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Biosynthesis of the Azoxycarboxamide Lyophyllin and Formation of Some of its Unnatural Analogues in Fruit-bodies of *Lyophyllum connatum*

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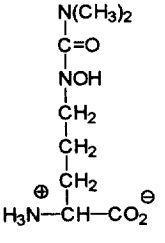
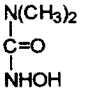
Abstract: The azoxy compound lyophyllin (**3**) is formed in fruit-bodies of the toadstool *Lyophyllum connatum* by oxidative condensation of *N*-hydroxy-*N'*,*N'*-dimethylurea (**2**) with *N*-methylhydroxylamine (**4**). The condensing enzyme is remarkably unspecific and transforms a variety of hydroxyureas and *N*-alkyl-hydroxylamines into the corresponding lyophyllin analogues **5**. © 1997 Elsevier Science Ltd.

The toadstool *Lyophyllum connatum* Fr. (Agaricales) is found in clusters in grassy areas along forest roads. It can be easily recognized by its characteristic violet colour reaction with aqueous ferric chloride for which the hydroxamic acid derivatives connatin (**1**) and *N*-hydroxy-*N'*,*N'*-dimethylurea (**2**) are responsible.[1] A third metabolite lyophyllin (**3**)[1] attracted our attention because of its unusual azoxycarboxamide structure.



1



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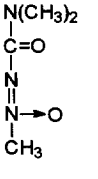
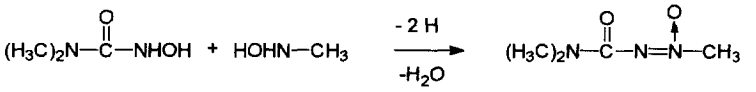


3

Naturally occurring azoxy compounds are known from streptomycetes,[2a] bacteria,[2b] basidiomycetes,[2c] and higher plants.[2d] The biosynthesis of elaiomycin and valanimycin has been studied by Parry and coworkers.[3,4]

We report herein our observations regarding the biosynthesis of lyophyllin (**3**). The structural similarity of lyophyllin (**3**) and hydroxyurea **2** suggested a close biosynthetic relationship between both compounds. Indeed, feeding of carbonyl ^{13}C-labelled **2** to young fruit-bodies of *L. connatum* in their natural habitat and harvesting the mushrooms after 3-4 days resulted in 50% incorporation of **2** into lyophyllin (**3**). The compound was isolated as described before[1] and showed the respective enhancement of the carbonyl signal at δ 158.2 in the

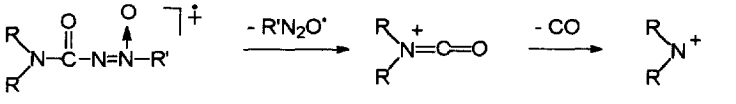
^{13}C NMR spectrum. In another feeding experiment the precursor of the second half of the lyophyllin molecule was identified as *N*-methylhydroxylamine (**4**). The ^{13}C-labelled compound **4** was incorporated into lyophyllin in a rate of up to 95%. This indicates, that lyophyllin (**3**) is formed by oxidative condensation of *N*-hydroxy-*N',N'*-dimethylurea (**2**) with *N*-methylhydroxylamine (**4**).



2 **4** **3**

The reaction resembles Bamberger's well known synthesis of azoxybenzene from nitrosobenzene and phenylhydroxylamine. Different to our findings, the azoxy unit of valanimycin is formed by oxidation of a hydrazine intermediate, which is in turn produced by coupling of a hydroxylamine unit with an amino component.[4] In our case, neither labelled methylamine nor *N,N,N''*-trimethylsemicarbazide was incorporated into **3**.

In order to study the specificity of the condensing enzyme, we fed several analogues of hydroxyurea **2** and *N*-methylhydroxylamine (**4**) to *L. connatum*. The unnatural precursors (40 mg) were applied in aqueous NaH_2PO_4/Na_2HPO_4 buffer solution (3 ml, pH 7) with a syringe to 3-4 young fruit-bodies. After 3-4 days the toadstools were harvested and extracted with methanol. Cautious evaporation of the solvent and extraction of the aqueous residue with $CHCl_3$ yielded standard solutions for the GC/MS analyses.[5] The newly formed analogues **5** and lyophyllin **3** were identified by their characteristic MS fragmentation pattern given in the following scheme and by high resolution of their molecular ions.[6]



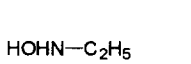
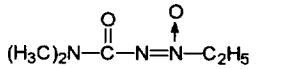
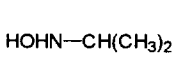
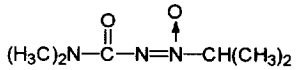
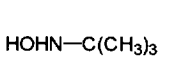
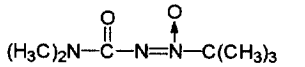
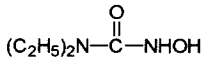
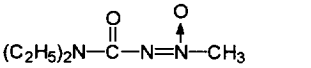
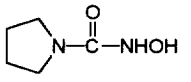
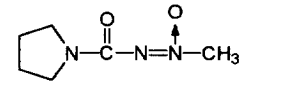
5 fragment A fragment B

The *N*-hydroxyurea derivatives and *N*-alkylhydroxylamines given to the toadstools and the resulting lyophyllin analogues are shown in Table 1. In the case of $(CH_3)_2NCO\text{-}N{=}N(O)\text{-}C_2H_5$ (**5a**) the feeding experiment was repeated on a larger scale (0.2 g *N*-ethylhydroxylamine/175 g fruit-bodies). The ethyl analogue of lyophyllin **5a** (6 mg) and lyophyllin (**3**) (19 mg) were isolated, separated by column chromatography on silica gel (petroleum ether/acetone 4:1) and identified by their MS, ^1H and ^{13}C NMR spectra.[6]

Simultaneous feeding of *N*-ethylhydroxylamine and *N,N*-diethyl-*N'*-hydroxyurea to *L. connatum* led to the formation of the triethyl analogue of lyophyllin $(C_2H_5)_2NCO\text{-}N{=}N(O)\text{-}C_2H_5$ (**5f**) in admixture with lyophillin (**3**) and minor amounts of $(C_2H_5)_2NCO\text{-}N{=}N(O)\text{-}CH_3$ (**5g**) and $(CH_3)_2NCO\text{-}N{=}N(O)\text{-}C_2H_5$ (**5h**).

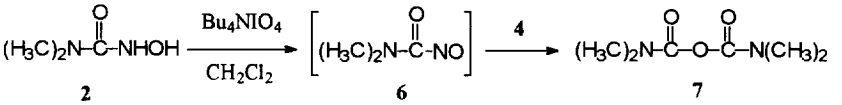
The results shown in Table 1 indicate that fruit-bodies of *L. connatum* are able to convert a variety of *N*-alkylhydroxylamines and *N,N*-dialkyl-*N'*-hydroxyureas into the corresponding azoxycarboxamides **5**. This indicates a remarkable substrate unspecifity of the lyophyllin forming enzyme.[7] The lack of transformation of aromatic hydroxylamine derivatives like PhNHOH and $PhCH_2NHOH$ displays the limits of this conversion.

Table 1. Conversion of unnatural precursors into lyophyllin analogues **5** by *L. connatum*

precursor	lyophyllin analogue **5**	relative amount [%] (lyophyllin = 100%)
	**5a** 	156
	**5b** 	35
	**5c** 	69
	**5d** 	9
	**5e** 	10

In order to mimic the biosynthesis of lyophyllin (**3**) a solution of *N*-hydroxy-*N',N'*-dimethylurea (**2**) and *N*-methylhydroxylamine (**4**) in aqueous $NaHCO_3$ was stirred with an excess of MnO_2 for 16 h at 25 °C. Purification of the resulting mixture yielded 1.5% of lyophyllin (**3**), which was characterized by GC/MS and its ^1H NMR spectrum.[1] Minute amounts of **3** were also formed by treatment of hydroxyurea **2** with the dimer of nitrosomethane in refluxing dichloromethane.

On the other hand, reaction of the acylnitroso compound **6** with *N*-methylhydroxylamine (**4**) could not be observed. The only product isolated was the carbamic acid anhydride **7**, formed in over 50% yield by reaction of **6** with water produced in the oxidation of hydroxyurea **2** with tetrabutylammonium periodate.[8]



2 **6** **7**

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5. GC/MS was carried out on a Varian Model 3400 GC coupled to a Finnigan MAT 95Q sector mass spectrometer using EI at 70 eV. The helium flow was 1 ml/min, the injector and coupling temp. 250 °C. 1 µl solution was injected with a split of 1:10. Fused-silica capillary columns were used: J&W DB-1701 (15 m × 0.25 mm id). Column temp. programming: maintained at 50 °C for 2 min, then heating to 300 °C (10 °C/min), then maintained at 300 °C for 3 min.

6. **5a**: EI-MS *m/z* (rel. int.): 145 (2) [M^+], 72 (100, fragment A), 60 (9); ^{1}H NMR ($CHCl_3$): δ 1.53 $\underline{H_3C}CH_2$, 2.77, 2.98 [$N(CH_3)_2$], 4.24 $H_3C\underline{CH_2}$; ^{13}C NMR ($CDCl_3$): δ 13.28, 35.79, 35.91, 65.79, 194.03. **5b**: 159 (1) [M^+], 117 (4) [M-C_3H_6]$^+$, 73 (11), 72 (100, A). **5c**: 173 (0.1) [M^+], 117 (28) [M-C_4H_8]$^+$, 72 (100, A), 57 (62). **5d**: 159 (3) [M^+], 144 (10), 142 (23), 100 (88, A), 87 (15), 72 (100, fragment B), 58 (18) 56 (15). **5e**: 157 (0.7) [M^+], 140 (7), 98 (100, A), 70 (41, B), 56 (51), 55 (92). The stereochemistry of lyophyllin (**3**) and its analogues **5** is unknown.

7. A similar range of 'unnatural' precursors was accepted by fruit-bodies of *Hebeloma sacchariolens* in their conversion of anthranilic acid to 2-aminobenzaldehyde: v. Nussbaum, F.; Spahl, W. and Steglich, W. *Phytochemistry* **1997**, *46*, 261-264.

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