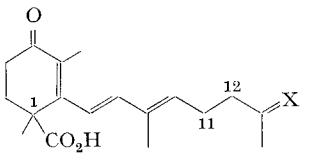
was fed to mated *B. trispora* the percentage incorporations and dilutions indicated that this was a more efficient precursor than was 2-^{14}C-mevalonate under comparable conditions.

Percent incorporation (and dilution factor) in:

	(Ib)	(Ia)
from ^{14}C-β-carotene	2.5% (× 220)	0.9% (× 3,700)
from 2-^{14}C-mevalonate	0.03% (× 8,800)	0.6% (× 11,200)

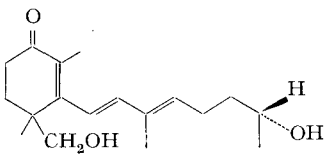
Such data also suggest that the ketone (Ib) is a precursor of the alcohol (Ia).

Through the courtesy of the Hoffman-La Roche Company of Basle, a sample of 11,12-$^3H_2$-retinyl acetate was available. Retinal is known to occur in Phycomyces and seemed a possible intermediate between β-carotene and (II). The compound was fed to mated *B. trispora* and the isotope was very effectively incorporated into trisporol and the trisporic acids: in (II), incorporation 1.3%, dilution × 50; in (Ia), incoporation 1.9%, dilution × 450. The purified methyl ester from (Ia) was degraded by ozonolysis of the acetate followed by $LiAlH_4$ reduction[8], purifying the resultant diols as the *p*-nitrobenzoates. The propan-1,2-diol derivative [from $C_{(8)}$, $C_{(9)}$ and 9-Me of (Ia)] was virtually inactive and the (–)-4R-pentan-1,4-diol derivative[9], from $C_{(10)}$-$C_{(14)}$ of (Ia), contained 48% of the ^{3}H originally found in (Ia). After conversion of the pentandiol into 1-trityloxypentan-4-one and base-catalysed exchange in D_2O, 21% of the original ^{3}H [of (Ia)] was retained. It follows that in the original (Ia) at least 21% of the total ^{3}H was at $C_{(11)}$ (or, less plausibly, $C_{(10)}$) and at least 27% was at $C_{(12)}$ (or less plausibly, $C_{(13)}$ or $C_{(14)}$).



(Ia) X = H, OH

(Ib) X = :O



(II)

The nominal distribution of ^{3}H in the retinyl acetate which was fed is equal between $C_{(11)}$ and $C_{(12)}$, but since the synthesis includes *cis-trans* isomerisation of a double bond at $C_{(11)}$ the actual ^{3}H distribution may well have been unequal. An unequal distribution of ^{3}H between these 2 positions may alternatively have arisen during the biosynthesis of (Ia). Furthermore, in the degradation of (Ia), during $LiAlH_4$ reduction of the ozonide, $C_{(11)}$ is transiently α to aldehyde C=O and γ to an alkoxyl anion, and hence very liable to ^{3}H loss (as H_2). Thus, although some aspects of the data will only be clarified by more detailed experiments, our failure to account for some 52% of the ^{3}H present in (Ia) is explicable and does not affect the main conclusion that the retinyl acetate was converted into trisporic acids by a direct route. In the unperturbed system, we presume that retinal is the more probable intermediate and that this is formed by the normal breakdown of β-carotene.

The overall biosynthesis of the trisporic acids, and incidentally the 'positive feedback' nature of trisporate-mediated carotenogenesis, are therefore established. The mechanism now provides a clear chemical basis for understanding observations that, in mutants of Phycomyces with defective β-carotene synthesis, in which retinal is also absent[10], both 'gamone' production[11] and the resultant sexual differentiation[12] are impaired[13].

Zusammenfassung. Aus *Blakeslea trispora* wird eine neue Verbindung, Trisporol-C isoliert, ihre Struktur aufgeklärt und die Biosynthese der verwandten Trisporinsäuren aus β-Carotin und retinal untersucht.

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Characterization of By-Products of Fusicoccin in Culture Filtrates of *Fusicoccum amygdali* Del.

Fusicoccin is a highly active phytotoxic metabolite produced by submerged cultures of the phytopathogenic fungus *Fusicoccum amygdali* Del.[1]. Its structure (I) has been elucidated in these laboratories[2], and independently confirmed at Imperial College, London[3].

Thin-layer chromatography (silica gel G, 8% propan-2-ol in chloroform) of culture filtrates of *F. amygdali* consistently shows, besides fusicoccin, small amounts of 6 substances with lower Rf values. In what follows these are indicated as F II, F III, . . . F VII, in order of decreasing chromatographic mobility. 4 of them, namely F II, F III, F IV and F VII, are also formed when dilute solutions of fusicoccin are incubated at room temperature in buffer having the same pH (about 7.0) of the culture brew during the production phase, and probably arise non-enzymically from fusicoccin in the course of the fermentation[4]. Among

the 6 by-products, F III, F IV and F VII are by far the most abundant, the other 3 substances being present only in trace amounts.

The present paper reports the chemical characterization of compounds F III, F IV and F VII, and conclusively demonstrates their identity with three products prepared from pure fusicoccin[5].

The brown oily residue left in ethyl acetate after crystallization of fusicoccin[4] was dissolved in chloroform and fractionated on a 'Florisil' column by extended washing with chloroform. Each fraction was tested by thin layer chromatography (silica gel G, 8% propan-2-ol in chloroform, sulphuric acid spray). After fusicoccin, which emerged first, a very small amount of F II appeared, followed by F III and F IV, and finally by a mixture of F V, F VI and F VII, neatly separated from F III and F IV. Appropriate fractions were pooled; those containing mixtures of F III and F IV were further fractionated by chromatography under the same conditions described above, whereas F VII was obtained pure after repeated chromatography on silica gel (Machery and Nagel S-HR, 50% benzene in acetone). By these procedures F III, F IV and F VII were obtained chromatographically pure.

Compounds F III, F IV and F VII gave triacetylfusicoccin (II)[2] on treatment with acetic anhydride in dry pyridine at room temperature; F III and F IV, as well as fusicoccin itself, were converted into F VII by 0.1*N* NaOH (30 min at room temperature). These results indicated that the 3 substances isolated from the culture brew have a close structural relation with fusicoccin.

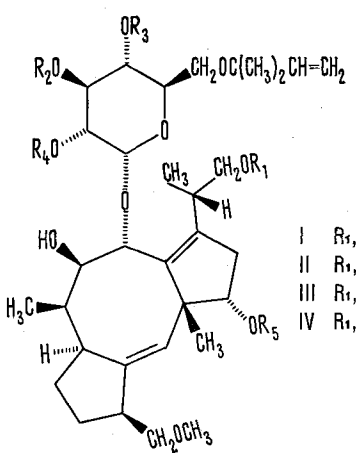
Compound F III[7]: $C_{36}H_{56}O_{12}$, $[\alpha]_D^{25}+11$ ($c=0.15$ in ethanol), $\lambda_{max}<215$ nm. The IR-spectrum in the region 850–3700 cm^{-1} was superimposable upon that of fusicoccin, whereas it was quite different in the region 720–850 cm^{-1}. The NMR-spectrum clearly indicated the same features observed in fusicoccin, namely a vinyl on a quaternary carbon, an olefinic proton on a *tri*substituted double bond, an *O*-Me, two *O*-Ac, 2 secondary and 3 tertiary C-Me groups. The mass spectrum was also very similar to that of fusicoccin, showing the molecular peak at *m/e* 680 and strong signals at *m/e* 408 (aglycone), 205 (monoacetylglucosyl), 69 ($C_5H_9^+$), and 43 (CH_3CO^+). The substance, which is an isomer of fusicoccin, has been named *isofusicoccin*.

Isofusicoccin was also prepared, together with monodeacetylfusicoccin (see below), on treatment of fusicoccin (100 mg), dissolved in methanol (5 ml), with 0.05*M* borate buffer pH 8.8 (8 ml). After 4 h at room temperature, the mixture was extracted with chloroform and the extract fractionated by chromatography on a column of silica gel (Machery and Nagel S-HR). Propan-2-ol (8% in chloroform) eluted first some unreacted fusicoccin; this was followed by isofusicoccin and last by monodeacetylfusicoccin. Frations containing isofusicoccin were pooled, evaporated in vacuo, and the residue dissolved in acetone and reprecipitated with light petroleum (bp 30–50 °). The amorphous solid was crystallized from ethyl acetate-ciclohexane: mp 89–92°; $[\alpha]_D^{25}+10$ ($c=0.79$ in ethanol). Rf in 4 solvent systems, IR-, NMR- and mass spectra were identical to those of compound F III. Calc. for $C_{36}H_{56}O_{12}\cdot 1H_2O$: C, 61.87; H, 8.30; found: C, 61.50; H, 8.19.

Compound F IV: $C_{34}H_{54}O_{11}$, $[\alpha]_D^{25}+18.5$ ($c=0.12$ in ethanol), $\lambda_{max}<215$ nm. The IR-spectrum, as with the preceding compound, was again very similar to that of fusicoccin. The NMR-spectrum indicated the occurrence of the same groups observed in fusicoccin, with the only difference that a single *O*-Ac group was present in this product. This was confirmed by the mass spectrum which showed the molecular peak at *m/e* 638. Furthermore, the aglycone peak at *m/e* 408 was still present, thus indicating that the missing acetyl group is that on the glucosyl moiety. This was corroborated by the observation that F IV, contrary to fusicoccin, is oxidizable with periodate. NMR-spectra yielded evidence that the *O*-Ac group cannot be located on either of the secondary alcoholic functions of the aglycone moiety. Compound F IV, therefore, corresponds to *monodeacetylfusicoccin* (III).

Monodeacetylfusicoccin was prepared on treatment of fusicoccin (100 mg), dissolved in methanol (5 ml), with aqueous 0.2*M* sodium bicarbonate (8 ml). After 30 min at 28 °C the mixture was worked up as reported above for the preparation of isofusicoccin. Only small quantities of unreacted fusicoccin and of isofusicoccin were obtained by this procedure, and the conversion to monoacetylfusicoccin amounted to 60–70%. After crystallization from cyclohexane, the compound had mp 78–80°; if heated above the mp (15 min at 110 °C) this rose to 102–104°. $[\alpha]_D^{25}+19.5$ ($c=0.72$ in ethanol). Rf in 4 solvent systems, IR-, NMR- and mass spectra were identical with those of compound F IV. Calc. for $C_{34}H_{54}O_{11}$: C, 63.95; H, 8.46. Found: C, 64.16; H, 7.92.

Compound F VII: $C_{32}H_{52}O_{10}$, $[\alpha]_D^{25}+9.0$ ($c=0.9$ in ethanol), $\lambda_{max}<215$. The IR-spectrum showed the absence of *O*-Ac groups; this was confirmed by the NMR-spectrum, which also indicated that the other features characteristic of fusicoccin were still present. The mass spectrum showed the molecular peak at *m/e* 596, and



I $R_1, R_2=COCH_3$; $R_3, R_4, R_5=H$
II $R_1, R_2, R_3 R_4, R_5=COCH_3$
III $R_1,=COCH_3$; $R_2, R_3, R_4, R_5=H$
IV $R_1, R_2, R_3, R_4, R_5=H$

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[7] IR-spectra were recorded in KBr pellets with a Beckman IR-9-spectrophotometer. NMR-spectra were recorded on a Varian HA-100 apparatus (internal reference TMS). Optical rotations were measured with a Perkin Elmer 14 polarimeter. Mass spectra were recorded on a MS-902 (GEC-AEI) spectrometer. Melting points are uncorrected.

strong peaks at *m/e* 366 (deacetylaglycone) and 69 ($C_5H_9^+$). Compound F VII must therefore be *dideacetylfusicoccin* (IV).

Dideacetylfusicoccin was prepared on treatment of fusicoccin (200 mg) in methanol (4 ml) with 0.1 *N* NaOH in methanol (4 ml). After 30 min at room temperature, the solution was neutralized with HCl, the methanol removed in vacuo and the residue extracted with chloroform. The chloroform solution, washed several times with water, was evaporated and the residue crystallized from dry ethyl acetate to give a solid with mp 183–187°; if the crude compound was heated only briefly with ethyl acetate, or if the solvent was not dry, the crystals had mp 116–117° (microanalysis showed a content of 0.5 moles of water per mole of compound). $[\alpha]_D^{25} + 9.5$ ($c = 0.76$ in ethanol). Rf in 4 solvent systems, IR-, NMR- and mass spectra were identical with those of compound F VII. Calc. for $C_{32}H_{52}O_{10}$: C, 64.43; H, 8.77. Found: C, 64.20; H, 8.84.

The structures of compounds F IV and F VII therefore appear to be firmly established, but further work is required to obtain full elucidation of that of F III (isofusicoccin). It is quite possible that the formation of isofusicoccin from fusicoccin only involves a reversible rearrangement concerning the acetyl group present in the glucose moiety; it was in fact observed that on brief treatment at room temperature with 0.2 *M* sodium bicarbonate, isofusicoccin yields not only monodeacetylfusicoccin but also some fusicoccin.

While isofusicoccin is nearly as phytotoxic as fusicoccin in the assay with tomato plants, monodeacetylfusicoccin and dideacetylfusicoccin are respectively 12 and 100 times less active[8,9].

Riassunto. Dai brodi di coltura di *Fusicoccum amygdali* Del. sono stati isolati un isomero della fusicoccina (isofusicoccina), una monodeacetilfusicoccina, in cui manca l'acetile sul residuo del glucosio, e la dideacetilfusicoccina.

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(Italy), 5 November 1969.

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[10] On leave from the Istituto di Chimica Farmaceutica e Tossicologica dell'Università di Perugia.

Chitin in the Cephalochordata, *Branchisotoma floridae*

Although chitin has been reported in all the major invertebrate phyla except in the Protozoa and Echinodermata, it seems to be totally absent in the Chordata[1–5]. During the course of an investigation of the skeletal tissues of protochordates, we detected chitin in the gill bars of the cephalochordate, *Branchisotoma floridae* (Figure 1).

Material and methods. Gill bars were isolated from surrounding tissues by a modification of the procedure outlined by Phillis[6]. Whole thoraces of *B. floridae* were macerated in a waring blender with an equal volume of 5% KOH solution for 24 h. The creamy suspension was passed through graded screens with openings of 1.165, 0.589, 0.295 and 0.147 mm with the aid of suction. Standard Tyler screens, customarily used for geological investigations, were fitted to a Buchner funnel with the aid of masking tape. The suspension after this filtration consisted of gill bars, fine tissue debris and dissolved constituents. Centrifugation of the suspension at approximately 1000 rev/min for 15 min isolated the gill bars, and they were washed with 12 *N* HCl to free them of all soluble materials. Differential centrifugation for periods of 15 min in a small international clinical centrifuge completed the purification procedure. The gill bars were then repeatedly washed with water and boiling methanol until they were no longer positive to histochemical tests for protein and lipid. The gill bars thus obtained were treated as required for demonstrating the presence of chitin.

Results. Gill bars withstood treatment for 15 min in saturated aqueous KOH solution at 100 °C. After washing in distilled water, the alkali-treated bars were coloured brown by iodine in KI solution, becoming violet when this was replaced by dilute H_2SO_4 (Figure 2)[7]. Alkali-treated bars were soluble in mineral and acetic acids. Under these conditions, both cellulose and chitin are relatively stable in alkali, but the above colour reaction and solubility in acids are properties shown only by chitin.

Chitin, a polymer of 2-acetamido-2-deoxy-α-D-glucopyranose (N-acetyl-D-glucosamine), yields D-glucosamine on acid hydrolysis and N-acetyl-D-glucosamine on enzymic degradation[8]. The theoretical value of nitrogen for purified chitin is 6.9%[2]. The gill bars in the specimens that we examined displayed these reactions.

The materials were hydrolyzed in sealed tubes with 6 *N* HCl at 160 °C for 6 h. After drying the hydrolysate over P_2O_5 and KOH, the residue was taken up in water and run on a partition chromatogram against D-glucose and D-glucosamine hydrochloride, using 6 different solvent mixtures. The chromatograms were sprayed with aniline hydrogen phthalate, silver nitrate, or the Elson and Morgan reagents. A substance behaving like glucosamine (or galactosamine) was present in large amounts, but no glucose was detected. On conversion to the corresponding pentose by the Stoffyn and Jeanloz procedure B[9], the

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