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Studies on the Biosynthesis of Bovilactone-4,4 and Related Fungal Meroterpenoids

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Dedicated to Professor Heinrich Nöth on the occasion of his 80th birthday

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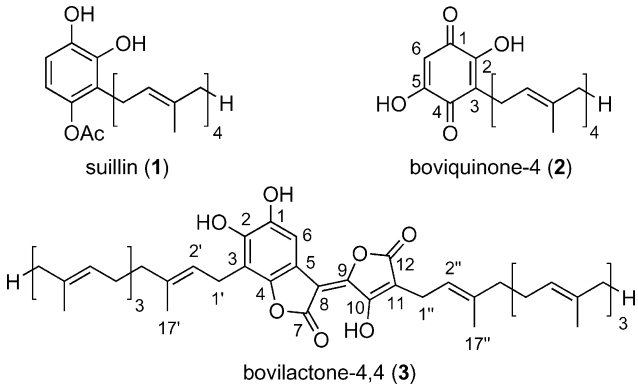
The initial step in the biosynthesis of suillin (**1**), boviquinone-4 (**2**) and bovilactone-4,4 (**3**) in *Suillus* species is the geranylgeranylation of 3,4-dihydroxybenzoic acid at the 2-position. Feeding experiments with advanced precursors have identified boviquinone-4 and deacetylsuillin (**9**) as building blocks for the dilactone and catechol moieties, respectively, of bovilactone-4,4 (**3**). In order to explain the failure of boviquinone-4 (**2**) to incorporate side-chain-labelled deacetylsuillin (**9#**), an alternative sequence for the formation of **2** is proposed. During these experiments an interesting change in metabolism was noticed: after administration of larger quantities of aromatic carboxylic acids, the boviquinone-4 present in the fruit bodies disappeared and de novo synthesis of bovilactone-4,4 occurred.

Introduction

Mushrooms of the genus *Suillus* produce characteristic meroterpenoids derived from 4-hydroxybenzoic acid and geranylgeranyl diphosphate.[1,2] Most widely distributed is suillin (**1**), which was first isolated from *S. granulatus* and also occurs in several related species.[3] Suillin has strong tumour-inhibiting and cytotoxic activities,[4] and also exhibits immunosuppressive properties.[5] The closely related boviquinone-4 (**2**), which is responsible for the brown-orange colour of *S. bovinus* (L.: Fr.) Roussel (German: Kuhröhrling),[6] has also been found in the North American *S. spraguei* (= *S. pictus*).[7] One single fruit body of *S. bovinus* can contain up to 20 mg of this pigment. The dimeric bovilactone-4,4 (**3**) occurs in *S. americanus*, *S. bovinus* and *S. collinitus*,[8] where it is concentrated in the pink mycelium covering the stipe base of these mushrooms.[9]

Previous studies with *S. bovinus* have shown that [1-^{13}C]-labelled 4-hydroxybenzoic acid (**10***) and 3,4-dihydroxybenzoic acid (**11***) are incorporated into boviquinone-4 (**2**) and bovilactone-4,4 (**3**) with high efficiency.[1] Similarly, acids **10** and **11** have been identified as specific precursors of suillin (**1**) in *S. variegatus*.[1] Here we report these and further experiments in detail and describe new insights into the biosynthesis of boviquinone-4 and bovilactone-4,4.



Results and Discussion

NMR Assignment of Bovilactone-4,4

For an interpretation of the labelling experiments, the unambiguous assignment of the ^{13}C NMR signals of bovilactone-4,4 (**3**) was necessary. The signals for 1′-H and 1′′-H could readily be distinguished on the basis of HMBC connectivities between the methylene protons 1′′-H and the carbonyl atoms C-10 and C-12, and the corresponding ^{13}C NMR signals were determined by HMQC measurements. In this manner, the assignment of all carbon signals of the bovilactone core unit was ascertained (Table 1). The

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results were in good agreement with the NMR spectroscopic data reported for several di-*n*-alkyl homologues of bovilactone-4,4.[10] Within the side chains, HMBC experiments enabled differentiation between CH-2′ and CH-2′′ and between C-3′ and C-3′′, whereas the assignment of CH_3-17′ and CH_3-17′′ followed from the H,H-COSY spectrum.

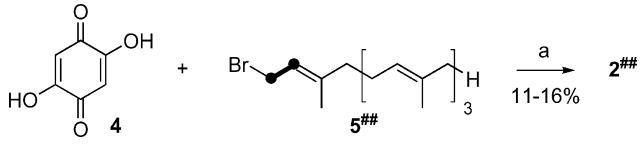
Table 1. Selected NMR spectroscopic data for **3** (600 and 151 MHz, $CDCl_3$).

Position	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm), mult., $J_{H,H}$ [Hz]	HMBC[a]
1	141.5		
2	146.2		
3	112.5[b]		
4	147.2		
5	112.8[b]		
6	109.0	7.29, s	1, 2, 4, 5, 8, 9
7	173.3		
8	107.2		
9	150.8		
10	161.5	11.73, s (OH)	
11	107.7		
12	167.8		
1′	23.1	3.45, d, 7.4	2, 3, 4, 2′, 3′
1′′	20.9	3.08, d, 7.1	9, 10, 11, 12, 2′′, 3′′
2′	119.7	5.30, t, 6.6	1′, 17′
2′′	118.1	5.25, t, 6.7	1′′, 17′′
3′	139.5		
3′′	138.4		
17′	16.4	1.81, s	2′, 3′
17′′	16.3	1.74, s	2′′, 3′′

[a] HMBC correlations are from the proton(s) stated to the carbon indicated. [b] Signals interchangeable.

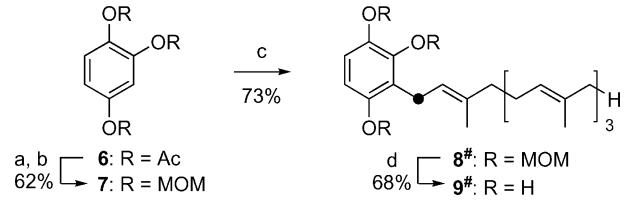
Synthesis of the Precursors Used for the Feeding Experiments

[1′,2′-$^{13}C_2$]Boviquinone-4 (**2**$^{\#\#}$) was prepared in low yield by heating (*E*,*E*,*E*)-[1,2-$^{13}C_2$]geranylgeranyl bromide[11] (**5**$^{\#\#}$) with excess 2,5-dihydroxybenzoquinone (**4**) in THF in the presence of triethylamine (Scheme 1).



Scheme 1. Synthesis of **2**$^{\#\#}$. Reagents and conditions: a) NEt_3, THF, 50 °C, 5 h.

The synthesis of ^{13}C-labelled deacetylsuillin (**9**$^{\#}$) commenced from 1,2,4-tris(methoxymethoxy)benzene (**7**), which was obtained from triacetate **6** by cleavage with hydrazine hydrate[12] and protection of the resulting free triol with MOMCl[13] (Scheme 2). Upon application of 1.2 equiv. of *n*BuLi at room temperature, the anion-stabilizing effect of two neighbouring methoxymethyl groups[14] allowed the predominant lithiation of **7** in the 3-position; the selectivity over the 6-position was 83:17 as shown by quenching experiments with [D_4]methanol. Lithiation in the 5-position was negligible. The desired regioisomer **8**$^{\#}$ was obtained exclusively when 1 equiv. of CuCl was added prior to the addition of the prenyl bromide. Finally, treatment of **8**$^{\#}$ with HCl in MeOH gave **9**$^{\#}$ in good yield.



Scheme 2. Synthesis of **9**$^{\#}$. Reagents and conditions: a) $N_2H_4{\cdot}H_2O$, MeOH; b) MOMCl, *i*Pr_2NEt, CH_2Cl_2; c) *n*BuLi, THF, room temp., 0.5 h; then CuCl (1 equiv.), 0 °C, 0.5 h; then [1-^{13}C]geranylgeranyl bromide[20] (**5**$^{\#}$), –20 °C; d) AcCl, MeOH.

Feeding Experiments with [^{13}C]-Labelled Precursors

Suillus bovinus

Solutions of the [1-^{13}C]-labelled hydroxybenzoic acids **10***[15] or **11***[16] in DMSO were administered by syringe to young fruit bodies of *S. bovinus*. After 4–7 d, the mushrooms, including the mycelium adherent to the stipe basis, were harvested, and their EtOAc extract was applied to a column of acetylated polyamide-6. Elution with *n*-hexane yielded crude boviquinone-4 (**2***), and subsequent elution with toluene provided much smaller amounts of bovilactone-4,4 (**3***). Both pigments were purified by high-speed countercurrent chromatography (HSCCC). In the case of dihydroxyquinone **2***, the incorporation rate was determined by ^{13}C NMR analysis of the corresponding dimethyl ether.[1]

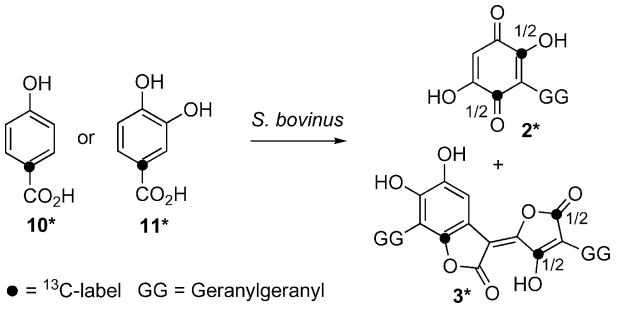
The outcome of these experiments was critically dependent on the amount of precursor administered. Application of [1-^{13}C]-labelled 4-hydroxy- (**10***) or 3,4-dihydroxybenzoic acid (**11***) in doses of 5 mg per fruit body yielded mainly boviquinone-4 (**2***), with incorporation rates (^{13}C atom-% excess) of 1.1 and 2.6%, respectively (Table 2, Entries 1 and 3, and Scheme 3).[1] Due to rapid equilibration of the two hydroxyquinone tautomers, the ^{13}C-label becomes equally distributed between carbon atoms 2 and 4.

Increasing the amount of precursor to 15–30 mg per fruit body caused diminished growth of the fungi, together with a remarkable alteration of the metabolism. The colour of the mushrooms changed to dark red, and the fungal extract no longer contained the main pigment **2**, but rather slightly increased amounts of bovilactone-4,4 (**3***, Table 2, Entries 2 and 4). This change could also be brought about by metabolically inert aromatic carboxylic acids such as benzoic or salicylic acid, but not by injuring the fruit body or by citric acid or DMSO alone.[1] The bovilactone-4,4 (**3***) formed in these experiments exhibited the labelling pattern depicted in Scheme 3 with labelling of the benzene ring at C-4 (50% incorporation) and the hydroxylactone moiety at carbon atoms 10 and 12 (each 25%, Scheme 3). This 1:1 distribution of carbon-13 reflects the situation found in labelled boviquinone-4 and makes this quinone a plausible precur-

Table 2. Feeding experiments with fruit bodies of *S. bovinus*.

Entry	Precursor (mg,[a] solvent)	Number of fruit bodies	Boviquinone-4 (**2**) Isolation [mg][a,b]	^{13}C enrichment	Bovilactone-4,4 (**3**) Isolation [mg][a,b]	^{13}C enrichment
1	**10*** (5, DMSO)	9	3.3	C-2, C-4 (each 1.1%)	0.3	C-4, C-10, C-12[c]
2	**10*** (16, DMSO)	6[d]	not detectable	–	1	C-4 (50%), C-10, C-12 (each 25%)
3	**11*** (5, DMSO)	18	3	C-2, C-4 (each 2.6%)	0.05	C-4, C-10, C-12[c]
4	**11*** (30, DMSO)	7[d]	not detectable	–	0.6	C-4 (50%), C-10, C-12 (each 25%)
5	**2**$^{\#\#}$ (5[e]) + **10*** (15[e])	2	16	C-1′, C-2′ (each 1.9%), C-2, C-4 (each 9%)	2.7	C-1′′, C-2′′ (each 4%), C-4 (25%), C-10, C-12 (each 8.3%)
6	**9**$^{\#}$ (10[f])	4	21	0%	0.8	C-1′ (25%)[g]
7	**12**$^{\#}$ (10[f])	5	20[h]	0%	0.6	0%

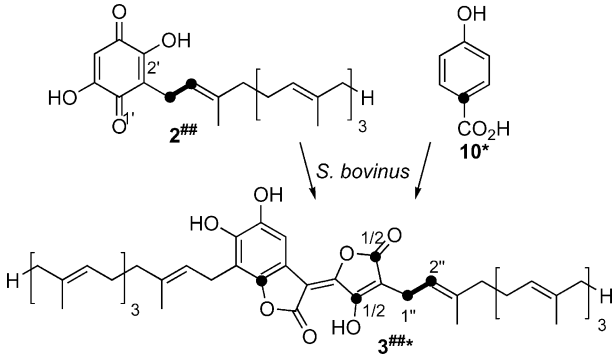
[a] Amount per fruit body. [b] The mushrooms were harvested 4–7 d after administration of labelled precursor. [c] Not quantified, due to limited amount. [d] Colour change to dark red. [e] Solubilized in water with 2-hydroxypropyl-β-cyclodextrin (Aldrich), average degree of substitution: 7. [f] Liposomal formulation. [g] Determined by ^{1}H NMR spectroscopy. ^{13}C NMR spectroscopy gave 15%. This aberration is likely to be a consequence of insufficient ^{13}C spin relaxation. [h] Extrapolated (only a fraction of the crude **2** was purified).



Scheme 3. Labelling of **2*** and **3*** after feeding of precursors **10*** or **11*** to fruit bodies of *S. bovinus*.

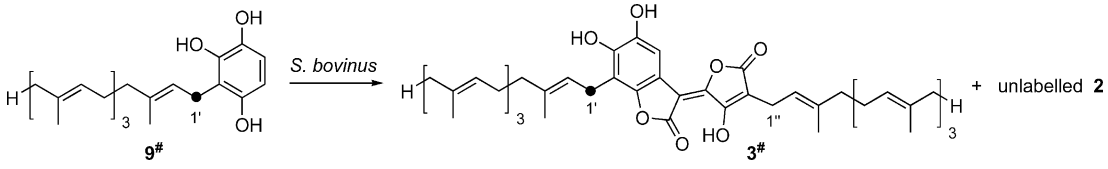
sor of the hydroxylactone portion of bovilactone-4,4. At the same time, the formation of **3** from two molecules of boviquinone-4 – a reaction that has been used for a one-step synthesis of bovilactone-4,4[8] and related dilactones[8,10b,17] – is excluded.

In order to verify the role of boviquinone-4 as a precursor of bovilactone-4,4, a mixture of [1′,2′-$^{13}C_2$]boviquinone-4 (**2**$^{\#\#}$) and 4-hydroxy-[1-^{13}C]benzoic acid[15] (**10***) was administered to young fruit bodies of *S. bovinus* (Scheme 4). The labelled acid **10*** was added to induce the formation of bovilactone-4,4, as well as to determine its de novo synthesis (Table 2, Entry 5). Both precursors were solubilized in water by addition of 2-hydroxypropyl-β-cyclodextrin[18] as a highly water-soluble host.[19] NMR investigation of the resulting bovilactone-4,4 (**3**$^{\#\#}$*) revealed a 4% ^{13}C enrichment of each side-chain carbon C-1′′ and C-2′′ and no detectable enhancement of the signals for C-1′ or C-2′ (Scheme 4). This confirms the role of boviquinone-4 as precursor of the dilactone portion of bovilactone-4,4. As expected, the [^{13}C]-labelled 4-hydroxybenzoic acid (**10***) was incorporated into both halves of **3**, the catechol ring showing 25% incorporation and the hydroxybutenolide ring 17%, equally distributed between C-10 and C-12 (Table 2, Entry 5, and Scheme 4).



Scheme 4. Simultaneous feeding of precursors **2**$^{\#\#}$ and **10*** to *S. bovinus*.

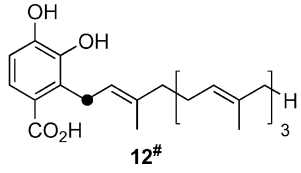
In a second experiment, [1′-^{13}C]deacetylsuillin (**9**$^{\#}$) was administered to *S. bovinus* as a liposomal formulation (Table 2, Entry 6, and Scheme 5). Investigation of the resulting bovilactone-4,4 (**3**$^{\#}$) revealed exclusive labelling of the polyprenyl residue attached to the benzene ring (incorporation rate 25%). The second polyprenyl chain in **3**$^{\#}$, as well as that of the accompanying boviquinone-4 (**2**), remained unlabelled. This result points to deacetylsuillin (**9**)



Scheme 5. Carbon-13 enrichment of **3**$^{\#}$ after feeding of [1′-^{13}C]deacetylsuillin (**9**$^{\#}$) to *S. bovinus*.

as the building block for the aromatic portion of bovilactone-4,4 and excludes **9** as the precursor of boviquinone-4.

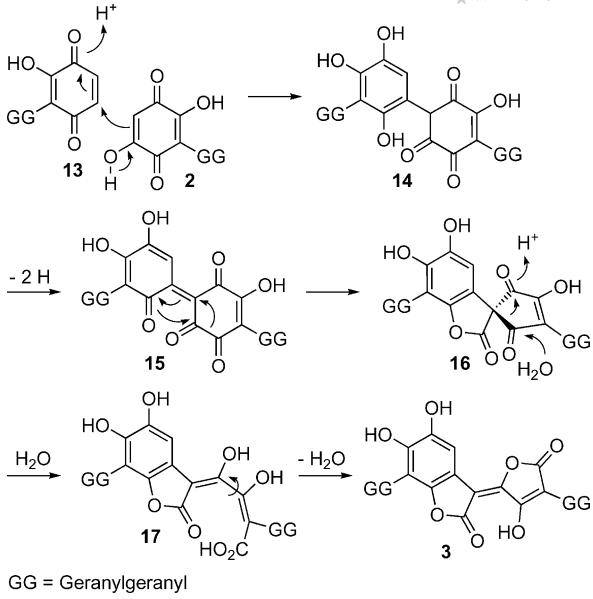
The administration of a liposomal formulation of 2-(*E*,*E*,*E*)-[1′-^{13}C]geranylgeranyl-3,4-dihydroxybenzoic acid[20] (**12**$^{\#}$) to fruit bodies of *S. bovinus* gave no detectable incorporation into **2** and **3** (Table 2, Entry 7). This negative result allows no conclusions, since difficulties encountered by this lipophilic precursor in reaching the site of biosynthesis cannot be ruled out.[2]



12$^{\#}$

The formation of bovilactone-4,4 (**3**) from deacetylsuillin (**9**) and boviquinone-4 (**2**) can be explained by the mechanism depicted in Scheme 6. After oxidation of deacetylsuillin to the corresponding benzoquinone **13**, this could then act as a Michael acceptor for boviquinone-4 (**2**). The resulting adduct **14** could then be dehydrogenated to tetraketone **15**, which could rearrange to spirodione **16**.[21] Hydrolytic ring-cleavage of the 1,3-dicarbonyl moiety, followed by lactonization of the resulting carboxylic acid **17**, would then afford bovilactone-4,4 (**3**).[21,22] Similar reactions are probably involved in the biosyntheses of gomphilactone,[23] ochroleucin A_2,[21] the *Embelia*[10a] and *Aegiceras*[24] lactones and the parathesilactones,[10c] and in the chemical syntheses of bovilactone-4,4[8] and related dilactones.[8,10b,17] The rearrangement of dibenzoquinones of type **14** into 4-ylidene butenolides was first described by Posternak.[25]

Boviquinone-4 (**2**) and bovilactone-4,4 (**3**) were also formed in submerse cultures of *S. bovinus*. The former pigment could only be detected in young cultures. On aging of the cultures, its concentration decreased steadily in favour of bovilactone-4,4 (**3**), and after 4 weeks only the latter compound was present. After addition of the [^{13}C]-labelled hydroxybenzoic acids **10*** or **11*** (Table 3, Entries 2 and 3), the cultures produced exclusively dilactone **3**, an effect resembling that observed with the fruit bodies. Dilactone **3*** exhibited the usual labelling pattern and was also obtained when [1′-^{13}C]tyrosine[16] was used as precursor (Table 3, Entry 1).



Scheme 6. Mechanism proposed for the formation of **3**.

Suillus variegatus

Unlike *Suillus bovinus* collected in Japan,[5] our European specimens did not contain HPLC-detectable amounts of suillin (**1**), neither in the fruit bodies nor in submerse cultures. In order to study the biosynthesis of this important meroterpenoid, we instead used fruit bodies and cultures of the closely related *S. variegatus* (Sw.: Fr.) O. Kuntze (German: Sandröhrling). Feeding of the [1-^{13}C]-labelled hydroxybenzoic acids **10*** or **11*** to young fruit bodies yielded suillin (**1***) with significant labelling at C-4 (Table 4, Entries 1 and 2, Scheme 7).[1] The position of the label is in agreement with the biosynthetic sequence given in Scheme 8, below.

Suillin (**1**) could also be isolated from submerse cultures of *S. variegatus*. To our surprise, however, this metabolite disappeared after addition of DL-[1′-^{13}C]tyrosine, 3,4-dihydroxy-[1-^{13}C]benzoic acid (**11***), or [4-^{13}C]benzene-1,2,4-triol, and significant amounts of bovilactone-4,4 (**3***), a compound not previously present, were formed (Table 3, Entries 4–6). In the first two experiments, bovilactone-4,4 showed the same labelling pattern as that reported for *S. bovinus*. [4-^{13}C]-Labelled benzene-1,2,4-triol[16] was not

Table 3. Feeding experiments with submerse cultures of *S. bovinus* and *S. variegatus*.

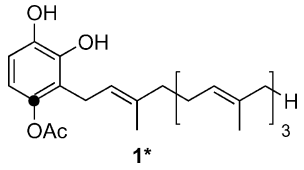
Entry	Species	Precursor [mg]	Bovilactone-4,4 (**3**) Isolation [mg][a,b]	^{13}C enrichment
1	*S. bovinus*	DL-[1′-^{13}C]Tyr (65)	8	C-4 (9%), C-10, C-12 (each 5%)
2	*S. bovinus*	**10*** (84)	3	C-4 (2%), C-10, C-12 (each 1%)
3	*S. bovinus*	**11*** (85)	8	C-4, C-10, C-12[c]
4	*S. variegatus*	DL-[1′-^{13}C]Tyr (50)	10	C-4, C-10, C-12[c]
5	*S. variegatus*	**11*** (82)	50	C-4 (9.2%), C-10, C-12 (each 5%)
6	*S. variegatus*	[4-^{13}C]benzene-1,2,4-triol (60)	52	0%

[a] Amount of isolated bovilactone-4,4 (**3**). [b] After 2 months incubation time. [c] Not quantified.

Table 4. Feeding experiments with fruit bodies of *S. variegatus*.

Entry	Precursor (mg,[a] solvent)	Number of fruit bodies	Suillin (**1**) Isolation [mg][a,b]	^{13}C enrichment
1	**10*** (25, DMSO)	4	11	C-4 (21%)
2	**11*** (40, DMSO)	5	9	C-4 (36%)
3	[4-^{13}C]benzene-1,2,4-triol (25, DMSO)	2	8.5	0%

[a] Amount per fruit body. [b] The mushrooms were harvested 4–7 d after administration.



Scheme 7. Labelling of **1*** after feeding of precursors **10*** or **11*** to fruit bodies of *S. variegatus*.

incorporated into suillin or bovilactone-4,4, neither in fruit bodies nor in cultures of *S. variegatus*, an indication that this potential intermediate[2] does not participate in the biosynthesis of these *Suillus* meroterpenoids.

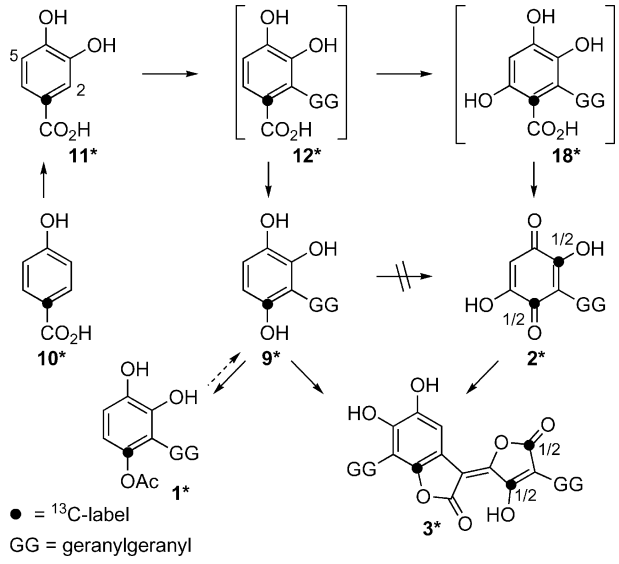
Biosynthetic Conclusions

The results of the feeding experiments can be explained by the biosynthetic sequence depicted in Scheme 8. Suillin (**1**) and the aromatic part of bovilactone-4,4 (**3**) are produced from deacetylsuillin (**9**), which is in turn derived from 3,4-dihydroxybenzoic acid (**11**) through geranylgeranylation at C-2 and oxidative decarboxylation of the resulting carboxylic acid **12**. Since boviquinone-4 (**2**) and the dilactone part of bovilactone-4,4 (**3**) are not formed from deacetylsuillin (**9**), their biosynthesis must follow a different route. As a plausible intermediate we propose trihydroxybenzoic acid **18**, which could be formed by hydroxylation of carboxylic acid **12** and transformed into boviquinone-4 (**2**) on oxidation.

Remarkably, feeding of [1-^{13}C]-labelled hydroxybenzoic acids **10*** and **11*** to fruit bodies of *S. bovinus* (Scheme 3) yielded bovilactone-4,4 (**3***) with equal incorporation of the labelled precursors into both halves of the molecule (Table 2, Entries 2 and 4), despite the fact that large amounts of unlabelled boviquinone-4 are present in the mushrooms. Incorporation of this would have decreased the isotope levels for C-10 and C-12. This finding is consistent with the restriction of **3** to the basal mycelium, in which the two necessary intermediates **2** and **9** can only be derived by de novo synthesis. For the biosynthesis of **3**, the boviquinone-4 pool in the fruit bodies thus remains largely unused.

The reason for the metabolic changes after the addition of the aromatic carboxylic acids is still unclear and needs further investigation.

In summary, we describe a common pathway for the formation of suillin (**1**), boviquinone-4 (**2**) and bovilactone-4,4 (**3**) in *Suillus* species, based on the polyprenylation of 3,4-dihydroxybenzoic acid (**11**) at C-2 as the primary biosynthetic step. In contrast, *S. tridentinus* produces the meroterpenoids bolegrevilol, tridentoquinone and tridentorubin by a different route, starting with polyprenylation of **11** at C-5.[2] The exceptional position of *S. tridentinus* and some related species within the genus *Suillus* is also expressed in the special pattern of its grevillin pigments.[26]



Scheme 8. Proposed sequence for the formation of meroterpenoids **1***, **2*** and **3*** and labelling pattern after feeding of [1-^{13}C]-labelled **10*** or **11*** to *S. bovinus* and *S. variegatus*.

Experimental Section

General: Elemental analyses: Microanalytical Laboratory, Ludwig-Maximilians-Universität München. IR spectra: Perkin–Elmer FT-IR 1000 spectrometer. Intensity of the bands: ss (very strong), s (strong), m (medium), and w (weak); sh: shoulder. UV/Vis spectra: Perkin–Elmer Lambda 16 spectrometer. NMR spectra: Bruker ARX 300 and AMX 600 instruments, with the solvent peak as internal standard ($CDCl_3$: δ_H = 7.26, δ_C = 77.1). Numbering of the chain carbon atoms of geranylgeranyl as in ref.[20] The ^{13}C NMR spectra for ^{13}C enrichment determinations were measured inverse-gated with a delay time of 8.0 s. MS: Finnigan MAT 90 and MAT 95Q instruments (direct inlet, 70 eV). Analytical TLC: silica gel 60 F_{254} aluminium foils (Merck); solvent system A: toluene/HCO_2Et/HCO_2H (10:5:3). Flash chromatography: silica gel 60, 40–63 μm (Merck). Column chromatography: acetylated polyamide-6 (Polyamide SC-6AC, 50–160 μm, Macherey–Nagel). High-speed countercurrent chromatography (HSCCC) was performed with an apparatus from P.C. Inc., Potomac, MD, USA, consisting of a multi-layer coil, a counter-weight/triple coil, and a Rainin Dynamax SD-200 pump. Liposome extrusions: Thermobarrel Extruder (10 mL), Northern Lipids Inc., Vancouver, Canada. Membrane:

polycarbonate, 400 nm (Osmotics). Drain Disc: polyethylene, 25 mm (Whatman). All solvents were distilled prior to use. Air- and moisture-sensitive compounds were handled under argon with use of standard Schlenk techniques. THF was distilled under argon from Na/benzophenone. CH_2Cl_2 was distilled under argon from Sicapent (Merck).

Mushrooms: The feeding experiments were carried out in September/October 1993–2001. *S. bovinus* was collected in a pine wood near Offenstetten, Bayern (Germany). *S. variegatus* was collected near Nassereith, Tirol (Austria). Harvested fruit bodies were frozen in liquid N_2 and stored at –20 °C.

Submerse Cultures: *S. bovinus* strain 824 and *S. variegatus* strain 615 were obtained from the Department of Biology, University of Regensburg (Germany). Both species were cultured on modified Moser's medium B {aneurin (50 g), biotin (1 g), inositol (50 mg), KH_2PO_4 (0.5 g), $MgSO_4$ (0.5 g), aq. $ZnSO_4$ [0.002% (*w*/*v*), 0.5 mL], aq. $FeCl_3$ [1% (*w*/*v*), 1 mL], aq. $CaCl_2$ (0.1 M, 5 mL), aq. $MnSO_4$ [1% (*w*/*v*), 0.5 mL], yeast extract (0.2 g), maltose (20 g), glucose (10 g), peptone (2 g), demineralised water up to 1 L of total volume}.

Isolation of Metabolites

Isolation Procedure from Fruit Bodies of *S. bovinus*: The frozen fruit bodies of *S. bovinus* (300 g, wet weight) were minced and extracted exhaustively with several portions of EtOAc. The aqueous phase was discarded, and the combined organic phases were dried (Na_2SO_4) and concentrated to a small volume under reduced pressure. The resulting solution was pre-purified by passage through a short pad of acetylated polyamide-6. After evaporation of the solvent, the residue was taken up in a small amount of *n*-hexane and chromatographed on acetylated polyamide-6. Elution with *n*-hexane yielded crude **2** (250 mg); subsequent elution with toluene afforded crude **3** (60 mg). Upon chromatography of the crude **2** by HSCCC (mobile phase: *n*-hexane, 4 volume parts, stationary phase: AcOH/MeOH (1:1) volume parts; 325 mL column, forward rotation mode, 825 rpm, flow: 1.5 mL min^{-1}), the pure compound collected in the stationary phase, which was fractionated. The yellow fractions were combined, diluted with Et_2O and washed with water to remove most of the AcOH. Concentration of the organic phase and drying under reduced pressure afforded pure **2** as an orange solid. Chromatography of the crude **3** by HSCCC (mobile phase: *n*-hexane, 4 volume parts, stationary phase: AcOH/MeOH (1:1) volume parts; 230 mL column, forward rotation mode, 850 rpm, flow: 1.5 mL min^{-1}) yielded pure bovilactone-4,4 (**3**) with the mobile phase. The orange fractions were combined, diluted with Et_2O and washed with water. The organic phase was concentrated, and the residue was dried under reduced pressure.

Boviquinone-4 (2): Orange solid. Yield up to 20 mg per fruit body (up to 0.75% of dry weight). R_f (TLC) = 0.70 (solvent system A), yellow to orange spot, +NH_3 violet. For m.p., IR, UV and MS data, see ref.[6] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.56–1.61 (m, 9 H, 18′-H, 19′-H, 20′-H), 1.67 (s, 3 H, 16′-H), 1.74 (s, 3 H, 17′-H), 1.92–2.11 (m, 12 H, 6 × CH_2), 3.16 (d, J = 7.1 Hz, 2 H, 1′-H), 5.02–5.20 (m, 4 H, 4 × CH_{vinyl}), 6.00 (s, 1 H, 6-H), 7.69 (br. s, 2 H, 2 × OH) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.06, 16.09 (C-18′ and C-19′), 16.2 (C-17′), 17.8 (C-20′), 21.7 (C-1′), 25.8 (C-16′), 26.5 (C-5′), 26.7, 26.8 (C-9′ and C-13′), 39.76 (2 ×) and 39.78 (C-4′, C-8′, C-12′), 102.3 (C-6), 116.1 (C-3), 119.1 (C-2′), 124.0, 124.3, 124.5 (3 × CH_{vinyl}), 131.3 (C-15′), 135.0, 135.2 (C-7′ and C-11′), 137.7 (C-3′) ppm.

Signals for C-1, C-2, C-4 and C-5 were not visible, due to broadening. The ^{13}C-incorporation was therefore determined after transformation of **2** into the corresponding dimethyl ether.[6] Yellow oil. R_f (TLC) = 0.37 (hexanes/EtOAc, 5:1). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.57, 1.58, 1.59 (each s, 3 H, 18′-H, 19′-H, 20′-H), 1.67 (s, 3 H, 16′-H), 1.73 (s, 3 H, 17′-H), 1.92–2.08 (m, 12 H, 6 × CH_2), 3.14 (d, J = 7.3 Hz, 2 H, 1′-H), 3.79, 4.05 (both s, 3 H, 2 × OCH_3), 5.02–5.09 (m, 4 H, 4 × CH_{vinyl}), 5.72 (s, 1 H, 6-H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 16.1 (2 ×), 16.2 (C-17′, C-18′, C-19′), 17.8 (C-20′), 22.3 (C-1′), 25.8 (C-16′), 26.6, 26.7, 26.8 (C-5′, C-9′, C-13′), 39.8 (3 × CH_2, C-4′, C-8′, C-12′), 56.4, 61.4 (each OCH_3), 105.5 (C-6), 119.8 (C-2′), 124.1, 124.3, 124.5 (3 × CH_{vinyl}), 129.6 (C-3), 131.3 (C-15′), 135.0, 135.2 (C-7′ and C-11′), 137.3 (C-3′), 155.6 (C-2), 158.8 (C-5), 182.3 (C-4), 183.8 (C-1) ppm.

Bovilactone-4,4 (3): Auburn oil. Yield approx. 0.02% of dry weight from whole fruit bodies including the mycelium adherent to the stipe base.[9] R_f (TLC) = 0.80 (solvent system A), red spot, becomes grey upon standing, +NH_3 grey. ^{1}H NMR (600 MHz, $CDCl_3$; only signals omitted from Table 1 given): δ = 1.57–1.61 (m, 18 H, 6 × CH_3), 1.67 (s, 6 H, 16′-H, 16′′-H), 1.92–2.13 (m, 24 H, 12 × CH_2), 5.05–5.14 (m, 6 H, 6 × CH_{vinyl}), 6.15 (br. s, 1 H, OH) ppm; 1 × OH obscured. ^{13}C NMR (151 MHz, $CDCl_3$; only signals omitted from Table 1 given): δ = 16.08 (2 ×), 16.11, 16.16 (C-18′, C-19′, C-18′′, C-19′′), 17.8 (2 × CH_3, C-20′ and C-20′′), 25.8 (2 × CH_3, C-16′ and C-16′′), 26.4, 26.5, 26.7, 26.76, 26.84 (2 ×, C-5′, C-9′, C-13′, C-5′′, C-9′′, C-13′′), 39.70–39.84 (m, 6 × CH_2, C-4′, C-8′, C-12′, C-4′′, C-8′′, C-12′′), 123.6, 124.0, 124.2, 124.3, 124.48, 124.51 (6 × CH_{vinyl}), 131.35, 131.41 (C-15′ and C-15′′), 135.0, 135.1, 135.3, 135.8 (4 × C_{vinyl}) ppm. IR (CCl_4): $\tilde{\nu}$ = 3360 (br), 2920 (ss), 1795 (w), 1760 (ss), 1730 (ss), 1645 (ss), 1620 (ss) cm^{-1}. UV/Vis (MeOH): λ_{max} (log ε) = 275 (4.06), 382 (3.92), 460 (3.95) nm. EI-MS: *m*/*z* (%) = 806 (0.15) [M]$^+$, 411 (1.1), 409 (1.5), 343 (2), 342 (2), 341 (3), 289 (3), 288 (3), 287 (5), 259 (5), 217 (6), 205 (7), 203 (5), 191 (9), 149 (10), 147 (9), 137 (11), 135 (20), 133 (9), 123 (16), 121 (23), 119 (13), 109 (23), 107 (21), 105 (16), 95 (25), 93 (27), 91 (20), 81 (57) $[C_6H_9]^+$, 69 (100) $[C_5H_9]^+$, 55 (16), 44 (33), 41 (11) $[C_3H_5]^+$.

Isolation Procedure from Fruit Bodies of *S. variegatus*: Lyophilized fruit bodies (8 g) were pulverized and extracted exhaustively with EtOAc. The extract was dried (Na_2SO_4), concentrated under reduced pressure and chromatographed on Sephadex LH-20 (eluent: MeOH). The fractions containing suillin (**1**) were further purified by chromatography on acetylated polyamide-6 (eluent: *n*-hexane, followed by toluene) to afford pure **1**.

Suillin (1): Colourless oil. Yield 15–50 mg (0.2–0.6% of dry weight). R_f (TLC) = 0.62 (solvent system A). For m.p., IR, UV and MS data, see ref.[3] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.59 (s, 9 H, 18′-H, 19′-H, 20′-H), 1.68 (s, 3 H, 16′-H), 1.78 (s, 3 H, 17′-H), 1.97–2.12 (m, 12 H, 6 × CH_2), 2.28 (s, 3 H, $COCH_3$), 3.25 (d, J = 6.9 Hz, 2 H, 1′-H), 5.05–5.12 (m, 3 H, 3 × CH_{vinyl}), 5.21 (t, J = 6.1 Hz, 1 H, 2′-H), 6.50 (d, J = 7.7 Hz, 1 H, 5-H), 6.72 (d, J = 7.7 Hz, 1 H, 6-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.06, 16.09 (C-18′ and C-19′), 16.2 (C-17′), 17.7 (C-20′), 20.8 ($COCH_3$), 24.0 (C-1′), 25.7 (C-16′), 26.3, 26.6, 26.8 (C-5′, C-9′, C-13′), 39.6 (2 ×) and 39.7 (C-4′, C-8′, C-12′), 112.8 (C-6), 113.8 (C-5), 120.1 (C-3), 120.8 (C-2′), 123.6, 124.2, 124.6 (3 × CH_{vinyl}), 131.3 (C-15′), 135.0, 135.8 (C-7′ and C-11′), 139.1 (C-3′), 142.0 (C-4), 142.2 (C-1), 142.9 (C-2), 170.3 ($COCH_3$) ppm.

Synthesis of Biosynthetic Precursors

[1′,2′-$^{13}C_2$]Boviquinone-4 (2$^{\#\#}$): A solution of (*E*,*E*,*E*)-[1,2-$^{13}C_2$]geranylgeranyl bromide[11] (**5**$^{\#\#}$, 178 mg, 0.50 mmol) in THF (1 mL) was slowly added at 50 °C to a solution of 2,5-dihydroxybenzoquinone (**4**, 140 mg, 1.00 mmol) and triethylamine (70 μL, 0.50 mmol) in THF (2.5 mL). After 5 h, the volatiles were removed,

and the residue was distributed between EtOAc and water. The organic phase was washed with water (3×), dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by chromatography on acetylated polyamide-6, followed by HSCCC (see isolation of **2**). Yield 27 mg (13%), orange solid. ^{1}H NMR (300 MHz, $CDCl_3$, only data differing from those of **2** given): δ = 1.74 (d, $^3J_{C,H}$ = 5.1 Hz, 3 H, 17′-H), 3.15 (ddd, $^1J_{C,H}$ = 130 Hz, $^2J_{C,H} \approx {}^3J_{H,H} \approx$ 7.2 Hz, 2 H, 1′-H), 5.02–5.13 (m, 3 H, 3×CH_{vinyl}), 5.13 (dm, $^1J_{C,H} \approx$ 156 Hz, 1 H, 2′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, only data differing from those for **2** given): δ = 16.18–16.28 (m, C-17′), 21.7 (d, $^1J_{C,C}$ = 43.0 Hz, ^{13}C-1′), 26.5 (d, $^3J_{C,C}$ = 3.0 Hz, C-5′), 116.1 (dd, $^1J_{C,C}$ = 44.1 Hz, $^2J_{C,C}$ = 4.0 Hz, C-3), 119.1 (d, $^1J_{C,C}$ = 43.0 Hz, C-2′), 137.6 (d, $^1J_{C,C}$ = 74.3 Hz, C-3′) ppm.

1,2,4-Tris(methoxymethoxy)benzene (7): A solution of 1,2,4-triacetoxybenzene (**6**, 5.04 g, 20 mmol) in MeOH (50 mL) was saturated with argon. Hydrazine monohydrate (3.9 mL, 80 mmol) was then added, and the mixture was stirred for 15 min at room temperature under argon. After removal of the volatiles under reduced pressure, the resulting residue was treated with HCl (2 N, 50 mL, saturated with argon), and the benzene-1,2,4-triol was extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with brine, dried (Na_2SO_4) and concentrated. The crude benzene-1,2,4-triol (2.45 g, yield 97%) was suspended in dry CH_2Cl_2 (100 mL) under argon and treated with chloro(methoxy)methane (6 M in EtOAc,[13b] 19 mL, 114 mmol) and *N*,*N*-diisopropylethylamine (26 mL, 152 mmol). After the mixture had been stirred overnight, NaOH (2 N, 100 mL) was added, and the phases were separated. The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic phases were washed with water and brine and dried (Na_2SO_4). The product was purified by flash chromatography (hexanes/EtOAc, 3:1). Yield 3.21 g (62%), yellowish oil. R_f (TLC) = 0.35 (hexanes/EtOAc, 3:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.46, 3.50, 3.51 (each s, 3 H, 3×OCH_2OCH_3), 5.10, 5.14, 5.20 (each s, 2 H, 3×OCH_2OCH_3), 6.64 (dd, ^3J = 8.9, ^4J = 2.9 Hz, 1 H, 5-H), 6.88 (d, ^4J = 2.9 Hz, 1 H, 3-H), 7.05 (d, ^3J = 8.9 Hz, 1 H, 6-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 56.0, 56.1, 56.2 (3×OCH_2OCH_3), 95.1, 95.5, 96.3 (3×OCH_2OCH_3), 106.6, 109.4 (C-3 and C-5), 118.3 (C-6), 142.3 (C-1), 148.3 (C-2), 153.0 (C-4) ppm. IR (KBr): $\tilde{\nu}$ = 2994 (m), 2956 (s), 2901 (s), 2827 (m), 2787 (w), 1609 (m), 1596 (m), 1505 (s), 1469 (m), 1442 (m), 1402 (m), 1261 (m), 1223 (s), 1194 (s), 1153 (s), 1126 (m), 1080 (s), 1008 (s, br), 923 (s), 850 (m), 811 (m), 767 (w), 708 (w), 666 (w), 631 (w) cm^{-1}. EI-MS: *m*/*z* (%) = 259 (4) $[M + 1]^+$, 258 (35) $[M]^+$, 213 (2) $[M - C_2H_5O]^+$, 195 (2), 182 (28), 152 (52), 137 (7), 123 (2), 121 (2), 79 (2), 45 (100) $[C_2H_5O]^+$. $C_{12}H_{18}O_6$ (258.27): calcd. C 55.81, H 7.02; found C 55.82, H 7.26.

3-(*E*,*E*,*E*)-[1′-^{13}C]Geranylgeranyl-1,2,4-tris(methoxymethoxy)-benzene (8$^{\#}$): A solution of **7** (65 mg, 0.25 mmol) in dry THF (2.5 mL) in a Schlenk tube was treated under argon at 0 °C with *n*BuLi[27] (1.7 M in hexanes, 0.176 mL, 0.30 mmol), and the mixture was stirred at room temperature for 0.5 h. After the system had been cooled to 0 °C, CuCl (99.995+%, Aldrich, 30 mg, 0.30 mmol) was added, and the stirring was continued at 0 °C for 0.5 h. The mixture was then cooled to –20 °C and treated over 0.5 h with a solution of [1-^{13}C]geranylgeranyl bromide[20] (**5$^{\#}$**, 89 mg, 0.25 mmol) in THF (2.5 mL). The mixture was stirred for an additional 0.5 h at –20 °C and subsequently warmed to room temperature. Saturated aq. NH_4Cl was added, and the product was extracted with Et_2O. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Compound **8$^{\#}$** was purified by flash chromatography (hexanes/EtOAc, 10:1). Yield 97 mg (73%), colourless oil. R_f (TLC) = 0.23 (hexanes/EtOAc, 10:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.58, 1.59, 1.60 (each s, 3 H, 18′-H, 19′-H, 20′-H), 1.68 (s, 3 H, 16′-H), 1.78 (s, 3 H, 17′-H), 1.91–2.12 (m, 12 H, 6×CH_2), 3.44 (dd, $^1J_{C,H}$ = 128, $^3J_{H,H}$ = 6.6 Hz, 2 H, 1′-H), 3.46, 3.50, 3.59 (each s, 3 H, 3×OCH_2OCH_3), 5.05–5.16 (m, 3 H, 4′-H, 8′-H, 12′-H), 5.10 (s, 2 H, OCH_2OCH_3), 5.12 (br. s, 4 H, 2×OCH_2OCH_3), 5.17–5.26 (m, 1 H, 2′-H), 6.78, 6.92 (2×d, ^3J = 9.1 Hz, 2×1 H, 5-H, 6-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1 (s, 2×CH_3, C-18′ and C-19′), 16.3 (d, $^3J_{C,C}$ = 3.8 Hz, C-17′), 17.7 (C-20′), 23.7 (^{13}C-1′), 25.8 (C-16′), 26.72, 26.75, 26.84 (C-5′, C-9′, C-13′), 39.7–40.0 (m, 3×CH_2, C-4′, C-8′, C-12′), 55.9, 56.2, 57.6 (3×OCH_2OCH_3), 94.9, 95.9, 99.3 (3×OCH_2OCH_3), 110.1 (C-5), 114.7 (C-6), 122.9 (d, $^1J_{C,C}$ = 42.9 Hz, C-2′), 124.31, 124.33, 124.5 (3×CH_{vinyl}), 126.2 (d, $^1J_{C,C}$ = 44.0 Hz, C-3), 131.3 (C-15′), 134.9 (2×C_{vinyl}), 135.0 (C_{vinyl}), 145.0 (C-1), 145.8, 150.97 (C-2 and C-4) ppm. IR (KBr):[28] $\tilde{\nu}$ = 2956 (s, sh), 2925 (s, br), 2849 (s), 2826 (s, sh), 1666 (w), 1593 (w), 1485 (s), 1440 (s), 1399 (m), 1382 (m), 1250 (s), 1206 (m), 1154 (s), 1089 (s), 1046 (ss, br), 944 (s), 926 (s), 805 (m), 717 (w), 679 (w) cm^{-1}. EI-MS: *m*/*z* (%) = 531 (6) $[M]^+$, 486 (2) $[M - C_2H_5O]^+$, 455 (5), 454 (5), 354 (3), 286 (8), 236 (9), 218 (22), 192 (15), 190 (19), 152 (27), 140 (15), 135 (12), 121 (15), 109 (11), 107 (13), 95 (13), 93 (11), 81 (25) $[C_6H_9]^+$, 69 (56) $[C_5H_9]^+$, 45 (100) $[C_2H_5O]^+$, 41 (16) $[C_3H_5]^+$. HR EI-MS:[28] *m*/*z* = 530.3617 $[M]^+$ (calcd. for $C_{32}H_{50}O_6$: 530.3607).

3-(*E*,*E*,*E*)-[1′-^{13}C](Geranylgeranyl)benzene-1,2,4-triol ([1′-^{13}C]Deacetylsuillin, 9$^{\#}$): Acetyl chloride (23 μL, 0.32 mmol) was added under argon to a solution of **8$^{\#}$** (85 mg, 0.16 mmol) in MeOH (2 mL). After completion of the reaction (3 h, TLC monitoring), H_2O (25 mL) was added, and the crude product was extracted with EtOAc (3 × 15 mL). The combined organic phases were washed with water (2 × 15 mL) and concentrated under reduced pressure. Flash chromatography (hexanes/acetone, 3:1) afforded **9$^{\#}$** (43.5 mg, 68%) as a yellowish waxy solid. R_f (TLC) = 0.19 (hexanes/acetone, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.60 (s, 9 H, 18′-H, 19′-H, 20′-H), 1.68 (s, 3 H, 16′-H), 1.82 (s, 3 H, 17′-H), 1.91–2.17 (m, 12 H, 6×CH_2), 3.44 (dd, $^1J_{C,H}$ = 128, $^3J_{H,H}$ = 7.0 Hz, 2 H, 1′-H), 5.04–5.16 (m, 3 H, 3×CH_{vinyl}), 5.24–5.33 (m, 1 H, 2′-H), 5.60 (br. s, 1 H, OH), 6.27 (dd, $^3J_{H,H}$ = 8.6, $^4J_{C,H}$ = 1.0 Hz, 1 H, 5-H), 6.61 (d, ^3J = 8.6 Hz, 1 H, 6-H) ppm; 2×OH obscured. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.08, 16.14 (C-18′ and C-19′), 16.3 (d, $^3J_{C,C}$ = 3.9 Hz, C-17′), 17.8 (C-20′), 23.0 (^{13}C-1′), 25.8 (C-16′), 26.4, 26.7, 26.9 (C-5′, C-9′, C-13′), 39.7–39.9 (m, C-4′, C-8′, C-12′), 106.9 (C-5), 113.1 (C-6), 114.6 (d, $^1J_{C,C}$ = 43.4 Hz, C-3), 121.3 (d, $^1J_{C,C}$ = 42.0 Hz, C-2′), 123.6, 124.3, 124.5 (3×CH_{vinyl}), 131.4 (C-15′), 135.1, 135.8, 137.5 (3×C_{vinyl}), 139.5 (C-1), 143.1, 148.5 (C-2 and C-4) ppm. EI-MS: *m*/*z* (%) = 400 (18) $[M + 1]^+$, 399 (60) $[M]^+$, 263 (10), 259 (11), 207 (10), 195 (14), 191 (11), 180 (36), 178 (30), 140 (100), 139 (45), 123 (21), 121 (15), 109 (18), 107 (13), 95 (17), 93 (12), 81 (30) $[C_6H_9]^+$, 69 (39) $[C_5H_9]^+$, 41 (20) $[C_3H_5]^+$. HR EI-MS:[28] *m*/*z* = 398.2828 $[M]^+$ (calcd. for $C_{26}H_{38}O_3$: 398.2821).

Feeding Experiments

Feeding Experiments with Fruit Bodies (Table 2 and Table 4): DMSO solutions and cyclodextrin preparations of the potential precursors were injected by syringe deep into the stalks of young specimens (cap diameter: approx. 2 cm) of *S. bovinus*. For the feeding of the liposome preparations, a deep hole was gouged into a young fruit body; if necessary, the reservoir volume was extended by insertion of an appropriate short glass tube. The resulting cavity was soaked with the liposome preparation (1 mL). The toadstools were harvested after 4–7 d, frozen in liquid nitrogen and kept at –20 °C until workup and isolation of the metabolites.

Cyclodextrin Preparations: 4-Hydroxy-[1-^{13}C]benzoic acid (**10***, 30 mg, 0.22 mmol) was mixed with 2-hydroxypropyl-β-cyclodextrin, average degree of substitution: 7[18] (0.34 g, 0.22 mmol, $M_{average}$ = 1541, Aldrich). After the addition of water (0.34 mL), the mixture was stirred until a clear viscous liquid was obtained. Quinone **2**$^{\#\#}$ (10 mg, 24 μmol) was separately solubilized with approximately 10 equiv. (0.4 g) of 2-hydroxypropyl-β-cyclodextrin and water (0.3 mL).

Liposome Preparations: Preparation was by the film method.[29] All steps were performed under argon. Lipid mixture: labelled precursor [**9**$^{\#}$ or **12**$^{\#}$, 10% (*w*/*w*)], egg phosphatidylcholine (Lipoid E-PC, Lipoid GmbH, Ludwigshafen, Germany, 74%), cholesterol (15%), DL-α-tocopherol (1%). The appropriate lipid mixture (300 to 600 mg) was dissolved in $CHCl_3$ in a 50 or 100 mL round-bottomed flask. The solvent was removed with a rotary evaporator to obtain a lipid film, which was dried under reduced pressure. MLV [10% (*w*/*v*) lipid-in-water ratio] formed by suspending with aqueous glucose solution [5% (*w*/*v*)]. The mean size of the liposomes containing **12**$^{\#}$ was limited to 400 nm by extrusion.

Feeding Experiments with Submerse Cultures (Table 3): Cultures were grown on cotton pads in modified Moser's medium B (250 mL, heat sterilization). The appropriate precursor was added as a solid before heat sterilization. For the isolation of **3**, the culture broth was filtered. The residual mycelium was minced and exhaustively extracted with EtOAc. The combined organic phases were dried (Na_2SO_4) and concentrated. Further purification of **3** followed the procedure described for the fruit bodies.

Supporting Information (see also the footnote on the first page of this article): 2D NMR spectra of **3**, and NMR spectra of **3**$^{\#\#*}$ and **3**$^{\#}$ (for Table 2, Entries 5 and 6) are provided.

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