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HYPOSTREPSILALIC ACID FROM A CULTURED LICHEN MYCOBIONT OF *STEREOCAULON JAPONICUM*

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Key Word Index—*Stereocaulon japonicum*; lichens; isolated mycobiont; dibenzofuran.

Abstract—A new dibenzofuran, named hypostrepsilalic acid, was isolated from a spore-derived mycobiont culture of the lichen, *Stereocaulon japonicum*, and structurally characterized. Hypostrepsilalic acid was not detected in the natural lichen sample, which produced both atranorin and stictic acid. © 1997 Elsevier Science Ltd

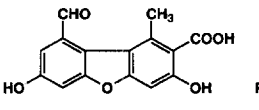
INTRODUCTION

It is well known that lichens produce a number of unique metabolites. The production of such compounds is thought to be a result of the secondary metabolism associated with the fungal portion (mycobiont) of the symbiotic system, which constitutes the lichen body together with the algal counterpart (photobiont). Recent studies in our laboratories have focused on examining the metabolic capacity of isolated mycobionts and have shown that, in some cases, isolated lichen mycobionts are capable of producing new compounds when cultured under osmotically-stressed conditions. These compounds are not detected in the natural lichen specimens [1, 2]. In a continuation of our studies in this area, we have discovered that a spore-derived mycobiont from *Stereocaulon japonicum* produces a new dibenzofuran. This paper describes the isolation and structural characterization of this compound.

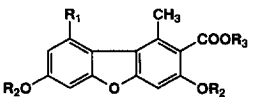
RESULTS AND DISCUSSION

The isolated mycobiont from *Stereocaulon japonicum* was cultured on a malt-yeast extract medium, supplemented with 10% sucrose. After a 7-month culture period, the cultivated colonies were extracted with acetone. The extract, which contained large quantities of a compound, as evidenced by TLC and/or HPLC analyses, was then purified chromatographically.

The molecular formula of the compound obtained



1



2 R_1 = CHO, R_2 = $COCH_3$, R_3 = CH_3
3 R_1 = CHO, R_2 = R_3 = CH_3
4 R_1 = CH_3, R_2 = R_3 = H
5 R_1 = CH_3, R_2 = R_3 = CH_3
6 R_1 = CH_2OH, R_2 = R_3 = H

(**1**) was established as $C_{15}H_{10}O_6$, based on the HR-EI mass spectrum, which gave a $[M]^+$ at m/z 286.0482 (calcd 286.0477). The degree of unsaturation for **1** was calculated to be 11. The presence of an aldehyde group was deduced from characteristic signals observed in the ^{13}C (δ_C 192.8) and ^1H NMR (δ_H 10.62, (1H, *s*)) spectra. Treatment of **1** with acetic anhydride in pyridine, followed by diazomethane, gave a diacetyl methyl ester (**2**), as confirmed by mass spectrometry and ^1H NMR, thus indicating the presence of two hydroxyl groups and one carboxylic acid moiety in **1**. The carboxylic acid moiety gave rise to a ^{13}C NMR signal at δ_C 170.2. In addition, signals assignable to an aryl methyl group were observed by ^1H (δ_H 2.58, 3H, *s*) and ^{13}C NMR (δ_C 21.4).

The signals corresponding to the remaining 12 carbons resonated in the region from 100 to 150 ppm. These data allowed the formulation of a dibenzofuran ring which contained these carbons and a residual oxygen atom, whose structure is consistent with the degree of unsaturation calculated for this compound.

Among the total of three aromatic protons, those observed at δ_H 7.25 (*d*, J = 1.8 Hz) and 7.19 (*d*, J = 1.8 Hz) were coupled with each other in a 1, 3-relationship, while the remaining one at δ_H 6.96 (*s*) showed no spin-interaction. Moreover, an NOE was observed between the aldehyde proton and one of the doublet

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aromatic protons (δ_H 7.25), as well as between the protons of the aldehyde and the methyl group. These couplings and NOE data are consistent with an arrangement of substituents on the dibenzofuran ring as shown for **1**.

The structure **1** is closely related to that of hypostrepsilic acid (**4**), which was previously isolated from a culture of the mycobiont of *Evernia esorediosa* [1]. To confirm this, **1** was converted to its trimethyl derivative **3**, by prolonged treatment with diazomethane, and was then reduced with zinc amalgam (Clemmensen reduction). A product was isolated from the reaction mixture, which was identical with the trimethyl derivative of hypostrepsilic acid (**5**), as evidenced by their HPLC chromatographic behaviour and EI mass spectrometry. These data clearly show that the arrangements of the substituents on the dibenzofuran ring are identical in **1** and **4**, except that one of the methyl groups in **4** is replaced with an aldehyde group in **1**. The position of the aldehyde group was determined on the basis of the NOE data described above. Thus, the structure of **1** was established as 9-formyl-3, 7-dihydroxy-1-methyldibenzofuran-2-carboxylic acid. This compound has not been described in the literature to date and we refer to it as hypostrepsilalic acid.

As the structural relationships mentioned above indicate, hypostrepsilalic acid (**1**) appears to be derived biosynthetically from **4** *via* the oxidation of the 9-methyl group in the latter compound. The oxidation of the 1-methyl group in **4** leads to strepsilin, a known, lichen dibenzofuran. Substantial amounts of **4**, the assumed precursor of **1**, could not be detected in this study. Similarly, neither atranorin nor stictic acid were detected in this culture, although these substances were produced by *S. japonicum* when growing under lichened conditions at the collection site. As was observed in the case of *E. esorediosa* [1], the culture conditions in this study, especially the osmotic stress, could have modified the metabolic capacity of the mycobiont to enhance the formation of the dibenzofuran. Recently, another dibenzofuran, isostrepsilic acid (**6**), was obtained from the cultured mycobiont of *Usnea orientalis* [3]. Isostrepsilic acid represents an intermediate compound in the putative oxidation pathway from **4** to **1**. Interestingly, the source of all three of these dibenzofuran compounds are mycobionts isolated from fruticose lichens.

EXPERIMENTAL

Biological material. Specimens of *Stereocaulon japonicum* Th. Fr. were collected on a rock at Kamikochi, Nagano Prefecture, Japan (*ca* 1800 m altitude) in September 1993. The specimens were identified by Nobuo Hamada and are deposited at Osaka City Institute of Public and Environmental Sciences under registration No. NH93953. Atranorin and stictic acid were detected by TLC in the thallus.

Isolation of cultured mycobiont product. The mycobiont of the lichen specimen was obtained from spores from the apothecia in the thallus and were cultured in test tubes containing MY10 medium (malt extract 10 g, yeast extract 4 g, agar 15 g in 1 l H_2O, pH 7), supplemented with sucrose at a conc. of 100 g l^{-1}. The culture was maintained at 15° in the dark for *ca* 7 months. The culture media along with the entire mycelia were then extracted with Me_2CO. The extract was filtered and concd under red. pres. The concentrate was applied to a SEPPAK C18 cartridge and eluted with a stepwise gradient solvent mixt. of MeOH–H_2O–HCO_2H by increasing the ratio of MeOH. The material eluting with MeOH–H_2O–HCO_2H (12:8:1) was then purified $\times 2$ by reversed-phase HPLC, using a Cosmosil C18 column (20 × 250 mm) and solvent systems composed of MeOH–H_2O–HCO_2H (35:15:1; 1st) and MeCN–THF–H_2O–HCO_2H (25:10:65:1; 2nd) to obtain hypostrepsilalic acid as a yellow solid material. The yield was 7.1 mg g^{-1} culture including both mycelia and the medium. TLC (Kieselgel 60 F254) R_f values ($\times 100$) of hypostrepsilalic acid and hypostrepsilic acid (int. standard) were; 39:48 in *n*-hexane–Et_2O–HCO_2H (13:8:2), 27:41 in C_6H_6–dioxane–HOAc (36:9:1) and 4:7 in toluene–HOAc (20:3).

Hypostrepsilalic acid (**1**). Yellow crystals from C_6H_6–Me_2CO, mp 256–257° (decomp). EIMS (70 eV): *m/z* (rel. int.): 286.0482 $[M]^+$, calcd for $C_{15}H_{10}O_6$; 286.0477, (26), 268 (67), 242 (100), 213 (38), 128 (27). UV λ_{max}^{MeCN} nm (log ε): 372 (3.84), 287 sh (4.05), 241 (4.54). IR ν_{KBr} cm^{-1}: 3550–2800 (br), 1660, 1600. ^1H NMR (300 MHz, DMSO-d_6): δ 10.62 (1H, *s*, NOE was observed when the signal at δ 2.58 was irradiated), 10.26 (0.64 H, *br s*), 7.25 (1H, *d*, J = 1.8 Hz), 7.19 (1H, *d*, J = 1.8 Hz, NOE was observed when the signal at δ 10.62 was irradiated), 6.96 (1H, *s*), 2.58 (3H, *s*, NOE was observed when the signal at δ 10.62 was irradiated). ^{13}C NMR (300 MHz, DMSO-d_6): δ 192.8 (*s*), 170.2 (*s*), 157.9 (*s*), 157.5 (*s*), 156.4 (*s*), 155.7 (*s*), 131.2 (*s*), 131.1 (*s*), 120.1 (*s*), 115.8 (*s*), 114.8 (*s*), 110.8 (*d*), 103.3 (*d*), 96.6 (*d*), 21.4 (*q*). Signal multiplicities were determined by INEPT expts.

O, O-*diacetylhypostrepsilalic acid methyl ester* (**2**). A small amount of **1** was treated with CH_2N_2 in THF followed by Ac_2O in dry pyridine. After chromatographic work-up, the product was analysed by EIMS and ^1H NMR. EIMS *m/z* (rel. int.): 384 $[M]^+$ (3), 342 (28), 300 (27), 268 (100), 43 (41). ^1H NMR (300 MHz, $CDCl_3$): δ 10.75 (1H, *s*), 7.63 (*m*, 2H), 7.36 (1H, *s*), 3.96 (3H, *s*), 2.73 (3H, *s*), 2.37 (3H, *s*), 2.33 (3H, *s*).

Conversion of hypostrepsilalic acid (**1**) *to* O, O-*dimethylhypostrepsilic acid methyl ester* (**3**). Hypostrepsilalic acid was dissolved in THF, into which CH_2N_2 in Et_2O was added. After reaction, the soln was kept overnight and the product purified chromatographically to give **3** nearly quantitatively. EIMS *m/z* (rel. int.): 328 $[M]^+$ (100), 300 (23), 297 (29), 285 (11), 279 (9), 268 (15), 253 (23). ^1H NMR (300 MHz, $CDCl_3$): δ 10.78 (1H, *s*), 7.43 (1H, *d*, J = 2.4 Hz), 7.30 (1H, *d*, J = 2.4 Hz), 7.01 (1H, *s*), 3.97 (3H, *s*), 3.95

(3H, *s*), 3.93 (3H, *s*), 2.64 (3H, *s*). The obtained **3** (2 mg) was then dissolved in EtOH (100 μl), which was added into HCl (30 μl) containing Zn amalgam (prepd from 45 mg of Zn and 18 mg of $HgCl_2$). After a portion of conc. HCl (90 μl) was added with stirring, the soln was kept for 1 hr at room temp. The reaction mixt. was then extracted with toluene and the extract subjected to HPLC using a Cosmosil $5C_{18}$ column and a mobile phase composed of MeOH–H_2O (4:1). A peak identical with standard *O*, *O*-dimethylhypostrepsilic acid methyl ester (**5**) [1] in terms of the chromatographic behaviour was detected, and the compound in the peak was collected and then analysed by EIMS. This spectrum was identical to that of the authentic sample. EIMS *m/z* (rel. int.): 314 $[M]^-$ (100), 299 (10), 283 (20), 267 (13).

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