Pergamon

PII: S0031-9422(97)00277-X

Phytochemistry, Vol. 46, No. 2, pp. 341–345, 1997

Printed in Great Britain
0031 9422/97 $17.00+0.00

A PHLOROGLUCINOL DERIVATIVE FROM THE BROWN ALGA *SARGASSUM SPINULIGERUM*

MICHAEL KEUSGEN,* MICHAEL FALK,† JOHN A. WALTER† and KARL-WERNER GLOMBITZA‡

Institut für Pharmazeutische Biologie, Nußallee 6, D-53115 Bonn, Germany; † Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia, B3H 3Z1, Canada

(*Received in revised form* 17 *February* 1997)

Key Word Index—*Sargassum spinuligerum*; Phaeophyceae; Sargassaceae; phloroglucinol derivative; ascorbic acid; structural elucidation.

Abstract—In addition to a large number of fuhalols and phlorethols previously identified in an ethanolic extract of *Sargassum spinuligerum*, a novel type of phloroglucinol derivative was isolated as its peracetyl derivative and identified by means of spectral analysis. In addition to a phloroglucinol unit, the new compound contains an ascorbic acid moiety. The stereochemistry of this compound was elucidated by NOE experiments in combination with molecular modelling. © 1997 Elsevier Science Ltd

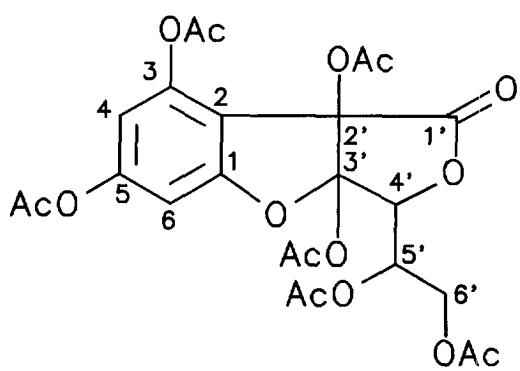
INTRODUCTION

Sargassum spinuligerum (Sond.) contains a great variety of different phlorotannins [1–4]. All major compounds belong to the fuhalol class. Usually phlorotannins consist of phloroglucinol units linked in various ways [5]. The new compound being reported here contains an ascorbic acid moiety, in addition to a phloroglucinol unit. Phloroglucinol and ascorbic acid have both been found in a number of brown algae [5–7].

RESULTS AND DISCUSSION

The acetylated crude mixture of phlorotannins was separated by flash chromatography and HPLC [1]. The fractions obtained after a multi-step HPLC separation were examined by mass and NMR spectroscopy. The compound under discussion was found in a fraction containing typical phlorotannins consisting of two phloroglucinol units.

EI- and FAB-mass spectra of phloroscorbinol hexaacetate **1** gave a M_r of 552 ($C_{24}H_{24}O_{15}$). Both spectra showed several ketene elimination series, which indicated the presence of six acetoxyl groups. Twice a loss of 59 or 60 mu was observed in the FAB-mass spectrum (*m/z* 552 → *m/z* 493 and *m/z* 451 → *m/z* 391), and may be explained by the release of aliphatic-bonded acetoxyl groups, like the acetoxyl groups at C-5′ and C-6′ of **1**. The ^{1}H NMR spectrum showed



1

signals for a total of six acetoxyl groups in the range between δ 2.0 and δ 2.4, which were well resolved in the spectrum recorded in acetone-d_6 (Table 1). Taking the elimination series obtained from the mass spectra into account, four of these acetoxyl groups are linked to carbons which are involved in aromatic or heterocyclic ring systems.

The ^{1}H NMR spectra showed signals at δ 6.67 and δ 6.72 (chloroform-*d*) forming an AB-system with a coupling constant of $J = 2.0$ Hz. This is typical of aromatic protons in a *meta*-position. Significant NOEs were observed between these protons and the protons of two acetoxyl groups. A nearly equal NOE between both aromatic protons and an acetoxyl group characterized by a chemical shift of δ 2.28 was found (Table 2). Hence, this acetoxyl group is placed between the two aromatic protons (Ac at C-5). An NOE between δ 6.72 and δ 2.33 (chloroform-*d*). stron-

* From the dissertation of M. Keusgen, 1993, Bonn D5.

‡ Author to whom correspondence should be addressed.

Table 1. ^{1}H NMR spectral data for compound **1**

	$CDCl_3$			Me_2CO-d_6		
H	Shift	*J*	System	Shift	*J*	System
C4	6.72	2.0	*d*	6.75	2.0	*d*
C6	6.67	2.0	*d*	6.73	2.0	*d*
C4′	4.98	4.6	*d*	5.22	4.7	*d*
C5′	5.67	4.3/4.6/6.3	*ddd*	5.66	4.3/4.7/6.7	*ddd*
C6′	4.27	12.0/6.3	*dd*	4.26	12.0/6.7	*dd*
	4.41	12.0/4.3	*dd*	4.50	12.0/4.3	*dd*
Me of:						
Ac at C-3	2.33			2.28		
Ac at C-5	2.28			2.26		
Ac at C-2′	2.11			2.11		
Ac at C-3′	2.16			2.18*		
Ac at C-5′	2.16			2.12*		
Ac at C-6′	2.10			2.03		

* Assignments may be interchanged.

Table 2. NOEs between protons of compound **1**

	Observed enhancement at: [% NOE]*					
Irradiation at:	H at C-4 δ 6.72	H at C-6 δ 6.67	H at C-4′ δ 4.98	H at C-5′ δ 5.67	H at C-6′ δ 4.41	H at C-6′ δ 4.27
H at C-6 δ 6.67			0.4			
H at C-4′ δ 4.98		0.4		5.8	1.4	0.7
H at C-5′ δ 5.67			5.7		2.4	2.5
H at C-6′ δ 4.41			2.9	5.6		19.7
H at C-6′ δ 4.27			1.4	5.3	18.1	
Ac at C-3 δ 2.33	0.6					
Ac at C-5 δ 2.28	0.8	0.6				
Ac at C-3′† Ac at C-5′† δ 2.16			0.4	0.8	0.2	0.4

* The minimum measurable NOE and error in small NOEs (< 1%), is *ca* 0.1%. Larger NOEs have errors of *ca* 0.2 to 0.3% (up to 6% NOE) or 1% (for > 18% NOE).

† Together.

ger than those between δ 6.67 and δ 2.33, indicated that the second acetoxyl group at C-3 of the aromatic moiety. The shifts for the aromatic protons and the corresponding carbons were confirmed by a proton-carbon coupled ^{13}C NMR and HMQC, as well as HMBC experiments (C-4: δ 111.8; C-6: δ 102.6, measured in chloroform-*d*, Table 3).

Suggesting a phloroglucinol element, C-1, C-3 and C-5 must be linked to oxygens. As expected, the ^{13}C NMR showed three signals for aromatic carbons, consistent with oxygen substitution (δ 148.1, δ 154.3 and δ 157.8) [8]. HMBC demonstrated that the carbon giving the signal δ 148.1 is exclusively coupled with the proton at C-4 (δ 6.72). Consequently, δ 148.1 was assigned to C-3. In the same experiment, the shift of C-1 was determined to be at δ 157.8 and that of C-5 at δ 154.3. The resonance of C-2 was found to be at δ 109.4. The interpretation of the ^{13}C NMR data, the HMQC and the HMBC gave evidence, that C-2 is exclusively linked to quaternary or aromatic carbons.

Table 3. ^{13}C NMR spectral data of compound **1**

	$CDCl_3$			CD_6
C	Shift	*J*	System	Shift
C1	157.8	5.4	*d*	159.3
C2	109.4	5.9	*dd*	110.9
C3	148.1	4.2	*d*	149.9
C4	111.8	169.0/4.7	*dd*	113.0
C5	154.3	5.1	*dd*	155.9
C6	102.6	170.2/4.8	*dd*	103.5
C1′	165.7	3.8	*d*	166.8
C2′	81.9		*s*	83.2
C3′	109.9	4.6	*d*	111.5
C4′	83.7	159.4/*ca* 3	*dd*	84.7
C5′	67.6	153.3/*ca* 2	*dd*	68.6
C6′	62.5	*ca* 149/*ca* 2	*ddd*	63.7
Carbonyl functions of acetoxyl groups				
Ac at C-3	167.9	*	*	168.5†
Ac at C-5	168.0	*	*	168.7†
Ac at C-2′	166.1	*ca* 7	*ddd*	166.9
Ac at C-3′	167.9	*ca* 7	*ddd*	168.1†
Ac at C-5′	169.4	*ca* 7	*ddd*	170.1
Ac at C-6′	170.1	*ca* 7	*ddd*	170.4
Methyl functions of acetoxyl groups				
Ac at C-3	20.8	130,5	*d*	21.0
Ac at C-5	21.1	130,5	*d*	21.2
Ac at C-2′	20.9	131,0	*d*	21.1
Ac at C-3′	19.9‡	130,7	*d*	20.0§
Ac at C-5′	20.8‡	130,5	*d*	21.0§
Ac at C-6′	20.7	130,0	*d*	20.8

* Could not be determined.
†, ‡, § Assignments may be interchanged.

In the ^1H NMR spectrum, the ascorbic acid moiety of **1** was characterized by signals for four protons in the range between δ 4.2 and δ 5.7. These values are typical for oxygen-carrying structural elements, such as sugars. The coupling constants and the spin systems for these protons allowed an unambiguous assignment (H at C-4′: δ 4.98, H at C-5′: δ 5.67 and H at C-6′: δ 4.27/4.41; Table 1). The resonances for the corresponding carbons were obtained by HMQC (C-4′: δ 83.7, C-5′: δ 67.6, C-6′: δ 62.5, Table 3). The HMBC showed evidence that the proton at C-4′ coupled with two quaternary carbons represented by signals at δ 109.9 (C-3′) and δ 165.7 (C-1′). The carbon shift of C-1′ indicated a lactone structure [8]. This was confirmed by strong IR absorption at 1775 cm^{-1}, typical of five-membered lactone ring systems [9].

The shift of C-2′ gave a very weak signal at δ 81.9. This weakness may be explained by a lack of any nearby hydrogen atoms and therefore the relaxation time for this carbon is long. To verify that no signals of **1** were hidden by those of the solvent, and to confirm the resonance at δ 81.9, a ^{13}C NMR spectrum was recorded in benzene-d_6. The spectrum showed a signal at δ 83.2 and no significant resonance in the range between δ 70 and δ 80. A theoretical shift of *ca* δ 81 was found for the assumed substitution pattern of C-2′ [10]. The weakness of this signal may be explained by high shielding of this carbon.

The downfield-shifted signal for C-3′ (δ 109.9) indicates a substitution with two oxygen functions at this carbon. In view of the M_r of 552 and the presence of six acetoxyl groups, one of these oxygen functions must be an acetoxyl group, the second one, an oxygen connected to C-1. In effect, this oxygen, C-1 to C-6, C-2′ and C-3′ form a dihydrobenzofuran ring system.

The proton shifts for the acetoxyl groups attached to the aromatic moiety are described above. The shifts for the corresponding carbonyl function are δ 167.9 (Ac at C-3) and δ 168.0 (Ac at C-5). Because of coupling of the protons at C-5′ and C-6′ with the carboxyl carbons of the attached acetoxy functions, the shifts for these carbons were confirmed by HMBC (Ac at C-5′: δ 2.16/δ 169.4; Ac at C-6′: δ 2.10/δ 170.1). Also the acetoxyl group at C-3′ is characterized by a signal at δ 2.16. The resonance for the corresponding carbon of the carboxyl function was obtained at δ 167.9.

Carbons C-2′, C-3′, C-4′ and C-5′ are chiral. Analysis by X-ray crystallography was not possible because **1** could not be recrystallized. But in further NMR experiments, significant NOEs were obtained between several protons (Table 2). There was no evidence for relayed NOE effects. The NOE between the protons at C-6 and C-4′ is of special interest because it indicates a distance between these protons shorter than 4 Å.*

The recorded NOEs were used to derive the relative configuration of **1** by means of ConGen. This recently developed molecular modelling procedure searches the configuration space of a molecule using high-temperature molecular dynamics under H...H distance constraints derived from NOE data [11]. The model of **1** was constructed using SYBYL molecular modelling software and the Tripos force field [12]. ConGen scrambles the initial configuration to avoid bias and then subjects the molecule to repeated cycles of dynamics at 8000 K, where chiral sites are frequently inverted and distance constraints guide the molecule towards configurations consistent with the NOE data. The configurations generated by ConGen are sorted, the correct one being identified by (1) its most frequent occurrence, (2) best agreement with the NOEs and (3) relatively low energy [11].

The results of a typical ConGen search on **1** are shown in Table 4. Configurations *RRSS* and *RRSR* (C-2′, C-3′, C-4′, C-5′) and their enantiomers *SSRR* and *SSRS* are generated with high frequency, with a distance between protons at C-6 and C-4′ as low as 3.7 Å and energy as low as 22.6 kcal mol^{-1}. In contrast, the other 12 configurations are generated only one tenth as often, with the distances between protons at C-6 and C-4′ never shorter than 5.2 Å, and

* A rough estimate of the internuclear distance can be made by assuming that NOE is proportional to R^{-6} and using the distance between the two H6′ protons, 1.8 Å, as calibration.

Table 4. Configurations of compound **1** generated by a typical ConGen search (420 cycles)

Configuration (C-2′, C-3′, C-4′, C-5′)	Number of times	Shortest *d** [Å]	Lowest energy [kcal mol^{-1}]
RRRR and *SSSS*	9	5.24	28.1
RRRS and *SSSR*	12	5.31	25.0
RRSR and *SSRS*	194	3.74	22.8
RRSS and *SSRR*	188	3.86	22.6
RSRR and *SRSS*	2	5.46	47.7
RSRS and *SRSR*	13	5.40	39.7
RSSR and *SRRS*	1	5.67	47.3
RSSS and *SRRR*	1	5.72	41.3
Summary:			
*RRS*x and *SSR*x	382	3.74	22.6
all others	38	5.24	28.1

* *d* is distance between the protons at C-6 and C-4′.

with energy always above 25.0 kcal mol^{-1}. This result was reproduced many times, either with the one constraint (distance between protons at C-6 and C-4′) or with several additional NOE-based constraints. We conclude that the configuration of **1** at C-2′, C-3′, C-4′ can only be *RRS* or *SSR*. The configuration at C-5′ could not be deduced from NOE data.

Naturally occurring ascorbic acid has the *RS*-configuration for the carbons which are analogous to C-4′ and C-5′. Under the assumption that **1** is derived from ascorbic acid, the relative configuration for the whole molecule would be *SSRS* (C-2′, C-3′, C-4′, C-5′). This result is in full accordance with the results obtained by molecular modelling. The lowest-energy structure with the configuration *SSRS* is shown in Fig. 1.

EXPERIMENTAL

EI-MS. 70 eV, 200–300°. Positive ion FAB-MS: Xe gun, 3-nitrobenzylalcohol as matrix.

NMR. ^{1}H spectra (90 and 300 MHz) and ^{13}C spectra (75 MHz) were recorded using solvents as int. standards. 2D C/H spectra and NOE expts were recorded at 500 MHz.

Molecular modelling. SYBYL software was used (version 6.1, Tripos, Inc., St Louis, U.S.A.), running on a Personal Iris 4D/35 TurboGraphic workstation (Silicon Graphics, Mountain View, U.S.A.).

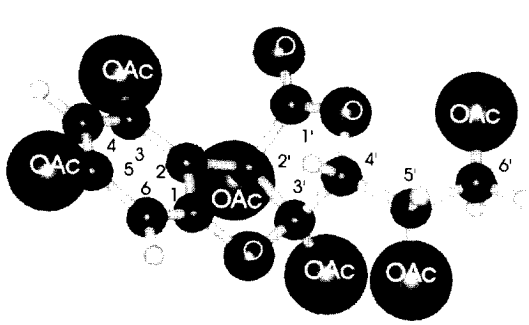


Fig. 1. Structure of compound **1** giving a low-energy *SSRS*-configuration for C-2′, C-3′, C-4′, C-5′.

Extraction and isolation. Extraction and presepn was carried out as described in ref. [1]. Highly purified **1** was obtained by HPLC using a LiChrosorb Si-60 column (5 μm, 250 × 8 mm) with an isocratic solvent system containing $CHCl_3$–*n*-hexane–EtOH (53:46:1) and a flow of 2.5 ml min^{-1}. Compounds were detected at 275 nm. Using this system, **1** showed a R_t of 25.2 min. On TLC plates (Merck silica gel F_{254}, $CHCl_3$–Me_2CO 9:1, 15 cm), **1** gave a red-coloured spot at R_f 0.58 after spraying with a soln of 1% vanillin in conc. H_2SO_4 and heating at 120° for 10 min.

Phloroscorbinol hexaacetate (**1**). 1-(1,2-Diacetoxyethyl)-3-oxy-3*a*,4,6,8*a*-tetraacetoxyfurano [3,4-*b*] benzofuran. Yield 5 mg (from 20 kg frozen algae). EI-MS elimination series: *m/z* 552 → 426, 450 → 366, 308 → 182, 290 → 206, 279 → 153. FAB-MS ketene elimination series: *m/z* 575 $[M+Na]^+ \rightarrow 533$, 552 $[M]^+ \rightarrow 426$, 493 $[M-OAc]^+ \rightarrow 367$, 391 $[M-H-20Ac\text{-}Ac]^+ \rightarrow 349$. IR ν_{max}^{KBr} cm^{-1}: 2820 (CH_3, CH_2) *m*, 1775 (λ lactone) *s*, 1745 (>C=O) *s*, 1625 >C=C< *m*, 1490 (C-H) *m*, 1435 (C-H) *m*, 1370 (-OCOCH$_3$) *s*, 1260 (C-O) *s*, 1220 (C-O) *s*, 1190 (CO-O) *vs*, 1165 (C-O) *s*, 1110 (C-O) *s*, 1080 (C-O) *s*, 1045 (C-O) *s*, 1015 (C-O) *s*, 945 *m*, 890 *m*, 800 *m*, 755 *m*, 700 *w*, 660 *w*, 600 *m*, 576 *m*. ^{1}H NMR: Table 1. ^{13}C NMR: Table 3.

Acknowledgements—We thank Dr Dromgoole (University of Auckland) for introducing us to the New Zealand algae and for identifying the species. Thanks for MS, NMR and IR are due to the Central Analytic of the Chemical Institutes, the NMR department of the Pharmaceutical Institute, Dr R. Hartman at the Institute for Physiological Chemistry and Mr J. Streich at the Institute for Food Chemistry, University of Bonn. We are grateful to Messrs Ping Seto and Peter Spierenburg at the Institute for Marine Biosciences,

Halifax, for carrying out the NOE measurements and computing. This research was supported by the Ministerium für Wissenschaft und Forschung, NRW.

REFERENCES

1. Glombitza, K.-W. and Keusgen, M., *Phytochemistry*, 1995, **38**, 987.
2. Keusgen, M. and Glombitza, K.-W., *Phytochemistry*, 1995, **38**, 975.
3. Keusgen, M. and Glombitza, K.-W., *Phytochemistry* (submitted for publication).
4. Glombitza, K.-W., Keusgen, M. and Hauperich, S. (1996) (in preparation).
5. Ragan, M. A. and Glombitza, K.-W., in *Progress in Phycological Research*, Vol. 4, ed. F. E. Round and D. J. Chapman. Biopress, Bristol, 1986, p. 129.
6. Munda, I. M., *Hydrobiologia*, 1987, **151–152**, 477.
7. Skare, M. and Topalovic-Avramov, R., *Hrana Ishrana*, 1967, **8**, 719.
8. Hesse, M., Meier, H. and Zeeh, B., *Spektroskopische Methoden in der Organischen Chemie*, 4th edn. Georg Thieme, Stuttgart, 1991, p. 152.
9. Hesse, M., Meier, H. and Zeeh, B., *Spektroskopische Methoden in der Organischen Chemie*, 4th edn. Georg Thieme, Stuttgart, 1991, p. 46.
10. Bremser, W., Franke, B. and Wagner, H., *Chemical Shift Ranges in Carbon-13 NMR Spectroscopy*. Verlag Chemie, Weinheim, 1982, p. 41.
11. Falk, M., Spierenburg, P. F. and Walter, J. A., *Journal of Computational Chemistry*, 1996, **17**, 409.
12. Clark, M., Cramer, III, R. D. and Van Opdenbosch, N., *Journal of Computational Chemistry*, 1989, **10**, 982.