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CONVERSION OF VELUTINAL ESTERS IN THE FRUIT BODIES OF *RUSSULA CUPREA*

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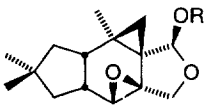
Key Word Index—*Russula cuprea*; Russulaceae; Basidiomycetes; marasmane sesquiterpenoids; velutinal esters; isovelleral; isovellerol; isovellerdiol; cupreal.

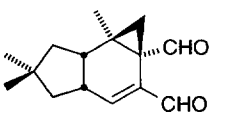
Abstract—The only sesquiterpenoid present in intact fruit bodies of *Russula cuprea* was found to be velutinal, as a mixture of stearic, oleic, linoleic and palmitic acid esters. As a response to injury, the velutinal esters are converted to the marasmane sesquiterpenes isovelleral, isovellerol, isovellerdiol and the new aldehyde cupreal [(2*S*, 3*R*, 6*R*, 7*S*, 8*S*, 9*R*)-8,13-dihydroxy-5-oxo-marasmane]. The structure of cupreal was determined by a combination of spectroscopic and computational methods.

INTRODUCTION

The hot pungent taste of the fruit bodies of several species belonging to Russulaceae (*Lactarius* and *Russula*) has been shown to be caused by sesquiterpenoid unsaturated 1,4-dialdehydes [1, 2]. These are formed from fatty acid esters of velutinal (**1a**), present in the intact fruit bodies, by rapid enzymatic conversions initiated by physical injury. Velutinal and the velutinal esters are mild-tasting, and do not possess antimicrobial activity, while the unsaturated 1,4-dialdehydes, e.g. isovelleral (**2**), are strongly pungent as well as antimicrobial [3]. It has been suggested that these mechanisms constitute a chemical defence against parasites and predators. While the genus *Lactarius* has been thoroughly investigated [1, 4], relatively little is known about the much larger genus *Russula*. However, as was shown for *R. queletii* [5], it seems that the defence mechanism in this genus is basically the same as in *Lactarius*, but in view of the variety of the enzymatic conversions in the *Lactarius* species additional investigations of *Russula* species were warranted. In addition, such investigations could prove valuable for the chemotaxonomy of the Russulaceae. We have, therefore, performed a chemical investigation of *R. cuprea* Krombh. ex Lange (also named *R. cinnamomicolor* Krombh. by some authorities [6]). It belongs to the section Urentinae [7], in which the species typically have an initially mild taste which develops into a strongly acrid taste after approximately one minute. This could be due to the enzymatic formation of pungent compounds as a response to injury, and we have investigated both the initial contents of *R. cuprea* (in intact fruit bodies) as well as the products found in specimens that have been injured (by grinding).



1a R = H
1b R = stearoyl
1c R = oleoyl
1d R = linoleoyl
1e R = palmitoyl



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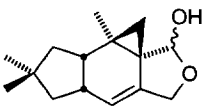

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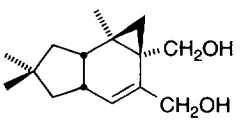

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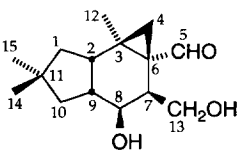

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Structure 1.

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RESULTS AND DISCUSSION

Velutinal esters can be isolated from the intact fruit bodies of Russulaceae by very careful extraction and chromatographic separation. However, velutinal is chemically labile and is rapidly transformed to an array of products by traces of acids (e.g. by silica gel chromatography or by reagent grade solvents) [8]. From *R. cuprea*, a fraction containing a mixture of different fatty acids of velutinal (**1a**) was obtained. Velutinal was identified by comparison of the NMR spectra of this mixture with the spectra of stearoylvelutinal (**1b**) isolated from *L. vellereus*, and the fatty acids were identified by hydrolysing the mixture and analysing the methyl esters of the fatty acids by GC-mass spectrometry. In this way we were able to establish that velutinal (**1a**) in intact fruit bodies of *R. cuprea* is present as esters of stearic, oleic, linoleic and palmitic acid (**1b**–**1e**), and that the stearic and oleic esters are the major components.

A similar set of fatty acids, and in addition 6-ketostearic acid, was recently reported to esterify the drimane alcohols drimenol and uvidin A in *L. uvidus* [9]. However, no trace of 6-ketostearic acid was found in the hydrolysate of the velutinal fraction of *R. cuprea*, confirming the suggestion that this fatty acid (also named lactarinic acid) is unique to *Lactarius* [4]. It has previously been shown that velutinal is present as several fatty acid esters in the fruit bodies of several species [10, 11], but to our knowledge this is the first time that the oleic acid, linoleic acid and palmitic acid moieties have been identified.

By TLC analysis, it was obvious that four metabolites, not present in the extracts of the intact fruit bodies, were formed from the velutinal esters as a response to injury. Two of these, isovelleral (**2**), which is responsible for the pungency of the injured fruit body, and isovellerol (**3**), were easily identified as they had previously been isolated from several *Lactarius* species (e.g. *L. vellereus* and *L. rufus*). In addition, isovellerdiol (**4**) was obtained, and although **4** has been prepared by chemical reduction of isovelleral (**2**) [12], this is the first time it is reported as a natural product. The diol **4** isolated in this investigation was in all respects identical to the diol obtained by reducing isovelleral with $NaBH_4$.

The most polar of the compounds formed as a response to injury, compound **5**, was isolated in relatively large amounts. The MS data (EI, CI and HREI) suggested that it was a sesquiterpene, and the intense IR band at 1681 cm^{-1}, a ^1H NMR signal at δ 9.2 and a ^{13}C NMR signal at δ 202 m indicated the presence of an aldehydic function. A characteristic AX system with the coupling constant 5 Hz at high field in the ^1H NMR spectrum corresponded to the presence of a cyclopropane ring, suggesting that **5** also possessed a marasmane skeleton. The structure of **5** was determined by 2D NMR spectroscopy, including COSY, NOESY, HMQC and HMBC experiments, and a summary of the NOESY and HMBC data is given in

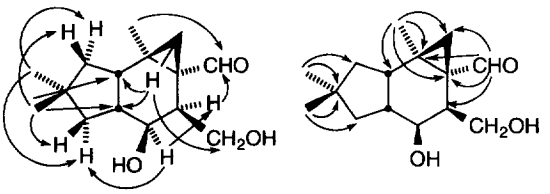

Fig. 1. Significant NOESY (left) and HMBC (right) correlations observed for cupreal (**5**) in $CDCl_3$.

Fig. 1. It is a new sesquiterpene [(2*S*, 3*R*, 6*R*, 7*S*, 8*S*, 9*R*)-8,13-dihydroxy-5-oxo-marasmane], for which we suggest the name cupreal. The absolute configuration of isovelleral (**2**) is as shown in Structure 1 [13], and that of cupreal (**5**) must consequently be the same.

Although the NOESY data support the suggested structure of cupreal (**5**), it was desirable to confirm it by comparing the theoretical ^1H-^1H coupling constants of the C-2/C-9/C-8/C-7 spin system of the most stable conformer (obtained by computational techniques) with the experimental data. Molecular mechanics calculations by the MM3(92) force-field show that the preferred conformation of cupreal (**5**) (Fig. 2) indeed has a *trans*-diaxial disposition of H-8 and H-9 (dihedral angle $-179°$), in agreement with the observed large vicinal coupling constant (10.2 Hz). The dihedral angle between H-7 and H-8 is $-44°$ in this conformation, and the coupling constant suggested by the MacMimic software (6.4 Hz) is close to the experimental value (6.7 Hz). The theoretical $J_{2\text{-}9}$ (6.6 Hz) is in agreement with the observed value (7.3 Hz), and both H-2 and H-9 give strong NOESY correlations to Hβ-4 (see Fig. 1).

The biosynthesis of cupreal (**5**) from the velutinal esters is unclear. The configuration of C-8 is the same as in velutinal (**1a**), indicating that **5** may be formed by the reduction of C-7 of **1a**, however, it cannot be excluded that it is formed by, for example, hydration of isovellerol (**3**). It is reasonable to assume that it is formed by enzymatic processes, as so far it has been

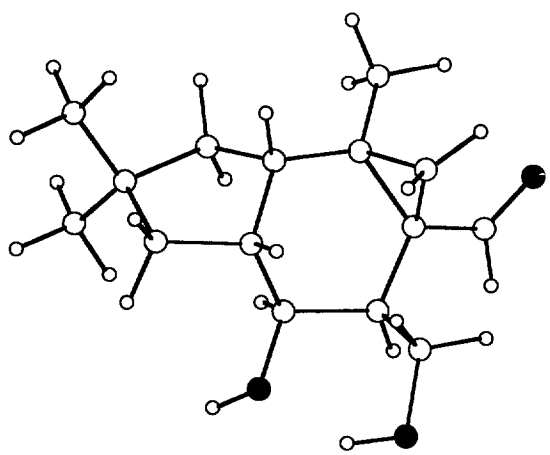

Fig. 2. The most stable conformation of cupreal (**5**), obtained by molecular mechanics calculations (see Experimental for details).

found only in *R. cuprea*, and this should be investigated further by the use of labelled precursors.

EXPERIMENTAL

NMR spectroscopy. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz): room temp., with an inverse 5 mm probe equipped with a shielded gradient coil; COSY, HMQC and HMBC experiments were performed with gradient enhancements using sine shaped gradient pulses, and for the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH} = 145$ Hz and $^2J_{CH} = 10$ Hz. Chemical shifts were given in δ units relative to TMS with the solvent signals (7.26 ppm (^{1}H) and 77.0 ppm (^{13}C) for $CDCl_3$, 1.94 ppm (^{1}H) for CD_3CN) as reference.

Mushroom material. Fruit bodies of *Russula cuprea* (*ca.* 500 g) were collected in Lund, growing under *Tilia* sp. Voucher specimens have been deposited at the Chemical Center, Lund University.

Extraction and isolation. The velutinal esters were obtained from an EtOAc extract of the intact fruit bodies (100 g) prepd at 0°, by flash chromatography on silica gel pretreated with di-isopropylamine. The remaining fruit bodies (400 g) were minced at room temp. and left for 20 min before extraction with EtOAc. The extract (500 mg) was fractionated by chromatography on silica gel eluted with EtOAc–heptane mixts, and the sesquiterpenes **2** (3 mg), **3** (25 mg), **4** (10 mg) and **5** (15 mg) were isolated by RP-HPLC (250 × 10 mm column eluted with MeCN–H_2O mixts).

Computational conformation analysis. The molecular mechanics calculations and the estimation of the vicinal ^{1}H-^{1}H coupling constants were performed with the MacMimic/MM3(92) package, obtained from InStar Software, Ideon Research Park, S-22370 Lund, Sweden. The torsional parameters of the cyclopropane system that were not provided by the programme were set to 0.

Isovellerdiol (**4**). White needles, mp 106–107°, $[\alpha]_D^{22}$ +1° ($CHCl_3$; *c* 0.25)*. UV λ_{max}^{MeCN} nm (log ε): 215 (3.68, sh), 194 (3.90); CD $\Delta\varepsilon_{218}+3.7$ (sh), $\Delta\varepsilon_{199}+6.2$ (MeCN); IR ν_{max}^{KBr} cm^{-1}: 3300 (-OH), 2950, 2920, 2860, 1000; EIMS (probe) 70 eV, *m/z* (rel. int.): 236 $[M]^+$ (11), 218 $[M-H_2O]^+$ (26), 203 (36), 187 (87), 175 (43), 159 (45), 119 (74), 105 (100), 94 (94), 91 (90), 84 (56), 79 (51), 41 (56); ^{1}H NMR (500.135 MHz, $CDCl_3$); δ 5.22 (1H, *s*, H-8), 4.35 (1H, *d*, $J_{13\alpha\text{-}13\beta} = 11.5$ Hz, H-13β), 4.21 (1H, *d*, H-13α), 4.17 (1H, *d*, $J_{5\alpha\text{-}5\beta} = 12.4$ Hz, H-5β), 3.50 (1H, *d*, H-5α), 2.43 (2H, *m*, $J_{2\text{-}1\alpha} = 1.4$ Hz, $J_{2\text{-}1\beta} = 7.8$ Hz, H-2 and H-9), 1.76 (1H, *dd*, H-1β), 1.63 (1H, *m*, H-10β), 1.34 (1H, *dd*, H-1α), 1.29 (3H, *s*, H-12), 1.28 (1H, *s*, H-10α), 1.01 (6H, *s*, H-14 and H-15), 0.87 (1H, *d*, $J_{4\alpha\text{-}4\beta} = 4.2$ Hz, H-4β), 0.61 (1H, *d*, H-4α); ^{13}C NMR (125.759 MHz, $CDCl_3$); δ 139.0 (C-7), 130.4 (C-8), 67.1 (C-13), 64.4 (C-5), 48.2 (C-10), 45.2 (C-1), 42.5 (C-9), 38.6 (C-2), 37.8 (C-11), 32.2 (C-15), 32.0 (C-14), 28.2 (C-6), 27.2 (C-4), 26.7 (C-3), 20.8 (C-12).

Cupreal (**5**). White crystals, mp 135–136°, $[\alpha]_D^{22}$ −41° ($CHCl_3$; *c* 0.30). UV λ_{max}^{MeCN} Me nm (log ε): 286 (2.17), 212 (3.69); CD: $\Delta\varepsilon_{291}-2.9$, $\Delta\varepsilon_{216}+5.0$ (MeCN); IR ν_{max}^{KBr} cm^{-1}: 3400 (-OH), 3290 (-OH), 2975, 2950, 2940, 2900, 2865, 1681 (>C = O), 1460, 1440, 1050, 1020, 1000, 910, 900, 890; EIMS (probe) 70 eV, *m/z* (rel. int.): 252 $[M]^-$ (0.2) 234 $[M-H_2O]^+$ (75), 216 $[M-2H_2O]^+$ (27), 203 (50), 187 (63), 173 (47), 161 (50), 123 (89), 111 (100), 105 (70), 95 (73), 81 (75), 69 (47), 55 (52), 41 (57). High-resolution on the peak 234 $[M-18]^+$: 234.1610; $C_{15}H_{22}O_2$ requires 234.1620. CIMS (CH_4, probe), 70 eV, *m/z* (rel. int.): 253 $[M+H]^+$ (28), 235 $[M+H-H_2O]^+$ (60), 217 $[M+H-2H_2O]^+$ (100), 189 (37); ^{1}H NMR (500.135 MHz, $CDCl_3$); δ 9.18 (1H, *s*, H-5), 3.86 (1H, *dd*, $J_{13\alpha\text{-}13\beta} = 11.1$ Hz, $J_{13\beta\text{-}7} = 8.2$ Hz, H-13β), 3.79 (1H, *dd*, $J_{8\text{-}7} = 6.7$ Hz, $J_{8\text{-}9} = 9.7$ Hz, H-8), 3.51 (1H, *dd*, $J_{13\alpha\text{-}7} = 4.4$ Hz, H-13α), 3.28 (1H, *ddd*, H-7), 2.58 (1H, *ddd*, $J_{2\text{-}9} = 7.1$ Hz, $J_{2\text{-}1\beta} = 7.0$ Hz, $J_{2\text{-}1\alpha} = 12.8$ Hz, H-2), 2.00 (1H, *m*, $J_{9\text{-}10} = 6.3$ Hz, H-9), 1.63 (1H, *dd*, $J_{1\alpha\text{-}1\beta} = 12.6$ Hz, H-1β), 1.59 (2H, *d*, H-10), 1.41 (1H, *dd*, H-1α), 1.40 (1H, *d*, $J_{4\alpha\text{-}4\beta} = 5.1$ Hz, H-4β), 1.30 (1H, *d*, H-4α), 1.25 (3H, *s*, H-12), 1.10 (3H, *s*, H-15), 1.01 (3H, *s*, H-14); ^{13}C NMR (125.759 MHz, $CDCl_3$): 202.2 (C-5), 70.7 (C-8), 63.5 (C-13), 45.3 (C-1), 45.0 (C-2), 43.3 (C-10), 40.5 (C-9), 39.7 (C-6), 37.1 (C-11), 35.4 (C-7), 31.9 (C-15), 31.0 (C-14), 30.3 (C-3), 23.7 (C-4), 20.3 (C-12); ^{1}H NMR (500.135 MHz, CD_3CN) δ 9.19 (1H, *s*, H-5), 3.73 (1H, *dd*, $J_{13\alpha\text{-}13\beta} = 10.9$ Hz, $J_{13\beta\text{-}7} = 7.6$ Hz, H-13β), 3.64 (1H, *dd*, $J_{8\text{-}7} = 6.6$ Hz, $J_{8\text{-}9} = 10.2$ Hz, H-8), 3.27 (1H, *dd*, $J_{13\alpha\text{-}7} = 5.9$ Hz, H-13α), 3.12 (1H, *ddd*, H-7), 2.56 (1H, *ddd*, $J_{2\text{-}9} = 7.3$ Hz, $J_{2\text{-}1\alpha} = 6.9$ Hz, $J_{2\text{-}1\beta} = 12.8$ Hz, H-2), 1.90 (1H, *m* H-9), 1.63 (1H, *dd*, $J_{9\text{-}10\beta} = 3.1$ Hz, $J_{10\alpha\text{-}10\beta} = 13.6$ Hz, H-10β), 1.59 (1H, *dd*, $J_{1\alpha\text{-}1\beta} = 12.3$ Hz, H-1β), 1.49 (1H, *dd*, $J_{9\text{-}10\alpha} = 7.6$ Hz, H-10α), 1.45 (1H, *dd*, H-1α), 1.29 (1H, *d*, $J_{4\alpha\text{-}4\beta} = 4.8$ Hz, H-4β), 1.24 (1H, *d*, H-4α), 1.17 (3H, *s*, H-12), 1.07 (3H, *s*, H-15), 0.99 (3H, *s*, H-14).

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* The optical rotation of **4** at room temp. in $CHCl_3$ is close to zero, and the value −2 was previously reported for the diol obtained by reduction of isovelleral **2** [12]. The optical rotation of isovelleral (**2**) isolated in this investigation is identical to that reported for **2** obtained from various *Lactarius* species [2, 12].

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