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INCORPORATION OF SHIKIMIC ACID INTO *p*-HYDROXYBENZOIC ACID IN *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; cell culture; biosynthesis; ^{13}C-labelling; naphthoquinones; shikonin, *p*-hydroxybenzoic acid; shikimic acid

Abstract—[1,7-$^{13}C_2$]Shikimic acid was fed to cell cultures of *Lithospermum erythrorhizon* which accumulate *p*-hydroxybenzoic acid as its *β*-*O*-D-glucoside. ^{13}C NMR analysis of *p*-hydroxybenzoic acid *β*-*O*-glucoside showed that incorporation of shikimate proceeds with complete loss of the carboxy group, i.e. exclusively via the prephenate-cinnamate pathway.

INTRODUCTION

Benzoic acids in plants can be formed by side-chain degradation of cinnamic acids [1] (Fig. 1a). In bacteria, however, an alternative pathway has been demonstrated (Fig. 1b) which proceeds by the direct conversion of chorismic acid (**2**) to *p*-hydroxybenzoic acid (**6**) and pyruvate [2]. Cell cultures of *Lithospermum erythrorhizon* Sieb. et. Zucc. (Boraginaceae) are capable of producing large amounts of shikonin [3, 4], a naphthoquinone pigment which is biosynthesized via cinnamic acid and *p*-hydroxybenzoic acid [5, 6]. Depending on culture conditions, they can also form *p*-hydroxybenzoic acid *β*-*O*-D-glucoside [7] instead of shikonin. In the course of our studies on the regulation of shikonin biosynthesis [8, 9], we wanted to establish whether the *p*-hydroxybenzoic acid *β*-*O*-glucoside is formed entirely via cinnamic acid, or whether the direct conversion of chorismic acid to *p*-hydroxybenzoic acid also occurs.

The two routes differ in the fate of the carboxyl group of shikimate, which gives rise to the carboxyl group of *p*-hydroxybenzoic acid in the direct conversion of **2**, but which is eliminated in the aromatization of prephenic acid on the route via cinnamic acids. Hence, the relative contribution of the two pathways in the formation of *p*-hydroxybenzoic acid can be assessed by using as precursor a shikimic acid sample carrying a label in the carboxyl group and a reference label in the ring. We chose to use two ^{13}C labels in adjacent positions to enhance the sensitivity of the experiment by virtue of ^{13}C–^{13}C coupling.

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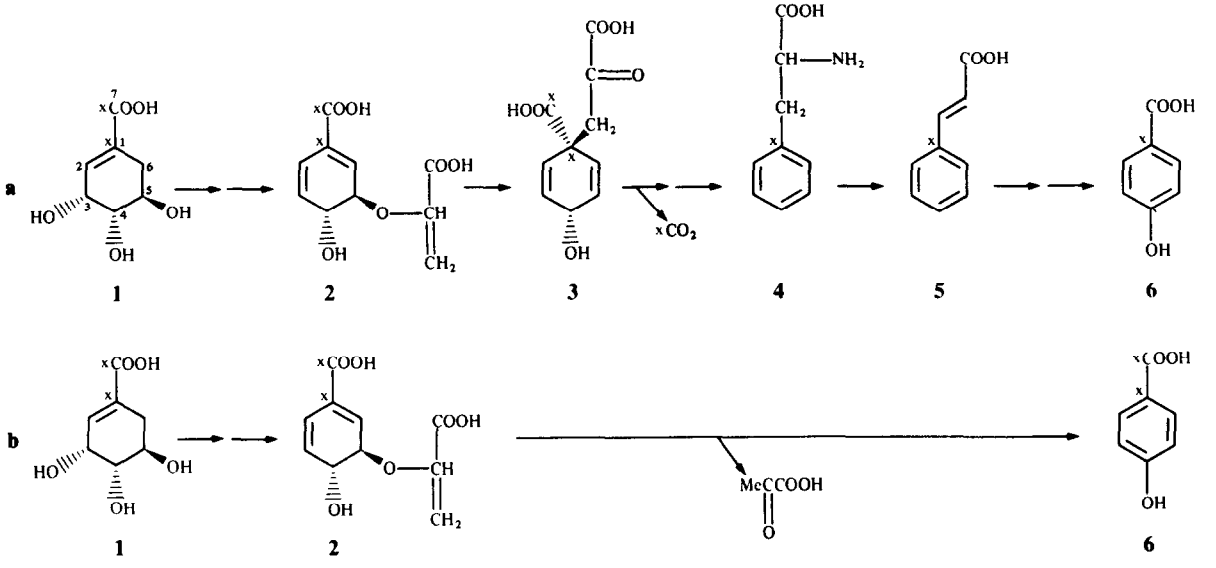

Fig. 1. Biosynthetic routes to *p*-hydroxybenzoic acid.

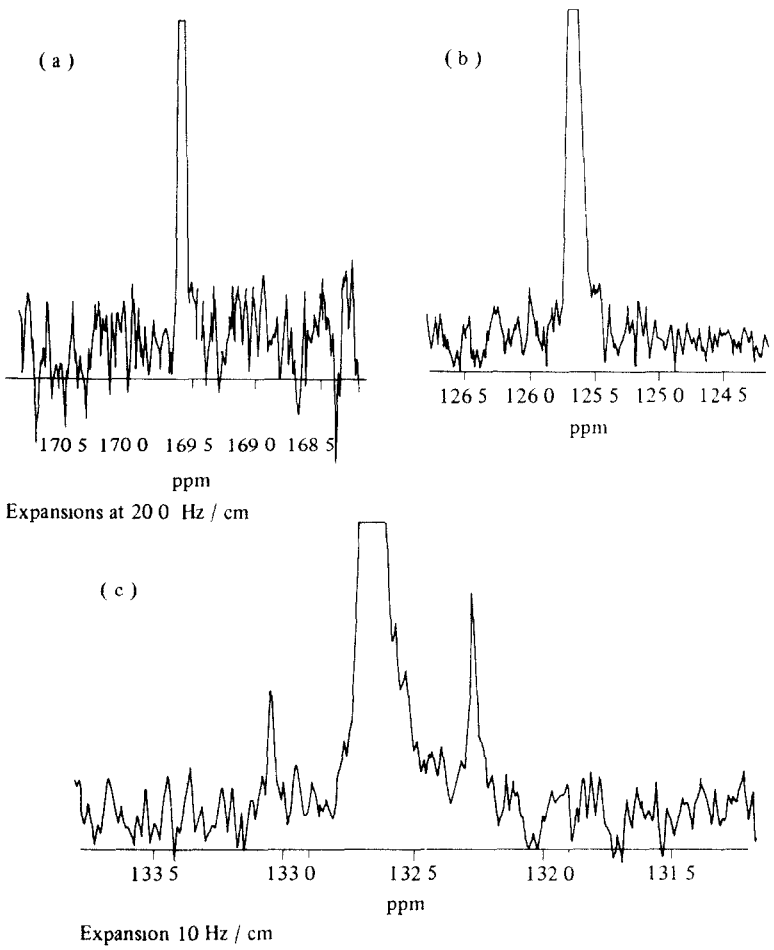

Fig. 2. Signals (scale expansion) for C-7 (a), C-1 (b) and C-2,6 (c) from the ^{13}C NMR spectrum of *p*-hydroxybenzoic acid *β*-*O*-D-glucoside biosynthesized from [1,7-$^{13}C_2$] shikimic acid

RESULTS AND DISCUSSION

D-(−)-[1,7-$^{13}C_2$]Shikimic acid (Fig. 1, **1**) was synthesized from [1,2-^{13}C]-acetate (Cambridge Isotopes, Inc., 99 atom % ^{13}C) according to the method of Fleet *et al.* [10, 11]. The overall yield was 24.6%, and the position of the label was confirmed by ^{13}C NMR spectroscopy [11]. Ten μmol of the labelled acid were added to each of five flasks of *Lithospermum erythrorhizon* cell cultures, M 18 strain [12], in 50 ml liquid LS medium [13,14]. After seven days of incubation, cells (77 g) were extracted with methanol *p*-Hydroxybenzioc acid *β*-*O*-glucoside was isolated by column chromatography on silica gel (EtOAc–MeOH–H_2O–HCOOH, 20 3:1:1) and a Lobar RP-8 column (H_2O–MeOH–HCOOH, 168:30:1), and crystallized from water to yield 6.3 mg of material.

The ^{13}C NMR spectrum of the product* showed labelling only at C-1 (2 88% enrichment) of the *p*-hydroxybenzoic acid moiety Satellite peaks due to ^{13}C–^{13}C coupling could not be detected (Fig 2)† for either C-1 or C-7

Thus, specific incorporation of shikimic acid into *p*-hydroxybenzoic acid was demonstrated, confirming earlier studies [6] Incorporation proceeds with complete (>98%) loss of the carboxyl group of shikimic acid, showing for the first time that only the prephenate-cinnamate pathway (Fig 1a) is used for the biosynthesis of *p*-hydroxybenzoic acid A direct conversion of chorismic acid does not seem to occur in this plant system One cannot rule out the possibility that different pools of **6** exist in the plant which are formed by different pathways However, if any **6** were formed directly from **2**, that pool must not exchange at all with the pool feeding the synthesis of the *p*-hydroxybenzoic acid *β*-D-glucoside We consider this a rather unlikely scenario

*^{13}C NMR (75 MHz, CD_3OD, ^{13}C-depleted) δ62 4, 71 3, 74 8, 77 9, 78 3, 101 6 (sugar carbons), 117 1 (C-3, C-5), 125 7 (C-1), 132 7 (C-2, C-6), 162 8 (C-4), 169 6 (C-7)

†Detection limit 0 06% abundance of coupled species, estimation based on intensity of coupled satellites for the signal at δ132 7

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