Phytochemistry, Vol. 22, No. 2, pp. 453–456, 1983.
Printed in Great Britain.

0031-9422/83/020453-04$03.00/0

FORMATION OF STEREOISOMERIC MIXTURES OF NAPHTHOQUINONE DERIVATIVES IN *ECHIUM LYCOPSIS* CALLUS CULTURES

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(*Received* 21 *May* 1982)

Key Word Index—*Echium lycopsis*; *Lithospermum erythrorhizon*; Boraginaceae; callus cultures; biosynthesis; shikonin; alkannin; naphthoquinone pigments; stereoisomers.

Abstract—Callus cultures of *Echium lycopsis* were shown to produce a large amount of a mixture of red pigments consisting of five esterified derivatives of 5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone. Examination of the absolute configuration of these compounds revealed that the cultures produced both the *R*-form (shikonin) and the *S*-form (alkannin) in various ratios depending upon the esterified derivative, although the overall ratio for the total derivatives was *ca* 1:1. On the other hand, all the corresponding derivatives produced by *Lithospermum* cultures were primarily of the *R*-form. It was also demonstrated that pigment formation in *Echium* cultures was inhibited by either white or blue light as well as by the synthetic auxin 2,4-D as in the case of *Lithospermum* cultures.

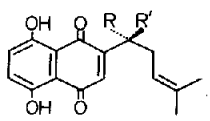
INTRODUCTION

It has been demonstrated that callus cultures of *Lithospermum erythrorhizon* are capable of producing the same naphthoquinone pigments (shikonin derivatives) as those accumulated in the root bark of the original plant and that the pigment content of the cultures could be increased greatly to surpass that of the root through the regulation of environmental factors and the selection of variant cell lines [1–4]. In the present study, we have established callus cultures of *Echium lycopsis* L., another boraginaceous plant containing red pigments in the root bark, and used them to compare the culture conditions required for pigment formation and the structures of these pigments, including their stereochemistry, with those of *Lithospermum* callus cultures.

RESULTS

Red pigments of callus cultures

Undifferentiated callus tissues of *E. lycopsis*, which were cultured on basal agar medium containing 10^{-6} M IAA and 10^{-5} M kinetin for 35 days in the dark, were found (TLC, mp, UV, IR, ^{1}H NMR, and/or GC) to contain the β,β-dimethylacryl, β-hydroxyisovaleryl, acetyl, isobutyl and isovaleryl derivatives of 5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1, 4-naphthoquinone [so-called shikonin (**1**) or alkannin (**2**)].



1 Shikonin R = OH, R' = H
2 Alkannin R = H, R' = OH

In all of these esters, the fatty acids were always esterified with the hydroxy group at C-1 of the C_6 side chain. Although similar pigments were present in the root of the intact plant cultivated for 1 year in the field, the total pigment content of callus cultures (12.3% on the basis of dry wt) was 350-times higher than that of the root (Table 1). In callus cultures, the acetyl derivative was produced in a larger quantity than the other derivatives, whereas its quantity in *Lithospermum* callus cultures was always smaller than that of the β,β-dimethylacryl derivative [2].

Stereochemistry of naphthoquinone derivatives

Shikonin (**1**) (*R*-form) and alkannin (**2**) (*S*-form) are stereochemically distinguished from each other by the difference in the configuration of the hydroxyl group at C-1 of the C_6 side chain attached to C-2 of 1,4-naphthoquinone [5, 6]. Since shikonin is dextrorotatory and alkannin laevorotatory, the distinction between the two enantiomers used to be made by the measurement of

Table 1. Pigment content in the callus culture and the root of *E. lycopsis*

Derivative	Pigment content (mg/g dry wt)	
	Callus	Root
Shikonin (alkannin)	trace	trace
Deoxyshikonin	trace	trace
β,β-Dimethylacryl-*	24	0.14
β-Hydroxyisovaleryl-	35	0.07
Acetyl-	64	0.14
Total	123	0.35

* β,β-Dimethylacryl, isovaleryl and isobutyl derivatives move as one spot on TLC and the mixture of these derivatives was determined as β,β-dimethylacryl derivative.

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optical rotation using a cadmium lamp as the illuminating source [7]. In the present study, however, the stereochemistry of each compound was determined by CD data based on our finding that shikonin shows a positive maximum at 358 nm ($[\theta] = +7580$) and a negative maximum at 308 nm ($[\theta] = -4920$), whereas this relationship is almost symmetrically inverted for alkannin. Furthermore, a quantitative estimation of the proportion of enantiomers was made possible by the method described in the Experimental.

Table 2 shows the results of the CD measurements on the β,β-dimethylacryl, β-hydroxyisovaleryl and acetyl derivatives as well as the total pigments. Unexpectedly, each derivative produced by *Echium* cultures was found to be a mixture of the *R*-form and the *S*-form. Moreover, the ratio *R*–*S* varied with the derivative, although the mixture of total pigments extracted from the callus, or its alkaline hydrolysate, showed no optical activity. The proportion of the *S*-form was greater than that of the *R*-form in the β,β-dimethylacryl and β-hydroxyisovaleryl derivatives, whereas the reverse was the case with the acetyl derivative. These facts have been confirmed by repeated experiments. Furthermore, similar results were obtained from callus tissues cultured on the medium containing 10^{-6} M indole-3-butyric acid (IBA) in place of IAA.

The pigment components isolated from the roots of *E. lycopsis* harvested at the flowering stage were also found to be a mixture of two enantiomers. All the components examined including the acetyl derivative, and also an alkaline hydrolysate of the total pigments, contained more *S*-form than *R*-form, although the ratio of the two enantiomers varied with the derivative as in the case of the callus tissues.

A stereochemical comparison of the pigments from *E. lycopsis* and *L. erythrorhizon* revealed that the pigments in the roots of the latter, which have hitherto been regarded as pure shikonin derivatives, contained small amounts of alkannin derivatives. Similarly, the naphthoquinone pigments, especially the acetyl derivative, produced by *Lithospermum* callus cultures contained significant amounts of alkannin derivatives (Table 2).

Factors controlling pigment formation

Figure 1 shows the time-course of growth and pigment production in *Echium* callus during a culture period of 5 weeks on agar medium containing 10^{-6} M IBA and 10^{-5} M kinetin. The total content of pigments increased almost in parallel with growth from week 2 until week 4 when growth reached the stationary phase. As regards the individual pigments, the formation of the β-hydroxyisovaleryl derivative was delayed *ca* 1 week as compared with that of the β,β-dimethylacryl or acetyl derivative.

It is known that irradiation of *Lithospermum* callus cultures with either white or blue light strongly inhibits the formation of naphthoquinone pigments, while it does not affect cell growth [1]. In *Echium* cultures, however,

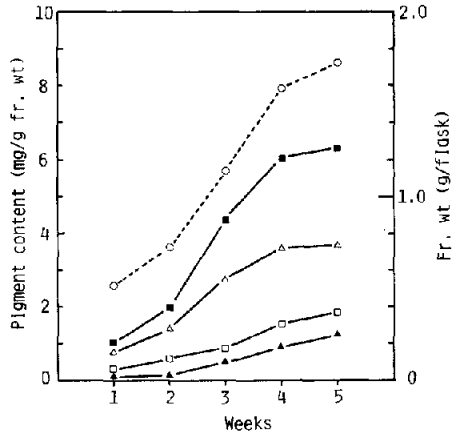

Fig. 1. The time-course of growth and pigment production in *E. lycopsis* callus cultures grown on basal agar medium containing 10^{-6} M IBA and 10^{-5} M kinetin in the dark at 25°. O---O, growth; ■———■, total pigments; △———△, β,β-dimethylacryl derivative; □— —□, acetyl derivative; ▲———▲, β-hydroxyisovaleryl derivative.

Table 2. Shikonin–alkannin ratios* of the naphthoquinone derivatives produced by the roots and callus cultures of *E. lycopsis* and *L. erythrorhizon*

Plant	Material	β,β-Dimethylacryl-	β-Hydroxyisovaleryl-	Acetyl-	Total pigments
E. lycopsis	Callus				
	Root				
L. erythrorhizon	Callus				
	Root				

* The white portion of each circle indicates the proportion of shikonin (*R*-form) and the black portion the proportion of alkannin (*S*-form). The proportions were determined from the CD curve of the mixture of napthoquinones released on alkaline hydrolysis of each pigment.

both pigment formation and cell growth were inhibited under continuous illumination with white or blue light (Table 3). Red light had little effect upon either pigment formation or cell growth as observed in *Lithospermum* cultures.

It is also known that the formation of red pigments in *Lithospermum* cultures is completely inhibited by replacing the 2,4-D in the medium with either IAA or IBA [1]. The effects of various auxins and kinetin on *Echium* cultures are shown in Table 4. The addition of kinetin to the medium was necessary for promoting both growth and pigment formation. Naphthalene-1-acetic acid (NAA) and 2,4-D were more effective in stimulating cell growth than either IAA or IBA, but 2,4-D was highly inhibitory to pigment formation. Although IBA was least effective in promoting cell growth, it was most effective in increasing the pigment content of callus tissues.

Table 3. Effects of white and blue light on growth and pigment production in *E. lycopsis* callus cultures grown on basal agar medium containing 10^{-6} M IAA and 10^{-5} M kinetin for 35 days

Light condition	Fr. wt of callus (g/test tube)	Content of pigment calculated as shikonin (mg/g fr. wt)
Dark	0.79	3.30
White light	0.08	0.86
Blue light	0.07	1.09

Table 4. Effects of various auxins and kinetin on growth and pigment production in *E. lycopsis* callus cultures grown on the basal agar medium (40 ml/flask) for 35 days

Auxin (10^{-6} M)	Concn of kinetin (M)	Fr. wt of callus (g/flask)	Total pigment content (mg/flask)
IAA	0	0.50	0.78
	10^{-5}	4.60	4.85
IBA	0	0.32	2.33
	10^{-5}	2.26	6.59
NAA	0	0.52	0.93
	10^{-5}	6.36	4.68
2,4-D	0	0.75	0.60
	10^{-5}	6.36	0.52

DISCUSSION

The present experiments have shown that callus cultures of *E. lycopsis* produce naphthoquinone pigments which are actually mixtures of two stereoisomers, the ratios of which depend upon the derivative. Furthermore, the proportion of the two enantiomers for a particular compound was found to be different between the callus cultures and the intact root.

The presence of enantiomers in different ratios in the same plant has recently been reported by Inouye *et al.* [8], who proved that the two naphthoquinones, 3-hydroxy-dehydroiso-α-lapachone and dehydro-iso-α-lapachone, isolated from *Radermachera sinica* Hemsl. (Bignoniaceae), were mixtures of D- and L-enantiomers in the ratios of 1:4.2 and 4.5:1, respectively, based on the measurements of ^{1}H NMR spectra using a chiral shift reagent, while another compound, 3-hydroxy-6-methoxy-dehydro-iso-α-lapachone was a racemate. Certain monoterpenes are also known to be present as pairs of stereoisomers in the same plant, e.g. a mixture of (−)-menthol and (+)-neomenthol in a varying ratio was found in the leaves of *Mentha piperita* [9]. However, the situation in *Echium* and *Lithospermum* is unique in that the ratio between a pair of enantiomers differs depending on the fatty acid esters of the naphthoquinone even in the same plant or callus tissues

The red naphthoquinone pigments hitherto isolated from various species of Boraginaceae were described either as shikonin or as alkannin derivatives, except in the case of *Arnebia hispidissima* which was reported to contain shikalkin (a racemate) [10]. The presence of shikonin was reported in certain species of *Arnebia*, *Echium*, *Lithospermum*, *Macrotomia* and *Onosma*, and that of alkannin in some other species of *Alkanna*, *Arnebia*, *Echium*, *Macrotomia* and *Onosma* [11]. Recently, we have confirmed the presence of acetylalkannin in the leaves of *Plagiobotrys arizonicus* by CD measurement [unpublished data]. In many of the previous studies, however, the pigments were tentatively called shikonin or alkannin without examination of the optical activity or the absolute configuration of each constituent. Therefore, an overall re-examination of the stereochemistry of the pigments found in various boraginaceous species should provide chemotaxonomically interesting information concerning the distribution of stereoisomers in a plant family.

Nothing is known about the reason for the formation of enantiomers in various ratios in *Echium* and *Lithospermum*. However, it seems likely that the absolute configuration of a particular derivative is liable to be changed from the *R*-form to the *S*-form, or vice versa by a specific isomerase whose activity might be controlled, not only by genetic factors, but also by the physiological conditions of the cells, as is suggested by the difference in the ratios of enantiomers between the intact plant and cultured cells.

EXPERIMENTAL

Plant material and culture method. Callus tissues used in the present study were originally induced in 1976 from germinating seeds of *E. lycopsis* L. (= *E. plantagineum* L.) on Linsmaier-Skoog basal agar medium [12] containing 10^{-6} M 2,4-D and 10^{-5} M kinetin. The callus tissues were subcultured on this medium containing 10^{-6} M IAA in place of 2,4-D at 1-month intervals for 4 years at 25° in the dark. For studying the effect of light on pigment formation, callus cultures were irradiated continuously throughout the culture period with white (4800 lx), red (620 lx, 610–710 nm), or blue (2300 lx, 380–560 nm) light from fluorescent lamps.

Analysis of naphthoquinone pigments. Extraction and separation of the naphthoquinone pigments from callus tissues were carried out by the methods described elsewhere [1]. Acetyl and β-hydroxyisovaleryl esters of shikonin (or alkannin) isolated from the $CHCl_3$ extracts of callus tissues were identified by comparison (TLC, mp, UV, IR, ^{1}H NMR and CD) with authentic samples. On the other hand, β,β-dimethylacryl, isobutyl and isovaleryl derivatives, which were inseparable from each other by

TLC, were confirmed by GC analysis of the corresponding fatty acids obtained on alkaline hydrolysis of the mixture as reported previously [1]. The quantitative estimation of the pigments was carried out spectrophotometrically according to the methods described earlier [1, 2].

CD of red naphthoquinone pigments. The molecular ellipticity $[\theta]$ in the CD curve is defined by:

$$[\theta] = \frac{[\psi] \cdot MW}{100},$$

where the specific ellipticity $[\psi]$ is defined by:

$$[\psi] = \frac{\psi}{l \cdot c}$$

in which ψ is measured in degrees, l is the path length in decimeters and c is the concn (g/ml) of the soln [13].

For both shikonin and alkannin, a linear relationship was observed between c and ψ, giving the same absolute values for $[\theta]$ and $[\psi]$ at any concn tested. Furthermore, the molecular amplitude,

$$a = \frac{[\theta]_{358} - [\theta]_{308}}{100},$$

for shikonin decreased linearly with the addition of alkannin. Since both shikonin and alkannin have a single asymmetric carbon in their molecules, the molecular amplitude, a, in the CD spectrum of a soln containing both *R*- and *S*-isomers directly gave their ratio in the soln. Accordingly, the percentage of the *R*-isomer or shikonin was estimated by the following equation:

$$\% \text{ shikonin} = 50\left(1 + \frac{a}{125}\right),$$

where the value of 125, the largest molecular amplitude observed among the pigment samples isolated from various tissues, was obtained from an alkaline hydrolysate of the β,β-dimethylacryl derivative from the root of *L. erythrorhizon*; hence this value was considered as that of pure shikonin.

Samples subjected to CD measurement were prepared by the following procedure. Fresh roots or callus tissues were homogenized in a mortar with $CHCl_3$. The $CHCl_3$ extract was dried over $MgSO_4$ and concd under red. pres. to give a dark red residue. The residue was subjected to repeated prep. TLC (Si gel, $CHCl_3$) to separate each naphthoquinone derivative, the purity of which was tested on TLC (Si gel, C_6H_6). Visualization of spots on TLC was carried out by irradiating with UV light (254 and 365 nm), spraying with 50% H_2SO_4 followed by heating at 120° for 4 min. The derivative tested for purity was hydrolysed with 1 N KOH. The hydrolysate was acidified with 1 N HCl and extracted with $CHCl_3$. The extract was dried and concd under red. pres. to give a red pigment, which was purified by repeated prep. TLC (Si gel, $CHCl_3$). Its purity was checked by TLC (Si gel, C_6H_6). The derivative or its hydrolysate was quantitatively estimated by its UV absorption at 520 nm and subjected to CD measurement. The stereochemistry of the β,β-dimethylacryl, isobutyl and isovaleryl esters of the naphthoquinone could not be determined individually because they were inseparable from each other by TLC. Therefore, these esters were subjected to CD measurement as a mixture after alkaline hydrolysis and this mixture was tentatively called 'β,β-dimethylacryl derivative' in the present study. Furthermore, the acetyl derivative isolated from *Lithospermum* cultures was found to be contaminated with a small amount of a neutral yellow pigment specific to the culture strain, so that the following procedure was used to remove the latter from the sample. The mixture of acetyl derivative and yellow pigment was dissolved in Et_2O and the soln shaken at room temp. with 1 N KOH to extract and hydrolyse the acetyl derivative. The aq. layer containing the naphthoquinone was acidified with 1 N HCl and extracted with $CHCl_3$. After evaporation of $CHCl_3$ under red. pres., the residue was subjected to prep. TLC (Si gel, $CHCl_3$) to isolate the red pigment, which was submitted to CD measurement.

Effect of alkaline hydrolysis on the absolute configuration of naphthoquinone derivatives. It was confirmed by the following two expts that the absolute configuration of neither shikonin nor alkannin is changed to the opposite configuration by treating the derivative with alkali or organic solvents. (1) Shikonin (51 mg), obtained by alkaline hydrolysis of the naphthoquinone derivatives isolated from *Lithospermum* cultures, was acetylated with pyridine (2 ml) and Ac_2O (2 ml) to yield a yellow syrup (60 mg). The syrup was purified by repeated prep. TLC (Si gel, $CHCl_3$) to isolate an acetate as an oil (25 mg). UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 242 (4.13) and 355 (3.49); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740–1720, 1638 and 1562; ^1H NMR (60 MHz, $CDCl_3$): δ 2.10 (3H, *s*, MeCOO–) and 2.42 (6H, *s*, MeCOO– × 2). The acetate (20 mg) was hydrolysed with 1 N KOH to yield a red residue which was purified by repeated prep. TLC (Si gel, $CHCl_3$). The red compound isolated was identical with the starting material (TLC, UV-visible, IR and CD). (2) The ratio of shikonin to alkannin for each naphthoquinone derivative isolated from the roots or callus tissues of *E. lycopsis* and *L. erythrorhizon* showed little change after alkaline hydrolysis. Therefore, unless otherwise stated, the CD measurements were carried out after alkaline hydrolysis of each naphthoquinone derivative.

Acknowledgements—We wish to thank Professor K. Hiromi, Faculty of Agriculture, Kyoto University, for allowing us to use an apparatus for CD measurements (Union Giken Ltd.) in his laboratory. We are also grateful to Professor M. H. Zenk, Institut für Pharmazeutische Biologie der Universität München, for providing us with the seeds of *Echium lycopsis*, and to Miss N. Arao for excellent assistance. This work was supported in part by a Grant-in-Aid (No. 337 044) from the Ministry of Education, Science and Culture.

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