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The Conversion of [12-$^2H_3$]-Labelled Velutinal in Injured Fruit Bodies of *Lactarius vellereus*. Further Insight into the Biosynthesis of the Russulaceae Sesquiterpenes

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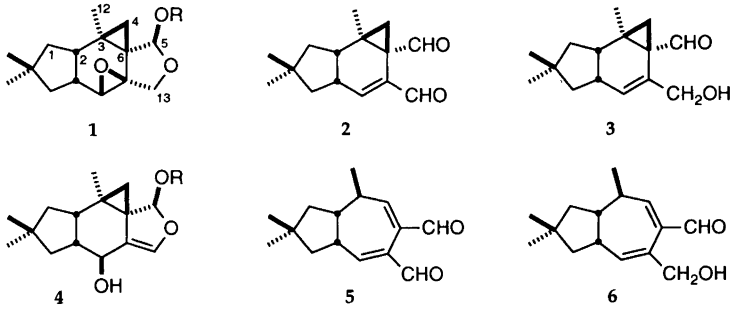
[12-$^2H_3$]-Labelled velutinal (**8**) has been prepared from isovellerol (**3**), and fed to injured fruit bodies of *Lactarius vellereus*. Analysis of the enzymatic conversion products by ^2H NMR spectroscopy showed that free velutinal is a good substrate for the conversions, and established the biosynthetic linkage between velutinal and the lactarane sesquiterpenes. Based on investigations with labelled compounds, a mechanism for the enzymatic conversion of velutinal (**1a**) to the lactaranes in the *Lactarius* species is proposed.

Enzymatic conversions of sesquiterpenes in the fruit bodies of pungent *Lactarius* species (Russulaceae, Basidiomycotina) as a response to physical injury have been proposed to constitute a chemical defence system that protects the mushrooms from parasites.[1] The intact fruit bodies contain only one sesquiterpene, the marasmane velutinal (**1a**), as various fatty acid esters (e.g., stearoylvelutinal **1b**) emulsified in the latex of the fruit bodies.[2] Upon injury to the fruit bodies, the velutinal esters are rapidly converted into, for example, the marasmane isovelleral (**2**), the lactaranes velleral (**5**), piperdial (**11**) and *epi*-piperdial (**12**), and *seco*-lactarane sesquiterpenes. The conversions are considered to be enzymatic as the corresponding transformations have never been observed *in vitro*, and because different sesquiterpenes are produced by different *Lactarius* species. In *L. vellereus*, the unsaturated dialdehydes **2** and **5** possessing antimicrobial, cytotoxic and anti-feedant activities are formed, while **5** and **11** are formed in *L. piperatus*[3] and **5** and **12** in *L. necator*.[4] The two dialdehydes **2** and **5** are subsequently (minutes to hours) reduced enzymatically by the injured *L. vellereus* to isovellerol (**3**) and vellerol (**6**),[1] respectively, which lack most of the biological activities of the dialdehydes.

We have recently shown that isovelleral (**2**) in *L. vellereus* is formed by an enzymatic β-elimination of the epoxide in velutinal (**1a**) (or stearoylvelutinal **1b**) via the enol acetal **4**.[5] By feeding injured fruit bodies of *L. vellereus* [12-$^2H_3$]-labelled **2** and analysing the ^2H content of the vellerol (**6**) eventually formed in the mush, we also showed that isovelleral (**2**) is not a precursor to the lactaranes.[5] Hence, separate biosynthetic routes from the velutinal esters to the marasmanes and the lactaranes in the fruit bodies of the *Lactarius* species exist. The study of the biosynthesis of the lactaranes, which comprise the majority of the Russulaceae sesquiterpenes, would be facilitated if a precursor in a labelled form were available. However, so far we have not been able to prepare stearoylvelutinal (**1b**) synthetically. In this study we show a way of circumventing this problem, and present results that indicate the mechanism of formation of the lactaranes in *L. vellereus*.

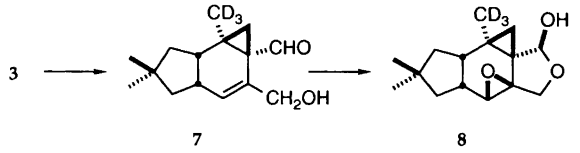


1 2 3 4 5 6

Scheme 1. **a**, R = H; **b**, R = $CO(CH_2)_{16}CH_3$.

Results and discussion

In order to investigate whether velutinal (**1a**) actually has to be esterified (as for example **1b**) to be enzymatically converted in the injured fruit bodies, [12-$^2H_3$]-labelled velutinal (**8**) was prepared and fed to a mush of *L. vellereus* whereafter the ^2H content of the sesquiterpenes formed was analysed.

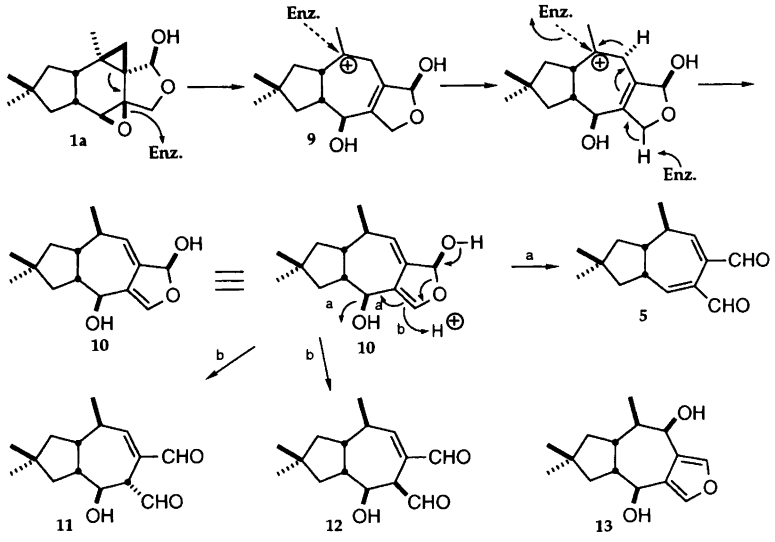


Scheme 2.

The [12-$^2H_3$]-labelled velutinal (**8**) was prepared according to Scheme 2, by a Sharpless epoxidation of C-12 deuteriated isovellerol (**7**)[6] essentially following a known procedure for undeuteriated material.[7] 100 mg of **8** were fed to 500 g freshly ground fruit bodies of *L. vellereus*, and the mush was kept for 30 min at room temperature before it was extracted with ethyl acetate. Isovellerol (**3**) (301) mg) and vellerol (**6**) (62 mg) were isolated from the extract by column chromatography, and ^2H NMR analysis showed that both the isovellerol and the vellerol fractions contained a C-12 deuteriated compound (7.4 and 1.1 %, respectively). There were no traces of free velutinal (**1a**) in this extract (according to TLC analysis). This experiment shows that the conjugation of velutinal (**1a**) with a fatty acid not is necessary for it to be a substrate in the bioconversions. As labelled **1a** is easily prepared from labelled isovellerol (**3**) as described above, or via one of the total syntheses of isovelleral **2** reported recently,[8] all Russulaceae sesquiterpenes that derive from the velutinal esters are available in labelled form. This may facilitate studies of the biosynthetic pathways for the Russulaceae sesquiterpenes, and enable investigations of the molecular mechanisms by which they exert their biological activities. In addition, the experiment unambiguously shows that velutinal (**1a**) (and presumably also stearoylvelutinal **1b**) is biosynthetic precursor to the lactaranes velleral (**5**) and vellerol (**6**) in *L. vellereus*.

The conversion of velutinal derivatives to velleral (**5**) in *L. vellereus* involves, among other things, the opening of the cyclopropane ring and the introduction of a 3-H not present in the marasmane skeleton. In order to examine the origin of this proton, fruit bodies were freeze dried, pulverised, and reconstituted with ^2H_2O. The enzymatic machinery necessary for the conversions of sesquiterpenes in *L. vellereus*, as well as stearoylvelutinal (**1b**) itself, survive this treatment, the dried specimens still contain only **1b**, which is converted in the usual way when water is added to pulverised specimens. If 3-H in **5** comes from water, or from a source in which the proton is exchangeable, one would expect to obtain [3-^2H]-labelled velleral (**5**) and vellerol (**6**) from this experiment. However, no traces of 3-^2H in the vellerol (**6**) isolated from an extract made after 30 min were detected by ^2H NMR analysis. Although this is a negative proof, it nevertheless suggests that 3-H in the lactaranes originates from **1a** itself. Bearing in mind that the neighbouring C-4 methylene in **1a** is transformed into a methine group in velleral (**5**), a hydride shift from C-4 to C-3 appears to be likely.

The *in vitro* transformation of the chemically quite labile velutinal (**1a**) in, for instance, reagent-grade solvents, takes place via an acid-catalysed epoxide ring opening leading to the intermediate cation **9** which is immediately transformed into lactarane dihydrohydroxyfurans eventually forming furans.[9] An opening of the epoxide ring (enzyme-catalysed) is also the first step in the



Scheme 3.

conversion of velutinal into isovelleral (**2**).[5] The interception of the enzymatically formed cation **9** by the enzyme followed by a C-4 to C-3 hydride shift induced by the enzymatic abstraction of an allylic 13-H, would produce an enol acetal (**10**) with the correct oxidation pattern and C-3 configuration (the suggested mechanism for the conversion of **1a** into the lactaranes is depicted in Scheme 3). Compound **10** could easily generate both **5** via path a, effective also in the formation of isovelleral (**2**),[5] and the two lactarane dialdehydes **11** and **12** via path b. This proposal is supported by the co-isolation of small amounts of the furan **13** with the lactarane dialdehydes[10] (**13** is not formed in *Lactarius* species which produce only isovelleral **2**). Compound **13** had the expected stereostructure, if it is formed by a C-4 to C-3 hydride shift in the cation **9** accompanied by addition of H_2O to C-4 instead of an enzymatic abstraction of an allylic 13-H to form **10**.

Experimental

Fruit bodies of *Lactarius vellereus* Fr. were collected in beech woods in the vicinity of Lund, and the sesquiterpenes were isolated[1] and analysed by ^1H and ^2H NMR spectroscopy as described previously.[5] Injury was induced by grinding intact specimens in a meat grinder, to produce a mush that was used directly in the feeding experiments. Isovellerol (**3**) was isolated from *L. vellereus* and was [12-$^2H_3$]-labelled according to the method published for isovelleral (**2**);[6] ^1H NMR analysis showed ca. 95% deuterium incorporation. This product was used for preparing [12-$^2H_3$]-labelled velutinal (**8**) according to the procedure published previously.[7] However, in order to obtain consistently good yields (>70%) the following modification of the original procedure was made. A stoichiometric amount of (+)-diethyl tartrate, $Ti(OiPr)_4$ and *tert*-butyl hydroperoxide was stirred in dry CH_2Cl_2 with activated 3 Å molecular sieves at −23°C for 30 min. The mixture was then cooled to −40°C and a solution of [12-$^2H_3$]-labelled isovellerol (**7**) in CH_2Cl_2 was added. After 3 h of stirring at −40°C the mixture was worked up according to the original procedure.

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