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THE STRUCTURE OF GRIFOLIN, AN ANTIBIOTIC FROM A BASIDIOMYCETE

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Abstract—Structure of grifolin was deduced as 2-*trans*,*trans*-farnesyl-5-methylresorcinol (I).

IN 1948, Kubo and Mizuno[1] found that *Grifola confluens* (Japanese name, shiromaitake) had antibiotic activity. One of us (Y. H.) and Nakanishi[2] isolated from the fungus a white crystalline antibiotic, m.p. 43°, named grifolin, the structure of which is presented in this paper.

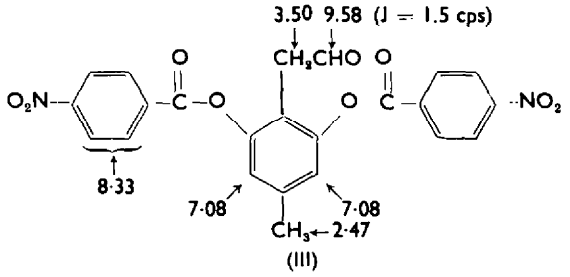
Elemental analysis and molecular weight determination by mass spectroscopy* (parent peak at 328) established the formula $C_{22}H_{32}O_2$ for grifolin (I). The presence of two hydroxyl groups was shown by conversion of the antibiotic to a di-*p*-nitrobenzoate (II), m.p. 62°. The fact that the *p*-nitrobenzoate (II) has a single carbonyl absorption at 1760 cm^{-1} in carbon tetrachloride attributable to an enol or phenol ester band, coupled with the presence of UV absorption of grifolin at 275 (log ε = 2·97) and 281 mμ (2·96) similar to that of benzene derivatives suggests that the antibiotic (I) has a dihydroxybenzene chromophore. Among the chromophores of the three isomeric dihydroxybenzenes, the hydroquinone type is excluded because even the parent compound, hydroquinone, absorbs at a longer wave length (299 mμ) than that of grifolin. That the antibiotic has a resorcinol type chromophore rather than catechol type was demonstrated from an IR analysis. Thus, in dilute chloroform solution, grifolin (I) shows a sharp band of unbonded hydroxyl groups at 3600 and a broad band of intermolecular hydrogen-bonded ones at 3430 cm^{-1}, but no intramolecular hydrogen-bond was detected. Catechol should have an intramolecular hydrogen-bond, and indeed shows three hydroxyl bands, i.e. free, intramolecular hydrogen-bonded, and intermolecular hydrogen-bonded bands at 3600 (sharp), 3560 (sharp), and 3270 (broad), respectively, whereas the IR spectrum of 4-n-hexylresorcinol lacks an intramolecular hydrogen-bonded hydroxyl band and has only two bands at 3600 (sharp) and 3340 cm^{-1} (broad).

Ozonolysis of the *p*-nitrobenzoate (II) afforded, beside acetone and levulinic aldehyde (more than 1 mole, characterized as the 2,4-dinitrophenylhydrazone), colorless needles having a formula $C_{23}H_{16}O_9N_2$, which must be a resorcinol di-*p*-nitrobenzoate derivative. Its structure was deduced as 5-methyl-1,3-bis-(*p*-nitrobenzoyloxy)phenyl-2-acetaldehyde (III) mainly from the assignments of its NMR spectrum, which shows the signals for two aromatic protons, two *p*-nitrobenzoyloxy groups, a methyl on an aromatic ring, and a $-CH_2CHO$ group as shown in the formula

* We thank Atlas-Werke AG for taking mass spectrum of grifolin.

[1] H. Kubo and T. Mizuno, *Botan. Mag. Japan*, **61**, 64 (1948).
[2] Y. Hirata and K. Nakanishi, *J. Biol. Chem.* **184**, 135 (1949).

below. Since the signal of two aromatic protons is a singlet, the protons must be

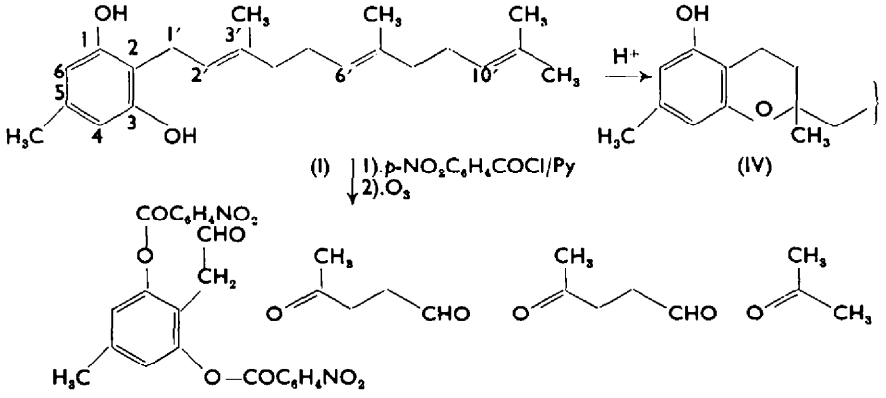


(III)

situated symmetrically on the benzene ring. This is possible only in the case that they occupy the 4-and 6-positions. That the methyl group is attached to the 5- rather than the 2-position was deduced from a comparison of the methyl signal (2·42 ppm) with that of orcinol di-*p*-nitrobenzoate (2·43) and methylphloroglucinol tri-*p*-nitrobenzoate (2·15 ppm).

p-Nitrobenzoyl groups deshield a *m*-methyl group but have little effect on an *o*-methyl. This is understandable by a consideration of an anisotropic effect of the *p*-nirtobenzoyl groups. The benzene ring and the carboxyl in the *p*-nitrobenzoyl group are forced to be in a plane by resonance between them, and with this restriction it is clear from models that in the most favourable conformations, the aromatic rings in the benzoyl groups of grifolin di-*p*-nitrobenzoate (II) are approximately perpendicular to the other aromatic ring, as shown in Fig. 1. Thus, according to the calculations of Johnson and Bovey,[3] the *o*-methyl group, if present, would lie on a neutral surface (isoshielding surface of $\delta' = 0$), shown by a dotted line, of magnetic effects of the *p*-nitrobenzoyl group, but the *m*-methyl group in a paramagnetic field.

Since the ozonolysis afforded acetone and levulinic aldehyde (2 moles), coupled with the molecular formula of grifolin, it is evident (invoking the isoprene rule) that the —CH_2CHO group of the phenylacetaldehyde (III) must have been a farnesyl group before ozonolysis, Thus, grifolin should have the structure (I): 2-farnesyl-5-methyl-



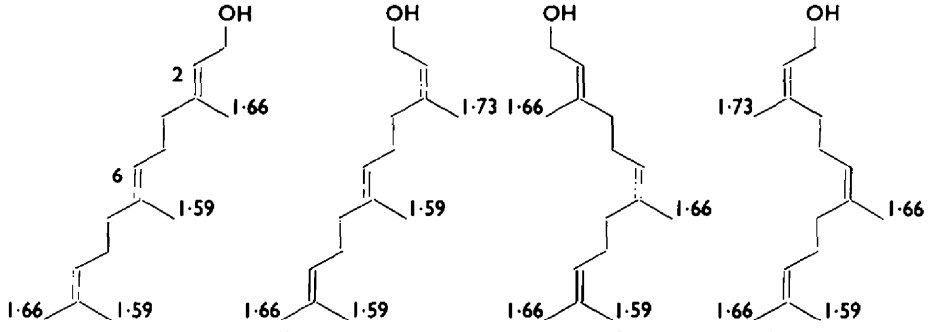
resorcinol. The configurations of the double bonds in the side chain will be discussed later.

The positions of the methyl and the farnesyl groups were further confirmed in the

[3] C. E. Johnson and F. A. Bovey, *J. Chem. Phys.* **29**, 1012 (1958).

following way. When heated in methanol containing hydrochloric acid, grifolin was isomerized to isogrifolin (IV). The *p*-nitrobenzoate of isogrifolin showed no hydroxyl absorption in its IR spectrum and has a signal at 8·22 ppm in its NMR spectrum corresponding to four aromatic protons in a *p*-nitrobenzoyl group. Therefore, isogrifolin (IV) has only one hydroxyl group. This result is interpretable by a formation of a cyclic ether from one of the phenolic hydroxyl groups and a double bond in the farnesyl side chain. This means that the farnesyl group must be *ortho* to one of the hydroxyl groups. That the NMR spectra of grifolin (I; Fig. 2) and the *p*-nitrobenzoate (II; Fig, 3) reveal always a sharp singlet for the two aromatic protons either in deuteriochloroform or in a mixture of deuteriochloroform and benzene suggests also that the aromatic protons are in the same magnetic environment. Isogrifolin (IV) has two signals for the aromatic protons since they are no longer equivalent.

The configuration of the double bonds in the farnesyl side chain were assigned as *trans-trans* from the following evidence. Bates, *et al.*,[4] examined the NMR spectra of four isomeric farnesols and assigned the methyl signals as shown in the following formulas.



Positions of methyl protons in farnesol NMR spectra (in ppm)[4].

The methyl group attached at the $C_{3'}$ of grifolin (I) would be in a different magnetic environment from the corresponding methyl group in the farnesols due to the strong anisotropy of the benzene ring (see below), but the other three methyl groups should give their signals at the same positions as the farnesols. Then, that grifolin (I) has two signals at 1·55 (6 protons) and 1·63 ppm (3 protons) corresponding to the three methyl groups in the farnesyl side chain suggests the *trans* configuration of the $C_{6'}$ double bond (Fig. 2). A signal at 1·77 ppm is then attributable to the $C_{3'}$ methyl group. When the solvent was changed from deuteriochloroform to an 1:2 mixture of deuteriochloroform and benzene, this signal moved 0·1 ppm upfield, whereas other methyl and methylene signals of the farnesyl group remained almost unchanged. This means that only this methyl group is affected by the paramagnetic anisotropy of the aromatic ring. This could be interpreted only by the *trans* configuration of the $C_{2'}$ double bond. Thus, protons of the methyl group at $C_{3'}$ are, on the average, in the plane (dashed line in Fig. 1) of the benzene ring; the latter must deshield the former strongly.

[4] R. B. Bates, D. M. Gale and B. J. Gruner, *J. Org. Chem.* **28**, 1086 (1963).

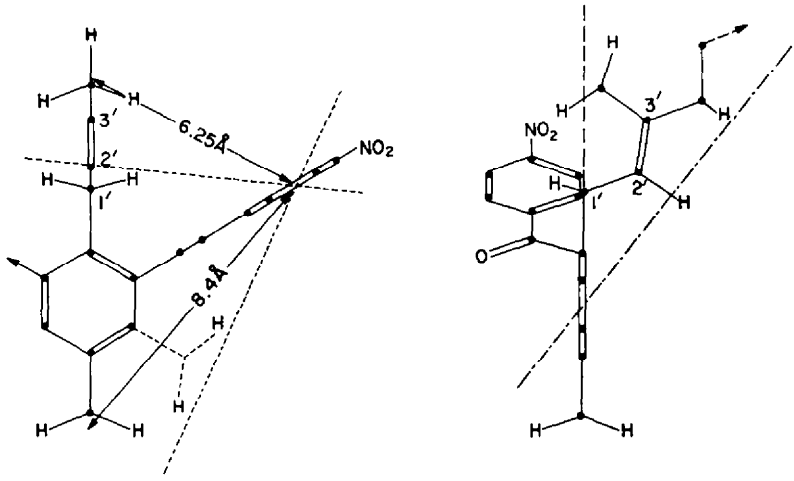


FIG. 1. The most favorable conformation of grifolin di-p-nitrobenzoate (II)

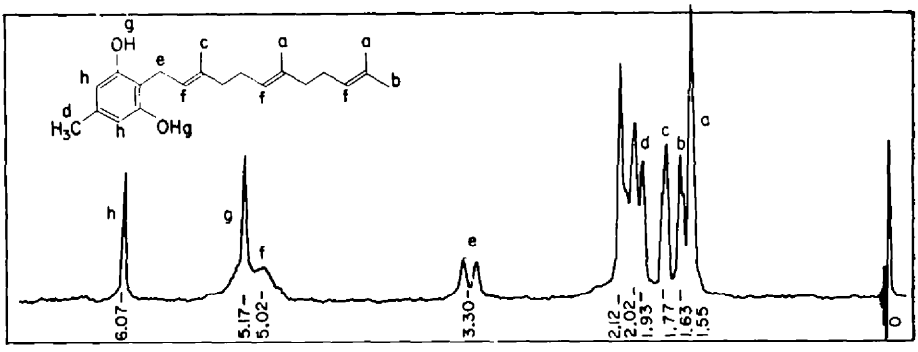


FIG. 2. The NMR spectrum of grifolin(I) in $CDCl_3$

Assignment	h	g	f	e		d	c	b	a
$CDCl_3$	6·07,	5·17,	5·02,	3·30,	2·12,	2·02,	1·93,	1·77,	1·63,	1·55
1:2 $CDCl_3$-C_6H_6	5·92,	4·55,	5·12 ,	3·33,	2·05,	1·96,	2·02,	1·67,	1·67,	1·53

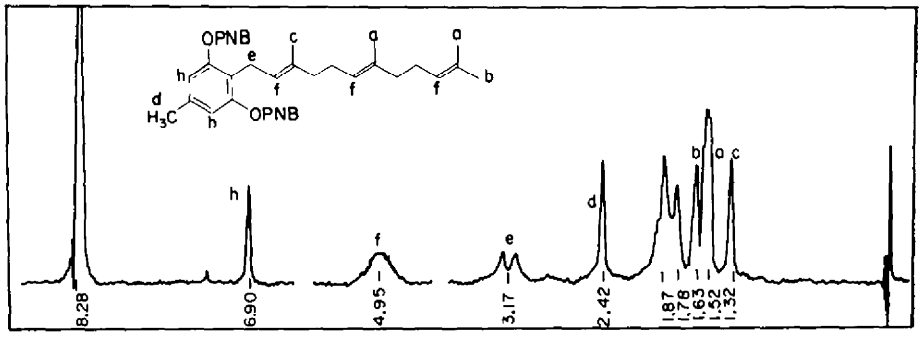


FIG 3. The NMR spectrum of grifolin di-p-nitrobenzoate (II) in $CDCl_3$

Assignment	PNB	h	f	e	d			b	a	c
$CDCl_3$	8·28,	6·90,	4·95,	3·17,	2·42,	1·87,	1·78,	1·63,	1·52,	1·32
1:2 $CDCl_3$-C_6H_6	7·98,	6·82,	5·12,	3·26,	2·10,	1·93,	1·83,	1·62,	1·48,	1·27

This is further supported by the fact that in the NMR spectrum of the *p*-nitrobenzoate (II; Fig. 3), whereas other farnesyl signals were not much changed in their positions compared with that of grifolin, the methyl signal was displaced 0·45 ppm upfield. When the $C_{2'}$ double bond is *trans*, the methyl group is sandwiched between the aromatic rings of the *p*-nitrobenzoyl groups, and a strong diamagnetic effect could be expected on it (Fig. 1). The favorable conformations of the *p*-nitrobenzoate groups are already discussed (see above). The calculated value from the diagram of Johnson and Bovey[3] is ca. 0·36 ppm, which is in good agreement with the observed value.

EXPERIMENTAL[5]

Grifolin (I). Recrystallizations from pet. ether of the crude antibiotic extracted from sporophores[4] gave colorless needles m.p. 43°; λ_{max}^{MeOH} 275, 281 mμ (ε 935, 906), ν^{CCl_4} 3620, 3460, 2920, 1635, 1585, 1450, 1170, 1044 cm^{-1}, mass spectrum (e/m) 328 (parent peak, 3), 201 (18), 191 (21), 175 (100), 137 (100), 69 (74). (Found: C, 80·38, 80·35; H, 9·78, 9·97. $C_{22}H_{32}O_2$ requires: C, 80·44; H, 9·83%).

Grifolin di-p-nitrobenzoate (II). Grifolin was treated with *p*-nitrobenzoyl chloride and pyridine at room temp[2]. The crude product was crystallized from pet. ether to give colorless needles; m.p. 62°, ν^{CCl_4} 1760, 1535, 1263, 1087. (Found: C, 68·97, 68·80; H, 6·09, 6·19; N, 4·30, 4·73. $C_{36}H_{36}O_8N_2$ requires: C, 68·99; H, 6·11; N, 4·47%).

*Ozonolysis of grifolin di-*p*-nitrobenzoate* (II). A solution of 200 mg of the benzoate (II) in 5 ml of ethanol-free chloroform was ozonized at −10° and then evaporated to dryness at room temp under vacuum. Water (7 ml) and small quantities of powdered zinc were added to the residue and the mixture was heated under a reflux condenser at 55° for 15 min and then at 100° for 1·5 hr. After addition of more water, the aqueous layer was decanted from a resinous residue and introduced into a saturated solution of 2,4-dinitrophenylhydrazine in 1N HCl, and a mixture of hydrazones precipitated. It was collected and extracted with hot methanol and the residue (73 mg, m.p. 228–231°) was crystallized from nitrobenzene to give yellow needles of levulinic aldehyde bis-2,4-dinitrophenylhydrazone, m.p. 234–236°. Acetone 2,4-dinitrophenylhydrazone was obtained from the methanol extract.

*5-methyl-*1,3-*bis-*(p-*nitrobenzoyloxy) phenyl-2-acetaldehyde* (III). The water-insoluble resinous residue obtained above was dissolved in chloroform, dried (Na_2SO_4), and chromatographed on silica gel (12 g, Mallinckrodt). From chloroform eluates colorless needles, m.p. 190–198°, was obtained. After recrystallizations from ethanol, it melted at 197–199°. The yield was 50 mg; ν^{KBr} 1735, 1715, 1520, 1100, 724 cm^{-1}, NMR 2·47 (3), 3·50 (2), 7·08 (2), 8·33 (8), 9·58 (1) ppm (in 1:1 mixture of $CDCl_3$ and CCl_4). (Found: C, 59·20, 59·53; H, 3·44, 3·53; N, 6·05. $C_{23}H_{16}O_8N_2$ requires: C, 59·48; H, 3·47; N, 6·03%).

Isomerization of grifolin (I). A solution of grifolin (100 mg) in methanol (5 ml) containing 5 drops of conc. HCl was refluxed for 2 hr. Dilution with water and extraction with ether gave oily *isogrifolin* (IV), which was directly *p*-nitrobenzoylated as usaul, and the product was purified by chromatography on alumina (Merck). Though it was not crystallized, the product, *isogrifolin p-nitrobenzoate*, seemed homogeneous and was used for IR and NMR measurements; ν^{CCl_4} 1750, 1535, 1270, 1092 (no absorption near 3600), NMR 1·05, 1·18, 1·48, 2·17, 3·02, 5·00, 6·45, 8·22 (4) (in CCl_4).

[5] M.ps were determined on a micro hot stage. UV absorption spectra were determined by means of a Beckman DK-2 Spectrophotometer, and IR spectra by means of a Japan Spectroscopic Co. DS-402G grating Spectrophotometer. NMR spectra were taken by means of Nihondenshi model JNM-3 Spectrometer (60 mc) and are expressed in ppm unit from internal tetramethylsilane.