New 3-Methoxyflavones in the Roots of Yellow Lupin
(Lupinus luteus L. cv. Topaz)

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An isopropenyl (=3,3-dimethylallyl) 3-methoxyflavone (1) and its hydrate (5) were isolated from the roots of yellow lupin, Lupinus luteus L. cv. Topaz. Their structures were unambiguously determined to be 5,7,4'-trihydroxy-3-methoxy-6-(3,3-dimethylallyl)flavone (1) and 5,7,4'-trihydroxy-6-(3-hydroxy-3-methylbutyl)-3-methoxyflavone (5) by a combination of chemical and spectroscopic methods, and the new flavones were named topazolin and topazolin hydrate, respectively.

Antifungal tests against the growth of Cladosporium herbarum indicated that, in spite of its phenolic nature and the possession of an isopentenyl sidechain, topazolin (1) had only weak fungitoxic activity.

During the course of earlier studies,1,2 the roots of yellow lupin, Lupinus luteus L. were found to contain numerous simple and complex isoflavones, many of which possessed fungitoxic properties.3,4 On silica gel thin-layer plates sprayed with Gibbs reagent and fumed with NH₃, all of those Gibbs-test-positive compounds exhibited a rather bluish color (clear blue, purple-blue, blue-green or dull blue).4,5 However, some column chromatographic fractions of the yellow lupin root extractives were found to contain an unknown compound which was located between luteone3 [5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone] and wighteone (=2'-deoxy-luteone) on silica gel TLC plates developed in CHCl₃-acetone-conc. ammonia water (CAAm)=35:30:1, and which gave a noticeably clear purple Gibbs test color. The unknown compound named topazolin (1) in the 30% EtOAc in benzene eluate from the first column chromatography was eventually isolated by preparative TLC (PTLC) in CAAm, and subsequently purified by re-PTLC in benzene-EtOAc=4:1. The content of 1 was approximately estimated to be 10 mg/kg of the fresh roots of L. luteus cv. Topaz, and the presence of this compound in the roots of L. luteus cv. Barpine was also confirmed.

Topazolin (1), with a molecular formula C₂₁H₂₀O₆ (HR-MS, M⁺ 368.125), on silica gel TLC plates viewed under UV₃₆₅ nm light exhibited a dark fluorescence, as did 5-hydroxylated isoflavone which fluoresced dark red. Although the presence of C-5-OH was confirmed by UV spectroscopy (a bathochromic shift of the methanolic UV maxima at 272 nm by 8 nm in the presence of AlCl₃)6 and ¹H-NMR detection of a lower-field signal at δ 7.8-8.1 that was assignable to C-2-H of isoflavone7 was observed. The flavone skeleton for 1 was deduced from the positive result of a Shinoda test,8 which gave an anthocyanidin product with a decisive reddish color if the test compound did not have an isoflavonoidal but flavonoidal structure.

The UV spectroscopic evidence that the methanolic UV maxima of 1 were shifted bathochromically by the addition of NaOAc (λmax at 272 nm → 274 nm and λmax at 336 → 339 nm → 380 nm), and that these shifts were reversible with H₃BO₃, inferred the pres-
ence of another hydroxyl group at C-7.6) A set of aliphatic \( ^1 \text{H-NMR} \) signals \([\delta 1.65 \text{ and } 1.79 \text{ (both 3H, two s)}\], 3.36 (2H, br. d, \( J = 7.3 \text{ Hz} \)) and 5.28 (1H, br. t, \( J = 7.3 \text{ Hz} \)]) was characteristic of an isopentenyl group,3,4) and the remaining \( ^1 \text{H-NMR} \) absorptions were assigned to a methoxy group \([\delta 3.86 \text{ (3H, s)}\], an isolated aromatic proton \([\delta 6.56 \text{ (1H, s)}\]) and 1,4-disubstituted benzene protons \([\text{as the B-ring of flavone, } \delta 7.01 \text{ (2H, br. d, } J = 9.0 \text{ Hz, 3'}- \text{ and } 5'-\text{H} \) and 8.01 (2H, br. d, \( J = 9.0 \text{ Hz, 2'}- \text{ and } 6’-\text{H} ))\]. Together with the \( ^1 \text{H-NMR} \) evidence, the part structure of the 4’-hydroxylated B-ring became feasible by MS detection of a B-ring fragment characteristic of flavone at \( m/z \) 121 (Fig. 1).9)

Recently, Voirin has reported a systematic and effective method for UV spectroscopic differentiation of 5-hydroxy- and 5-hydroxy-3-methoxyflavones with mono-(4’), di-(3’,4’) or tri-(3’,4’,5’)-substituted B-rings.10) According to his criteria, topazolin (1) falls into the group consisting of 6-O- or 6-C-alk(en)yl-3-methoxyflavones. Furthermore, the relatively large value of \([\Delta \text{max} - \Delta \text{min}] / \Delta \text{max} = 269.5 \text{ nm shifted (+4 nm) in the presence of NaOAc (C-7-OH free), but was unchanged by the addition of AlCl}_3 (C-5-OH converted)\].

The acid-catalyzed cyclization of 1 by heating in 88% HCOOH11) yielded approximately equal amounts of \( \alpha \)-isotopazolin (4a) [an angular-type chromane derivative, whose UV \( \lambda_{\text{max}} \) at 269.5 nm was shifted (+4 nm) in the presence of NaOAc (C-7-OH free), but was unchanged by the addition of AlCl\(_3\) (C-5-OH converted)] and \( \beta \)-isotopazolin (4b) [a linear-type chromane derivative, whose UV \( \lambda_{\text{max}} \) at 271 nm was shifted (+9 nm) in the presence of AlCl\(_3\) (C-5-OH free), but was unchanged by the addition of NaOAc (C-7-OH converted)]. The result apparently indicates that the isopentenyl group must be placed at C-6 of the A-ring (ortho to both C-5- and C-7-OH).

The Gibbs-test-positive nature of 1 already mentioned requires C-8 (\( \text{para to C-5-OH} \)) to be unsubstituted, because the remaining phenolic OH’s at C-4’ and C-7 were both unreactive with Gibbs reagent. The methoxy group was thus located not at C-8 but at C-3. A singlet proton detected at \( \delta 6.56 \) was finally assigned to C-8-H (cf. C-3-H in flavone: near \( \delta 6.37 \)). The whole structure 1 in Fig. 2 for topazolin was thus unambiguously concluded.
The second compound exhibiting a purplish Gibbs test color was found in the more polar fraction eluted with 55% EtOAc in benzene from the silica gel column, and subsequently isolated by PTLC. The topazolin hydrate structure (5) for the second isolate was immediately apparent by the UV, MS and ¹H-NMR analyses. First, the UV spectroscopic properties closely resembling those of 1 were indicative of a 6-substituted isokaempferide structure for 5, as with 1. Secondly, the MS of 5 [M⁺ 386.133 (C₂₁H₂₂O₇=1+H₂O); fragments m/z 368 (M⁺-H₂O), 325 (M⁺-H₂O-C₃H₇) and 313 (M⁺-H₂O-C₄H₇) characteristic of flavonoids containing a 3-hydroxy-3-methylbutyl part structure, and m/z 121 (from the B-ring as shown in Fig. 1)] and the ¹H-NMR signals for aliphatic protons of 5 at δ 1.26 (6H, s, two methyl groups on a carbinol carbon), 1.63~1.79 (2H, m, a methylene beta to OH and the benzene ring) and 2.70~2.86 (2H, m, a methylene alpha to the benzene ring) showed the presence of a 3-hydroxy-3-methylbutyl part structure¹,¹² in 5 in place of the isopentenyl sidechain of 1.

The chemical shift values of the aromatic protons of 5 were expectedly quite similar to those of 1, in accordance with our earlier observations¹,¹²) that hydration at the isopentenyl sidechain of the flavonoid hardly affected the chemical shift value of the aromatic proton. Other properties of the second compound (UV₃₆₅ nm fluorescence: dull black; Gibbs test: (+); slow response; and Shinoda test: (+), reddish pink) are well compatible with the deduced structure (5).

Seven or more C-alk(en)yl 3-methoxyflavones have been reported so far,¹⁰,¹³) and three of these are 5,7,4'-trihydroxy-3-methoxyflavone (=isokaempferide) derivatives, i.e., an 8-methyl derivative (sylpin) from Pinus silvestris (Pinaceae),¹⁴) and 6-methyl¹⁵) and 6,8-dimethyl derivatives from Alluaudia dumosa (Didiereaceae).¹³) However, topazolin (1) and its hydrate (5) are the first examples of the isopentenyl or the modified isopentenyl isokaempferides.

The antifungal activity of 1 was determined by means of TLC plate bioassays,¹²,¹⁶) using Cladosporium herbarum AHU 9262 as the test fungus, and the results were compared with that of the antifungal isoflavone luteone.³) Less than 2 μg of luteone was sufficient to affect the fungal growth, whilst 1 revealed considerably less activity than luteone. Although a growth retardation effect of 1 on the fungus was
observed at applied levels of 25, 50 and 100 μg, the growth inhibition was incomplete even at the highest dose level.

**EXPERIMENTAL**

**General methods.** Melting points (mp) were determined by the micro hot-plate method and are uncorrected.

**Instrumentation:** Mass spectra were obtained on a JEOL JMS-D300X instrument (direct inlet system, 70 eV ionization potential), and 1H-NMR spectra on a JEOL FX-100 spectrometer. UV spectra were recorded according to the procedure presented by Mabry et al., by using a Hitachi EPS-3T instrument.

**TLC:** Analytical TLC and PTLC separations were carried out on Merck pre-coated Silica Gel 60 plates (F254; layer thickness, 0.25 mm or 0.5 mm). Flavone derivatives were detected by inspecting developed TLC/PTLC plates under long (365nm) and short (254nm) wavelength UV light, and by the characteristic colors formed with Gibbs reagent. The compounds of interest were eluted from the silica gel with EtOAc.

**Gibbs test:** See refs. 4 and 5 for details.

**Antifungal test.** The antifungal activity of topazolin (1) against Cladosporium herbarum AHU 9262 was examined by using the TLC plate bioassay method outlined in our earlier paper. An antifungal isoflavone luteone which gave complete inhibition zones at dose levels of 25, 50 and 100 μg (each dose/14 mm dia. zone of the silica gel layer, thickness 0.25 mm) was used as a reference compound.

**Shinoda test:** The test compound (ca. 200 μg) was transferred in MeOH (0.1 ml) to a specimen tube, and two small Mg metal granules were added before conc. HCl (1 drop). Rapid effervescence accompanied by the development of a deep reddish pink color indicates that the compound is a flavone derivative.

**Isolation of 3-methoxyflavones.** The defatted neutral and phenolic fraction (ca. 88 g) prepared from the methanolic extracts of yellow lupin roots (*Lupinus luteus* L. cv. Topaz, 12.95 kg) was initially subjected to column chromatography over silica gel (500 g). The charged constituents were successively eluted with benzene, and benzene plus increasing amounts of EtOAc. After eluting with benzene, and 5% and 15% EtOAc in benzene (1 liter each), the successive eluate with 30% EtOAc in benzene (1 liter) was collected as three equi-volume fractions denoted by YF-7, YF-8 and YF-9. A new flavone, topazolin (1) was detected in both YF-8 and YF-9. TLC Rf values in CHCl3-acetone-conc. ammonia water (CAAm)=35: 30: 1 for luteone, 1 and wighteone were 0.21, 0.30 and 0.48, respectively. Although the amount of 1 in YF-8 was approximately equal to that in YF-9, the former fraction was used in the present study to isolate 1, because of its relatively high concentration of 1. After the separation of luteone by crystallization, the residue of YF-8 was subjected to PTLC in CAAm (35: 30: 1) to separate I. Crude I was purified by re-PTLC in EtOAc–benzene = 1: 4 using the multiple development technique (x 3), and the eluate from the silica gel with EtOAc was concentrated to give 51 mg of 1.

The more polar constituents in the root extract were successively eluted from the column with 40% EtOAc in benzene (ca. 330 ml x 3; YF-10, YF-11 and YF-12), and 55% EtOAc in benzene (ca. 330 ml x 3; YF-13, YF-14 and YF-15). The second flavone showing a purplish Gibbs test color was found in the latter eluate. The concentrated YF-15 (2.5) was further fractionated by PTLC using the following solvent systems successively: CAAm (70: 60: 1, Rf 0.08), CHCl3–methyl ethyl ketone (1: 1, Rf 0.53) and hexane-acetone–n-BuOH (8: 1: 1, Rf 0.37) to yield 5.3 mg of topazolin hydrate (5).

**Topazolin (1).** Pale yellow rods from acetone/EtOAc, mp 227 ~ 228.5°C. UV365 nm fluorescence: dull black and unchanged with weak NH3 fuming, but irreversively turning yellow with strong NH3 fuming. Gibbs test: (+), the corresponding spot on TLC plates became brownish yellow immediately after spraying by Gibbs reagent, and the color gradually turned clear purple under NH3 vapor. Shinoda test: (+), rapidly gave a reddish pink color. HR-FABMS: M+ 368.125 (C21H20O6 requires 368.126). El-MS (350°C) m/z (%): 369 (M+ +1, 15), 368 (M+, 63), 367 (10), 353 (M+–C2H4, 16), 326 (18), 325 (M+–C2H5, 86), 314 (20), 313 (M+–C2H5, 100), 311 (13), 310 (17), 169 (10), 121 (24). UV λmax nm (ε or rel. int.): 215.5 (31,100), 272 (25,600), 290sh (15,500), 337 (br. 19,000); +NaOMe, 223sh (br. 100), 277 (93), 330 (br. 52), 401 (91); +AlCl3, 236 (100), 280 (69), 308 (55), 362 (67), 402sh (34) with the spectrum unchanged by further addition of HCl; +NaOAc, 274 (100), 297sh (br. 53), 337sh (45), 380 (38) with the MeOH spectrum regenerated by the addition of H2BO3. 1H-NMR δH 6.0 (100 MHz): 1.65 (3H, d, J=1.0Hz, 5'-H3), 1.79 (3H, s, 4'-H3), 3.36 (2H, br. d, J=7.3Hz, 1'H3), 3.86 (3H, s, 3-OCH3), 5.28 (1H, br. t, J=7.3Hz, 2'H), 6.56 (1H, s, 8-H), 7.01 (2H, br. d, J=9.0Hz, 3' and 5'-H), 8.01 (2H, br. d, J=9.0Hz, 2'- and 6'-H), 13.08 (s, 5-OH).

**Derivation of topazolin (1).**

a) **Acetylation of 1.** Topazolin (1, 5 mg) was treated with acetic anhydride–pyridine (1: 1, 1 ml) for 2 hr at 80°C. The reaction mixture was diluted with tolune and concentrated in vacuo. The two major products were purified by PTLC in CHCl3–MeOH (CM)=50: 1.

**Topazolin diacetate (2a):** Silica gel TLC Rf 0.39 in hexane–EtOAc (HE)=4: 1. Pale yellow needles (0.8 mg), mp 140 ~ 141°C. UV365 nm fluorescence: dull black. Gibbs test: (+), slow, blue-green. MS m/z (%): 452 (M+, 23), 409 (30), 397 (14), 368 (12), 367 (53), 355 (44), 337 (12), 325 (31), 313 (26), 121 (30), 69 (13), 43 (100). UV λmax nm:
Gibbs test: (+), slow, blue-green. MS m/z (%): 397 (10 ml). The combined extracts were successively washed solved in acetone (10 ml), to which was added 200 mg of br.d., 7 = 9.0 Hz, 2'- and 6'-H). br.d., 7 = 9.0 Hz, 3'- and 5'-H), 7.46 (1H, s, 8-H), 8.18 (2H, OCOCH₃), 3.32 (2H, br.d, 7 = 7.1 Hz, 1'-H₂), 3.84 (3H, s, 3-OCH₃), 5.16 (1H, br.t, 7 = 7.1 Hz, 2''-H), 7.00 (1H, s, 4'-H), 1.79 (3H, s, 4''-H₃), 3.39 (2H, br.d, 7 = 6.8 Hz, 5''-H₃), 1.79 (3H, s, 4''-H₃), 1.63-1.79 (2H, m, 2''-H₂), 3.79 (3H, s, 3-OCH₃), 6.55 (1H, s, 8-H), 6.96 (2H, br d, J = 8.9 Hz, 3'- and 5'-H'), 8.02 (2H, br.d, J = 8.8 Hz, 2' and 6'-H).

β-Isotopazolin (4b): Colorless needles (4.1 mg), mp 259 ~ 260°C. UV 365 nm fluorescence: dull black; and Gibbs test: (+), slow, dull blue. MS m/z (%): 369 (M+ 1, 25), 368 (M+ 100), 367 (45), 349 (19), 325 (29), 314 (18), 313 (84), 312 (35), 311 (23), 283 (11), 270 (13), 269 (36), 121 (59), 15 (13). UV λ max nm: 214, 226, 263, 271, 297 sh, 308 sh, 340 br.), 350 sh; +NaOMe, 234, 262 sh, 271, 306 sh, 397; +AlCl₃, 213, 235, 280, 303 sh, 310.5, 360, 399 sh. The MeOH spectrum was unchanged by the addition of AlCl₃. 1H-NMR δ TMS 100% (100 MHz): 1.37 (6H, s, 4'- and 5'-H'), 1.82 (2H, t, J = 6.8 Hz, 2''-H₂), 2.70 (2H, t, J = 6.8 Hz, 1'-H₂), 3.87 (3H, s, 3-OCH₃), 6.41 (1H, s, 8-H), 7.02 (2H, br d, J = 9.0 Hz, 3' and 5'-H'), 8.04 (2H, br.d, J = 9.0 Hz, 2' and 6'-H). 13.13 (s, 5-OH).

Topazolin hydrate (5): Pale yellow rods from EtOAc/ hexane, mp 239 ~ 241°C. UV 365 nm fluorescence: dull black. Gibbs test: (+), slow, brown → yellow-green → purple. Shinoda test: (+), rapidly gave a reddish pink color. HR-MS: M+ 386.133 (C₂₁H₂₂O₇ requires 386.136). EI-MS (280°C) m/z (%): 387 (M+ 1, 72.8), 386 (M+ 34), 371 (7.8), 368 (14), 367 (9.1), 353 (7.3), 328 (8.7), 327 (27), 326 (12), 325 (41), 314 (18), 313 (100), 312 (21), 311 (14), 297 (7.5), 270 (8.1), 269 (12), 123 (11), 121 (28), 105 (7.6), 93 (9.4), 69 (13), 65 (9.1), 59 (15). UV λ max nm (rel. int.): 214.5 (100), 263 sh, 271.5 (67), 285 ~ 310 (br. 42), 339 (64); +NaOMe, 236 (88), 276.5 (92), 313 sh (51), 330 (58), 398 (100); +AlCl₃, 236 (100), 262 sh (71), 282 (75), 308 (61), 363 (83), 402 sh (43) with further addition of HCl not changing the spectrum; +NaOAc, 274 (100), 301 (54), 314 sh (49), 376 (72) with further addition of H₃BO₃ regenerating the MeOH spectrum. 1H-NMR δ TMS 100% (100 MHz): 1.26 (6H, s, 4''- and 5'-H'), 1.63 ~ 1.79 (2H, m, 2''-H₂), 2.70 ~ 2.86 (2H, m, 1'-H₂), 3.87 (3H, s, 3-OCH₃), 6.54 (1H, s, 8-H), 7.01 (2H, d, J = 8.9 Hz, 3' and 5'-H'), 8.02 (2H, br.d, J = 8.8 Hz, 2' and 6'-H). 13.07 (s, 5-OH).

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