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# INTERMEDIATE SYMMETRY IN LAWSONE BIOSYNTHESIS

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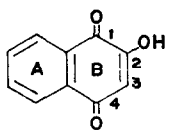
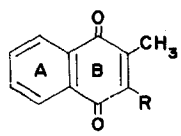
**Abstract**—Feeding experiments in *Impatiens balsamina* have established that 2-$^{14}$C-acetate predominantly labels C-2 of lawsone. This finding confirms previous biosynthetic postulates, indicates that no symmetrical intermediate is involved in lawsone biosynthesis, and sheds light on the final steps in that process. The experimental methods that have been employed in this series of studies are also described in detail.

## INTRODUCTION

THE PLANT product lawsone (I) has proved not only an interesting biosynthetic problem in its own right but also a valuable model system from which to derive information concerning the biosynthesis of the naphthoquinone ring systems of the phyllo- and menaquinones




(I)

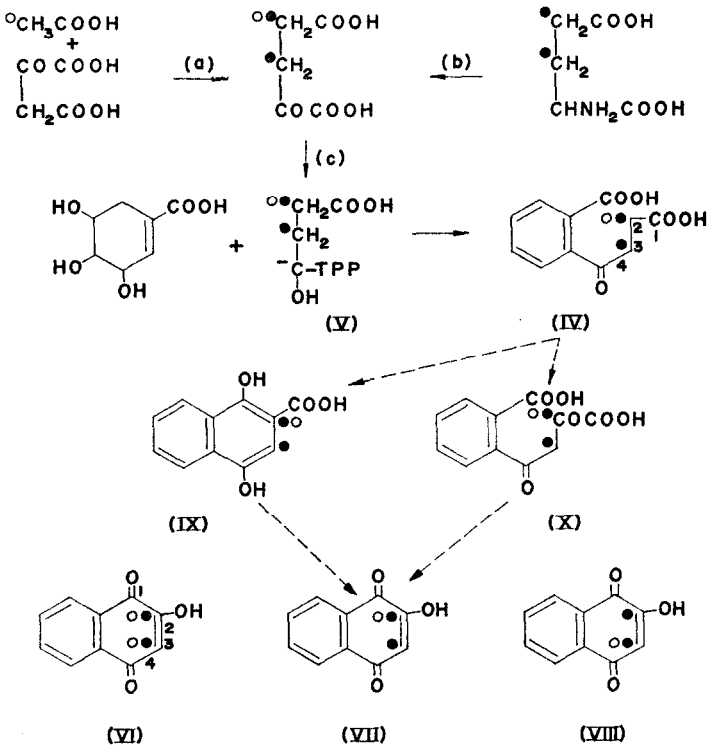



(II) R = phytyl
(III) R = polyprenyl

(II, III). As a result of the combined efforts of several groups, it can now be affirmed that the naphthalene nucleus of all these materials derives from shikimate and the three non-carboxyl carbon atoms of glutamate.[1–6] The substance 4-(2′-carboxyphenyl)-4-oxobutyrate (IV, Scheme 1) has been shown to be an important intermediate,[3] thus lending credence to the original proposal[4] that the initial step in the construction of ring B of the nucleus is the condensation of shikimate with the thiamine pyrophosphate complex of succinyl semi-aldehyde (V). The latter complex can be derived from glutamate by transamination and the action of the Krebs cycle enzyme 2-ketoglutarate dehydrogenase on the derived keto acid.

Of considerable interest in exploring the reactions subsequent to the formation of compound IV is the question of whether symmetrical intermediates such as 1,4-naphthaquinone are involved in the anabolic pathway. Direct feeding experiments with the latter compound in the menaquinone producing microorganism, *Mycobacterium phlei*, yielded only negative results.[6] A more definitive answer, however, should be derivable from the plant system using a somewhat different approach. As shown in Scheme 1, 2-$^{14}$C-acetate

[1] M. GUÉRIN, M. M. LEDUC and R. G. AZERAD, *Eur. J. Biochem.* **15**, 421 (1970).
[2] M. M. LEDUC, P. M. DANSETTE and R. G. AZERAD, *Eur. J. Biochem.* **15**, 428 (1970).
[3] P. DANSETTE and R. AZERAD, *Biochem. Biophys. Res. Commun.* **40**, 1090 (1970).
[4] I. M. CAMPBELL, *Tetrahedron Letters* 4777 (1969).
[5] D. J. ROBBINS, I. M. CAMPBELL and R. BENTLEY, *Biochem. Biophys. Res. Commun.* **39**, 1081 (1970).
[6] I. M. CAMPBELL, D. J. ROBBINS, M. KELSEY and R. BENTLEY, *Biochem.* **10**, 3069 (1971).



SCHEME 1. LAWSONE BIOSYNTHESIS. (a) *pro-S* CITRATE SYNTHASE, ACONITASE AND ISOCITRATE DEHYDROGENASE; (b) TRANSAMINASE; (c) A MODIFIED 2-KETOGLUTARATE DEHYDROGENASE. TPP = THIAMINE PYROPHOSPHATE.

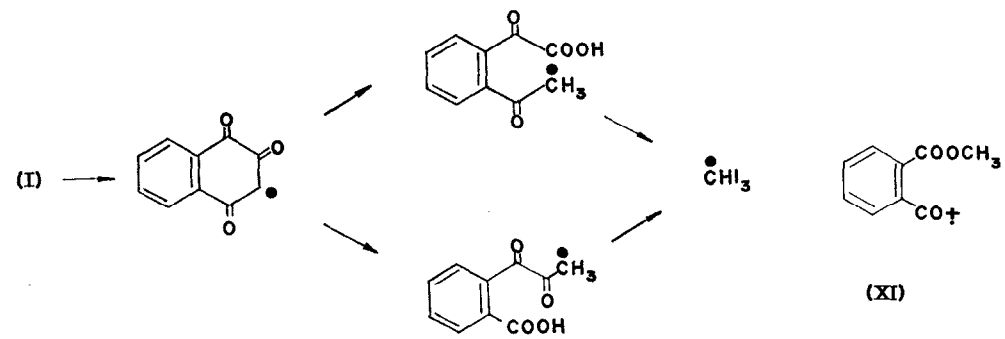
specifically will label C-2 of compound IV. Therefore, it can be argued that if a symmetrical intermediate intervenes between compound IV and lawsone, this acetate derived activity will be equally distributed between C-2 and C-3 of lawsone (VI, Scheme 1). If no such intermediate is involved the activity in question will be located either in C-2 or C-3 (VII or VIII) the outcome depending on whether the hydroxyl group is attached to the carbon atom that was C-2 or C-3 of compound IV. Since in lawsone the activity in C-3 and the sum of the activities in C-2 and C-3 can be measured readily, a simple answer to the symmetry question is at hand.

## RESULTS

Two degradative reactions were used to probe the radioactivity distribution in C-2 and C-3 of lawsone. Treatment of the material with alkaline hydrogen peroxide yields phthalic acid and $CO_2$. The specific activity difference, lawsone-phthalate, gives a measure of C-2 + C-3. In principle, the simultaneously generated $CO_2$ (trapped as $BaCO_3$) also gives a measure of this sum. However, since the reaction is not quantitative and since there is no *a priori* reason to assume that both C-2 and C-3 will be converted to $CO_2$ with equal efficiency, the specific activity of the isolated $BaCO_3$ can be expected to be an accurate measure of the quantity (C-2 + C-3)/2 only if the lawsone is symmetrically labeled in C-2 and C-3 to begin with. If initially the lawsone is unsymmetrically labeled in C-2 and C-3, the $BaCO_3$ result may diverge from the value (C-2 + C-3)/2; the extent and direction of this divergence

depending on which carbon is predominantly labeled and which is preferentially eliminated as $CO_2$.

To determine the activity present in C-3 of lawsone, use was made of the fact that in basic solution the $\beta$-diketone system of this compound, C-2/C-4, opens in two possible ways, both of which yield C-3 as the methyl group of a methyl ketone. Thus, in basic solution lawsone reacts with iodine to give iodoform, the carbon atom of which is C-3 of lawsone (Scheme 2).




SCHEME 2. LAWSONE → IODOFORM CONVERSION.

The results of degrading lawsone samples derived from feeding 2-$^{14}$C-acetate and the control substance 3,4-$^{14}$C-L-glutamate to excised stems of *Impatiens balsamina* are shown in Table 1. The distribution of activity from 3,4-$^{14}$C-L-glutamate clearly indicates that our experimental approach is a valid one. As shown in Scheme 1, this precursor should label C-2 and C-3 of lawsone to the same extent. The experimental results so prove. They also confirm anew the fact that glutamate is involved in naphthalene ring construction.

The result of the 2-$^{14}$C-acetate feeding is equally conclusive. By far the majority of the label is found in C-2. The small amount of activity found in C-3 is attributable to label

TABLE 1. ACTIVITY DISTRIBUTION IN BIOSYNTHESIZED LAWSONE SAMPLES

| | 3,4-$^{14}$C-L-glutamate | | 2-$^{14}$C-acetate | |
|---|---|---|---|---|
| Amount of precursor added | 100 $\mu$C | | 100 $\mu$C | |
| Per cent incorporation | 0·42 | | 0·008 | |
| Dilution value | 1652 | | 6090 | |
| Activity distribution | dis./min/$\mu$mole | % of I | dis./min/$\mu$mole | % of I |
| lawsone (I) | 1002 | 100 | 196 | 100 |
| phthalate | 209 | 21 | 35 | 18 |
| iodoform | 448 | 45 | 31 | 16 |
| $BaCO_3$ | 415 | 42 | 112 | 56 |
| Activity location in lawsone | | | | |
| C-3 (iodoform) | | 45 | | 16 |
| $\frac{\text{C-2} + \text{C-3}}{2}$ [$\frac{\text{I-phthalate}}{2}$] | | 39·5 | | 41 |
| $BaCO_3$ | | 42 | | 56 |

scrambling brought about by operation of the Krebs cycle. Each time the cycle 'turns' 2-$^{14}$C-acetate activity is incorporated into succinate and so eventually into C-3 and C-4 of compound IV and of lawsone. The disparity between the $BaCO_3$ specific activity and the (C-2 + C-3)/2 value determined from the difference (lawsone-phthalate), provides further evidence for labeling asymmetry. The sense of the disparity suggests that in the oxidation, C-2 is more efficiently eliminated as $CO_2$.

## DISCUSSION

The results described above demonstrate conclusively that symmetrical intermediates such as free 1,4-naphthaquinone cannot be involved in lawsone biosynthesis. While complexed, and therefore potentially asymmetric, forms of these materials may have a role to play, the possibility seems remote.

Since it has been clearly shown that 2-$^{14}$C-acetate preferentially labels C-2 of lawsone, we can deduce that the hydroxyl group found in lawsone is attached to that carbon atom which was C-2 in compound IV (alternative VII, Scheme 1). This fact sheds light on the mechanism by which the latter stages of lawsone biosynthesis might proceed. Two pathways seem feasible; both are shown in Scheme 1. The first involves cyclisation of compound IV to the 1,4-dihydroxy-2-naphthoic acid (IX) and subsequent oxidative decarboxylation. Although Dansette and Azerad have been unsuccessful in using this naphthoic acid to swamp out incorporation of activity from 4-(2′-carboxyphenyl)-4-oxobutyrate into lawsone,[3] and although we have been unable to detect this acid in *I. balsamina* extracts by combined GLC–MS,[7] the involvement of this compound still has to be excluded positively.

An alternative pathway of lawsone biosynthesis could involve conversion of compound IV to the α-ketoacid (X) by successive dehydrogenation, hydration and oxidation in the fashion of fatty acid biosynthesis. Decarboxylative closing of the ketoacid in a manner analogous to the coupling of shikimic acid with the glutamate derived unit would yield lawsone. While this route seems, *prima facie*, unduly lengthy it would account for the problems with the naphthoic acid derivative. It would also explain why combined GLC–MS reveals the presence in methylated extracts of the plant of several materials, in addition to compound IV, which have an *m/e* 163 base peak characteristic of the ion XI. The identification of these substances is being pursued presently, as is the extent to which these findings in the plant system can be transferred to the phyllo- and menaquinone fields.

## EXPERIMENTAL

*Feeding experiments.* In a typical run, four 4–6-week-old plants of *I. balsamina* var. Peppermint were cut at ground level, were quickly dipped in water, and were transferred to 5 ml beakers containing the requisite tracer in distilled water (*ca.* 1 ml). The cuttings were maintained at room temp. and in indirect sunlight for 12 hr, care being taken that the liquid volume in the feeding beaker remained at 1 ml. During the feeding period the cuttings remained healthy.

At the end of the feeding period, the unused tracer was recovered from the beakers, and the cuttings (16 g) were homogenized (60 sec) in the biphase EtOAc–$Me_2CO$–$H_2O$ (1:1:1) (150 ml). After centrifugal removal of the insoluble matter, the aqueous layer which contained the lawsone glycoside* was partially evaporated to remove acetone. Treatment of the resultant solution (100 ml) at 60° for 5 min with conc. HCl (10 ml) liberated lawsone which was extracted into EtOAc. The crude product obtained by evaporation of the EtOAc was purified by buffer extraction from $CHCl_3$ (Tris, pH 8), TLC on silica gel ($CHCl_3$–MeOH–HOAc, 90:5:0·5), sublimation (80–90°, 0·1 mmHg), and crystallization from EtOAc/isooctane then aq. MeOH. Yield, 11 mg. Radio purity was checked by crystallization to constant specific activity and gas flow proportional counting (methyl and trimethylsilyl derivatives of lawsone).

* In *I. balsamina*, lawsone occurs predominantly as a glycoside. Small amounts of a 2-methyl ether are also present. The latter material was not considered in the present study.

[7] E. GROTZINGER and I. M. CAMPBELL, unpublished observations.

*Synthesis of cold lawsone.* 1,4-Naphthaquinone-2,3-oxide was prepared by the method of Fieser *et al.*[8] with an average yield on the 25–50 mg scale of 63%. The material crystallized from EtOH as needles,* m.p., 134–136° (Lit.[8], 134·5–135·5°); NMR ($CF_3CO_2H$, TMS = 10$\tau$), 1·84–2·22 $\tau$ (multiplet, 4 aromatic H), 5·84 $\tau$ (singlet, 2 H on epoxide); parent molecular ion, *m/e* 174. Lawsone was prepared in 42% average yield by treating the oxide (25 mg) with ice-cold conc. $H_2SO_4$ (0·5 ml) for 5 min. At the end of this period, $H_2O$ (5 ml) is added and the resultant precipitate dissolved in $CHCl_3$. Extraction of this solution with Tris pH 8 buffer separates lawsone from unreacted starting material. Lawsone crystallized from EtOAc–isooctane then aq. MeOH as needles, m.p. 189–190° decomp. (Lit.[9], *ca.* 192°); NMR ($CF_3CO_2H$, TMS = 10 $\tau$) 1·72–2·23 $\tau$ (multiplet, 4 aromatic H), 3·49 $\tau$ (singlet, H-C-3); parent molecular ion, *m/e* 174.

*Degradation of lawsone.* (*a*) *To phthalic acid and carbon dioxide.* Typically, lawsone (52 mg) was dissolved in 2 N NaOH (8 ml) under a $N_2$ stream and cooled to 0°. $H_2O_2$ (50%, 4 ml) was carefully added and the reaction mixture warmed to 80° over 45 min. At the end of that period the mixture was cooled to 0° and a $Ba(OH)_2$ trap set in the $N_2$ effluent stream. Conc. HCl (2 ml) was added and the reaction mixture was flushed with $N_2$ for 1 hr. The collected $BaCO_3$ (66 mg, 56%) was washed, dried, and counted as described heretofore.[10] Phthalic acid was recovered from the reaction solution by evaporation, EtOAc extraction, and crystallization from dil. HCl. Yield, 15 mg 30%. (*b*) *To iodoform.* Typically, lawsone (20 mg) was dissolved in 2 N NaOH (2 ml) and treated with the reagent of Shriner *et al.*[11] till a slight red coloration persisted. The iodoform was extracted from the reaction mixture with $CHCl_3$, washed with a little water and crystallized from aq. MeOH to constant specific activity.

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* This material caused all those who came in contact with it to sneeze violently and persistently. We therefore urge caution in its handling.

[8] L. F. Fieser, W. P. Campbell, E. M. Fry and M. D. Yates, *J. Am. Chem. Soc.* **61**, 3216 (1939).
[9] E. H. Rodd, *Chemistry of Carbon Compounds*, Vol. III, 1320, Elsevier, Amsterdam (1956).
[10] A. T. Hudson and R. Bentley, *Biochem.* **9**, 3984 (1970).
[11] R. L. Shriner, R. C. Luson and D. Y. Curtin, *The Systematic Identification of Organic Compounds*, 5th Ed, p. 137, Wiley, New York (1967).

*Key Word Index—Impatiens balsamina*; Balsaminaceae; biosynthesis; lawsone.