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4-(2′-CARBOXYPHENYL)-4-OXOBUTYRATE: AN OBLIGATORY INTERMEDIATE IN LAWSONE BIOSYNTHESIS

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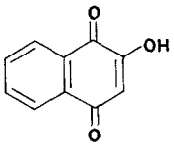
Key Word Index—*Impatiens balsamina*; Balsaminaceae; Garden balsam; 2-hydroxy-1,4-naphthoquinone; lawsone; biosynthesis; 3-(2′- carboxyphenyl)-3-oxopropionate

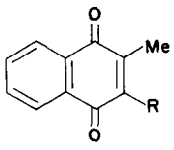
Abstract—4-(2′-Carboxyphenyl)-4-oxobutyric acid (**6**) has been detected in cuttings of *Impatiens balsamina*. It is labelled under conditions where activity from U-^{14}C-glutamate is incorporated effectively into lawsone (**1**). 3-(2′-Carboxyphenyl)-3-oxopropionic acid (**7**) has also been encountered in the cuttings.

INTRODUCTION

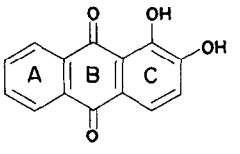
THE NAPHTHOQUINONE ring of lawsone (**1**), phylloquinone (**2**), and the menaquinones (**3**), together with rings A and B of the *Rubia* anthraquinones (e.g. alizarin, (**4**)), are derived from shikimate and the noncarboxyl carbon atoms of glutamate.[1–12] Shikimate is also incorporated as a seven carbon unit into juglone (**5**)[1,2,11] and it can be assumed that glutamate provides the remaining three carbon atoms there also.



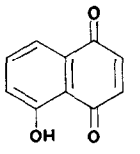
(**1**)



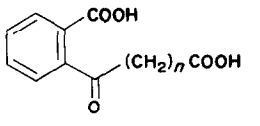
(**2**) R = phytyl
(**3**) R = polyprenyl



(**4**)



(**5**)



(**6**) $n = 2$
(**7**) $n = 1$

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[11] ZENK, M. H. and LEISTNER, E. (1968) *Lloydia* **31**, 275.
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There is strong evidence that 4-(2′-carboxyphenyl)-4-oxobutyric acid (**6**) is an intermediate in this naphthoquinone biosynthetic pathway. It is well incorporated into lawsone by *Impatiens balsamina*, into juglone by *Juglans regia*, into the anthraquinones of *Rubia peregrina* and *R. tinctorum*, and into the menaquinones of *Mycobacterum phlei*, *Aerobacter aerogenes*, and *E. coli*.[4,12,13] The oxobutyrate (**6**), however, has not been detected in any naphthoquinone producing system and, therefore, the obligatory nature of its involvement in the biosynthesis has still to be established. This paper reports not only the identification of the oxobutyrate (**6**) in *I. balsamina* cuttings but also its labelling from ^{14}C-glutamate.

RESULTS

U-^{14}C-glutamate (250 μCi, 200 μCi/μmol) was fed in aqueous solution to fresh *I. balsamina* cuttings for 5 hr. Glycosidically bound lawsone was isolated from an aqueous extract of the cuttings and found to have a sp. act. of $3{\cdot}02 \times 10^5$ dpm/μmol. Thus U-^{14}C-glutamate had been incorporated into bound lawsone to the extent of 0·43% with a dilution value of $1{\cdot}47 \times 10^3$. A non-bound form of lawsone (3% total lawsone) was isolated from an organic extract of the cuttings; its sp. act. was $1{\cdot}95 \times 10^5$ dpm/μmol; incorporation $7{\cdot}4 \times 10^{-3}$%; dilution value $2{\cdot}28 \times 10^3$. These findings prove that U-^{14}C-glutamate was being metabolized to lawsone in the cuttings under study.

We argued that if the oxobutyrate (**6**) was present in the tissue it would most likely be found in the organic extract. Thus, the base soluble portion of this extract was methylated and scanned on a combined GLC-gas flow proportional counter. The trace was dominated by fatty acid methyl esters. Recalling that the lipophilic gel filter, Sephadex LH20, retards the passage of aromatic compounds relative to aliphatic materials of comparable MW,[14] the methylated organic extract was passed through a column of LH20 in *iso*octane–$CHCl_3$–MEOH (2 : 1 : 1). The first five fractions were void while fractions 6 and 7 contained the fatty acid esters. The GLC-proportional counter trace of combined fractions 8 and 9 showed a mass and radioactivity maximum with retention time corresponding to that of methyl 4-(2′-carbomethoxyphenyl)-4-oxobutyrate: the MS taken at that point was identical with that of the authentic oxobutyrate ester. From the GLC-proportional counter trace it was estimated that the specific activity of the oxobutyrate was *ca* 2×10^6 dpm/μmol. Consequently its dilution value from glutamate is 2×10^2.

To corroborate this finding, a known amount of cold, authentic methyl 4-(2′-carbomethoxyphenyl)-4-oxobutyrate was added to combined fractions 8 and 9 followed by excess semicarbazide hydrochloride. The methyl 4-(2′-carbomethoxyphenyl)-4-oxobutyrate semicarbazone was prepared, isolated and crystallized to a constant sp. act. of 240·5 dpm/μmol. From this datum it follows that the incorporation of U-^{14}C-glutamate activity into the minute intracellular pool of the oxobutyrate was $1{\cdot}7 \times 10^{-3}$%.

Fractions 11–13 of the Sephadex column contained, as a methyl ether, the non-glycosidically bound lawsone previously mentioned. The GLC-proportional counter trace of combined fractions 11 and 12 showed in addition to methyl lawsone a small quantity of a faster moving labelled compound. Mass spectral comparison of this compound with methyl 3-(2′-carbomethoxyphenyl)-3-oxopropionate (**7**) established the identity of the

[13] LEISTNER, E. (1973) *Phytochemistry* **12**, 337.

[14] Sephadex LH-20 Technical bulletin issued by Pharmacia Fine Chemicals Inc., Piscataway, NJ 08854, U.S.A.

two. The GLC retention time of the fraction 11 and 12 component and the authentic propionate were identical. The sp. act. of this material was estimated to be $1{\cdot}0 \times 10^5$ dpm/μmol, its dilution value, $4{\cdot}4 \times 10^3$.

DISCUSSION

Since Dansette and Azerad[12] have demonstrated that the oxobutyrate (**6**) is incorporated efficiently into lawsone by *I. balsamina* (20%), and since the research reported herein established that (**6**) is present in *I. balsamina* tissue, and that it is labelled when activity from U-^{14}C-glutamate is being incorporated into lawsone, it follows that the oxobutyrate (**6**) can be considered a *bona fide* intermediate in lawsone biosynthesis. Moreover, since in the past lawsone biosynthesis has proved a realistic model for juglone, phylloquinone, menaquinone, and *Rubia* anthraquinone biosynthesis, it seems probable that the oxobutyrate will also figure obligatorily in these processes.

A valuable attribute of the combined GLC–MS-proportional counter scanning method used here, is that it has the capability to detect *all* the metabolites that derive from the fed precursor. The vast potential of this technique is being exploited and results will be published hereafter. Suffice it at present to note that 3-(2′-carboxyphenyl)-3-oxopropionate (**7**) was identified as a product of U-^{14}C-glutamate metabolism. From its estimated dilution value ($4{\cdot}4 \times 10^3$) we conclude that it is a catabolic product of lawsone formed presumably by oxidative cleavage of the 1,2 bond in the hydroxylated quinone.

EXPERIMENTAL

Feeding technique and tissue processing. In a typical run a 7-week-old plant of *Impatiens balsamina* var. Peppermint was cut at ground level, dipped in H_2O and transferred to a 4ml vial containing the labelled substrate in dist. H_2O (*ca* 0·75 ml) wherein it was maintained for 5 hr in artificial daylight at 28°. The cutting usually consumed the initial vol. of soln in 45 min whereupon additional vol. (3 × 1 ml) of dist. H_2O were added to the vial. Throughout all the feeding experiments the cutting remained healthy as judged by leaf turgor. At the close of the feeding period the cutting (*ca* 4 g) was homogenized (60 sec) in the ice-cold biphasic mixture, EtOAc–Me_2CO–H_2O (1 : 1 : 1) (90 ml). The insoluble cell debris was removed centrifugally leaving an upper non-polar soluble (NPS) layer and a lower polar soluble (PS) layer These layers were separated. The NPS layer (40 ml) was extracted with 1·0 M Tris buffer (pH = 8·0, 2 × 20 ml), and the extracts collected, acidified (conc. HCl), and treated with EtOAc (40 ml). The EtOAc solubles so formed were reacted with 10% methanolic HCl (15 ml, 1 hr at 60°), to yield the "NPS methylated acids." The PS layer (50 ml) was freed from acetone, treated with conc. HCl (0·1 ml), warmed for 15 min on a steam bath, then cooled and extracted with EtOAc (2 × 30 ml). The combined extracts were dried (rotary evaporator) and treated with 10% methanolic HCl (15 ml) for 2 hr at 60° to yield the "PS methylated acids."

Lawsone 2-methyl ether. (a) authentic. Lawsone is methylated with 10% methanolic HCl for 2 hr at 60° to give in 90% yield a product which on crystallization from EtOAc–*iso*octane gave needles, m.p. 184–185° (lit.[15] 183·5°); parent molecular ion (17 eV) *m/e* 188 : six largest ions (17 eV) *m/e* 188 > 89 > 103 > 158 > 173 > 85; NMR ($CDCl_3$, TMS = 10·00 τ) 6·39 (3H, OMe), 4·17 (1H, C(3)-H), 2·24–2·77 (4H, ring A hydrogens); λ_{max} (EtOH, nm (log ε) 2420 (4·08), 2470 (4·09, 2750 (4·00), 3320 (3·31). (b) from glycosidically bound *I. balsamina.* The "PS methylated acid" fraction, normally *ca* 5 mg was gel filtered on a column (4 × 40 mm) of Sephadex LH20, preswelled 24 hr in *iso*octane–MeOH–$CHCl_3$ (2 : 1 : 1). Elution with the same solvent system gave lawsone 2-methyl ether (*ca* 1·5 mg) in fraction 5 (fraction vol. 0·2 ml) as judged by m.p., GC retention time, MS and UV. (c) from nonglycosidically bound *I. balsamina.* Fractionation of the NPS methylated acids on Sephadex LH20 as described above gave lawsone 2-methyl ether (*ca* 0·05 mg).

Methyl 4-(2′-carbomethoxyphenyl)-4-oxobutyrate (**6**). (a) authentic. 4-(2′-Carboxyphenyl)-4-oxobutyric acid was prepared as described by Roser[16] m.p. 138–140° (lit.[16] 137°), then methylated with excess ethereal CH_2N_2 to give an oil, whose spectral properties were entirely consistent with it being methyl 4-(2′-carbomethoxyphenyl)-4-oxobutyrate: no parent molecular ion (17 eV); major ions (17 eV) at *m/e* 163 > 159 > 164 > 187 > 219. NMR ($CDCl_3$; DMSO, 1:1; TMS = 10·00 τ) centred at 7·25 (4H, A_2B_2 system of CH_2CH_2), 6·51 (3H, CO_2Me),

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[16] ROSER, W. (1884) *Berichte* 2770.

6·34 (3H, CO_2Me), 2·96–2·50 (4H, aromatic H): λ_{max} (EtOH) 2300 (3·98), 2755 (3·05), 2802 (3·04). It formed a semicarbazone m.p. 154–155°; no parent molecular ion; six largest ions (17 eV) *m/e* 173 > 172 > 232 > 201 > 130 > 200; NMR ($CDCl_3$–DMSO, 1:1; TMS = 10·00 τ) centred at 7·05 (4H, A_2B_2 system of CH_2CH_2), 6·80 (3H, CO_2ME), 6·44 (3H, CO_2Me); 2·56–1·87 (4H, aromatic H); λ_{max} (EtOH) 225 (4·19), 244 (3·91), 254 (3·95), 283 nm (3·86). (b) detection in *I. balsamina* extract by dilution analysis. To the combined fractions 8 and 9 of the Sephadex LH20 column on the NPS methylated acids, was added 10·0 mg of pure 4-(2′-carboxyphenyl)-4-oxobutyric acid. The composite was methylated with ethereal diazomethane and the semicarbazone of the oxobutyric dimethyl ester prepared as described by Vogel.[17] The product m.p. 154–155°, was crystallized 3 × to constant specific activity.

Methyl 3-(2′-carbomethoxyphenyl)-3-oxopropionate. Phthalylacetic acid was prepared from phthalic anhydride, Ac_2O and KOAc as described by Schroeder *et al.*[18] The product crystallized from dimethyl formamide, then aqueous pyridine as needles, m.p. 230–233° (sublimes), parent molecular ion 190·0260 a.m.u. ($C_{10}H_6O_4$ requires: 190·0266 a.m.u.). Treatment of the acid for 72 hr at room temp. with 10% BF_3 in CH_3OH and chromatography of the product on silicic acid gave an oil whose properties were completely consistent with those expected of methyl 3-(2′-carbomethoxyphenyl)-3-oxopropionate, viz. no parent molecular ion (17 eV); major ions (17 eV) at *m/e* 163 > 205 > 164 > 177 > 173. NMR ($CDCl_3$; TMS = 10·00 τ) 6·93 (3H, CO_2Me), 6·80 (2H, CH_2) 6·43 (3H, CO_2Me), 2·40–2·07 (4H, aromatic H); λ_{max} (EtOH) 230 (3·87), 273 (3·06), 281·5 nm (2·99).

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