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FORMATION OF EDULININE AND FUROQUINOLINE ALKALOIDS FROM QUINOLINE DERIVATIVES BY CELL SUSPENSION CULTURES OF *RUTA GRAVEOLENS**

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Abstract—The formation of furoquinoline alkaloids and of edulinine, elaborated by cell suspension cultures of *Ruta graveolens*, was found to occur by way of 4-hydroxy-2-quinolone. Other substrates transformed to furoquinolines included 4-hydroxy- and 4-methoxy-3-(3-methyl-2-butenyl)-2-quinolone, known earlier as natural precursors in studies with whole plants. Involvement of dictamnine as a natural precursor of 8-methoxydictamnine (γ-fagarine) and skimmianine was proved using ^{14}C-labelled compounds. Edulinine in the cell suspensions was formed from such substrates as 4-hydroxy-*N*-methyl-2-quinolone, 4-hydroxy-3-(3-methyl-2-butenyl)-*N*-methyl-2-quinolone and its 4- methyl ether; this is probably the natural biosynthetic sequence. Changes in alkaloid yields were noted upon prolonged subculturing.

INTRODUCTION

ALKALOIDS containing a quinoline nucleus are common in the Rutaceae;[1] many of these possess a third fused ring similar to that present in the coumarins and chromones, which are also widely distributed throughout this family, and this has led to the suggestion[2] that these added rings may be elaborated in exactly the same manner in all three classes of products.

Several workers have studied the formation of quinoline alkaloids in intact plant organs. Anthranilate and acetate were shown[3,4] to be the precursors of the quinoline ring, and 4-hydroxy-2-quinolone was demonstrated to be an intermediate.[5,6] Grundon's group showed[7,8] that 4-hydroxy-3-(3-methyl-2-butenyl)-2-quinolone and platydesmine were both good precursors of furoquinoline compounds. The derivation of the outer furano carbons from mevalonate has been reported.[9] We had at our disposal a suspension culture producing some furoquinoline alkaloids along with edulinine (1-methyl-3-(2,3-dihydroxy-3-methylbutyl)-4-methoxy-2-quinolone);[10] we undertook a study of the conversion of some substrates to these alkaloids by the cells.

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RESULTS AND DISCUSSION

The production of edulinine and furoquinoline alkaloids by cell suspension cultures of *Ruta graveolens* was observed over a period of 2 yr. Initially the cultures produced primarily furoquinoline derivatives (phase 1) but after repeated subculturing over several months yields of these compounds diminished, with a simultaneous rise in the production of edulinine (phase 2). After 1 yr some of the more complex furoquinolines, such as kokusaginine (XII) and skimmianine (XI), fell to very low levels, although substantial quantities of edulinine were still produced (phase 3). In the past year, no further changes in the alkaloid pattern of the cells have been noticed; all the present results date from this period.

TABLE 1. ALKALOID FORMATION FROM NON-LABELLED QUINOLONE DERIVATIVES

Compound administered, per kg fresh cells*	Dictamnine (IX) formation†	Edulinine (V) increase
4-Hydroxy-2-quinolone (I) 700 mg	++ (2–3%)	+
4-Hydroxy-3-(3-methyl-2-butenyl)-2-quinolone (VI) 480 mg	++	−
4-Methoxy-3-(3-methyl-2-butenyl)-2-quinolone (VII) 1200 mg	+++ (14–18%)	−
4-Hydroxy-*N*-methyl-2-quinolone (II) 450 mg	−	++
4-Methoxy-*N*-methyl-2-quinolone, 600 mg	−	−
4-Hydroxy-*N*-methyl-3-(3-methyl-2-butenyl)-2-quinolone (III) 480 mg	−	++
4-Methoxy-*N*-methyl-3-(3-methyl-2-butenyl)-2-quinolone (IV) 1800 mg	−	+++

* Inocula of 50–90 g fr. wt were used, in 8 times as much nutrient medium. The incubation period was 72 hr.

† Values in parentheses are yields of dictamnine based on amount of precursor offered.

The studies of the formation of these alkaloids used two complementary techniques. On one hand, cells were incubated in nutrient medium fortified with various test compounds, and the utilization of each compound for alkaloid synthesis was observed. On the other hand, possible precursors were synthesized with ^{14}C label and added to cell media in tracer amounts to provide more quantitative incorporation data. The results are summarized in Tables 1 and 2.

Following reports on the involvement of 4-hydroxy-2-quinolone ('2,4-dihydroxyquinoline') in quinoline alkaloid biogenesis[5,7] this compound was administered. A general stimulation of alkaloid formation resulted, with an especially dramatic rise in the cell content of dictamnine IX. In earlier experiments with labelled substrate, done at a time when the cells still produced large amounts of methoxylated furoquinolines, label was also well-distributed among all these products; but in more recent trials the label tended, after short metabolic periods, to remain primarily in dictamnine, with a substantial proportion in all cases going to edulinine (see Table 2).

Formation of Edulinine

Having established that 4-hydroxy-2-quinolone was a precursor both of furoquinolines and of edulinine, we next tested a number of non-labelled materials as possible specific precursors. Incubation of cells in medium containing 4-hydroxy-3-(3-methyl-2-butenyl)-2-

quinolone (VI) resulted in pronounced dictamnine formation but little or no increase in levels of edulinine. A branch point to edulinine may therefore exist at 4-hydroxy-2-quinolone. The main possibilities for the next step to edulinine would then be 4-methoxy-2-quinolone or 4-hydroxy-*N*-methyl-2-quinolone (II). Each of these compounds was tested, but only the latter raised the amounts of edulinine in the cells; neither gave rise to dictamnine or other furoquinoline derivatives. When this *N*-methyl compound was prepared labelled with ^{14}C and administered, good incorporation into edulinine was observed (see Table 2).

TABLE 2. INCORPORATION OF LABELLED PRECURSORS INTO CELL OR SHOOT ALKALOIDS OF *Ruta graveolens*

Labelled compound administered†	Metabolic period (hr)	Tissue‡	Specific activities, μCi/mmol (and incorporations*) in Dictamnine (IX)	γ-Fagarine (X)	Skimmianine (XI)	Kokusaginine (XII)	Edulinine (V)
Quinolones							
4-Hydroxy-[3-^{14}C]-2-quinolone (I) 1·04 μCi at 3·5 μCi/mmol	72	Cells	1·9	—	—	—	1·45
4-Hydroxy-[3-^{14}C]-2-quinolone (I) 11 μCi at 60 μCi/mmol	72	Cells	35	—	5·8	1·4	—
4-Hydroxy-[3-^{14}C]-2-quinolone (I) 18·6 μCi at 62 μCi/mmol	72	Cells	37 (4·3%)	—	—	—	—
4-Hydroxy-[3-^{14}C]-2-quinolone (I) 43·4 μCi at 155 μCi/mmol	240	Cells	—	125 (3·6%)	—	—	—
4-Hydroxy-N-methyl-[3-^{14}C]-2-quinolone (II) 32 μCi at 64 μCi/mmol	72	Cells	nil	—	—	—	52·5 (10%)
Furoquinolines							
[^{14}C]-Dictamnine (IX) 0·42 μCi at 31 μCi/mmol	48	Shoots	—	2·1	0·23	0·01	0·01
[^{14}C]-Dictamnine (IX) 2·2 μCi at 112 μCi/mmol	48	Shoots	112 (50%)	25 (12%)	2·0 (2%)	0·6 (0·2%)	0·4 (0·2%)
[^{14}C]-Dictamnine (IX) 0·073 μCi at 6·4 μCi/mmol	120	Cells§	6·4 (15%)	6·3 (14%)	3·1 (5%)	—	0·04 (<1%)
[^{14}C]-γ-Fagarine (X) 1·54 μCi at 125 μCi/mmol	120	Cells§	nil	130 (56%)	88 (10%)	—	—

* Per cent of label fed appearing in the compound recovered.
† With quinolones, dose was 0·25–0·50 mmol; with furoquinolines, 0·012–0·020 mmol.
‡ About 200 g fresh cell inoculum; or 100 g fresh shoots.
§ Inoculum wt 25 g.

The extremely low dilution values (~2·0) in these experiments may have been obtained because compound II is a natural precursor; however, dilution values are certainly not a reliable indicator of this. Natural or not, II could give rise to either 4-methoxy-*N*-methyl-2-quinolone or 4-hydroxy-*N*-methyl-3-(3-methyl-2-butenyl)-2-quinolone (III), but on incubation with cell suspensions only the latter stimulated edulinine formation. This further suggested 4-methoxy-N-methyl-3-(3-methyl-2-butenyl)-2-quinolone (IV) might be an effective precursor, and in fact this compound gave very pronounced enhancement of edulinine content in the cultures, nearly seven times more than in control cells. Attempts to prepare the hypothetical intermediate 4-methoxy-*N*-methyl-3-(3-methyl-2,3-epoxybutyl)-2-quinolone failed, so it was not possible to test this epoxide. Assuming that the tested precursors are natural and sequential,[7,8] there does not seem to be any more likely formative route to edulinine and furoquinolines than shown in Scheme 1. Boyd and Grundon[11] have recently shown an *in vitro* synthetic route from platydesmine to *N*-methylplatydesminium ion, and have shown its facile conversion to edulinine. This is not an evident *in vivo* route, and in fact Boyd and Grundon avoided any such implication. Theirs and other work[12,13] raises

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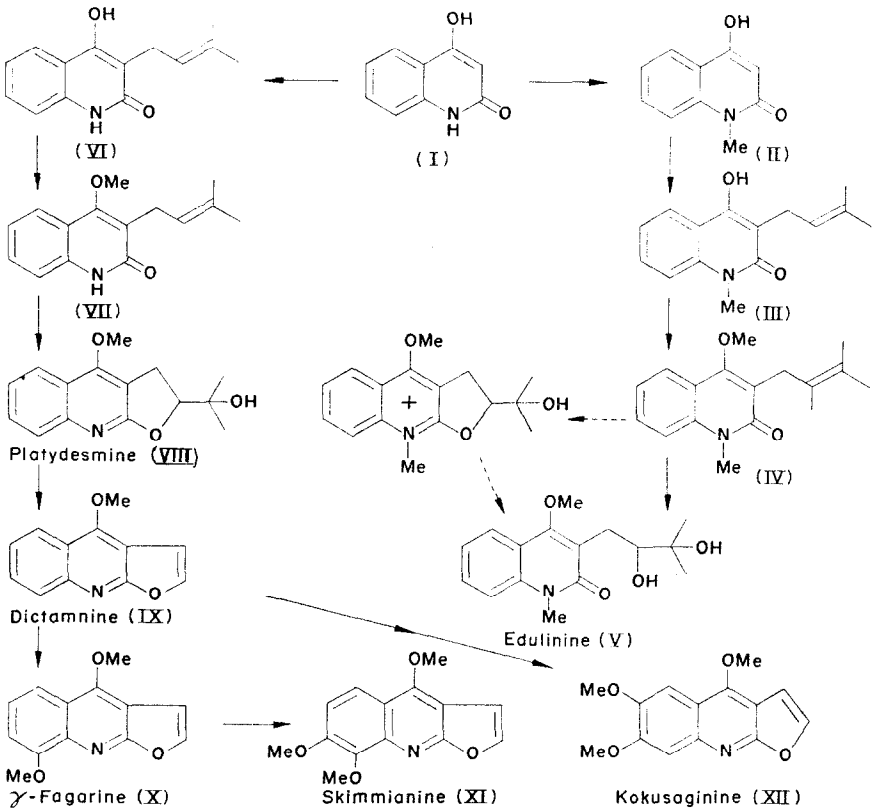
the vital question of whether the edulinine isolated from *Ruta* cells is an artefact of *N*-methylplatydesminium ion, produced by alkaline treatment. Since our method of isolation permits only the briefest contact with alkali (at pH < 11), we think not. Two pieces of evidence indicate that edulinine is indeed an authentic natural product: first, isolations using an alkaline extraction step gave no more alkaloid than usual; second, direct chromatographic isolation of edulinine from cells extracted with chloroform gave the usual quantity of edulinine. Nonetheless, we have consistently obtained *racemic* edulinine. While this edulinine does not seem to be an artefact, *N*-methylplatydesminium ion is not ruled out as an additional product of *Ruta* cells. If present, it would presumably arise *in vivo* from compound IV and might be either optically active or racemic. A biosynthetic scheme involving this ion is shown in Scheme 1.



SCHEME 1. APPARENT METABOLISM OF 4-HYDROXY-2-QUINOLONE IN CELL SUSPENSION CULTURES OF *Ruta graveolens*.

N-Methylation of the quinolone I, formed from acetate and anthranilate, thus leads on to edulinine in *Ruta* cells, and in whole rutaceous plants probably to such 4-quinolone alkaloids as those of the genera *Lunasia* and *Balfourodendron* (see Ref. 1). The observed variation of alkaloid elaboration during prolonged cell subculturing also is in harmony with a development of a competitive methylation mechanism diverting precursor from furoquinoline to edulinine pathways.

Formation of Furoquinoline Alkaloids

Neither dictamnine nor 8-methoxydictamnine (γ-fagarine, X) was normally detectable in unfed cells and for practical purposes these alkaloids can be considered absent from normal *Ruta* cells. The appearance of dictamnine after incubation of cells with various possible precursors was used as a gauge of these compounds' involvement in furoquinoline alkaloid biosynthesis. As expected,[8] 4-hydroxy-2-quinolone (I), its 3-(3-methyl-2-butenyl) congener (VI) and 4-methoxy-3-(3-methyl-2-butenyl)-2-quinolone (VII) all gave rise to dictamnine. The very large amount of dictamnine formed from this last substrate indicates that as in edulinine formation, the methylation of the 4-hydroxyl group follows directly the introduction of the 5-carbon chain at C-3. Therefore, such postulated compounds as norplatydesmine and nordictamnine[6] are probably not natural precursors of furoquinoline alkaloids; the key intermediate must indeed be platydesmine itself.[8] The biological mechanism of the platydesmine–dictamnine transformation is not known, but the chemical conversion which has been achieved[14] suggests a two-step process.

Dictamnine is not an 'end product' in *Ruta graveolens* but is slowly transformed, in part at least, into methoxylated derivatives. Labelled dictamnine obtained (4–5% yield) by 10-day incubation of 4-hydroxy-[3-^{14}C]-2-quinolone with *Ruta* cells was fed both to *R. graveolens* cells and to plant shoots. After 48 hr the shoot alkaloids were isolated and their specific activities determined. Cells were allowed 120 hr of metabolism. The results, summarized in Table 2, revealed that γ-fagarine (X) was very strongly labelled, skimmianine (XI) was radioactive, and kokusaginine (XII) had a low but significant ^{14}C content. Especially in the cells, where little if any 8-methoxydictamnine is normally accumulated, this compound is evidently the main product of dictamnine metabolism. In its turn it gave rise to skimmianine (Table 2) on refeeding to cells, showing that the enzyme systems necessary for the transformation: dictamnine $\rightarrow\rightarrow$ 8-methoxydictamnine (γ-fagarine) $\rightarrow\rightarrow$ 7,8-dimethoxydictamnine (skimmianine) are present in *Ruta graveolens* cells. We believe this is the natural biosynthetic route to skimmianine in rutaceous plants since all compounds in the chain are well-known natural products. The alternative hypothesis[15] requiring methoxylation before furan ring formation is not supported by comparable evidence; neither are methoxylated furocoumarins formed in that way.[16] On the other hand, involvement of dictamnine as a natural precursor of some more complex furoquinoline is consistent with the demonstration that skimmianine is formed from both 4-hydroxy-2-quinolone[7] and platydesmine[8] in *Skimmia japonica*.

The suggested biogenetic scheme for quinoline derivatives in *R. graveolens* cell suspension cultures is, then, in general agreement with pathways discovered in rutaceous plants. However, the relative amounts and the kinds of compounds accumulating in cultured cells differ from those in whole plants. The array of cell compounds also changes over a long time, probably because of minor genetic changes combined with gradual natural selection of cells with higher growth rates. Metabolite accumulation also varies between cultures of the same plant species. Attempts to establish new cultures of *Ruta* in this laboratory gave cells with a somewhat different composition of alkaloids and coumarins than that found in previous cultures. Thus, caution should be exercised in extrapolating to whole plants quantitative results obtained with cultured cells, since the two systems may not have

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comparable secondary products. On the other hand, there is no evidence to suggest that the biosynthetic pathways to cell products are qualitatively different from those to the same products in plant organs. We believe that the substrate biotransformations to edulinine observed in our cultures constitute a biosynthetic sequence consistent with known related pathways, probably operating generally in edulinine-producing plants; but this awaits confirmation. For the route to furoquinolines, confirmation of many of the transformed materials as natural precursors already exists.

EXPERIMENTAL

The cultures of *Ruta graveolens* used and the type of alkaloids present have already been described.[10,17] In the present experiments the cells were maintained and grown under the same conditions. The experiments were initiated by adding cell inoculum and medium to freshly autoclaved precursors. The precursors were synthesized either by novel procedures described below, or by published methods (4-methoxy-2-quinolone,[5] 4-hydroxy-*N*-methyl-2-quinolone,[18] 4-methoxy-*N*-methyl-2-quinolone,[18] 4-hydroxy-3-[3-methyl-2-butenyl]-2-quinolone,[19] 4-methoxy-3-[3-methyl-2-butenyl]-2-quinolone.)[19] Alkaloids were recovered from the cells by boiling and homogenizing the medium-free, washed culture in boiling EtOH. The resulting filtered solution was freed of solvent and partitioned once between hexane and 75% MeOH. The aqueous layer was concentrated, then diluted with H_2O and extracted with $CHCl_3$. The alkaloids were taken out of $CHCl_3$ with 3 N HCl, which on basification to pH 10 and immediate extraction with fresh $CHCl_3$ gave an organic solution of the alkaloids. This was then fractionated on a small column of silicic acid using C_6H_6–2-butanone (3:1) for elution, and each fraction was subjected to GLC. For this, a Varian aerograph 700 instrument (TC detection) was used, and a 0·4 × 150 cm column of 5% Apiezon L on Gaschrom Q (60–80 mesh). Column temp. was 240°, injector 255°, detector 270°, helium flow 100 ml/min. A second column of the same dimensions was 5% SE30 on Chromosorb W-DMCS (acid-washed, 60–80 mesh), operated at 195°, injector 240°, detector 255°, helium flow 60 ml/min. On both columns, even crude mixtures of cell alkaloids were well separated.

Standard compounds. 4-*Hydroxy*-3-(3-*methyl*-2-*butenyl*)-2-*quinolone* (VI). 1-Bromo-3-methyl-2-butene (18·0 g, 0·14 mol) was added at once to a solution of 4-hydroxy-2-quinolone (9·6 g, 0·06 mol) in 150 ml methanolic NaOMe (from 3·0 g Na), and the mixture was refluxed 1 hr. The MeOH was mostly evaporated and the residue poured into 500 ml N HCl. The resulting suspension was extracted with CH_2Cl_2, and the organic layer in turn extracted with 3 portions of 2 N Na_2CO_3. Acidification of this carbonate extract with HCl acid gave a ppt (1·6 g) which was recrystallized from dil. aq. alcohol to plates of product, m.p. 177–179° (lit.[20] m.p. 180–182°). Yield 11%. The NMR and UV spectra were identical with those of material prepared from diethyl 3-methyl-2-butenylmalonate and aniline in similar yield.

4-*Methyl*-N-*methyl*-3-(3-*methyl*-2-*butenyl*)-2-*quinolone* (IV). Diethyl 3-methyl-2-butenylmalonate[21] (0·7 g) and *N*-methylaniline (0·32 g) were refluxed together 2·5 hr in 5 ml phenyl ether under N_2, the EtOH formed being allowed to escape. The cooled mixture was charged to a 25-g column of silicic acid and eluted with Et_2O, collecting fractions of 15 ml. Fractions 4–6 deposited 4-hydroxy-*N*-methyl-3-(3-methyl-2-butenyl)-2-quinolone III (0·13 g, 18%), m.p. 161–163° (lit.[22] 162–163°), whose identity was confirmed by NMR spectroscopy. The compound was methylated with CH_2N_2 in CH_2Cl_2, giving a nearly quantitative yield of the 4-methoxy compound, IV, obtained as an oil showing a single peak on gas chromatograms. The identity was confirmed by the NMR spectrum (CDl_3), which showed signals at δ7·78 (m, H-5), 7·2–7·5 (*m*, H-6, H-7, H-8), 5·27 (*t q*, *J* 7 and 1 Hz resp., =CH), 3·90 (*s*, OMe), 3·71 (*s*, NMe), 3·41 (*d*, *J* 7 Hz, CH_2), 1·82 and 1·70 (*d*, *J* 1 Hz, sidechain methyls).

Dictamnine (IX). 4-Methoxy-3-(3-methyl-2-butenyl)-2-quinolone (132 mg) prepared by the action of CH_2N_2 on the 4-hydroxy compound, was dissolved in EtOAc and treated with 1·1 equivalents O_3. The solution of ozonide was hydrogenated over 5% Pd–C until rapid H_2 uptake ceased (about 0·85 equivalents). The reaction mixture was filtered and the colourless filtrate evaporated to a colourless residue which NMR spectroscopy indicated was the expected 3-acetaldehydo derivative. The material was heated 20 min at 80° in 2–3 ml polyphosphoric acid, then poured into H_2O. The solution was made alkaline with 5 N NaOH and extracted with 3 × 10 ml CH_2Cl_2. Washing and evaporation of this organic layer left 31 mg solid, shown by

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UV, NMR and GLC to be > 95% dictamnine. Recrystallization from alcohol or passage through a column of silicic acid gave 29 mg pure material, m.p. 131–132° (lit.[23] 133°). Overall yield 28%. A similar method has been reported very recently.[24]

Edulinine (V). 4-Methoxy-*N*-methyl-3-(3-methyl-2-butenyl)-2-quinolone (0·11 g) in 20 ml CH_2Cl_2 was treated with 3-chloroperoxybenzoic acid (0·13 g of 85% peracid), dissolved in the same solvent. After standing 2 hr at room temp. the solution was shaken successively with excess 2% NaOH and H_2O, then dried and evaporated to give crude edulinine. The material was purified by chromatography on silicic acid using C_6H_6–2-butanone (at first 3:1, then 1:3 to elute the edulinine). Final yield of pure racemic edulinine, m.p. 138–139° (lit.[11] 141–142°) was 0·07 g; the GLC and spectral (UV, NMR) properties were identical with those of authentic natural edulinine.

4-*Hydroxy*-[3-^{14}C]-2-*quinolone**. Diethyl-[2-^{14}C]-malonate (0·64 g, 4·0 mmol) and aniline (0·36 g, 3·9 mmol) were refluxed together 1 hr at 180° in an oil bath. The solid product was cooled and washed with pentane. The dried solid (0·51 g) was mixed with polyphosphoric acid (5 g) and heated at 150° for 1 hr. Cooling and dilution with H_2O gave a ppt which was filtered off and dissolved in hot 10% Na_2CO_3. The hot alkaline solution was filtered and the cooled filtrate was acidified with conc HCl, giving a white ppt of the quinolone product. It was filtered, washed and dried. Yields on two preparations: 0·18, 0·30 g; 60 and 62 μCi/mmol. The material melted above 320° and on chromatograms gave a single radioactive spot corresponding to authentic material. Material with specific activity 155 μCi/mmol was prepared similarly.

4-*Hydroxy*-N-*methyl*-[3-^{14}C]-2-*quinolone*. Diethyl-[2-^{14}C]-malonate (0·80 g, 5·0 mmol) and *N*-methylaniline (0·48 g, 5·0 mmol) were refluxed together 2 hr in 3 ml phenyl ether. On cooling, 0·47 g (57%) product deposited as yellow crystals. Two recrystallizations from pyridine–EtOH gave colourless needles, m.p. 262–268° (lit.[18] 272°); the m.p. could not be raised further, and the material showed only one radioactive spot on PCs, corresponding to authentic material. Final yield 40%, specific activity 64 μCi/mmol.

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