

## Regulation of Lithospermic Acid B and Shikonin Production in *Lithospermum erythrorhizon* Cell Suspension Cultures

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Cell suspension cultures of *Lithospermum erythrorhizon* produced a large amount of lithospermic acid B, a caffeic acid tetramer, as well as shikonin derivatives (each ca. 10% of dry wt.) when cultured in shikonin production medium M-9. Various culture factors for increasing the production of lithospermic acid B were investigated. Lithospermic acid B production was inhibited by 2,4-D or NH<sub>4</sub><sup>+</sup>, whereas it was stimulated by Cu<sup>2+</sup>. These regulatory patterns were similar to those for the production of shikonin derivatives in these cell cultures, suggestive of close relations and similar metabolic regulation between the production of these compounds. Cultivation under light illumination, however, showed that these metabolisms were independently regulated. In particular, blue light showed a stimulatory effect on lithospermic acid B production, while shikonin production was strongly inhibited, indicative of an effective condition for lithospermic acid B production.

**Key words** *Lithospermum erythrorhizon*; plant cell culture; lithospermic acid B; shikonin; light; nitrogen source

Caffeic acid oligomers such as rosmarinic acid and lithospermic acid B (LAB) are distributed in plants of Labiatae and Boraginaceae. Among these oligomers, LAB, the caffeic acid tetramer, has a variety of attractive pharmacological activities such as antioxidant and radical scavenging,<sup>1,2</sup> improvement of renal disease,<sup>3-6</sup> hypotensive,<sup>7-9</sup> improvement of myocardial infarction,<sup>1,10</sup> amplification of beating of myocardial muscles,<sup>11</sup> anti-hepatitis,<sup>12</sup> and aldose reductase inhibitory activity.<sup>13</sup> To utilize LAB as a medicinal resource, more than 30 plant sources were surveyed and only two medicinal plants, *Salvia officinalis* and *Lithospermum erythrorhizon* SIEB. et ZUCC. were found to contain this substance in an appreciable amount.<sup>14</sup> Many attempts to produce this compound in high quantities using cell or hairy root cultures have been unsuccessful.<sup>15-22</sup>

Recently, we found that *L. erythrorhizon* cell-suspension cultures, which were established for the industrial production of shikonin,<sup>23</sup> produced four caffeic acid oligomers, rosmarinic acid, LAB, a monoglucoside of LAB, and (+)-rabdosiin.<sup>24</sup> In particular, the production of LAB in the cultured cells was highly stimulated in shikonin production medium M-9,<sup>25</sup> reaching almost the same amount as that of shikonin (LAB: ca. 6—10% of dry wt, and total shikonin derivatives: ca. 6—12% of dry wt.).<sup>24,26</sup> Since both LAB and shikonin are formed through a common phenylpropanoid pathway (Chart 1), the counterbalance between two metabolic routes either to LAB or to shikonin would be regulated in *L. erythrorhizon* cells, suggesting that the selection of suitable regulatory factors would result in higher production of LAB. However, the metabolic linkage and the regulatory mechanism for the biosyntheses of phenylpropanoid-derived compounds of diverse types in *L. erythrorhizon* cell cultures remain to be clarified. In this study, we investigated the critical regulatory factors to control these two biosynthetic pathways separately and to produce LAB efficiently in the culture system of *L. erythrorhizon*.

### Experimental

**Cell Cultures** The culture strain M18TOM<sup>24</sup> of *Lithospermum erythrorhizon*, which had been maintained in Linsmaier-Skoog (LS) medium<sup>27</sup> containing IAA 1 μM and kinetin 10 μM, was used in the present study. Cell-suspension cultures in 100-ml flasks each containing 30 ml of medium were agitated on a rotary shaker (80 rpm) at 23 °C in the dark and subcultured at intervals of 2 weeks. The cells cultured in LS medium for 2 weeks were inoculated into M-9 medium,<sup>25</sup> modified M-9 or LS media containing 3% sucrose, and the same growth regulators as above (inoculum size: 1 g of fresh cells/30 ml of medium in a 100-ml flask) and cultured for 3 weeks under the above-mentioned culture conditions. For the investigation of the effect of light color on the production of secondary metabolites, each flask was covered with a commercially available colored cellophane sheet,<sup>28</sup> and irradiated with white light (10000 lx) from fluorescent lamps during the entire culture period. Under such conditions, light intensities in blue, red, and green cellophane-covered flasks were 600, 1000, and 900 lx, respectively.

**Quantitative Analysis of Secondary Metabolites** Sample preparations and quantitative analyses of secondary metabolites were carried out according to the methods described in the previous report,<sup>26</sup> and the sum of contents of β-hydroxyisovalerylshikonin, acetylshikonin, isobutylshikonin, β,β-dimethylacrylshikonin, isovalerylshikonin, and α-methyl-*n*-butylshikonin was expressed as shikonin. In addition to shikonin and caffeic acid oligomers, *L. erythrorhizon* cells produced several phenylpropanoid-derived secondary metabolites, including *p*-hydroxybenzoic acid 1-*O*-glucoside (PHBOG),<sup>29,30</sup> furobenzoquinone and furohydroquinone derivatives such as echinofurans B and C,<sup>31</sup> dihydroechinofuran,<sup>32</sup> deoxyshikonofuran, and shikonofuran E,<sup>33</sup> all of which were biosynthesized from *p*-hydroxybenzoic acid (PHB). In the present study, we determined the contents of all these compounds and found that the total content of furobenzoquinone and furohydroquinone derivatives was within about 10% of that of shikonin. The contents of caffeic acid oligomers, rosmarinic acid, LAB monoglucoside, and (+)-rabdosiin were also low compared with that of LAB, and were not affected by the supplements of modulators used. Therefore the contents of these compounds are not given in the Results section.

### Results

#### Time Course of Secondary Metabolite Production

Figure 1 shows the time courses of cell growth and production of phenylpropanoid-derived secondary metabolites in *L. erythrorhizon* cells cultured in LS medium (A) and M-9 medium (B). In LS medium, the production level of LAB transiently increased 2—4 days after inoculation, and then gradually decreased (Fig. 1A). Meanwhile, shikonin was hardly detectable, and the level of PHBOG, which is known

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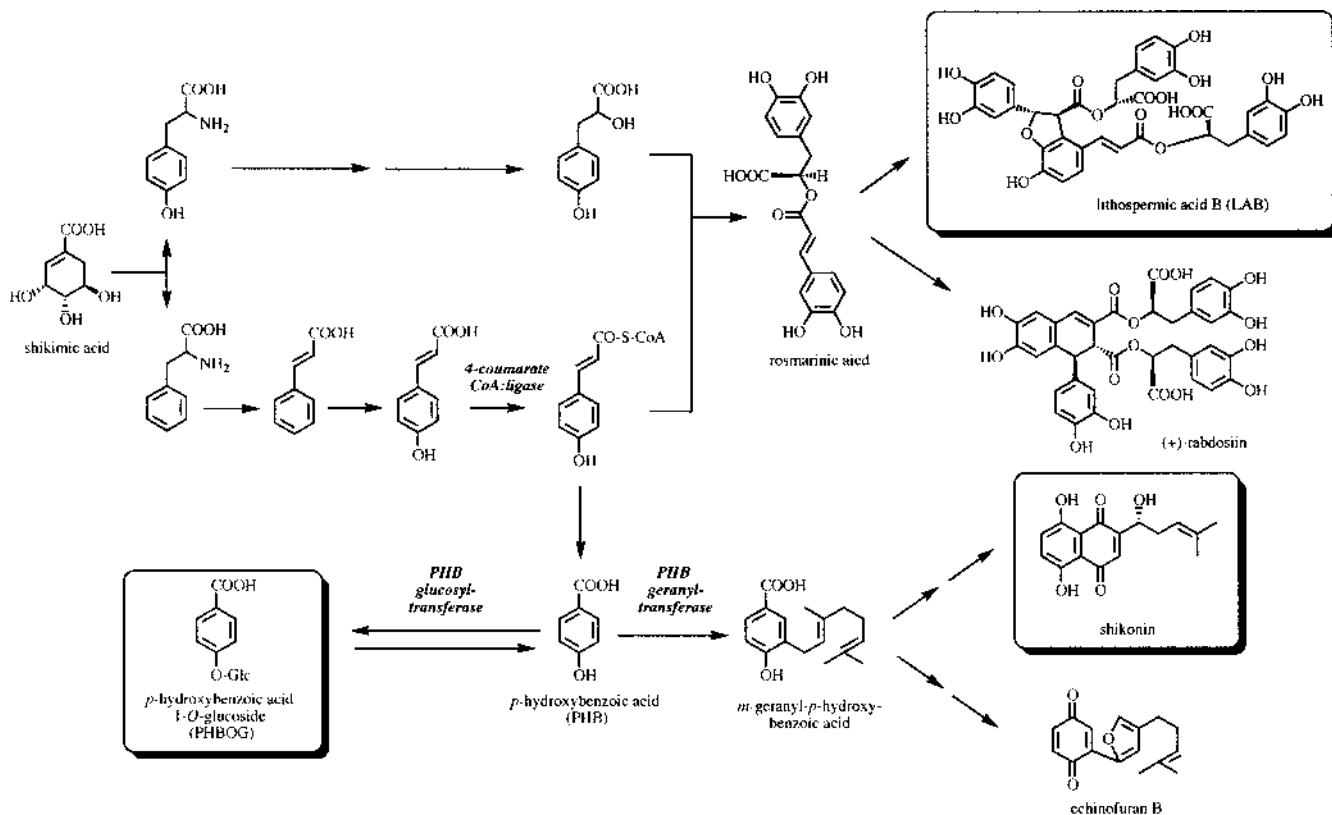


Chart 1. Biosynthesis of Phenylpropanoid-Derived Secondary Metabolites in *Lithospermum erythrorhizon* Cultured Cells

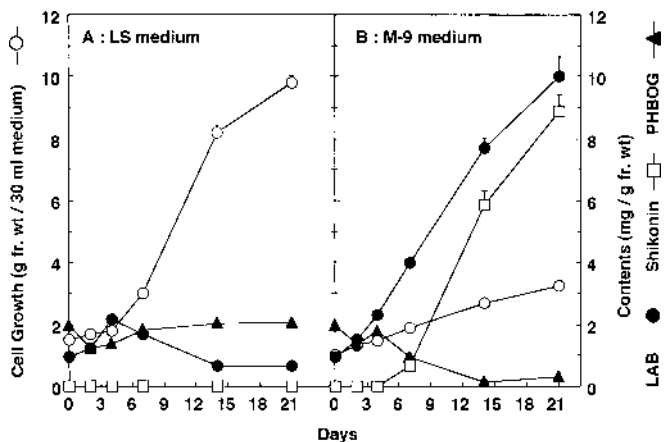


Fig. 1. Time Courses of Cell Growth and Production of Secondary Metabolites in *L. erythrorhizon* Cells Cultured in LS Medium (A) or M-9 Medium (B)

Cells (1 g) were inoculated in a 100-ml flask containing 30 ml of medium and cultured for 3 weeks in the dark. Values are means of triplicate results, and bars show standard errors.

to be a storage form of PHB, a biosynthetic precursor of shikonin,<sup>29,30</sup> was almost constant during the entire culture period (Fig. 1A). In M-9 medium, LAB production increased rapidly after inoculation and resulted in a linear increase toward the end of the culture period, reaching 10 mg/g fresh wt. on day 21 (Fig. 1B). Unlike the production pattern of LAB, the production of shikonin needed a lag phase of 4 d after inoculation and the level reached 9 mg/g fresh wt. on day 21. The production of PHBOG was inversely proportional to that of shikonin, and only a trace amount of

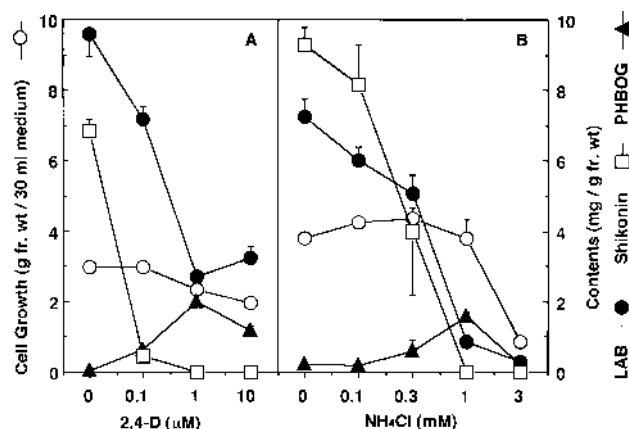


Fig. 2. Inhibitory Effects of 2,4-D (A) or  $\text{NH}_4\text{Cl}$  (B) on Phenylpropanoid-Derived Secondary Metabolites in *L. erythrorhizon* Cells Cultured in M-9 Medium (30 ml)

2,4-D or  $\text{NH}_4\text{Cl}$  was added to the medium before autoclaving. Values are means of triplicate results, and bars show standard errors.

PHBOG was detectable to the end of the culture period.

**Effects of Chemical Modulators in Shikonin Production on LAB Production** A number of modulators in shikonin production have been revealed in extensive studies made so far (for a recent review see ref. 34). In particular, shikonin production is clearly enhanced by the supplementation of  $\text{Cu}^{2+}$ ,<sup>35</sup> but strongly inhibited by either  $\text{NH}_4^+$ <sup>25,36</sup> or 2,4-D.<sup>37</sup> We examined the effects of such shikonin production modulators on LAB production. As shown in Fig. 2, contrary to our expectations, 2,4-D and  $\text{NH}_4^+$ , strong inhibitors of shikonin production, also inhibited LAB produc-

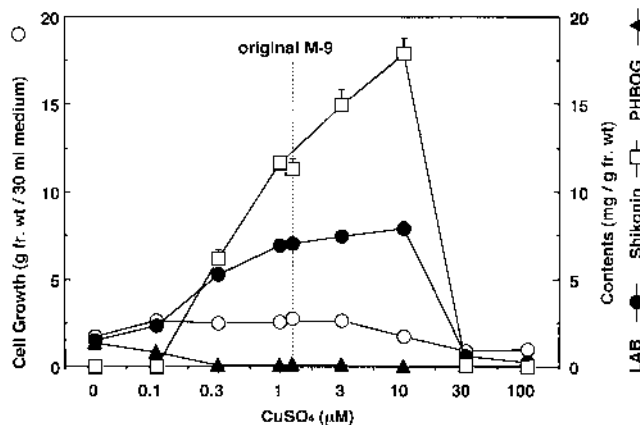


Fig. 3. Stimulatory Effect of  $\text{Cu}^{2+}$  on Phenylpropanoid-Derived Secondary Metabolites in *L. erythrorhizon* Cells Cultured in M-9 Medium (30 ml)

$\text{CuSO}_4$  was added to M-9 basal medium without any  $\text{Cu}^{2+}$ . Values are means of triplicate results, and bars show standard errors.

tion in M-9 medium in a dose-dependent manner. At 2,4-D  $1 \mu\text{M}$  or at  $\text{NH}_4^+$   $1 \text{ mM}$  at which shikonin production was completely inhibited, the LAB level declined to that in LS medium-cultured cells. Under these culture conditions, the level of PHBOG was restored to that in the cells cultured in LS medium (Fig. 1A). On the other hand, the supplementation of  $\text{Cu}^{2+}$ , a potent stimulator of shikonin production, also stimulated the production of LAB. Figure 3 shows the effect of added  $\text{Cu}^{2+}$  on LAB production in  $\text{Cu}^{2+}$ -free M-9 medium. Both LAB and shikonin production was enhanced by the addition of  $\text{Cu}^{2+}$ . However, the LAB level nearly reached a plateau at concentrations higher than  $\text{Cu}^{2+}$   $1 \mu\text{M}$ , whereas shikonin production was stimulated up to  $\text{Cu}^{2+}$   $10 \mu\text{M}$  in a dose-dependent manner.

LAB production was also affected by the  $\text{NO}_3^-$  concentration. When nitrogen sources in LS medium were replaced with  $\text{NO}_3^-$ , lower concentrations of  $\text{NO}_3^-$  tended to result in more LAB production (data not shown). This effect was reinforced by the addition of  $\text{Cu}^{2+}$   $1.2 \mu\text{M}$  (Fig. 4). At  $\text{NO}_3^-$   $6.7 \text{ mM}$  and  $\text{Cu}^{2+}$   $1.2 \mu\text{M}$ , the amount of LAB reached to 71% (5.3 mg/g fresh wt.) of that in M-9 medium cells. On the other hand, only a trace amount of shikonin production (0.2 mg/g fresh wt., 3% of that in M-9 medium) was observed under the same conditions.

$\text{PO}_4^{3-}$ , which influences the production of many secondary metabolites including polyphenols in plant cell cultures,<sup>38–40</sup> did not affect the production of LAB and shikonin in *L. erythrorhizon* cells cultured in either LS or M-9 medium (ranging from 0.12 to 1.25 mM, data not shown).

**Effect of Light on LAB Production** Light is a physiological regulatory element in shikonin biosynthesis. White or blue light strongly inhibits shikonin production by suppressing the gene expression of PHB geranyltransferase, a key enzyme in shikonin biosynthesis,<sup>41,42</sup> as well as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme responsible for the formation of the geranyl side chain of shikonin.<sup>43</sup> As shown in Fig. 5, LAB production was slightly stimulated by white light irradiation compared with that in cells cultured in the dark, whereas shikonin production was completely inhibited. It was noteworthy that cultivation under white light irradiation resulted in a high accumu-

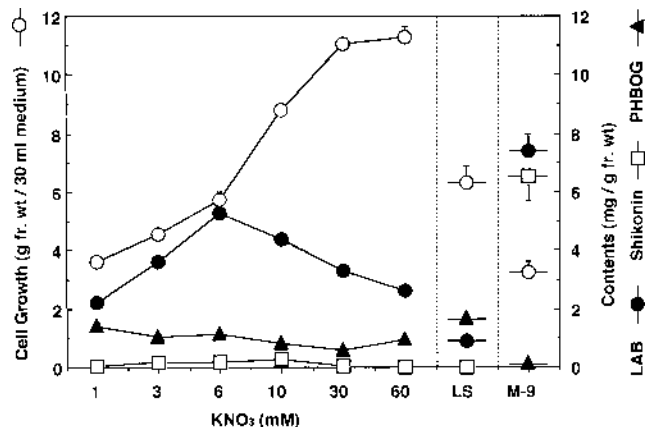


Fig. 4. Effect of  $\text{NO}_3^-$  on Phenylpropanoid-Derived Secondary Metabolites in *L. erythrorhizon* Cells Cultured in LS Basal Medium (30 ml)

$\text{KNO}_3$  was added to LS basal medium without any nitrogen source. The concentration of  $\text{Cu}^{2+}$  was adjusted to  $1.2 \mu\text{M}$ . Values are means of triplicate results, and bars show standard errors.

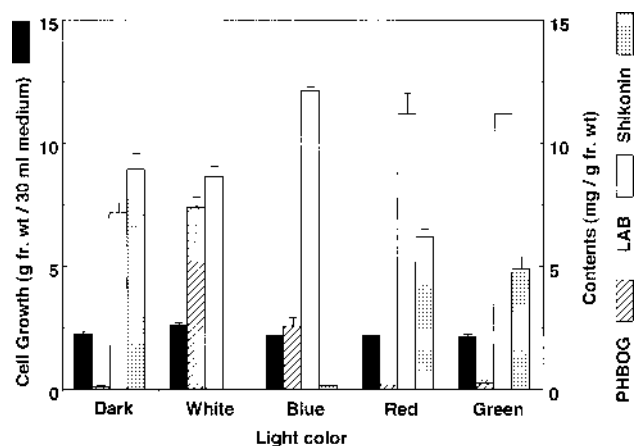


Fig. 5. Effect of Light on Phenylpropanoid-Derived Secondary Metabolites in *L. erythrorhizon* Cells Cultured in M-9 Medium (30 ml)

Each flask was placed on a rotary shaker and irradiated either with white, blue, red, or green light during the entire culture period. Flasks covered with aluminum foil were used as dark controls. Values are means of triplicate results, and bars show standard errors.

lation of PHBOG (7.4 mg/g fresh wt.), which was about equal to that of shikonin in the dark-grown controls (8.9 mg/g fresh wt.). When the cells were cultured under blue light, PHBOG was only 2.5 mg/g fresh wt., whereas the LAB content reached 12.1 mg/g fresh wt. (170% of the dark-grown controls). LAB production was also stimulated under red or green light (157% and 156% of the dark-grown controls, respectively). Under these conditions, shikonin production was not as inhibited (69% and 55% of the dark-grown controls, respectively), and the PHBOG content remained at a low level.

## Discussion

It is well known that shikonin derivatives are produced on industrial scale from *L. erythrorhizon* cell cultures.<sup>23</sup> In the present study, we demonstrated that LAB was produced almost specifically in a higher yield than that of shikonin, which was achieved by cultivation in the dark, in *L. erythrorhizon* cells by cultivation under blue light irradiation.

It has been demonstrated that the enzyme susceptible to

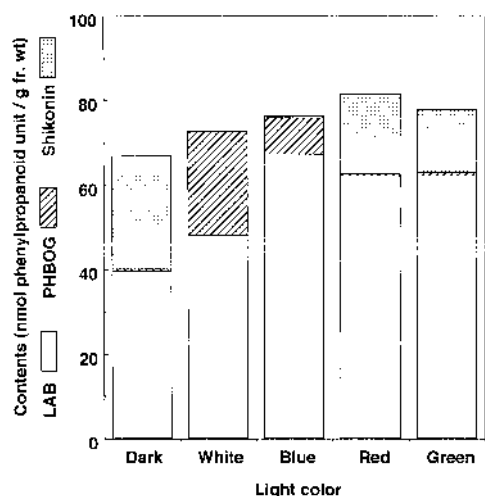


Fig. 6. Efficiency of Phenylpropanoid Unit Utilization in the Biosynthesis of LAB, PHBOG, and Shikonin in *L. erythrorhizon* Cells Cultured under Different Light Conditions

The molar amounts of phenylpropanoid unit in each metabolite were calculated from the results shown in Fig. 5. LAB, PHBOG and shikonin contain four, one, and one phenylpropanoid units in each one molecule, respectively.

inhibition by blue or white light in shikonin biosynthesis is PHB geranyltransferase.<sup>41)</sup> Furthermore, Gaisser and Heide<sup>44)</sup> reported that white light irradiation increased the activity of PHB-*O*-glucosyltransferase in *L. erythrorhizon* cells, while blue light did not affect it. No shikonin in cells cultured under white or blue light was produced. This led us to expect that the exclusive utilization of the phenylpropanoid unit under light irradiation could lead to higher production of LAB. To assess this assumption, the efficiency of utilization of the phenylpropanoid unit in either LAB or shikonin biosynthesis and in PHBOG formation was stoichiometrically analyzed in *L. erythrorhizon* cells cultured under the different light conditions (Fig. 6). Light irradiation did not increase the production of the phenylpropanoid unit but led to increased production of caffeic acid derivatives. The production of LAB was less affected by white light, although greater accumulation of PHBOG was observed. Meanwhile, cultivation under blue light illumination increased the flow of the phenylpropanoid unit into the formation of LAB, presumably owing to the inhibition of PHB geranyltransferase and the low activity of PHB glucosyltransferase.<sup>44)</sup> PHB, which was assumed to be cytotoxic,<sup>45)</sup> was not detectable under any culture conditions employed in the present study, as reported in previous papers.<sup>32,33)</sup> There might be a negative feedback regulation in the supply of PHB as a biosynthetic intermediate, although the conversion of 4-coumaric acid to PHB has not yet been completely clarified.<sup>46,47)</sup>

Under red or green light irradiation, LAB production was also stimulated but shikonin production was not completely inhibited (Fig. 5). These results might indicate that light qualities regulate LAB formation in different manners and also have independent effects on the biosynthesis of both compounds in *L. erythrorhizon* cells. The stimulation of 4-coumarate CoA:ligase expression by light in *L. erythrorhizon* cells<sup>48)</sup> may also contribute to the increase in LAB production, because this enzyme is responsible for LAB biosynthesis (Chart 1).

The inhibitory effects of 2,4-D and  $\text{NH}_4^+$  as well as the

stimulatory effect of  $\text{Cu}^{2+}$  on LAB and shikonin production were also demonstrated in this study (Figs. 2, 3). These data suggest that common earlier steps in the biosynthetic pathways of shikonin and LAB are controlled by similar regulation mechanisms. Cells cultured in modified LS medium containing nitrate and  $\text{Cu}^{2+}$  at the same concentrations as those in M-9 medium ( $\text{NO}_3^-$  6.7 mM and  $\text{Cu}^{2+}$  1.2  $\mu\text{M}$ ) produced some LAB (ca. 70% of that observed in M-9 medium), while they resulted in the poor production of shikonin (3% of the yield in M-9 medium) (Fig. 4). These results suggest that nitrate and  $\text{Cu}^{2+}$  stimulate the production of LAB but not of shikonin. Thus we have found an effective, although not yet sufficient, condition for high production of LAB. On the other hand, the production of (+)-rabdosiin, another caffeic acid tetramer presumably formed from rosmarinic acid like LAB (see Chart 1)<sup>26)</sup> was almost unaffected by the modulators connected with LAB production (data not shown). In intact *L. erythrorhizon* plants, LAB is mostly accumulated in the main roots whereas (+)-rabdosiin is mainly found in the aerial parts as well as in fibrous roots.<sup>26)</sup> The different distribution pattern of these two caffeic acid tetramers in intact plants might result in the different response to the above modulators in cultured cells. Further investigation of culture components as well as further biochemical and molecular biological studies on the enzymes responsible for LAB biosynthesis will contribute to more effective production of LAB in this cell culture.

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