

HOKKAIDO UNIVERSITY

Title	Use of P450 cytochrome inhibitors in studies of enokipodin biosynthesis
Author(s)	Ishikawa, Noemia Kazue; Tahara, Satoshi; Namatame, Tomohiro; Farooq, Afgan; Fukushi, Yukiharu
Citation	Brazilian Journal of Microbiology, 44(4), 1285-1290
Issue Date	2014
Doc URL	http://hdl.handle.net/2115/56115
Rights(URL)	http://creativecommons.org/licenses/by-nc/3.0/
Туре	article
File Information	bmj-44-4-1285.pdf



Research Paper

Use of P450 cytochrome inhibitors in studies of enokipodin biosynthesis

Noemia Kazue Ishikawa¹, Satoshi Tahara², Tomohiro Namatame², Afgan Farooq², Yukiharu Fukushi²

¹Division of Environmental Resources, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan. ²Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan.

Submitted: November 27, 2011; Approved: April 4, 2013.

Abstract

Enokipodins A, B, C, and D are antimicrobial sesquiterpenes isolated from the mycelial culture medium of *Flammulina velutipes*, an edible mushroom. The presence of a quaternary carbon stereocenter on the cyclopentane ring makes enokipodins A-D attractive synthetic targets. In this study, nine different cytochrome P450 inhibitors were used to trap the biosynthetic intermediates of highly oxygenated cuparene-type sesquiterpenes of *F. velutipes*. Of these, 1-aminobenzotriazole produced three less-highly oxygenated biosynthetic intermediates of enokipodins A-D; these were identified as (*S*)-(-)-cuparene-1,4-quinone and epimers at C-3 of 6-hydroxy-6-methyl-3-(1,2,2-trimethylcyclopentyl)-2-cyclohexen-1-one. One of the epimers was found to be a new compound.

Key words: Antimicrobial compound, cuparene-1,4-quinone, edible mushroom, enokitake, *Flammulina velutipes*.

Introduction

Flammulina velutipes (Curt. Fr.) Sing. (Enokitake in Japanese), in the family Physalacriaceae (Agaricales, Agaricomycetes), is one of the most popular edible mushrooms in Japan. Many bioactive metabolites have been isolated from this fungus, including proteins (Komatsu et al., 1963, Lin et al., 1974, Tsuda, 1979, Ko et al., 1995, Tomita et al., 1998), glycoproteins (Ikekawa et al., 1985), polysaccharides (Yoshioka et al., 1973, Leung et al., 1997, Yaoita et al., 1998, Wasser and Wess, 1999, Smiderle et al., 2006), sterols (Yaoita et al., 1998), and monoterpenetriol (Hirai et al., 1998). In a previous screen for antimicrobial secondary metabolites from edible mushrooms, we identified four highly oxygenated cuparene-type sesquiterpenes, enokipodins A-D (compounds 1-4), from F. velutipes (Ishikawa et al., 2000, 2001). Enokipodins A-D demonstrated antimicrobial activity against the fungus Cladosporium herbarum (Ishikawa et al., 2000, 2001) and the Gram-positive bacteria Bacillus subtilis and Staphylococcus aureus (Ishikawa et al., 2005). Following our report, several research groups synthesized these compounds (Srikrishna and Rao, 2004, Saito and Kuwahara, 2005, Srikrishna et al., 2006, Secci et al., 2007, Yoshida et al., 2009, Luján-Montelongo and Ávila-Zarraga, 2010, Srikrishna and Rao, 2010, Leboeuf et al., 2013). The influence of mycelial culture conditions on biosynthetic production by F. velutipes was also studied (Ishikawa et al., 2005, Melo et al., 2009). We speculated that the antimicrobial activity of enokipodins A-D correlates to a highly oxygenated cuparene nucleus. The involvement of cytochrome P450s in many complex bioconversion processes, including detoxification reactions and the production of secondary metabolites, has been established in fungi (van den Brink et al., 1998). Although these enzymes carry out a wide range of biocatalytic conversions, the general equation for all of these reactions may be summarized as RH + NAD(P)H + $H^+ + O_2 \rightarrow ROH + NAD(P)^+ + H_2O$ (van den Brink *et al.*, 1998). The presence of a quaternary carbon stereocenter on the cyclopentane ring has made enokipodins A-D attractive synthetic targets. However, considering the absence of biosynthetic studies involving these sesquiterpenes, the aim of the present study was to trap the biosynthetic intermediates of highly oxygenated cuparene-type sesquiterpenes of F. velutipes using cytochrome P450 inhibitors.

Send correspondence to N.K. Ishikawa. Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia, Av. André Araújo 2936, 69067-375 Manaus, AM, Brazil. E-mail: noemia@inpa.gov.br.

Materials and Methods

General notes

Merck Kieselgel 60 F₂₅₄, 0.25-mm thick TLC plates were used to purify the metabolites, while the spots were viewed under UV light (254 and 365 nm). IR spectra were recorded on a PerkinElmer 2000 FTIR, while mass spectra were recorded on a JEOL JMS-SX 102 mass spectrometer. ¹H- and ¹³C-NMR as well as 2D-NMR spectra were recorded on a Bruker AMX-500 spectrometer. Conformation analysis was assisted by MM2 calculations using the ChemBio3D molecular modeling program in ChemOffice (CambridgeSoft).

Cultivation of the fungus

Flammulina velutipes (Fv-4) was cultivated in a 300 mL volume in 22 Erlenmeyer flasks containing 100 mL of malt peptone broth (3% Difco malt extract and 0.3% Merck peptone in distilled water, pH 4.5; the medium was sterilized by autoclaving at 121 °C for 15 min). Each flask was inoculated with five disks (7 mm i.d.) of freshly grown mycelia on malt agar plates, and cultured for 30 days at 25 °C under stationary conditions.

Incubation with cytochrome P450 inhibitors

On day 20 of fermentation, a 1 mM ethanolic solution (1 mL) of each inhibitor was passed through a Millipore membrane filter (0.22 nm pore size) and added to two flasks under aseptic conditions. To investigate the mechanism of enokipodin oxygenation, the fungus was inoculated with nine cytochrome P450 inhibitors: 1-aminobenzotriazole, α -naphthoflavone, ancymidol, 1-benzylimidazole, chlorocholine chloride, ketoconazole, miconazole, SKF-525A, and xanthotoxin. Of these, 1-aminobenzotriazole produced three less highly oxygenated metabolites (compounds 5-7). The carbon atoms in compounds 5-7 were numbered on the basis of biosynthetic considerations. Two flasks inoculated with ethanol (1 mL each) and two uninoculated flasks were used as a negative control. Fermentation was carried out at 25 °C for an additional 10 days. The mycelia were filtered, washed with water and ethyl acetate (EtOAc), and the broth thus obtained was extracted with EtOAc (600 mL each). The extracts were concentrated in a vacuum and the crude extracts thus obtained were spotted on TLC plates in parallel with an aliquot of enokipodins A-D as references. The analysis suggested that 1-aminobenzotriazole produced two less-polar new spots (B-1 and -2). In this test, the Rf values using toluene-acetone (4:1), in order of polarity, were: enokipodin C (Rf 0.09), enokipodin D (Rf 0.23), compound B-2 (Rf 0.30), enokipodin A (Rf 0.43), enokipodin B (Rf0.75), and compound B-1 (Rf0.87). The experiment was therefore scaled up to 5 L and repeated using 1-aminobenzotriazole. Part (567 mg) of the gum (810 mg) thus obtained was chromatographed on a silica gel (toluene: acetone = 6:1) to give two fractions containing B-1 and

-2, respectively. The fractions containing B-1 were purified by TLC using hexane-EtOAc (20:1) as a mobile phase to obtain compound 5 (6.1 mg). Those fractions containing B-2 were purified by preparative TLC using toluene-acetone (15:1) and hexane-EtOAc (3:1) to give compounds 6 and 7 (14.0 mg) as a 3.7:1 mixture of epimers (¹H-NMR analysis).

Compound 5

M.p.: 68-75 °C (lit. 72-73 °C) (Matsuo et al., 1977). $[\alpha]_D^{24}$: -7° (*c* 0.01, CHCl₃), +10° for (*R*)-enantiomer (Matsuo et al., 1977). IR max (film) 2959, 1642, 1370 cm⁻¹. EIMS *m/z* (rel. int.): 233 (M+1+, 6), 232 (M+, 36), 217 (M+-15, 32), 202 (8), 189 (43), 164 (100), 150 (34), 149 (19), 137 (18), 95 (22), and 69 (28). HREIMS *m/z* 232.1486 (C₁₅H₂₀O₂ requires 232.1464). For ¹H and ¹³C spectral analysis, see Table 1.

A 3.7:1 mixture of compounds 6 and 7

 $[\alpha]_D^{24}$: -61° (*c* 0.01, CHCl₃), IR max (film) 3445, 1645 cm⁻¹. EIMS *m/z* (rel. int.): 237 (M++1, 9), 237 (M+, 50), 218 (M+-H₂O, 16), 203 (15), 180 (34), 135 (38), 121 (52), 109 (100), 91 (77), 79 (40), and 43 (81). HREIMS *m/z* 236.1770, (C₁₅H₂₄O₂ requires 236.1772). For ¹H and ¹³C spectral analyses of the major diastereomer compound 6, see Table 2.

Compound 7

¹H NMR (CDCl₃, 500 MHz): (Apparent signals were selected.) 1.97 (1H,ddd, Hβ-2), 2.10 (1H, ddd, Hα-2), 2.42 (1H, dddd, Hα-1), 2.59 (1H, ddd, Hβ-1), 3.63 (1H, s, OH), 6.00 (1H, d, H-5). ¹³C NMR δ (CDCl₃, 125 MHz) 19.3, 22.3, 24.0, 24.9, 26.3, 27.8, 35.7, 36.7, 40.6, 44.6, 52.7, 72.3, 122.2, 172.6, and 202.7.

Results and Discussion

1-Aminobenzotriazole inhibited the biosynthesis of enokipodins A-D (1-4) to produce two less-highly oxygenated metabolites (compounds 5-7) by inhibiting the activity of the fungal cytochrome P450 enzymes.

The EIMS of compound 5 showed a molecular ion peak at m/z 232, which was confirmed by recording the FDMS. HREIMS of the metabolite showed the precise molecular mass to be 232.1486, corresponding to the molecular formula $C_{15}H_{20}O_2$, and hence proved that the compound contained one less oxygen and two more protons than enokipodin B. The ¹H-NMR, ¹³C-NMR, and HMQC spectra of compound 5 exhibited the presence of four methyl, three methylene, two methane, and six quaternary carbons. Two quaternary carbons resonated at δ 188.2 and 188.5 due to the carbonyls of the quinone moiety. A quaternary ole-finic and an olefinic methine carbon were featured at δ 143.6 and 135.5, respectively. Assignments of all proton and carbon signals were made based on HMQC, HMBC,

Position	δC^a		$\delta H^{a}(J, Hz)$	¹ H- ¹ H COSY	HMBC	NOESY ^b
1	188.2	С	-	-	-	-
2	135.5	CH	6.50 d (2)	15	4, 6, 15	15
3	143.6	С	-	-	-	-
4	188.5	С	-	-	-	-
5	133.8	CH	6.65 s	-	1, 3, 7	8α(s), 8β(w), 12(w), 13(w), 14(w)
6	154.9	С	-	-	-	-
7	51.4	С	-	-	-	-
8	38.6	CH_2	α2.24 m	8β, 9	_ ^c	5(s), 8β, 9, 13(w)
			β1.60 m	8α, 9	10	8α, 14
9	19.9	CH_2	αβ ca. 1.7 m	8, 10	_c	8αβ, 10α, 12, 13, 14
10	41.6	CH_2	α1.54 m	9, 10β	_c	10β, 13
			β1.73 m	9, 10α	_c	8β, 10α, 12
11	44.1	С	-	-	-	-
12	25.3	CH_3	1.12 s	-	7, 10, 11, 13	5(w), 13(s), 14(s)
13	27.9	CH ₃	0.74 s	-	7, 10, 11, 12	5(w), 8α(w), 10α, 12
14	23.0	CH ₃	1.29 s	-	6, 7, 8, 11	5(w), 8β, 10β
15	14.9	CH ₃	2.01 d (2)	2	2, 3, 4	2

 Table 1 - ¹H and ¹³C NMR spectral data of compound 5 in CDCl₃.

^aFrom HMQC. ^bw; weak cross peak, s: strong cross peak. ^cAccumulation time was not enough.

Position	δC^a		$\delta \mathrm{H}^{\mathrm{a}}\left(J,\mathrm{Hz} ight)$	¹ H- ¹ H COSY	HMBC	NOESY ^b
1	27.1	CH_2	α2.39 dddd (2.5, 4, 13, 16)	1β, 2αβ	6	1β, 2αβ, 12 (s), 13, 15(s)
			β2.65 ddd (2, 4, 16)	1α, 2β	3, 5, 6	1α, 2αβ, 8α, 12, 13(w), 14 (w)
2	36.7	CH_2	α2.13 ddd (2, 4, 12)	1αβ, 2β	3, 4, 6, 15	1α, 2β, 15
			β1.95 ddd (4, 12, 13)	1αβ, 2α	1, 3, 4, 6, 15	1αβ, 2α
3	72.3	С	-	-	-	-
4	202.7	С	-	-	-	-
5	122.1	С	5.98 d (2.5)	-	1, 3, 7	8αβ(s), 13(w), 14, 15(w)
6	172.5	С	-	-	-	-
7	52.7	С	-	-	-	-
8	36.1	CH_2	α2.22 m	8β, 9	14	8β, 9, 13
			β1.53 m	8α, 9	7, 10, 14	8α, 14
9	18.9	CH_2	αβ ca. 1.69 m	8, 10	10	8α(s), 8β(w), 10α, 12, 13(w), 14
10	40.2	CH_2	α ca. 1.56 m	9, 10β	9, 11, 13	8α 10β, 12 13, 14
			β ca. 1.69 m	9, 10α	9	8β, 10α, 12, 13(w), 14
11	44.3	С	-	-	-	-
12	24.3	CH_3	1.08 s	-	7, 10, 11, 13	$1\alpha(s), 1\beta(w), 5, 13(s)$
13	26.1	CH_3	0.82 s	-	7, 10, 11, 12	$1\alpha(s), 1\beta(w), 5(w), 8\alpha(s), 12, 15(s)$
14	22.3	CH_3	1.10 s	-	6, 7, 8. 11	$1\alpha(w), 1\beta(s), 5(w), 8\beta, 10\beta$
15	23.9	CH_3	1.31	-	2, 3, 4	1α, 2α, 5(w), 13(s)
OH			3.65	-	2, 3, 4	15

Table 2 - ¹H and ¹³C NMR spectral data of compound 6 in CDCl₃.

^aFrom DEPT. ^bw; weak cross peak, s: strong cross peak.

¹H-¹H COSY, and NOESY spectra (Table 1) to give the structure of compound 5 as shown. Compound 5 was previously isolated from the liverworts *Jungermannia rosulans* (Matsuo *et al.*, 1977), *Radula javanica* (Asakawa *et al.*, 1991), *Lejeunea aquatic* (Toyota *et al.*, 1997), and *Lejeunea flava* (Toyota *et al.*, 1997). The ¹H and ¹³C spectral data for compound 5 were identical to those for synthesized racemic 5 (Paul *et al.*, 2003). Thus, we report here for the first time the complete ¹H- and ¹³C-NMR assignments of compound 5. The NOE data for compound 5 revealed the conformation of the main or averaged rotamer as shown in Figure 1. The ring current in quinone shows a deshielding effect on Hα-8 (δ 2.24) and shielding effect on H-13 (δ 0.74).



Figure 1 - A stable conformation of (S)-(-)-cuparene-1,4-quinone (5). Important NOE correlations is shown by arrow.





Figure 2 - Planar structure of compound 6, with ${}^{1}H{}^{-1}H$ connectivities represented by dotted lines, HMBC correlations indicated by bold-faced bonds and those of H-4 and H-14 indicated by arrow.



Figure 3 - (A) A stable conformation for compound 6. (B) Key NOEs observed of the cyclohexenone moiety in 6. (C) Key NOEs observed between the protonse of the cyclohexenone moiety and those of the cyclopentane moiety in 6. (D) A possible conformation for compound 7.

maining three carbons (122.1, 172.5, and 202.7) may form an α , β -unsaturated ketone moiety. An IR absorption at 3445 cm⁻¹, dehydration ion at m/z 218 by EIMS, and the presence of a tertiary carbonyl carbon resonating at δ 72.3 in the ¹³C-NMR spectrum indicated compound 6 to be a tertiary alcohol. A sharp signal corresponding to a hydroxy proton was observed at δ 3.65 in the ¹H-NMR spectrum, indicating the existence of intramolecular hydrogen bonding between the hydroxy proton and carbonyl oxygen. The HMBC spectrum revealed five- and six-membered rings in compound 6 (Figure 2). The HMBC correlations of H-5 to C-7 and H-14 to C-6 led to the assignment of a cuparene skeleton for compound 6. The relative stereochemistry of compound 6 as shown in Figure 3A is derived from several lines of data: i) the observed NOEs of H-15/H α -1 (1,3-diaxial), H-15/H-5, and H-15/Ha-2; ii) an allyl coupling (2.5 Hz) between H-5 and H α -1 (pseudoaxial) in Figure 3B; and iii) the observed NOEs of H-15/H-13, H-5/H $\alpha\beta$ -8, H $\alpha\beta$ -1/H-12, 13, and H-14/H β -1 in Figure 3C.

In light of the model of biogenesis shown in Figure 4, the configuration at C-7 in compound 6 must be S, as indicated. The IUPAC name for compound 6 will be, therefore, (S)-6-hydroxy-6-methyl-3-[(S)-1,2,2-trimethylcyclopentyl]-2-cyclohexen-1-one. Compound 7 is deduced, tentatively, to be an epimer of compound 6 at C-3 and a novel compound. Very recently, compounds 5 and 6 and related compounds were isolated from a solid culture of F. velutipes growing on cooked rice (Wang et al., 2012a, 2012b). The ¹H and ¹³C spectral data for compound 6 are identical to those reported for flamvelutpenoid C (Wang et al., 2012a). Compound 5 showed weak antibacterial activity against B. subtilis (Wang et al., 2012a). Flamvelutpenoid C showed weak antibacterial activity against Escherichia coli, B. subtilis, and methicillin-resistant S. aureus (Wang et al., 2012b). This means that appropriate strains of the fungus can produce a series of cuparene-type sesquiterpenes under



Figure 4 - Hypothetical biogenesis scheme for enokipodins $A \sim D(1 \sim 4)$ in *Flammulina velutipes*. Fpp = farnesyl pyrophosphate.

suitable culture conditions. The precise assignment of ¹H signals for flamvelutpenoid C is reported here.

Three intermediates, compounds 5-7, were isolated using 1-aminobenzotriazole as a cytochrome P450 inhibitor in this study. Of these, compounds 6 and 7 are likely key precursors of the phenolic ring in cuparene-type sesquiterpenes, including enokipodins A-D (1-4).

Acknowledgments

Many thanks to Dr. Kunihide Takahashi (Retired Professor, Faculty of Agriculture, Hokkaido University) for acting as N.K.I.'s Ph.D. advisor. We thank the Japan Society for the Promotion of Science and MEXT for providing a Ph.D. studentship and post-doctoral fellowship to N.K.I. and A.F., respectively. We also thank Mr. Kenji Watanabe, Dr. Eri Fukushi (GC-MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University) for recording the mass and 2D-NMR spectra. We thank Dr. Hideaki Oikawa for his invaluable suggestion concerning the P450 inhibitors.

References

- Asakawa Y, Kondo K, Tori M (1991) Cyclopropanochroman derivatives from the liverwort *Radula javanica*. Phytochemistry 30:325-328.
- Hirai Y, Ikeda M, Murayama T, Ohata T (1998) New monoterpene triols from fruiting body of *Flammulina velutipes*. Biosci Biotechnol Biochem 62:1364-1368.
- Ikekawa T, Maruyama H, Miyano T, Okura A, Sawasaki Y, Naito K, Kawamura K, Shiratori K (1985) Proframin, a new antitumor agent: preparation, physicochemical properties and antitumor activity. Jpn J Cancer Res (Gann) 76:142-148.
- Ishikawa NK, Yamaji K, Tahara S, Fukushi Y, Takahashi K (2000) Highly oxidized cuparene-type sesquiterpenes from a mycelial culture of *Flammulina velutipes*. Phytochemistry 54:777-782.
- Ishikawa NK, Yamaji K, Tahara S, Fukushi Y, Takahashi K (2001) Antimicrobial cuparene-type sesquiterpenes, enokipodins C and D, from a mycelial culture of *Flammulina velutipes*. J Nat Prod 64:932-934.
- Ishikawa NK, Yamaji K, Ishimoto H, Miura K, Fukushi Y, Takahashi K, Tahara S (2005) Production of enokipodins A, B, C, and D: a new group of antimicrobial metabolites from mycelial culture of *Flammulina velutipes*. Mycoscience 46:39-45.
- Ko JJ, Hsu CI, Lin RH, Kao CL, Lin JY (1995) A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. Eur J Biochem 228:244-249.
- Komatsu N, Terakawa H, Nakanishi K, Watanabe Y (1963) Flammulin, a basic protein of *Flammulina velutipes* with antitumor activities. J Antib (A) 16:139-143.
- Leboeuf D, Wright CM, Frontier AJ (2013) Reagent control of [1,2]-Wagner-Meerwein shift chemoselectivity following the Nazarov cyclization: application to the total synthesis of enokipodin B. Chem Eur J 19:4835-4841.
- Leung MYK, Fung KP, Choy YM (1997) The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. Immunopharmacology 35:255-263.

- Lin JY, Lin YJ, Chen CC, Wu HL, Shi GY, Jeng TW (1974) Cardiotoxic protein from edible mushrooms. Nature 252:235-237.
- Luján-Montelongo JA, Ávila-Zarraga JG (2010) Palladium (II) catalyzed 6-endo epoxynitrile cyclizations: total syntheses of enokipodins A and B. Tetrahedron Lett 51:2232-2236.
- Matsuo A, Terada I, Nakayama M, Hayashi S (1977) Cuprenenol and rosulantol, new cuparane class sesquiterpene alcohols from the liverwort *Jungermannia rosulans*. Tetrahedron Lett 43:3821-3824.
- Melo MR, Paccola-Meirelles LD, Faria TJ, Ishikawa NK (2009) Influence of *Flammulina velutipes* mycelia culture conditions on antimicrobial metabolite production. Mycoscience 50:78-81.
- Paul T, Pal A, Gupta PD, Mukherjee D (2003) Stereoselective total syntheses of (±)-1,14-herbertenediol and (±)-tochuinyl acetate and facile total syntheses of (±)- α -herbertenol, (±)- β -herbertenol and (±)-1,4-cuparenediol. Tetrahedron Lett 44:737-740.
- Saito M, Kuwahara S (2005) Enantioselective total synthesis of enokipodins A-D, antimicrobial sesquiterpenes produced by the mushroom, *Flammulina velutipes*. Biosci Biotechnol Biochem 69:374-381.
- Secci F, Frongia A, Ollivier J, Piras PP (2007) Convenient formal synthesis of (±)-cuparene, (±)-enokipodins A and B, and (±)-cuparene-1,4-quinone. Synthesis 7:999-1002.
- Smiderle FR, Carbonero ER, Mellinger CG, Sassaki GL, Gorin PAJ, Iacomini M (2006) Structural characterization of a polysaccharide and a β-glucan isolated from the edible mushroom *Flammulina velutipes*. Phytochemistry 67:2189-2196.
- Srikrishna A, Rao MS (2004) The first total synthesis of the antimicrobial sesquiterpenes (±)-enokipodins A and B. Synlett 2:374-376.
- Srikrishna A, Rao MS (2010) Total synthesis of enokipodins A-D cuparene-1,4-diol. Indian J Chem 49B:1363-1371.

- Srikrishna A, Vasantha Lakshmi B, Ravikumar PC (2006) The first total synthesis of (±)-lagopodin A. Tetrahedron Lett 47:1277-1281.
- Tomita T, Ishikawa D, Noguchi T, Katayama E, Hashimoto Y (1998) Assembly of flammutoxin, a cytolytic protein from the edible mushroom *Flammulina velutipes*, into a poreforming ring-shaped oligomer on the target cell. Biochem J 333:129-137.
- Toyota M, Koyama H, Asakawa Y (1997) Sesquiterpenoids from the three Japanese liverworts *Lejeunea aquatic*, *L. flava* and *L. japonica*. Phytochemistry 46:145-150.
- Tsuda M (1979) Purification and characterization of a lectin from the mushroom, *Flammulina velutipes*. J Biochem 86:1463-1468.
- van den Brink HJM, van Gorcom RFM, van den Hondel CAMJJ, Punt PJ (1998) Cytochrome P450 enzyme systems in fungi. Fungal Gen Biol 23:1-17.
- Wang Y-Q, Bao L, Yang X-L, Dai H-Q, Guo H, Yao X-S, Zhang L-X, Liu H-W (2012) Four new cuparene-type sesquiterpenes from Flammulina velutipes. Helvetica Chimica Acta 95:261-267.
- Wang Y-Q, Bao L, Yang,X-L, Li L, Li S, Gao H, Yao X-S, Wen H, Liu H-W (2012) Bioactive sesquiterpenoids from the solid culture of the edible mushroom Flammulina velutipes growing on cooked rice. Food Chem 132:1346-135.
- Wasser SP, Weis AL (1999) Therapeutic effects of substances occurring in higher basidiomycetes mushrooms: a modern perspective. Crit Rev Immunol 19:65-96.
- Yaoita Y, Amemiya K, Ohnuma H, Furumura K, Masaki A, Matsuki T, Kikuchi M (1998) Sterol constituents from edible mushrooms. Chem Pharm Bull 46:944-950.
- Yoshida M, Shoji Y, Shishido K (2009) Total syntheses of enokipodins A and B utilizing palladium-catalyzed addition of an arylboronic acid to an allene. Organic Lett 11:1441-1443.
- Yoshioka Y, Sano T, Ikekawa T (1973) Studies on antitumor polysaccharides of *Flammulina velutipes* (Curt. ex Fr.) Sing. I. Chem Pharm Bull 21:1772-1776.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.