Tigloylshikonin, a New Minor Shikonin Derivative, from the Roots and the Commercial Root Extract of *Lithospermum erythrorhizon*

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Tigloylshikonin, a new shikonin derivative esterified with tiglic acid ((*E*)-2-methylbut-2-enoic acid), was isolated as a minor pigment from a food colorant "Shikon color," a commercial root extract from *Lithospermum erythrorhizon* Siebold *et* Zuccarini. The structure of tigloylshikonin was elucidated using ^{1}H, ^{13}C, the difference nuclear Overhauser effect (NOE), and 2D NMR techniques. Its stereochemistry was determined by chiral-phase HPLC analysis. Tigloylshikonin was also found in the roots of *L. erythrorhizon*, which indicated that this new shikonin derivative is a typical component of naphthoquinone pigments in the roots of *L. erythrorhizon*.

Key words *Lithospermum erythrorhizon*; shikonin; tiglic acid; angelic acid; Shikon color

The red pigments from the roots of *Lithospermum erythrorhizon* Siebold *et* Zuccarini (Boraginaceae) have been used in Asia since ancient times as dyestuffs for fabrics, cosmetics, and food and as crude drugs for anti-inflammatory ointment.[1)] The pigments were naphthoquinone natural products, shikonin (**1**)[2)] and its ester derivatives. In many previous studies, various shikonin derivatives, such as those of β-hydroxyisovalerylshikonin (**2**),[3)] acetylshikonin (**3**),[4)] propionylshikonin (**4**),[5)] isobutyrylshikonin (**5**),[6)] β,β-dimethylacryloylshikonin (**6**),[6)] isovalerylshikonin (**7**),[7)] and α-methylbutyrylshikonin (**8**),[7)] were reported from the roots of *L. erythrorhizon* (Fig. 1). Shikonin derivatives have several biological activities such as anti-inflammatory,[8)] antiviral,[9)] antibacterial,[7,10)] antifungal,[9)] and antitumor activities,[11)] and recent studies showed that the level of these activities were affected by the structures of the derivatives.[12)] Therefore, the evaluation of various natural and synthetic shikonin analogues as new drug candidates has been eagerly reported.[1,8,13—15)]

In our continuous study to secure safe for the natural food additives, the commercial root extract of *L. erythrorhizon* used as food colorant (Shikon color) was analyzed using liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS). In addition to shikonin (**1**) and its ester derivatives (**2**—**8**), we found a new shikonin derivative, tigloylshikonin (**9**), as a minor pigment. Here we report for the first time the isolation and structural elucidation of **9** with mass spectrometry and NMR techniques.

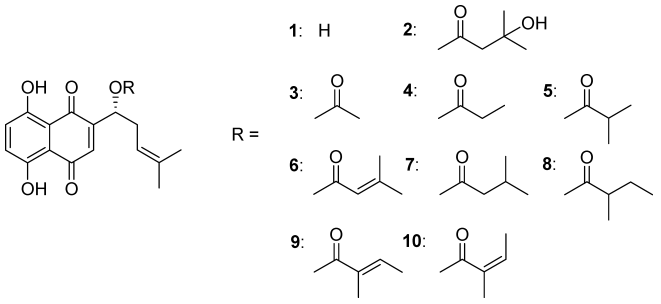

Fig. 1. The Structures of Shikonin (**1**) and Its Ester Derivatives (**2**—**10**)

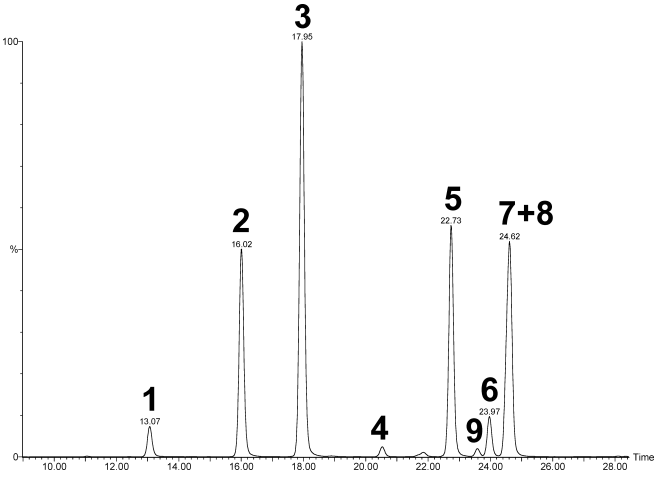

Fig. 2. The Reversed-Phase HPLC Chromatogram Detected at 515 nm of the Commercial Root Extract from *L. erythrorhizon*

Results and Discussion

The commercial root extract of *L. erythrorhizon*, "Shikon color," was diluted with MeOH and subjected to reversed-phase LC/ESI-MS analysis. The chromatogram detected at 515 nm indicated some pigment peaks (Fig. 2), and the positive-mode ESI-MS analysis of each peak showed ion peaks at *m*/*z* 311, 411, 353, 367, 381, 393, and 395 as $[M+Na]^+$, corresponding to shikonin (**1**), β-hydroxyisovalerylshikonin (**2**), acetylshikonin (**3**), propionylshikonin (**4**), isobutyrylshikonin (**5**), β,β-dimethylacryloylshikonin (**6**), and isovalerylshikonin (**7**) with α-methylbutyrylshikonin (**8**), respectively. Except for **7** and **8**, each compound was purified with preparative HPLC and its structure was identified with NMR experiments. Because **7** and **8** could not be clearly separated on HPLC, both compounds were purified and their structures elucidated as a mixture.

A minor shikonin derivative (**9**) was found between the peaks of isobutyrylshikonin (**5**) and β,β-dimethylacryloylshikonin (**6**) on the chromatogram (Fig. 2). The positive ESI-MS spectrum of **9** showed the ion peak at *m*/*z* 393 as $[M+Na]^+$, which was the same as that of **6**. Therefore, it was presumed that **9** was a structural isomer of **6** and a derivative esterified with tiglic acid ((*E*)-2-methylbut-2-enoic acid; *cis* form) or angelic acid ((*Z*)-2-methylbut-2-enoic acid; *trans* form) as a substitution of β,β-dimethylacrylic acid of **6**. The ^{1}H-NMR spectrum of **9** recorded in $CDCl_3$ showed the set of resonances about shikonin (**1**) as well as other derivatives. In

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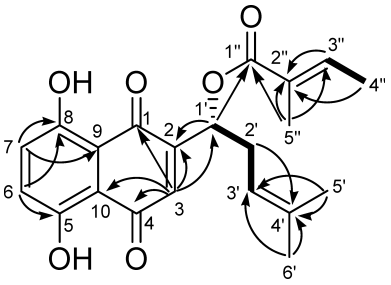

Fig. 3. Characteristic Correlations Observed in ^{1}H–^{1}H COSY (Bold Lines) and ^{1}H–^{13}C HMBC (Arrows) of **9**

addition, a doublet methyl signal at δ_H 1.83 ppm (H-4″), a singlet methyl signal at δ_H 1.86 ppm (H-5″), and a multiplet 1H signal at δ_H 6.97 ppm (H-3″) were observed. The shikonin moiety in **9** was confirmed by the ^{1}H–^{1}H correlation spectroscopy (COSY) and ^{1}H–^{13}C heteronuclear multiple bond coherence (HMBC) correlations (Fig. 3). The ^{1}H–^{1}H COSY experiment of **9** indicated the spin couplings between H-3″ and H-4″ and the ^{1}H–^{13}C HMBC experiment of **9** revealed the structure of a 2,3-dimethylacrylic acid moiety by the correlations from the H-5″ signal to the δ_C 167.1 ppm (C-1″), δ_C 128.3 (C-2″) and δ_C 138.6 (C-3″) signals as well as by the correlation from the H-3″ and H-4″ signals to the C-2″ signal (Fig. 3). The geochemistry of the acid was determined in the *cis* form (tiglate) by in the difference nuclear Overhauser effect (NOE) experiments between H-3″ and H-5″ and between H-4″ and H-5″. The chemical shift at H-3″ of **9** (δ_H 6.97 ppm) also supported this interpretation, because previous studies reported the chemical shift at H-3 of angelate was 6.08 ppm,[16] whereas that of tiglate was 6.95 ppm.[17] Finally, the chiral HPLC analysis of the saponificates of **9** revealed that the naphthoquinone structure of **9** was composed of 96.5% of **1** and 3.5% of alkannin, an enantiomer of **1**.[18] Recent studies showed that naphthoquinone pigments in the roots of Boraginaceae plants are a mixture of optical isomers, **1** and alkannin, and that the ratio of isomers varies with the species of plant.[1] In the case of *L. erythrorhizon*, it is known that the shikonin type is a major isomer. Thus, the structure of **9** was established as tigloylshikonin.

To confirm that tigloylshikonin (**9**) was not an artifact in commercial products, the roots of *L. erythrorhizon* as a crude traditional drug, designated as "Ko-shikon," were extracted with ethanol and the extract was subjected to LC/ESI-MS analysis. The obtained profile of shikonin derivatives in the extract was the same as that in the food colorant, and the presence of **9** was also observed. The purification of **9** from the extract was carried out in the same manner, and the structural identification of **9** was achieved by ^{1}H-NMR analysis. Thus, the results demonstrated that **9** was a typical shikonin derivative in the roots of *L. erythrorhizon*.

In the past, angeloylshikonin (**10**), a geometrical isomer of tigloylshikonin (**9**), was reported as a major pigment from the roots of *Alkanna hirsutissima* collected in northern Iraq.[19] As the optical isomer of **10**, angeloylalkannin was also reported from the roots of *Alkanna tinctoria*, a common source of alkannin derivatives.[20] However, there are no reports on the isolation and structural elucidation of **9** from any Boraginaceae plants, although HPLC analysis recently suggested the presence of **9** in addition to **10** in the roots of *Echium italicum* collected in Macedonia.[21] The root extract of *L. erythrorhizon* has been traditionally used as dye and crude drag in Japan, and the pigment constitution in it has been eagerly studied ever since Japanese scientists first purified shikonin (**1**) as its acetate (**3**) from the roots of *L. erythrorhizon*.[22] Therefore, the discovery of a new shikonin derivative was surprising and suggested the presence of unidentified minor pigments in the roots of this species.

Experimental

Instruments The NMR spectra were recorded on an ECA-500 spectrometer (JEOL, Tokyo, Japan). LC/ESI-MS analysis was performed on a Waters system (Waters, Milford, MA, U.S.A.) equipped with an Alliance 2695 (LC), a 2996 photodiode array (PDA) detector, and a Quattro micro (quadrupole ESI-MS). High-resolution mass spectrometry analysis was performed on LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, U.S.A.). HPLC purification was performed on a Shimadzu LC-10ADvp (Shimadzu, Kyoto, Japan) equipped with a SPD-M10Avp PDA detector. The IR spectrum was recorded on a Fourier transform (FT)/IR-4100 (JASCO, Tokyo Japan). The UV spectra were obtained in MeOH on a V-650 spectrophotometer (JASCO, Tokyo, Japan).

Standard Samples and Materials The shikonin standard sample (99%) and the mixture of optical isomers (shikonin : alkannin = about 6 : 1) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The commercial root extract of *L. erythrorhizon*, or Shikon color, was obtained from a food company in Japan. The commercial extract was prepared by the root extraction of *L. erythrorhizon* with ethanol, following evaporation. A crude drug "Ko-shikon" (the chipped roots of *L. erythrorhizon*) was purchased from a crude-drug company in Tokyo, which had collected in China.

LC/ESI-MS Analysis The commercial root extract (500 mg) was diluted with MeOH (10 ml) and filtrated with a polyvinylidene difluoride (PVDF) membrane syringe filter (pore size = 0.45 μm, Whatman International, Piscataway, NJ, U.S.A.). The crude drug "Ko-shikon" (50 g) was extracted with EtOH (500 ml) and the extract was filtrated. Each aliquot (10 μl) of both samples was injected to the LC/ESI-MS system. A COSMOSIL 5C_{18}-MS-II column (4.6 mm i.d. × 150 mm, Nacalai Tesque, Kyoto, Japan) was employed for separation at 40 °C. As the mobile phase, H_2O (solvent A) and MeOH (solvent B) were used. The gradient condition was linear from 50 to 100% of solvent B in solvent A for 25 min, followed by an isocratic condition of 100% (solvent B) continuously for 10 min. The flow rate was 0.5 ml/min. UV absorption was recorded at 515 nm on PDA. The retention times of **1**, **2**, **3**, **4**, **5**, **6**, **7** with **8** and **9** were 13.07, 16.02, 17.96, 20.56, 22.73, 23.97, 24.62 and 23.57 min, respectively. The ESI-MS was operated in positive ion mode of SCAN analysis. The source temperature was 120 °C and cone nitrogen gas flow was 50 l/h. Desolvation nitrogen gas flow was 500 l/h and desolvation temperature was 400 °C. The capillary voltage was 4.25 kV and the cone voltage was 30 V.

The Purification of Shikonin Derivatives (1—9) The commercial extract (2.0 g) was diluted with MeOH (20 ml) and purified with a reversed-phase HPLC to afford **1** (8.5 mg), **2** (24.7 mg), **3** (34.2 mg), **4** (3.7 mg), **5** (22.3 mg), **6** (14.3 mg), **7** with **8** (19.8 mg) and **9** (3.5 mg). A COSMOSIL 5C_{18}-MS-II column (20 mm i.d. × 250 mm) was employed for separation at 40 °C. As an eluent, H_2O containing 0.05% (v/v) trifluoroacetic acid (TFA) (solvent A) and acetonitrile containing 0.05% (v/v) TFA (solvent B) were used. The gradient condition was linear from 70 to 100% of solvent B in solvent A for 10 min, and then an isocratic condition of 100% (solvent B) was continuous for 5 min. The flow rate was 9.0 ml/min. UV absorption was recorded at 515 nm on PDA. Compounds (**1**—**9**) were manually collected. The red fraction containing shikonin derivatives were evaporated to remove volatile and then diluted with H_2O to settle the red pigments, which were extracted with EtOAc. The red EtOAc fraction was dried with anhydrous Na_2SO_4 and concentrated to dryness *in vacuo*. The obtained shikonin derivatives were stored at −20 °C.

Shikonin (1) Red purple solid. ESI-MS (positive mode) *m/z*: 311 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.65 (3H, s, H-6′), 1.76 (3H, s, H-5′), 2.37 (1H, m, H-2′), 2.63 (1H, m, H-2′), 4.91 (1H, m, H-1′), 5.20 (1H, m, H-3′), 7.04 (1H, s, H-3), 7.18 (1H, d, J=9.7 Hz, H-6 or H-7), 7.22 (1H, d, = 9.7 Hz, H-6 or H-7), 12.5 (1H, s, OH-5), 12.5 (1H, s, OH-8).

β-Hydroxyisovalerylshikonin (2) Red purple solid. ESI-MS (positive mode) *m/z*: 411 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.31 (3H, s, H-4″), 1.33 (3H, s, H-5″), 1.59 (3H, s, H-6′), 1.69 (3H, s, H-5′), 2.52 (1H, m, H-2′), 2.61 (1H, m, H-2′), 2.61 (2H, s, H-2″), 5.11 (1H, m, H-3′), 6.10 (1H, dd,

J=6.9, 4.6 Hz, H-1′), 7.03 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.4 (1H, s, OH-5), 12.6 (1H, s, OH-8).

Acetylshikonin (3) Red purple solid. ESI-MS (positive mode) *m/z*: 353 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.57 (3H, s, H-6′), 1.68 (3H, s, H-5′), 2.13 (3H, s, H-2″), 2.46 (1H, m, H-2′), 2.60 (1H, m, H-2′), 5.11 (1H, t, J=7.5 Hz, H-3′), 6.01 (1H, dd, J=6.9, 5.2 Hz, H-1′), 6.98 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.4 (1H, s, OH-5), 12.4 (1H, s, OH-8).

Propionylshikonin (4) Red purple solid. ESI-MS (positive mode) *m/z*: 367 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.57 (3H, s, H-6′), 1.68 (3H, s, H-5′), 2.40 (1H, d, J=7.7 Hz, H-2″), 2.41 (1H, d, J=7.7 Hz, H-2″), 2.48 (1H, m, H-2′), 2.59 (1H, m, H-2′), 5.11 (1H, t, J=9.4 Hz, H-3′), 6.02 (1H, dd, J=6.3, 4.6 Hz, H-1′), 6.97 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.42 (1H, s, OH-5), 12.4 (1H, s, OH-8).

Isobutyrylshikonin (5) Red purple solid. ESI-MS (positive mode) *m/z*: 381 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.20 (3H, d, J=6.8 Hz, H-3″), 1.20 (3H, d, J=6.8 Hz, H-4″), 1.58 (3H, s, H-6′), 1.68 (3H, s, H-5′), 2.48 (1H, m, H-2′), 2.60 (1H, m, H-2′), 2.61 (1H, m, H-2″), 5.11 (1H, t, J=7.4 Hz, H-3′), 6.01 (1H, dd, J=7.4, 4.6 Hz, H-1′), 6.97 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.43 (1H, s, OH-5), 12.43 (1H, s, OH-8).

β,β-Dimethylacryloylshikonin (6) Red purple solid. ESI-MS (positive mode) *m/z*: 393 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 1.57 (3H, s, H-6′), 1.68 (3H, s, H-5′), 1.93 (3H, s, H-5″), 2.15 (3H, s, H-4″), 2.48 (1H, m, H-2′), 2.60 (1H, m, H-2′), 5.14 (1H, dd, J=6.9, 5.7 Hz, H-3′), 5.78, (1H, s, H-2″), 6.00 (1H, dd, J=6.9, 5.8 Hz, H-1′), 6.97 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.43 (1H, s, OH-5), 12.43 (1H, s, OH-8).

Isovalerylshikonin (7) Red purple solid. ESI-MS (positive mode) *m/z*: 395 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 0.96 (3H, d, J=2.3 Hz, H-4″), 0.97 (3H, d, J=2.3 Hz, H-5″), 2.12 (1H, m, H-3″), 2.26 (2H, m, H-2″), 1.59 (3H, s, H-6′), 1.68 (3H, s, H-5′), 2.45 (1H, m, H-2′), 2.59 (1H, m, H-2′), 5.12 (1H, t, J=6.3 Hz, H-3′), 6.03 (1H, m, H-1′), 6.98 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.42 (1H, s, OH-5), 12.42 (1H, s, OH-8).

α-Methylbutyrylshikonin (8) Red purple solid. ESI-MS (positive mode) *m/z*: 395 $[M+Na]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 0.92 (3H, t, J=7.5 Hz, H-4″), 1.16 (3H, d, J=6.9 Hz, H-5″), 1.52 (1H, m, H-3″), 1.58 (3H, s, H-6′), 1.68 (3H, s, H-5′), 1.70 (1H, m, H-3″), 2.45 (1H, m, H-2′), 2.46 (1H, m, H-2″), 2.59 (1H, m, H-2′), 5.12 (1H, t, J=6.3 Hz, H-3′), 6.03 (1H, m, H-1′), 6.98 (1H, s, H-3), 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.42 (1H, s, OH-5), 12.42 (1H, s, OH-8).

Tigloylshikonin (9) Red purple solid. IR (KBr) ν_{max} cm^{-1}: 1715, 1610, 1455, 760. ESI-MS (positive mode) *m/z*: 393 $[M+Na]^+$, HR-ESI-MS (positive mode) *m/z*: 371.14968 $[M+H]^+$ (Calcd for $C_{21}H_{23}O_6$, 371.14946). UV–Vis. λ_{max} (MeOH) nm (log ε): 560 (3.6), 515.5 (3.8), 485 (3.6), 275.5 (3.9). ^{1}H-NMR ($CDCl_3$) δ: 1.57 (3H, s, H-6′), 1.68 (3H, s, H-5′), 1.83 (3H, d, J=8.0 Hz, H-4″), 1.86 (3H, s, H-5″), 2.53 (1H, m, H-2′), 2.64 (1H, m, H-2′), 5.14 (1H, t, J=8.0 Hz, H-3′), 6.04 (1H, dd, J=6.3, 5.1 Hz, H-1′), 6.95 (1H, s, H-3), 6.97 (1H, m, H-3″) 7.18 (1H, s, H-6), 7.18 (1H, s, H-7), 12.39 (1H, s, OH-5), 12.43 (1H, s, OH-8), ^{13}C-NMR ($CDCl_3$) δ: 12.2 (C-4″), 14.6 (C-5″), 18.1 (C-5′), 25.9 (C-6′), 33.0 (C-2′), 69.6 (C-1′), 111.7 (C-10), 111.9 (C-9), 117.9 (C-3′), 128.3 (C-2″), 131.5 (C-3), 132.6 (C-7), 132.8 (C-6), 136.1 (C-4′), 138.6 (C-3″), 149.0 (C-2), 166.5 (C-5), 166.8 (C-8), 167.1 (C-1″), 177.4 (C-1), 178.9 (C-4).

Chiral Analysis of the Saponificates of 9 Chiral analysis was based on the manner reported previously.[18] Briefly, the purified **9** (0.5 mg) was dissolved in MeOH (1 ml), and to this 1 M NaOH (3 ml) was added. After stirring for 12 h at room temperature, 1 M HCl (3 ml) was added for neutralization and extracted with $CHCl_3$ (10 ml), which was dried and evaporated *in vacuo*. The residue was dissolved in MeOH (1 ml) and subjected to reversed-phase LC/ESI-MS analysis as described above to check the achievement of saponification of **9**. Subsequently, the saponificate of **9** was subjected to chiral-phase HPLC analysis to determine the ratio of shikonin to alkannin on the peak areas of both compounds. For the chiral column, a Chiralcel-OJ-H column (4.6 mm i.d.×250 mm, Daicel Chemical Industry, Osaka, Japan) was used at 40 °C. For the mobile phase, *n*-hexane/2-propanol/acetic acid (95 : 5 : 0.3, v/v) was used. The flow rate was 1.0 ml/min. The UV absorption was recorded at 515 nm on PDA. The retention times of shikonin and alkannin were 12.3 min and 14.7 min, respectively.

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