Pergamon

Tetrahedron Letters 42 (2001) 7439–7442

TETRAHEDRON LETTERS

Isolation of (−)-cyatha-3,12-diene, a common biosynthetic intermediate of cyathane diterpenoids, from an erinacine-producing basidiomycete, *Hericium erinaceum*, and its formation in a cell-free system

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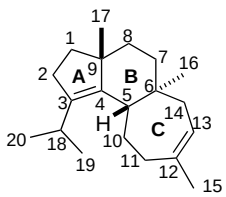
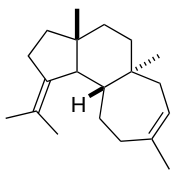
Received 14 June 2001; revised 24 July 2001; accepted 17 August 2001

Abstract—Cyatha-3,12-diene, a diterpene hydrocarbon having a 5–6–7-membered tricyclic skeleton, has been isolated for the first time from an erinacine-producing basidiomycete, *Hericium erinaceum* YB4-6237. This hydrocarbon had been formulated by Ayer et al. as a common biosynthetic intermediate of cyathane diterpenoids. Its stereo-structure, including the absolute configuration, has been unambiguously confirmed by its semi-synthesis from erinacine P, and its cell-free conversion from all-*trans*-geranylgeranyl diphosphate was demonstrated. © 2001 Elsevier Science Ltd. All rights reserved.

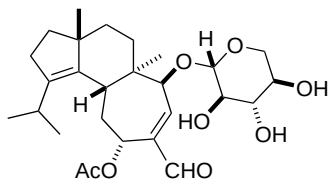
Cyathane diterpenoids[1–3] and their xylosides[4,5] are currently attracting much attention because of their unique biological activities. Biosynthetic studies on the cyathane skeleton, which does not follow the isoprene rule, had been carried out by Ayer et al., in the late 1970s.[6] They proposed a biogenesis in which cyatha-3,12-diene (**1**) is the initially-formed hydrocarbon. However, no cyathane hydrocarbons have been isolated from natural sources heretofore. In connection with our current interest[7,8] in isolating cDNAs encoding novel fungal diterpene cyclases, we attempted to isolate the cyathane hydrocarbon from *Hericium erinaceum*, the erinacine-producing basidiomycete. Here we report the isolation of cyatha-3,12-diene (**1**) and its isomer, which is most likely to be cyatha-3(18),12-diene (**2**), and cell-free conversion of the all-*trans*-geranylgeranyl diphosphate into cyatha-3,12-diene (**1**). The stereostructure of the former was further confirmed by its semi-synthesis from erinacine P (**3**) (Fig. 1).[9]



cyatha-3,12-diene (1)



cyatha-3(18),12-diene (2)



erinacine P (3)

Figure 1.

Keywords: biosynthesis; diterpene; structural elucidation; terpenes and terpenoids.

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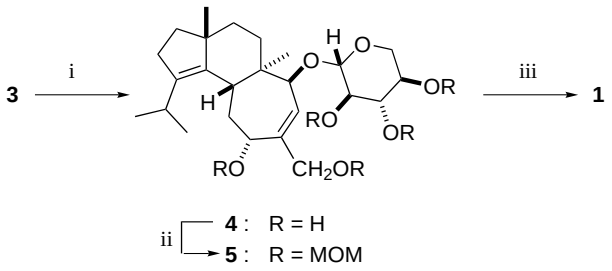
PII: S0040-4039(01)01550-7

In a previous paper,[9] we reported the isolation of a new and parental cyathane xyloside, erinacine P, from *Hericium erinaceum* YB4-6237. Investigation of the mycelial extract of this basidiomycete was continued to isolate cyathane hydrocarbons. The acetone extract of the mycelia (1.1 kg, wet weight) from the basidiomycete shake-cultured with Sakaguchi flasks gave a dark-brown residue (ca. 5.6 g), which was then successively purified by silica gel (Wakogel FC-40, hexane) flash chromatography and preparative reverse-phase HPLC (KUSANO Silicagel-S ODS, 300 mm×2, acetonitrile) to give two diterpenic hydrocarbons.

The more abundant hydrocarbon was isolated as a colorless oil {0.3 mg, $[\alpha]_D^{25}$ −103 (*c* 0.03, $CHCl_3$)}. It exhibited a molecular ion peak at *m/z* 272.2498 ($C_{20}H_{32}$ requires 272.2504) in the mass spectrum [*m/z* 272 (M^+, 25%), 257 (9), 229 (12), 204 (98), 203 (100), 189 (82), and 161 (40)] and showed characteristic ^1H NMR (400 MHz, $CDCl_3$) signals at δ_H 0.71 (3H, s; Me-16), 0.93 (3H, d, *J*=6.8 Hz; Me-19 or 20), 0.94 (3H, d, *J*=6.8 Hz; Me-20 or 19), 1.05 (3H, s; Me-17), 1.75 (3H, br s; Me-15), 3.00 (1H, septet, *J*=6.8 Hz; H-18), and 5.36 (1H, m; H-13). These data can be assignable for cyatha-3,12-diene (**1**), a putative hydrocarbon proposed by Ayer et al.[6] Although the limited amount of the isolated material prevented us from getting concrete proof for this speculation, the structure of this hydrocarbon has been unambiguously confirmed to be **1** by its semi-synthesis described below. If the structure of **1** is correct, then the planar structure of the less abundant hydrocarbon [a colorless oil, <0.1 mg, *m/z* 272 (M^+)] is most likely to be cyatha-3(18),12-diene (**2**) from the following characteristic signals in ^1H NMR, δ_H ($CDCl_3$, 400 MHz) 0.67 (3H, s; Me-16), 0.82 (3H, s; Me-17), 1.60, 1.65 (each 3H, br s; Me-19/20), 1.74 (3H, br s; Me-15), and 5.35 (1H, m; H-13). The stereochemistry of C4 could not be assigned at this moment, and the other stereogenic centers of **2** are tentatively adopted from those of **1**.

We then carried out the semi-synthesis of **1** to confirm the absolute stereo-structure of the isolated diterpene. As described, we have already obtained erinacine P (**3**), a cyathane xyloside, as a main metabolite produced by *H. erimaceum* YB4-6237.



Scheme 1. *Reagents and conditions*: (i) $LiAlH_4$, THF (79%); (ii) MOMCl, $^iPr_2NEt$, CH_2Cl_2 (79%); (iii) Li, Me_2N–$EtNH_2$ (80% with 90% isomeric purity).

Taking advantage of the fact that all oxygen functions on the diterpenic moiety of **3** locate at allylic positions, we could have easy access to the desired cyathadiene by dissolving metal reduction on an appropriate derivative of **3**. Thus, **3** was treated with $LiAlH_4$ to give **4** [HR-FABMS *m/z* 475.2669 ($C_{25}H_{40}O_7Na$ requires 475.2672)], of which all the hydroxy groups were then protected as MOM-ethers to afford **5** [HR-FABMS *m/z* 695.3983 ($C_{35}H_{60}O_{12}Na$ requires 695.3983)]. The dissolving metal reduction of **5** by Li metal in a mixture of Me_2N and $EtNH_2$ proceeded cleanly to give a hydrocarbon mixture in 80% yield (Scheme 1). GC–MS analysis of this fraction revealed one main product, of which the retention time and mass fragmentation pattern are coincident with those of natural **1**, with ca. 90% isomeric purity. By preparative reverse-phase HPLC, the main product {a colorless oil, $[\alpha]_D^{25}$ −115 (*c* 0.10, $CHCl_3$)} was further purified from the mixture. Direct comparison of the physicochemical properties of natural and semi-synthetic **1**, thus obtained, allowed us to conclude the identity of both compounds.

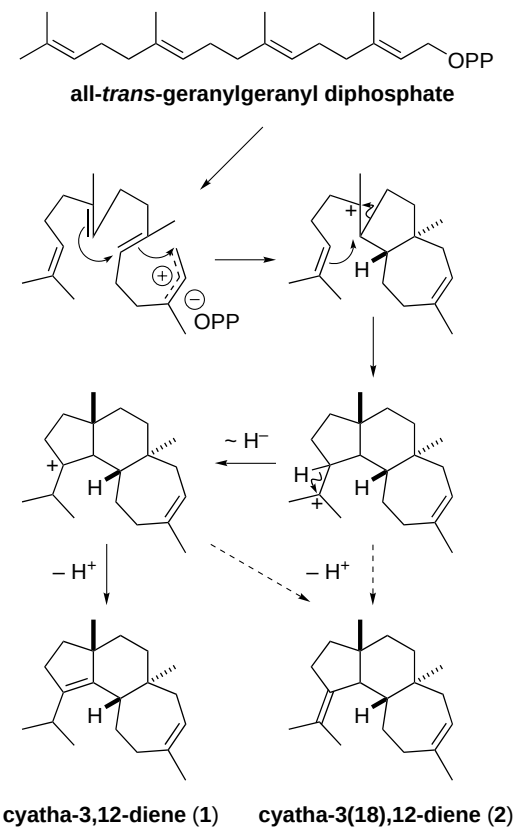
Since the structure of **3** had been established, including the absolute configuration, the stereochemistries of three stereogenic centers in **1** have been proven as depicted. However, some uncertainty regarding the structure of **1** remains. Although, from the biosynthetic point of view, it is most likely that the trisubstituted double bond in the C ring locates at the C12–C13 position, one should be aware that speculation without corroborative proof often leads to misunderstanding of the position of the double bonds in the biosynthetic intermediate as exemplified by the cases of taxadiene[10] and fusicoccadiene.[11] Additionally, from the mechanistic consideration on the dissolving metal reduction of **5**, the reductate could be cyatha-3,11-diene, which also has a trisubstituted double bond. To clarify the position of the double bond in the C ring, an extensive NMR analysis on semi-synthetic material was carried out including COSY, HMQC, and HMBC measurements. These allowed us to assign all ^1H and ^{13}C signals as summarized in Table 1. The coupling pattern of the C14 methylene protons, which have strong correlation with the olefinic proton in the COSY spectrum, suggests that the double bond locates at the C12–C13 position as expected. The clear enhancement of the olefinic proton by irradiation of Me-16 in the difNOE measurement also confirms that **1** is not cyatha-3,11-diene but -3,12-diene.

Occurrence of tentatively-assigned cyatha-3(18),12-diene (**2**) as a minor constituent in *H. erinaceum* is plausible from the biogenesis proposed by Ayer et al. (Scheme 2),[6] although no natural cyathane diterpenoids having such unsaturation pattern have been reported.

To confirm the enzymatic formation of **1** from the deuterated all-*trans*-geranylgeranyl diphosphate, preparation of cell-free extracts was carried out. The filtered mycelia (4 g, wet weight) from a 5-day old shaken-culture were homogenized in tris–HCl buffer (5 ml, 100 mM, pH 7.5) containing 5 mM dithiothreitol, 0.1 mM pepstatin A, 20% glycerol, 0.5 mM EDTA and

Table 1. ^{1}H and ^{13}C NMR assignments of semi-synthetic **1** in $CDCl_3$; 600 MHz for ^{1}H and 150 MHz for ^{13}C

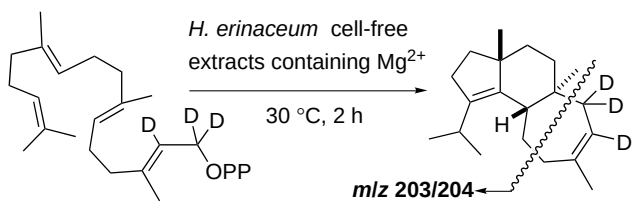
Position	^{1}H [ppm (multiplicity, J in Hz)]	^{13}C [ppm (type)]
1	1.465 (dt, 12.6, 8.1)	38.29 (CH_2)
	~1.56 (m)	
2	2.247 (2H, td like, <7.5, 1.8>)	28.51 (CH_2)
3	–	138.60 (C)
4	–	139.69 (C)
5	2.281 (dm, 12.1)	52.23 (CH)
6	–	38.19 (C)
7	1.115 (m)	39.81 (CH_2)
	~1.53 (m)	
8	1.355 (m)	37.46 (CH_2)
	~1.53 (m)	
9	–	49.40 (C)
10	~1.70 (m)	26.63 (CH_2)
	1.768 (m)	
11	1.993 (ddt, 14.3, 7.0, 1.6)	34.34 (CH_2)
	2.194 (br dd, 14.3, 11.2)	
12	–	141.61 (C)
13	5.364 (m)	122.52 (CH)
14	1.707 (dd, 14.1, 8.6)	42.97 (CH_2)
	2.115 (ddm, 14.1, 5.3)	
15	1.752 (3H, tm, 1.7)	25.08 (CH_3)
16	0.710 (3H, s)	16.87 (CH_3)
17	1.051 (3H, s)	24.22 (CH_3)
18	3.004 (septet, 6.8)	26.78 (CH)
19/20	0.934 (3H, d, 6.8)	21.91 (CH_3)
	0.949 (3H, d, 6.8)	21.82 (CH_3)



Scheme 2. Plausible pathway leading to **1** and **2** based on the biogenesis proposed by Ayer et al.[6]

proteinase inhibitor cocktail tablets (Complete™, Roche, one tablet used in 100 ml buffer) by using a porcelain mortar and pestle precooled by liquid nitrogen. One-milliliter aliquots of the supernatants from the centrifugation (20,000×g at 4°C for 40 min) were supplemented with $MgCl_2 \cdot 6H_2O$ (5 mM), and then were used for the conversion of the geranylgeranyl diphosphate. The all-*trans*-[1-$^{2}H_2$, 2-$^{2}H_1$]geranylgeranyl diphosphate (>95 atom% $^{2}H_2$ at C1 and ~50 atom% ^{2}H at C2) was synthesized from the corresponding geranylgeraniol via its chloride,[10,12] and it (20 μg) was converted by this cell-free system into **1** at 30°C for 2 h. The hexane extract from the reaction mixture was purified by silica gel chromatography (hexane) to give pure **1** (3.8 μg, 30% conversion), and then was analyzed by capillary full-scan GC–MS [m/z 275 (M^++3, 17%), 274 (M^++2, 14), 260 (8), 232 (13), 204 (88), 203 (100), 189 (60) and 161 (27)]. Comparison of the retention time and the fragment pattern with those of the natural product confirmed the hydrocarbon obtained here to be [13-$^{2}H_1$, 14-$^{2}H_2$]cyatha-3,12-diene (**1**-d_3). These observations demonstrated the presence of 'cyathadiene synthase' activity in mycelia of *H. erinaceum* YB4-6237 (Scheme 3).

Isolation of cyathadienes from an erinacine-producing basidiomycete, and conversion of the all-*trans*-geranylgeranyl diphosphate into cyatha-3,12-diene by a cell-free system prepared from this basidiomycete, reported here, are of primary importance in our current effort to characterize a gene encoding the corresponding fungal diterpene cyclase, 'cyathadiene synthase'.



Scheme 3. Formation of cyatha-3,12-diene (**1**) from geranylgeranyl diphosphate (D = ^{2}H).

Acknowledgements

We thank Messrs. Eiichi Kimura and Takashi Shigihara, Edible Fungi Institute of Kinox Co. Ltd., for providing *H. erinaceum* YB4-6237. This work was partially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan through Grant-in-Aid for Scientific Research (No. 13306009).

References

1. (a) Ayer, W. A.; Yoshida, T.; van Schie, D. M. J. *Can. J. Chem.* **1978**, *56*, 2113–2120; (b) Ayer, W. A.; Lee, S. P. *Can. J. Chem.* **1979**, *57*, 3332–3337; (c) Ayer, W. A.; Browne, L. M.; Fernádez, S.; Ward, D. E.; Yoshida, T. *Rev. Latinoamer. Quim.* **1978**, *9*, 177–184 and references cited therein.
2. (a) Shibata, H.; Tokunaga, T.; Karasawa, D.; Hirota, A.; Nakayama, M.; Nozaki, H.; Tada, T. *Agric. Biol. Chem.* **1989**, *53*, 3373–3375; (b) Shibata, H.; Irie, A.; Morita, Y. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 2450–2452.
3. (a) Ohta, T.; Kita, T.; Kobayashi, N.; Obara, Y.; Nakahata, N.; Ohizumi, Y.; Takaya, Y.; Oshima, Y. *Tetrahedron Lett.* **1998**, *39*, 6229–6232; (b) Kita, T.; Takaya, Y.; Oshima, Y.; Ohta, T.; Aizawa, K.; Hirano, T.; Inakuma, T. *Tetrahedron* **1998**, *54*, 11877–11886; (c) Obara, Y.; Nakayama, N.; Kita, T.; Takaya, Y.; Kobayashi, H.; Hosoi, S.; Kiuchi, F.; Ohta, T.; Oshima, Y.; Ohizumi, Y. *Eur. J. Pharmacol.* **1999**, *370*, 79–84.
4. (a) Hecht, H.-J.; Höfle, G.; Steglich, W.; Anke, T.; Oberwinkler, F. *J. Chem. Soc., Chem. Commun.* **1978**, 665–666; (b) Inchausti, A.; Yaluff, G.; Rojas de Arias, A.; Torres, S.; Ferreira, M. E.; Nakayama, H.; Schinini, A.; Lorenzen, K.; Anke, T.; Fournet, A. *Phytotherapy Res.* **1997**, *11*, 193–197.
5. (a) Kawagishi, H.; Shimada, A.; Shirai, R.; Okamoto, K.; Ojima, F.; Sakamoto, H.; Ishiguro, Y.; Furukawa, S. *Tetrahedron Lett.* **1994**, *35*, 1569–1572; (b) Kawagishi, H.; Shimada, A.; Shizuki, K.; Mori, H.; Okamoto, K.; Sakamoto, H.; Furukawa, S. *Heterocycl. Commun.* **1996**, *2*, 51–54; (c) Kawagishi, H.; Shimada, A.; Hosokawa, S.; Mori, H.; Sakamoto, H.; Ishiguro, Y.; Sakemi, S.; Bordner, J.; Kojima, N.; Furukawa, S. *Tetrahedron Lett.* **1996**, *37*, 7399–7402; (d) Saito, T.; Aoki, F.; Hirai, H.; Inagaki, T.; Matsunaga, Y.; Sakakibara, T.; Sakemi, S.; Suzuki, Y.; Watanabe, S.; Suga, O.; Sujaku, T.; Smogowicz, A. A.; Truesdell, S. J.; Wong, J. W.; Nagahisa, A.; Kojima, Y.; Kojima, N. *J. Antibiot.* **1998**, *51*, 983–990.
6. Ayer, W. A.; Lee, S. P.; Nakashima, T. T. *Can. J. Chem.* **1979**, *57*, 3338–3343.
7. (a) Kawaide, H.; Imai, R.; Sassa, T.; Kamiya, Y. *J. Biol. Chem.* **1997**, *272*, 21706–21712; (b) Kawaide, H.; Sassa, T.; Kamiya, Y. *J. Biol. Chem.* **2000**, *275*, 2276–2280.
8. (a) Oikawa, H.; Toshima, H.; Ohashi, S.; König, W. A.; Kenmoku, H.; Sassa, T. *Tetrahedron Lett.* **2001**, *42*, 2329–2332; (b) Oikawa, H.; Toyomasu, T.; Toshima, H.; Ohashi, S.; Kawaide, H.; Kamiya, Y.; Ohtsuka, M.; Shinoda, S.; Mitsuhashi, W.; Sassa, T. *J. Am. Chem. Soc.* **2001**, *123*, 5154–5155.
9. Kenmoku, H.; Sassa, T.; Kato, N. *Tetrahedron Lett.* **2000**, *41*, 4389–4393.
10. Lin, X.; Henzari, M.; Koepp, A. E.; Floss, H. G.; Croteau, R. *Biochemistry* **1996**, *35*, 2968–2977.
11. (a) Kato, N.; Zhang, C.-S.; Matsui, T.; Iwabuchi, H.; Mori, A.; Ballio, A.; Sassa, T. *J. Chem. Soc., Perkin Trans. 1* **1998**, 2473–2474; (b) Kato, N.; Zhang, C.-S.; Tajima, N.; Mori, A.; Graniti, A.; Sassa, T. *J. Chem. Soc., Chem. Commun.* **1999**, 367–368; (c) Sassa, T.; Zhang, C.-S.; Sato, M.; Tajima, N.; Kato, N.; Mori, A. *Tetrahedron Lett.* **2000**, *41*, 2401–2404.
12. (a) Corey, E. J.; Kim, C. U.; Takeda, M. *Tetrahedron Lett.* **1972**, *13*, 4339–4342; (b) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768–4779.