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GRACILIFORMIN AND ITS ACETATES IN *CLADONIA GRACILIFORMIS*

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Key Word Index—*Cladonia graciliformis*; *Cladonia bellidiflora*; lichen; lichen mycobiont; graciliformin; bellidiflorin; modified bianthraquinones; iron complex; structure determination.

Abstract—From the lichen *Cladonia graciliformis*, three new pigments A, B and C were isolated along with bellidiflorin. Pigment A was an epimer of (+)-rugulosin diacetate and pigment B and C its corresponding deacetylated derivatives. Bellidiflorin is probably an iron complex compound of A. The chemical constituents of the mycobionts of *Cl. graciliformis* and *Cl. bellidiflora* were also studied.

INTRODUCTION

Cladonia graciliformis is a fruticose lichen growing occasionally on the ground near sulphur containing hot springs in Japanese volcanoes. Asahina [1] reported that it contains usnic acid, squamatic acid and a dark brown coloured pigment, bellidiflorin. Bellidiflorin was first isolated by Zopf [2] in 1907 from *Cladonia bellidiflora* (Ach.) var. *coccocephala* (Ach.), but its chemistry is still obscure. This paper describes an investigation of the metabolites of the lichen *Cl. graciliformis* and a comparison with those of the mycobionts of *Cl. graciliformis* and *Cl. bellidiflora*.

RESULTS AND DISCUSSION

The acetone extracts of the lichen and the cultured mycobiont colonies were first examined by TLC on silica gel impregnated with 0·5 N oxalic acid. As bellidiflorin was firmly adsorbed on silica gel, the extracts were chromatographed on Sephadex LH-20 and then on silica gel treated with oxalic acid to obtain usnic acid, squamatic acid, skyrin [3] (newly isolated by the present experiment), bellidiflorin and three yellow pigments A, B and C.

Pigment A, which is the major yellow pigment, has two alcoholic acetyls as shown in its IR spectrum (1745, 1221 cm^{-1}) and MS (M^+-60, M^+-60-60). The UV and ORD curves indicated that A was a rugulosin-like compound, since (+)-rugulosin diacetate prepared from (+)-rugulosin [4] using acetyl chloride in HOAc gave almost the same IR, UV, ORD and MS spectral data as A, but some differences in the NMR spectra were observed. In the NMR spectrum of (+)-rugulosin diacetate, the proton signal at 3,3′ (δ 3·23) is coupled with that of the proton at 2,2′ (δ 5·39) having a coupling constant J 5·5 Hz, while in Pigment A, the signals from both protons at 3,3′ (δ 3·16) and 2,2′ (δ 4·90) are singlets. If A is an epimer at C-2 and C-2′ of (+)-rugulosin diacetate, the dihedral angles of $H_{1(1')}$-$H_{2(2')}$ and $H_{2(2')}$-$H_{3(3')}$ should be 78° and 85° respectively according to Dreiding models, and hence the signals from H-3(3′) and H-2(2′) should be singlets. Structure (**1**) is therefore proposed for Pigment A.

The MS and NMR spectra of Pigment B showed the presence of one alcoholic acetyl, and its NMR spectrum indicated that it is an unsymmetrical dimer. By decoupling experiments and comparison with (+)-rugulosin monoacetate, the structure of Pigment B has been formulated as (**2**). Mild acetylation of B gave a product which was identical to A.

The minor component, Pigment C, was isolated after repeating column chromatography and preparative TLC on silica gel impregnated with 0·5 N oxalic acid. The TLC R_f value, IR and MS spectra of C revealed that it is identical with the product prepared from A on treating with NaOH.

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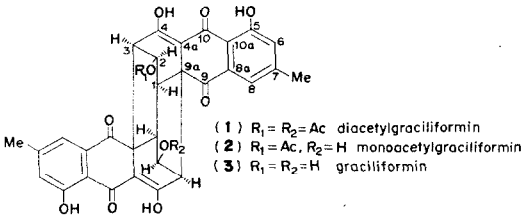
Pigment C has no acetyl group, and its IR, UV and MS spectra resemble those of (+)-rugulosin. The NMR spectrum showed that Pigment C is a symmetrical dimer and an epimer of (+)-rugulosin which has been formulated as (**3**). The Pigment C is now named graciliformin. Accordingly the Pigments A and B are diacetylgraciliformin and monoacetylgraciliformin, respectively.



Bellidiflorin, m.p. >270°, $[\alpha]_D$ −380° (dioxane), a dark brown colouring matter, was shown by X-ray fluorescence analysis to contain iron. It dissolved in 0·6 N NaOH to give an orange solution which turned yellow and liberated iron on acidification with 2 N HCl to afford graciliformin as the main product. The UV spectrum of bellidiflorin resembled that of graciliformin ($\lambda_{max}^{dioxane}$; 253, 387 nm) but exhibited an additional shoulder at 465 nm, while its ORD and CD curves were also very similar to those of graciliformin in the lower wavelength region, but different in the higher wavelength region showing three additional Cotton effects. The presence of acetyl groups in bellidiflorin was indicated by the IR absorptions at 1745 and 1226 cm^{-1}. As the NMR spectral measurement of bellidiflorin was unsuccessful because of the presence of iron in the molecule, the conclusive evidence for the structure of bellidiflorin has not been obtained, but it is quite probable that bellidiflorin is an iron complex of graciliformin acetate.

From the extracts of the mycobiont colonies of *Cl. graciliformis* and *Cl. bellidiflora* cultured on shaking in Lilly and Barnett's medium [5], bellidiflorin, skyrin and graciliformin and its acetates were isolated, whereas usnic acid and squamatic acid were not found. This is the first example of the isolation of rugulosin-like compounds from a cultured mycobiont, although (+)-rugulosin has already been found in the lichen, *Acroscyphus sphaerophoroides* [6].

Addition of excess iron salt to the medium used in the shake culture of *Cl. bellidiflora* produced a darker colour than the normal culture indicating an increased formation of bellidiflorin.

EXPERIMENTAL

Cladonia graciliformis Zahlbr was collected in 1972 at Honzawa spa in Mt. Yatsugatake, Nagano Pref. *Cl. bellidiflora* was collected in 1970 at Revelstoke in the Rocky mountains, British Columbia. The mycobionts were isolated by the test tube method and cultivated on Hamada's No. 117 medium or a malt–yeast extract medium for several months. The colonies were then homogenized in a blender and cultivated by shaking for 3 months on the modified Lilly and Barnett's medium (glucose 10 g, ammonium tartrate 2 g or 5 mmol/l. of L-alanine, KH_2PO_4 1 g, $MgSO_4 . 7H_2O$ 0·5 g, $Fe(NO_3)_3 . 9H_2O$ 0·2 mg, $ZnSO_4 . 7H_2O$ 0·2 mg, $MnSO_4$ $4H_2O$ 0·1 mg, $CaCl_2$ 0·2 g, thiamine 100 μg, biotin 5 μg, dist. H_2O 1 liter, pH 5·5).

Isolation and purification. Lichen thalli were extracted successively with *n*-hexane, C_6H_6 and Me_2CO. Mycelia of the mycobionts were filtered, air dried and extracted with hot Me_2CO. TLC of the Me_2CO extracts of lichen and mycobients on silica gel impregnated with 0·5 N oxalic acid in C_6H_6–Me_2CO (10:1) gave the following R_f values: usnic acid 0·84, pigment A 0·69, pigment B 0·49, skyrin 0·31, bellidiflorin 0·26, squamatic acid 0·16 and Pigment C 0·11. The culture filtrate was also extracted with EtOAc and added to the previous extracts. The C_6H_6 extract of lichen and the Me_2CO extract of mycobiont were chromatographed on Sephadex LH-20 and eluted with Me_2CO. The brown ppts separated from the first portion of elution were recrystallized from C_6H_6 or Me_2CO to obtain bellidiflorin. All other fractions were combined, mixed with $CaHPO_4$ powder and placed on the top of a silica gel column impregnated with 0·5 N oxalic acid. Eluting with a *n*-hexane–C_6H_6–Me_2CO system, some fatty acids, usnic acid, Pigment A, Pigment B, skyrin and Pigment C were obtained successively. Preparative-TLC was also used for the purification of Pigment C.

Identification of metabolites. Usnic acid, skyrin and squamatic acid were identified by comparing with the authentic samples using TLC, IR and MS. Diacetylgraciliformin (Pigment A): m.p. >270° (recryst. from Me_2CO–EtOH), $[\alpha]_D$ +392° (dioxane) (Found: C, 64·93; H, 4·11. $C_{34}H_{26}O_{12}$ requires: C, 65·17; H, 4·18%); UV $\lambda_{max}^{dioxane}$ nm (log ε): 253 (4·44), 278 (inf.) (4·29), 386·5 (4·41); IR υ_{max}^{KBr} cm^{-1}: 1745, 1703, 1608, 1221; ORD (in dioxane) nm (φ): 252 (+6·12 × 10^4), 257 (+4·54 × 10^4), 268 (+7·94 × 10^4), 280 (0), 287 (−4·76 × 10^4), 300 (0), 315 (+2·27 × 10^4), 343 (0), 378 (−3·90 × 10^4), 403 (0), 416 (+2·09 × 10^4); MS: 626 (M^+), 566, 506, 254 (base peak). Monoacetylgraciliformin (Pigment B); m.p. >270° (recryst. from Me_2CO–EtOH), UV $\lambda_{max}^{dioxane}$ nm (log ε): 251·5 (4·38), 278 (inf.) (4·26), 386·5 (4·35); IR υ_{max}^{KBr} cm^{-1}: 1748, 1704, 1611, 1226; MS: 584 (M^+), 524, 254 (base peak). Graciliformin (Pigment C); m.p. >270° (recryst. from Me_2CO–EtOH), UV $\lambda_{max}^{dioxane}$ nm (log ε): 251 (4·23), 275 (inf.) (4·07), 387·5 (4·19); IR υ_{max}^{KBr} cm^{-1}: 1703, 1618; MS: 542 (M^+), 270, 254 (base peak). Bellidiflorin; m.p. >270° (recryst. from C_6H_6 or Me_2CO), $[\alpha]_D$ −380° (dioxane) (Found: C, 62·71; H, 3·92. Ash was found. UV $\lambda_{max}^{dioxane}$ nm: 253, 387, 465 (*sh*); IR $\upsilon_{max}^{CHCl_3}$ cm^{-1}: 1745, 1702, 1615, 1226; ORD (in dioxane) nm (z): 253 (+7·64 × 10^3), 267 (+4·37 × 10^3), 272 (+5·68 × 10^3), 281 (0), 288 (−5·68 × 10^3), 296 (0), 306 (+4·59 × 10^3), 318 (+3·93 × 10^3), 333 (+5·90 × 10^3), 357 (0), 373 (−7·21 × 10^3), 384 (0), 407 (+1·09 × 10^4), 429 (*sh*) (+3·93 × 10^3), 444 (0), 474 (−4·10 × 10^3), 519 (0), 545 (+7·86 × 10^2), 580 (0), 628 (−1·07 × 10^3).

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