6–6,4	multiplet	6 protons	H's on glucose moiety	
7,98	singlet	6 protons	two acetate CH_3 groups	
7,95	singlet	3 protons	acetate CH_3 group	
7,97	singlet	3 protons	acetate CH_3 group	

6.0 PREPARATION OF EXTRACTS FOR TESTING FOR ANTITUMOUR ACTIVITY

300 g of dried ground plant material was soxhlet extracted with 2 l of petroleum ether for 8 hrs. The petroleum ether extract was then discarded and the plant material was air dried overnight, and then further extracted in the soxhlet with 2 l of 95% ethanol for 16 hrs. The plant material was then discarded and the ethanol extract was concentrated down to a paste (95,7 g). 2,0 g of this was put aside as sample A. The remainder was then partioned between 200 ml of chloroform and 200 ml of water. The water fraction (79,0 g) was discarded and the chloroform fraction concentrated under reduced pressure to a paste which was taken as sample B (7,2 g). Samples A and B were submitted to the National Cancer Institute in America for testing for antitumour activity. Sample B has been reported to be active but this has not yet been confirmed.

Tumour system: Mouse Leukemia P-388 system.

$T/c = 135$ (dose, 400 mg/Kg)

where T/c is the mean survival time of a test group of 6 mice divided by the mean survival time of a control group of 6 mice expressed as a percentage. A value of $T/c \geqslant 125$ demonstrates significant activity.

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