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# THE PHENYL- AND BIPHENYL-QUINOLIZIDINES OF IN-VITRO-GROWN *HEIMIA SALICIFOLIA*

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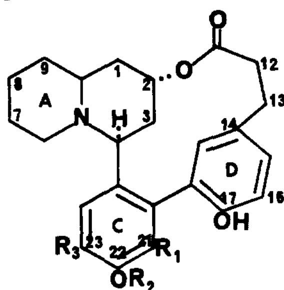
ABSTRACT.—Under the conditions delineated in this paper, alkaloid synthesis was not detectable in heterotrophic callus and suspension cultures of *Heimia salicifolia*. However, in vitro-grown shoots are active in alkaloid biosynthesis, yielding the biphenylquinolizidine lactones vertine (**1**), lythrine (**5**), and lyfoline (**7**), the ester alkaloids demethoxyabresoline (**14**) and epidemethoxylabresoline (**11**), the phenylquinolizidinols demethyllasubine-I (**10**) and demethyllasubine-II (**12**), and several minor alkaloids that had not been previously shown to occur in *H. salicifolia*.

The defined conditions of in vitro growth of plant cells and tissues present an ideal system in which to carry out investigations in the field of secondary plant products. However, alterations in the species' secondary metabolism are often observed when it is grown in culture (1-7), and it is not yet possible to predict, on the basis of chemical structure or biogenesis, under which conditions of medium composition, intensity of light, and other external factors a plant species would yield the products it synthesizes when it is grown in the field. Thus, the metabolic response, and consequently the best culture method, has to be experimentally determined for each species; this has been pointed out in recent reviews by authorities in the field (1-7). The great progress that has been made during the past decade in the production of secondary plant products by culture-grown species has been reviewed in these and other monographs (1-9). Recent work in the alkaloid field further supports these points: thus, reports from four laboratories dealt with the production of *Cinchona* alkaloids in callus (10, 11), cell suspension (12, 13) and organ (12, 14) culture of *Cinchona* species. This group of pharmacologically active natural products had previously not been isolated from tissue cultures (9). The effect of both media composition and degree of illumination on alkaloid production is shown in these reports (10, 11, 13). Further, protoberberine alkaloids have been produced in high yields in cell culture (15). New reports on the production of monoterpene indole alkaloids are good examples of the capacity of culture-grown species to produce secondary metabolites and also the altered metabolism—when compared with the differentiated species—that cultured cells often present (16, 18). Thus, *Tabernaemontana divaricata* cell cultures yield six monoterpenoid indoles belonging to four alkaloid groups (16), *Tabernanthe iboga* yields two alkaloids belonging to two alkaloid groups but typical *Iboga* alkaloids (17) are not produced. As the last example, the production of new dimeric indole alkaloids by *Voacanga africana* cell suspension cultures may be mentioned (18). This work (18) shows the influence of the composition of the medium on alkaloid yield.

In this communication, the alkaloid expression of in vitro-grown *Heimia salicifolia* Link and Otto is reported.

The alkaloids of *H. salicifolia* (Table 1) and other members of the *Lythraceae* have been recently reviewed by Golebiewski and Wróbel (19). This monograph demonstrates that, although the structure of the alkaloids has been firmly established, work is still needed to establish completely the biogenesis of the alkaloids. Although radiotracer incorporation studies (20-23) have shown that the biphenylquinolizidine lactones (as **1**, Table 1) are derived from one mole of lysine (**17**), (20,21), one mole of acetate or malonate (**18**) (21) and two moles of phenylalanine (**19**) (22,23), and that

TABLE 1. Alkaloids of Field-Grown *Heimia salicifolia*




| | Alkaloid | Stereochemistry at ring juncture | 12-13[a] | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|---|---|
| (**1**) | Vertine (Cryogenine[b,c]) . . . | *cis* | -CH=CH- | H | $CH_3$ | $OCH_3$ |
| (**2**) | Sinicuichine . . . . . . . | *cis* | -CH=CH- | $OCH_3$ | $CH_3$ | H |
| (**3**) | Anelisine (27) . . . . . . | *cis* | -CH=CH- | $OCH_3$ | H | H |
| (**4**) | Heimidine . . . . . . . . | *cis* | $-CH_2$-CHOH- | H | $CH_3$ | $OCH_3$ |
| (**5**) | Lythrine . . . . . . . . . | *trans* | -CH=CH- | H | $CH_3$ | $OCH_3$ |
| (**6**) | Nesodine . . . . . . . . | *trans* | -CH=CH- | $OCH_3$ | $CH_3$ | H |
| (**7**) | Lyfoline . . . . . . . . . | *trans* | -CH=CH- | H | $CH_3$ | OH |
| (**8**) | Dehydrodecodine . . . . . | *trans* | -CH=CH- | HO | $CH_3$ | H |
| (**9**) | Lythridine . . . . . . . . | *trans* | $-CH_2$-CHOH- | H | $CH_3$ | $OCH_3$ |

[a]We would like to refer to the reason for adopting only in its modified form (20) the numbering proposed by I.D. Spenser and his coworkers (19,22), a system that ingeniously eliminates the use of multiple primed numbers, and points to the biogenetic relation of the metacyclophane alkaloids of *Lythrum anceps* and *Lythrum lanceolata*, with the lactonic alkaloids of *Decodon verticillatus*, *L. lanceolata*, *Lagerstroemia indica*, *Lagerstroemia faurieri*, *Heimia myrtifolia* and *H. salicifolia*. The consistency in the biogenetic numbering, regretfully, breaks down with the simple phenylquinolizidines of *H. salicifolia* and *Lythrum subcostata*, for which either (a) a non-biogenetic modification as employed in (19), or (b) a discontinuity in the numbering to maintain the biogenetic relation must be used. Thus, considering that Spensers' system cannot be applied to all the *Heimia* alkaloids, together with the observation that any change in the numbering of the quinolizidine ring is prone to cause confusion as evidenced by the reference to the carbon atom bearing the lactonic oxygen in the biphenylalkaloids as C-3 but also inadvertedly as C-2 in (19), we opted for continuing to number the quinolizidine ring as previously, and discontinue the usage of multiple primed numbers, as shown in (20). [As a comment we would like to mention that the numbering system C (22) not adopted by I.D. Spenser would have been consistent for all the *Lythraceous* alkaloids.]

[b]The name for this alkaloid has variably appeared as vertine and cryogenine[c] (19).

[c]It should be noted that cryogenine (MW 435.2047) used in this study is the alkaloid isolated from *H. salicifolia* and not the trade name product cyrogénine (phenylsemicarbazide, MW 151.2), a speciality of Lumière of Lyons, France and distributed by Laboratories Sarbach of Châtillon, France.

cadaverine (1,5-diaminopentane) (**20**) (20,21), 1,2-dehydropiperidine (**21**) (21) and the phenylquinolizidinones **22** (20) are biosynthetic intermediates to the biphenyl alkaloids, knowledge is not available on the sequence of metabolic events and their regulation. The biphenylquinolizidine lactones represent the completion of a metabolic sequence involving three pathways of secondary metabolism-piperidine, acetate (or malonate), and phenylpropanoid—(20-23) and are a good model with which to study the regulation and coordination of these pathways in the cell.

The major alkaloids of *H. salicifolia* have been shown to possess promising anti-inflammatory properties and have been the subject of continued studies in the laboratories of M. Malone (24 and references cited in 19).

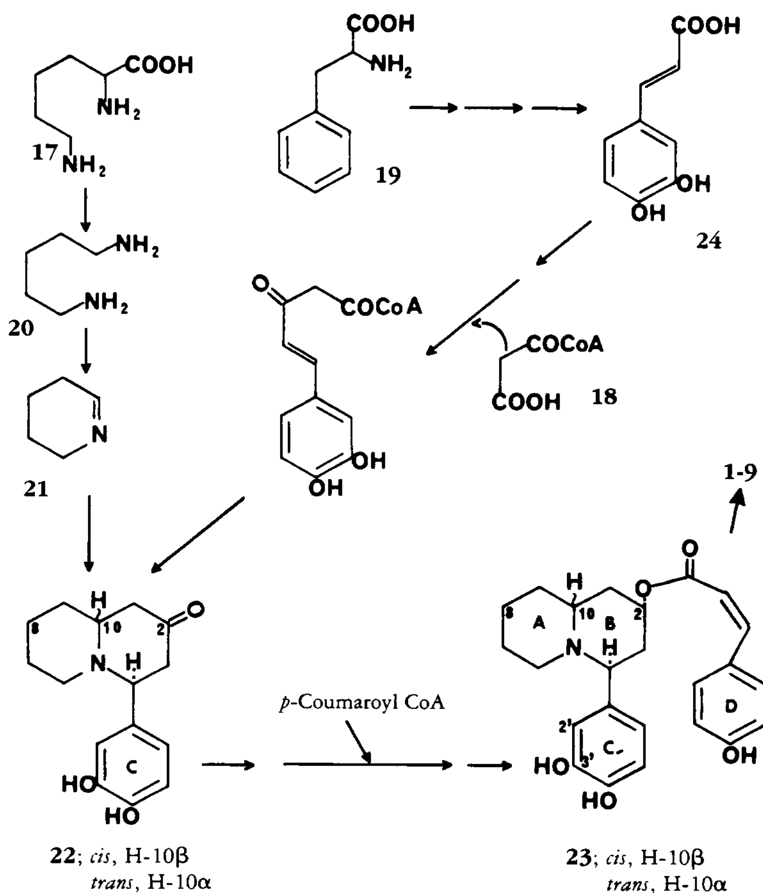
## RESULTS AND DISCUSSION

The alkaloids that have been shown to occur in field-grown *H. salicifolia* are listed in

TABLE 1. Continued

| Alkaloid | Stereochemistry at ring juncture | R |
|---|---|---|
| (**10**) Demethyllasubine-I | *cis* | H |
| (**11**) *Epi*demethoxylabresoline[d] | *cis* | *p*-hydroxycinnamoyl |
| (**12**) Demethyllasubine-II | *trans* | H |
| (**13**) Abresoline | *trans* | feruloyl |
| (**14**) Demethoxylabresoline[d] | *trans* | *p*-hydroxycinnamoyl |

[d]An error was inadvertedly included in the abstract of the publication (40) on the isolation and determination of structure of demethoxyabresoline and *epi*-demethoxyabresoline. Both alkaloids were shown to be minor components of mature *H. salicifolia* but not, as printed in the abstract, of young seedlings.




SCHEME 1. Biogenesis of the lactonic biphenylquinolizidines

Table 1. The results presented below have been summarized in Table 2. *H. salicifolia* grows as a friable brown-to-black callus on the 2,4- dichlorophenoxyacetic acid (2,4-D) containing (4.5 micromolar) media A-D and F. The tendency to browning is less pronounced on media C and D, which in addition to the auxin also contains benzyladenine (BA) (2 and 20 micromolar, respectively). Suspension cultures were readily developed with media A-D. Naphthaleneacetic acid (NAA) has a marked effect, when added in micromolar quantities (medium H), to cause root development with *H. salicifolia*. Any efforts to use this auxin for callus development were therefore discontinued.

Chromatographic analysis of the cultures developed on A-D and F did not reveal the presence of any phenolic alkaloids, although the cultures gave evidence for the formation of other phenolic compounds (diazotized *p*-nitroaniline test), and the presence of some alkaloidal non-phenolic material was also revealed (Dragendorffs' reagent). Efforts to determine the identity of these compounds has not yet been undertaken.

When grown on media containing kinetins, media E (BA, 2 micromolar) and G (BA, 1 micromolar plus kinetin, 2.3 micromolar), but without 2,4-D, *H. salicifolia* develops into actively multiplying alkaloid-producing shoots.

Preparative tlc resolution of the alkaloid mixture isolated from shoots grown on medium G (harvested 6 to 8 weeks after the last subculture) showed that the tissues synthesize the biphenylquinolizidine lactones vertine (**1**), lythrine (**5**), and lyfoline (**7**) together with the phenylquinolizidinols **10** and **12**, and the ester alkaloids de-

TABLE 2. Alkaloid Content and Growth Characteristics of in vitro-grown *Heimia salicifolia*

| | Callus & Suspension Cultures | | | | | Shoot Cultures | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | F | E | G[a] | G-3 | G-2 | G-1 | G-.5 | G-.2 | H-2 |
| Alkaloid | Alkaloid content (mg/g dry wt tissue)[b] | | | | | | | | | | | | |
| **1** | — | — | — | — | — | 1.5 | 1.1 | 1.5 | 1.5 | 0.6 | 0.3 | 0.2 | 0.4 |
| **2** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **3** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **4** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **5** | — | — | — | — | — | 0.5 | 0.25 | 0.5 | 0.2 | 0.5 | 0.2 | 0.1 | tr |
| **6** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **7** | — | — | — | — | — | 2 | 0.94 | 2 | 2.5 | 1.5 | 0.7 | 0.2 | 0.7 |
| **8** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **9** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **10** | — | — | — | — | — | 0.07 | 0.3 | 0.07 | 0.13 | tr | — | — | — |
| **11** | — | — | — | — | — | 0.02 | 0.15 | 0.02 | 0.15 | — | — | — | tr |
| **12** | — | — | — | — | — | 0.02 | 0.24 | 0.02 | 0.02 | tr | — | — | 0.02 |
| **13** | — | — | — | — | — | — | — | — | — | — | — | — | — |
| **14** | — | — | — | — | — | tr | tr | tr | 0.02 | — | — | — | tr |
| H-15 | — | — | — | — | — | — | 0.33 | tr | tr | tr | — | — | — |
| H-16 | — | — | — | — | — | — | 0.28 | tr | tr | — | — | — | — |
| | Growth characteristics[c] | | | | | | | | | | | | |
| | a | a | a | b | c | d,e,f | d,e,g | d,e,g | d,e,g | d,g,h | d,g,h | d,i | j |

[a]harvested 6-8 weeks after the last subculture.

[b]tr = trace; — = less than 200 ng/g.

[c]a = amber to brown cell aggregates; b = yellow to amber cell aggregates; c = suspension cultures were not developed; d = high rate of shoot multiplication; e = comparatively high rate of shoot growth; f = elongated shoots with relatively few leaves; g = shoots with relatively abundant leaves; h = low rate of shoot growth; i = shoot growth not observable; j = vigorous growth, shoots with multitude of rootlets.

methoxylabresoline (**14**) and *epi*-demethoxylabresoline (**11**). The yields have been recorded in Table 2. In addition, seven or eight minor compounds of different chromatographic mobility than the reference *Heimia* alkaloids are formed. From these, two alkaloids (H-15 and H-16) were isolated. The mass spectra of both are characteristic of the olefinic biphenylquinolizidine lactones, but the information obtained was insufficient for complete structural assignments.

No distinction in the alkaloid pattern was detectable between cultures grown on both Gamborg salts (25) with vitamins, BA, and sucrose (medium E) or an Murashige and Skoog salts (26) with vitamins, BA, kinetin, and either sucrose (media G2-G3) or glucose (medium G). However, more abundant shoot multiplication was observable with the latter (G) medium. At sucrose concentrations below 1%, shoot multiplication continues, but further growth is inhibited and the alkaloid content is reduced.

The alkaloid extraction-isolation procedure used in this study follows conventional methods. It is worth mentioning that partial alkaloid resolution was obtained by extracting an aqueous acidic (pH 1) solution containing the alkaloids with $CHCl_3$. The relatively less polar alkaloids **1, 5, 11, 14**, and also H-15 and H-16 partitioned into the organic phase (possibly as HCl salts) whereas the phenylquinolizidinols **10** and **12** and the majority of **7** remained in the aqueous phase.

The alkaloid spectrum of the in vitro-grown shoots of *H. salicifolia* is broad, comprising the major biphenylquinolizidines vertine (**1**), lythrine (**5**), and lyfoline (**7**), together with the esters demethoxyabresoline (**14**) and *epi*-demethoxylabresoline (**11**). These last are very minor constituents of the mature plants. In addition, the phenylquinolizidinols **10** and **12** are produced and have only been reported to occur in young, actively developing seedlings (19).

The in vitro-grown shoots constitute a continuously multiplying organ system, suggesting a possible relation between the state of morphologic development and the capacity to biosynthesize phenyl- vs. biphenyl-alkaloids. Further insight into this subject will be obtained by analyzing cultures at short time periods after both initiation and subculture. The previous observation (23) that roots are not required for de novo biosynthesis of vertine is confirmed in the work reported here. The alkaloid spectrum of the tissues grown in the presence of micromolar amounts of NAA (medium H-2), which show abundant adventitious root formation, is not different from the alkaloid pattern given by the in vitro-grown shoots.

In analogy with the field-grown plants both *cis*-fused and *trans*-fused quinolizidines accumulate in the shoot cultures; however, within the group of the biphenylquinolizidines only the 17, 22, 23-substituted [vertine (**1**), lythrine (**5**), and lyfoline (**7**)] (Table 1) but not the 17, 21, 22-substituted [sinicuichine (**2**), anelisine (**3**), (18) nesodine (**6**), dehydrodecodine (**8**)] alkaloids are formed in culture. Although the mechanism of biphenyl linkage formation in the *Heimia* alkaloids is not yet known, on the basis of current knowledge of the biochemistry of phenyl coupling in alkaloid biogenesis which points to a dual role of the oxidase in both the formation (28) and the conformational stabilization of the intermediate phenoxy radicals (29), one would expect that one oxidase is active in the generation of the *ortho-, para, meta-* as well as the *ortho-, ortho, meta-* substituted (with respect to the biphenyl bridge) alkaloids, and a priori, that if biphenyl alkaloid formation takes place at all in in vitro-grown tissues both substitution types would be produced in the cultures.

Assuming that the biogenetic pathway to the alkaloids is the same in both field-grown plants and in vitro-grown shoots, then the postulated trihydroxylated phenol ester **23** (20) (Scheme 1) is a precursor to the biphenyl alkaloids in both biological systems. In order to explain the different coupling patterns, one could propose that rotation of ring C of intermediate **23** is restricted in the shoot-culture systems in a manner

that allows oxidative coupling only *para* to the 3′-hydroxyl of **23**, i.e., leading to **1, 5,** and **7**. Vertine (**1**) and lyfoline (**7**) are the major alkaloids of the differentiated species (19) indicating that *para*-coupling is the preferred mode of ring closure.

One may consider that the exogenous growth factors, the photoheterotrophic growth and the constant humidity might influence the biochemical events in the cell; however, it would be an impossible task to rationalize at present the cause of the apparent change in the biochemistry of alkaloid metabolism in the in vitro-grown tissues.

The failure of the heterotrophic cultures to yield alkaloids in any detectable amounts and the capacity of the photoheterotrophic shoots to produce them leads to the suggestion that organ development may be required for alkaloid accumulation in *H. salicifolia*. These results, however, must be viewed with caution, because the media used for the development of the calli and cell suspension contain 2,4-D, an auxin inhibitory to the production of secondary metabolites in certain cultures (3). It has been often observed that the failure of production of secondary metabolites is dependent upon the composition of the growth medium (1-7,10,11,13,18).

Biogenetic consideration would suggest that some degree of morphologic differentiation, possibly involving chloroplast formation, might be required for alkaloid biosynthesis in *H. salicifolia*. It has been shown that in chlorophyllous tissue, lysine biosynthesis (30) as well as lysine decarboxylation (31,32)—the step that yields 1,5-diaminopentane (cadaverine) (**20**), which is the step diverting lysine from primary to alkaloid metabolism—is localized in the chloroplasts.

Indeed, work from the laboratories of T. Hartmann has also shown that one enzyme system for tetracyclic quinolizidine alkaloid biosynthesis in chlorophyllous tissues of *Lupinus polyphyllus* and *Lupinus albus* is localized in the chloroplasts (32-34) and that the metabolic sequence from 1,5-diaminopentane to the alkaloids is highly channeled (32,34)

Upon consideration of the biogenesis of the *Heimia* alkaloids (Scheme 1) which should involve the intermediacy of the reactive 1,2-dehydropiperidine (**21**) (21) derived from the lysine-branch as well as, possibly, the polar caffeic acid (**24**) (20,22) derived from the phenylalanine-branch, the requirement for metabolic channeling (35,36) becomes apparent. It is possible that the membrane-rich chloroplasts might constitute the degree of morphologic differentiation required for phenyl- and biphenylquinolizidine biosynthesis. Precedence for the location in the chloroplasts not only of tetracyclic quinolizidine biosynthesis (32-34) but also of phenylpropanoid biosynthesis is available (35). In future studies we would like to determine the function that chloroplasts may have in the biosynthesis of the alkaloids. We are presently developing chlorophyllous tissue cultures of *H. salicifolia*.

The in vitro-grown shoots gave evidence for the presence of minor alkaloids that had not been previously shown to occur in *H. salicifolia*, but the amount isolated did not permit a complete determination of their structure. Our continued work with the shoot cultures should provide identification of these alkaloids.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Analytical tlc was performed on pre-coated plates, E. Merck, Silica Gel 60-$F_{254}$. Preparative tlc used Baker-flex® Silica Gel IB-F, 20 cm × 20 cm flexible sheets. The following systems were employed throughout: I. $CHCl_3$ saturated with $NH_4OH$-MeOH (a) 15:1; (b) 10:3; (c) 15:2; (d) 6:1. II. $CHCl_3$-MeOH-$NEt_2$ (a) 300:3:14; (b) 150:5:4; (c) 150:5:7; (d) 300:11:16. III. $C_6H_6$ saturated with $NH_4OH$-MeOH (a) 15:2; (b) 9:2. IV. EtOAc-$NH_4OH$ (30%)-MeOH (15:0.01:3).

Alkaloids were routinely visualized with diazotized *p*-nitroaniline. This reagent was freshly prepared as follows (39): equal volumes of 0.1% *p*-nitroaniline in 0.1 N HCl and 0.2% aqueous $NaNO_2$ were mixed. The chromatogram was first sprayed with this solution, then with 20% aqueous $Na_2CO_3$. This reagent was used, together with Dragendorffs' reagent, as a criterion for the presence or absence of alkaloids.

The limit of detection of the spray reagents is 700 to 1500 ng with Dragendorffs' and 70-900 ng with the phenol spray. The sensitivity varies with the structure of the alkaloid.

In the preparative tlc the components of separated, uv-quenching bands were eluted with MeOH. Aqueous $NaHCO_3$ solution (2%) and a fourfold excess of $CHCl_3$ were added to the eluates and the alkaloids were extracted into $CHCl_3$-MeOH (× 3). The organic phase was dried ($Na_2SO_4$), filtered, and the solvent evaporated under reduced pressure. The residue was redissolved in either MeOH or $CHCl_3$ or a mixture of both depending upon the alkaloidal component of the sample, filtered, and evaporated to dryness.

Proof of the identity of the alkaloids isolated from the in vitro grown *H. salicifolia* was effected in each case by mass spectrometry and co-chromatography with the known alkaloids in systems I to IV. Vertine (**1**), lythrine (**5**), and lyfoline (**7**) were also identified by their ir spectrum. For a semiquantitative tlc determination, alkaloid standards and samples were compared after resolution and spraying with diazotized *p*-nitroaniline.

IN VITRO GROWTH CONDITIONS.—Tissue cultures were maintained at 23° to 26° with a 16 h daylight cycle: 500 fc, 40 watts, plant grow, cool-light fluorescent bulbs.

The following media were used: (1) Gamborg salts (B5 medium) and vitamins (2% sucrose) (25) containing the following organic supplements and growth factors (hormone concentrations are given in micromolarity):

A. 2,4-D: 4.5
B. 2,4-D: 4.5; coconut milk: 20 ml/liter; "Bacto casitone" (Pancreatic digent of casein): 2 g/liter
C. 2,4-D: 4.5; BA: 2
D. Medium C but BA: 20
E. BA: 2

(2) Murashige and Skoog salts containing thiamine HCl (0.1 mg/liter) and inositol (100 mg/liter) (26), 3% sucrose unless otherwise indicated and the following growth factors (hormone concentrations are given in micromolarity).

F. NAA: $5.6 \times 10^{-2}$; 2,4-D: 4,5; kinetin: 2.3
G-3: BA: 1; NAA: $5.6 \times 10^{-2}$; kinetin: 2.3
G-2: medium G-3 but 2% sucrose
G-1: medium G-3 but 1% sucrose
G-0.5: medium G-3 but 0.5% sucrose
G-0.2: medium G-3 but 0.2% sucrose
G; medium G-3 without any sucrose but 2% glucose
H-1: NAA: 1; H-2: NAA: 86

In vitro growth was initiated from seeds obtained from garden-grown *H. salicifolia*. The seeds were surface sterilized by stirring with EtOH containing 0.1% (v/v) Tween 80, two times for 5 min, followed by NaOCl (5%) ("Clorox") three times for 10 min each, and then washing three times with sterile distilled $H_2O$, filtering each time through sterile filter paper. Germination was on either sterile $H_2O$ or Gamborg salts (25) (2% sucrose).

Callus (media A to D and F, 0.7% agar) and shoot cultures (media E, G, G-3 and G-2) were developed from four- to six-week-old seedlings. Growth on G-1, G-0.5, and G-0.2 were initiated from cultures grown on G-2. These reduced-sugar-grown cultures were maintained for one year.

Attempts to reduce the degree of browning of the callus by addition of the antioxidants (37) dithiothreitol (28 mg/liter) or ascorbic acid (100 mg/liter) to media A and F did not give any apparent beneficial effect and were discontinued after three subcultures.

Shoot cultures were maintained on both solid and liquid media and subcultured every 4 (liquid medium) to 6 (solid medium) weeks. Callus was also subcultured every 4 to 6 weeks.

Suspension cultures were developed from tissues grown on A to D media upon omission of the agar and maintenance on an orbital shaker (120 rpm), with a bi-weekly transfer schedule for the first 2 months, and later weekly. Cultures grew in general as 0.1 to 3 $mm^3$ tissue clusters with great tendency to browning. Attempts were not made to obtain fine cell suspension cultures.

Prior to extraction, the harvested tissue was either frozen in liquid nitrogen and crushed in a mortar to reduce particle size, or first dried and then powdered.

ALKALOID EXTRACTION.—The method described by Dobberstein *et al.*, (38,39) was slightly modified as follows: The tissue was stirred with HCl (0.27 *N*, 20 ml/g dry tissue, or 0.35 *N*, 5 ml/g fresh tissue at 70° for 4 h and then at rt for 15 h. The mixture was filtered and the residue was reextracted with HCl (1%). The combined filtrates (pH ca. 1) were extracted (× 1) with $CHCl_3$ to give, after evaporation of the solvent under vacuum, extract I. The pH of the aqueous layer was adjusted to 8.5 and the remaining alkaloids were then extracted (× 3) with $CHCl_3$-MeOH (4:1), to yield, after removal of the solvent under vacuum, extract II. The contents of both I and II were analyzed by tlc.

In order to confirm the presence of the phenylquinolizidinols **10** and **12** in the tissues, two alternate procedures were employed: (1) Crushed, deep-frozen shoots were first extracted with $Me_2CO$ in a blender. The solvent was removed under vacuum and $NaHCO_3$ solution (2%) was then added to the aqueous residue which was extracted (× 3) with $CHCl_3$-MeOH (3:1). The contents of the mixture remaining upon evaporation of the organic solvent were analyzed by tlc. (2) The frozen shoots were stirred with $CHCl_3$-MeOH (1:1) at rt (48 h), filtered, and the solution was evaporated to dryness under vacuum. A few ml of $CHCl_3$-MeOH was added to the residue and the soluble compounds were analyzed by tlc. The shoot tissue, the callus, the cells separated from the media as well as the media were extracted.

ALKALOID RESOLUTION AND CHARACTERIZATION.—*H. salicifolia* that had been maintained for one year on medium G, was harvested (254 g fresh wt) 6 to 8 weeks after the last subculture, dried (21.8 g dry wt), and extracted as delineated in the preceding section to yield the alkaloidal fractions I and II.

*Resolution of fraction II.*—Chromatography in systems Ia gave a multitude of fluorescence-quenching bands. Five zones were eluted and further purified. Zone 2-1, rechromatographed in system IId gave rel-(2*S*, 4*S*, 10*R*)-2-hydroxy-4-(3-hydroxy-4-methoxyphenyl)-quinolizidine (**10**) (6.5 mg). Rechromatography of the eluate of zone 2-2 in system IIb gave an additional amount of **10** (1.1 mg), a major band which upon elution gave lyfoline (**7**) (20 mg), and a third phenolic band which upon rechromatography in Ic followed by elution and rechromatography in IIc yielded 10-*epi*-demethoxyabresoline (**11**) (3.1 mg). Resolution of eluates 2-3 and 2-4 in system IIc gave vertine (**1**) (8.6 mg) and 2(a)-hydroxy-4(e)-(3-hydroxy-4-methoxyphenyl) *trans*-quinolizidine (**12**) (5.1 mg); the latter was rechromatographed in system Ia before spectral analysis.

*Resolution of fraction I.*—Tlc showed that a relatively large amount of alkaloidal salts had been extracted with $CHCl_3$ at pH 1 together with non-alkaloidal material. Therefore, the components were first partitioned between aqueous $NaHCO_3$ solution (2%) and $CHCl_3$ (× 3). The organic phase containing the alkaloids was separated, and the solvent was then evaporated under vacuum. The alkaloid mixture so obtained was resolved by tlc, first in system Ib giving many uv-quenching zones. Four bands were separated, eluted, and rechromatographed as follows.

Elute 1-4 was combined with eluate 2-5 obtained from the first chromatography of extract II, and the mixture was resolved first in system IIa which was followed by elution of the alkaloidal band and rechromatography in system Ia to yield lythrine (**5**) (5.45 mg).

Vertine (**1**) (15.5 mg) was obtained upon rechromatography of eluate 1-3 in system IIb.

Resolution of the eluate of 1-(1+2) in system IIIb gave two alkaloidal bands (Rf 0.4 and 0.3), labeled H-15 and H-16, respectively, which were separated and eluated.

Eluate H-16 was purified by rechromatography in system Ic to give alkaloid H-16 (6.1 mg). Rf-(system): 0.33- (in Ic); 0.20- (in IIc); 0.31 (in IIIa); lavender color with diazotized *p*-nitroaniline. Ms *m/z* (rel. int.): 435:2036 ($M^+$, $C_{26}H_{29}NO_5$, calcd 435.2047) (100).

Eluate H-15 was purified by rechromatography in system IIc to give alkaloid H-15 (7.1 mg). Rf-(system): 0.28- (Ic); 0.10- (IIc); 0.40- (IIIa); 0.15- (IV): grey color with diazotized *p*-nitroaniline. Ms *m/z* (rel. int.): 421.1879 ($M^+$, $C_{25}H_{27}NO_5$, calcd 421.1891) (100).

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