Isolation and Structure Elucidation of Polivione, A Polyketide Co-metabolite of Citromycetin in *Penicillium frequentans*

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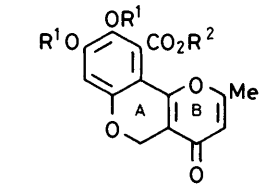
The isolation and characterization of polivione, a polyketide metabolite and probably a precursor of citromycetin (**1**), from *Penicillium frequentans* is reported. The structure elucidation of polivione is based on a detailed study of its high-field ^1H and ^{13}C n.m.r. spectra. Extensive use was made of two-dimensional homonuclear and heteronuclear correlation experiments in the assignment of the n.m.r. spectra. Some chemical transformations were also explored, both to confirm its structure and to investigate its relationship to citromycetin.

Citromycetin is a yellow, crystalline, characteristic metabolite of the fungus *Penicillium frequentans*. It was assigned the structure (**1**) as a result of a series of degradations carried out by Robertson *et al.*[1] Fulvic acid (**5**) was isolated, also from the culture medium of several *Penicillium* species, by Oxford *et al.*[2] and was later shown by Dean *et al.*[3] to possess the structure shown. Fulvic acid is readily dehydrated to anhydrofulvic acid (**6**) which is isomeric with citromycetin. The close resemblance between citromycetin and fulvic acid, both in carbon skeleton and oxygenation pattern suggests that they are probably built up using a common biosynthetic strategy. Another metabolite, lapidosin (**7**), recently isolated[4] from *P. lapidosum*, shares the same carbon skeleton (**8**) with compounds (**1**) and (**5**) and is probably related to them in its biosynthesis. The interest in this unusual group of fungal metabolites is also reflected in recent reports[5] of synthetic approaches to citromycetin and fulvic acid *via* supposedly biomimetic routes.

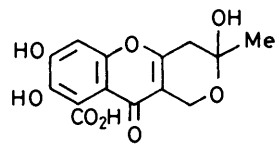
In the course of our studies on the biosynthesis of these heptaketide metabolites, we decided to search for possible biosynthetic intermediates in the cultures of *Penicillium frequentans*. To our surprise, thin layer chromatography (t.l.c.) of the crude ethyl acetate extract of the culture filtrate of the fungus revealed that citromycetin is only a trace component of the mixture, the major component being an unstable compound which forms a green spot at a higher R_F. Here we describe the isolation and purification of this new fungal metabolite. We also present spectroscopic and chemical evidence that this new natural product, named polivione, exists as a mixture of two slowly interconverting tautomers (**9**) and (**10**).[6]

Extraction of the acidified culture filtrate with ethyl acetate and evaporation of the solvent gave a red-brown gum. Purification of this crude extract by conventional chromatography proved to be extremely difficult. This is partly because of the acidic nature of the components and partly because of the insolubility of the oil in most organic solvents. A wide range of possible eluant systems was tried, but they invariably gave streaking on silica gel. This problem was solved by impregnating the silica gel with phosphoric acid to make it slightly acidic. Column chromatography was also carried out on acidified silica gel. Although ordinary column chromatography worked very well, flash chromatography[7] (SiO_2–H_3PO_4) proved to be more successful in separating the small amounts of citromycetin from the major component present in the crude extract than the conventional method.

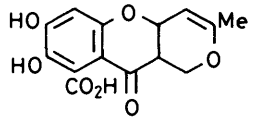
Pure polivione was obtained as a yellow glass, and was assigned the molecular formula $C_{14}H_{12}O_8$ on the basis of its elemental analysis and high resolution mass spectrum. The i.r. spectrum showed strong absorption bands in both the hydroxy and carbonyl regions ($\nu_{max.}$ 3 500, 1 726, and 1 668 cm^{-1} in $CHCl_3$), consistent with a mixture of compounds (**9**) and (**10**).



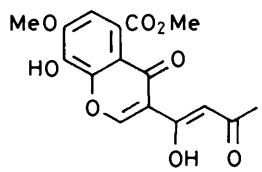
	R^1	R^2
(**1**)	H	H
(**2**)	Ac	Me
(**3**)	H	Me
(**4**)	Ac	H



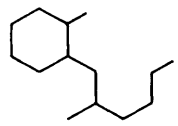
(**5**)



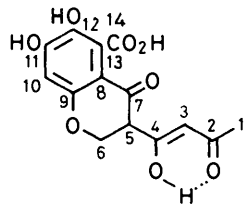
(**6**)



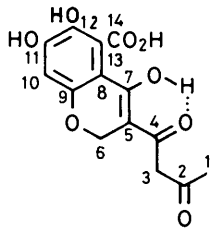
(**7**)



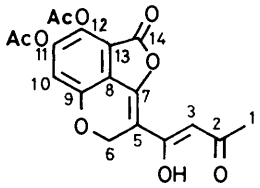
(**8**)



(**9**)



(**10**)



(**11**)

The ^1H n.m.r. (Table 1) can be divided into sets of CH peaks corresponding to the two isomers, the relative amounts of which can vary (from 1:1 to 4:1 in favour of compound (**9**) in $CDCl_3$ solution). The assignments made with the aid of n.O.e.

Table 1. ^{1}H N.m.r. spectroscopic data[a] of polivione tautomers (**9**) and (**10**) and diacetylanhydropolivione (**11**)

Hydrogen	(9)	(10)	(11)[b]
1	2.05	2.3	2.15
3	5.5	3.6	6.26
5	3.65 (t, *J* 6 Hz)		
6	4.53 (dd, *J* 6, 11 Hz)	4.8	5.46
	4.85 (dd, *J* 6, 11 Hz)		
10	6.7	6.65	6.82
OH	*c*	*c*	15.5

[a] At 250 MHz in $CDCl_3$ solution; all resonances singlets except where multiplets are specified. [b] Acetate resonances at 2.31 and 2.35. [c] Hydroxy resonances at 14.0, 14.5, and 15.0.

Table 2. Chemical shifts (δ) and couplings ($^1J_{CC}$) in the ^{13}C n.m.r. spectra[a] of polivione tautomers (**9**) and (**10**) and diacetylanhydropolivione (**11**)

Carbon	(9)	(10)	(11)[b]
1	23.9 (46.2)	30.8 (42.6)	24.4 (46.0)
2	189.56 (46.1)	200.3 (42.5)	189.9 (46.0)
3	100.2 (62.6)	48.9 (49.2)	101.37 (61.9)
4	189.54 (62.5)	172.9 (49.2)	179.7 (61.9)
5	55.0 (34.4)	105.0 (49.5)	103.26 (45.1)
6	67.4 (34.4)	65.9 (49.5)	68.8 (45.2)
7	193.0 (58.1)	185.0 (58.1)	144.6 (52.7)
8	108.7 (58.1)	110.0 (58.0)	123.9 (52.7)
9	162.7 (69.6)	160.0 (69.8)	150.3 (74.0)
10	107.6 (69.6)	107.7 (69.9)	115.8 (74.0)
11	157.6 (64.2)	155.9 (65.3)	147.4 (84.7)
12	152.2 (64.2)	151.4 (65.4)	133.6 (84.4)
13	108.9 (65.8)	109.5 (65.7)	113.9 (79.4)
14	173.0 (65.9)	173.2 (66.2)	161.7 (79.3)

[a] At 100 MHz in CD_2Cl_2 solution (δ relative to CD_2Cl_2 = 53.85 p.p.m.). [b] At 100 MHz in $CDCl_3$ solution (δ relative to $CDCl_3$ = 77.02 p.p.m.); the four acetate resonances are at δ 20.3, 20.5, 167.2, 167.6 p.p.m.

difference experiments[8] are consistent with intensity measurements. The presence of a three-proton ABX system in compound (**9**) was confirmed by homonuclear decoupling experiments. Thus irradiation of the triplet (5-H) at δ 3.65 p.p.m. led to the collapse of the two quartets (6-H) at δ 4.53 and 4.83 p.p.m. to doublets, thus simplifying the ABX system to an AB system. Each of the quartets was then irradiated in turn. In both cases the triplet collapsed to a doublet whilst the non-irradiated quartet also decoupled to a doublet. Addition of a drop of D_2O to a $CDCl_3$ solution resulted in immediate loss of strong OH signals at δ 14.0, 14.5, and 15.0 p.p.m.; there was also a more gradual loss of those for 3-H (δ 5.5 p.p.m.) and 5-H (δ 3.65 p.p.m.) accompanied by expected changes elsewhere in the spectrum.

Supporting evidence came from the ^{13}C n.m.r. spectrum of polivione (Table 2). The multiplicities of the different ^{13}C resonances were determined by generating the proton-decoupled CH, CH_2, and Me subspectra using the DEPT sequence.[9] Furthermore, chemical shift considerations, and the multiplicities observed in the ^{1}H-coupled ^{13}C n.m.r. spectrum allowed the assignment of the resonances at δ 23.9 and 30.8 p.p.m. to methyl carbons, the signals at δ 48.9, 65.9, and 67.4 p.p.m. to methylene carbons, and finally the resonances at δ 55.0, 100.2, 107.6, and 107.7 p.p.m. to methine positions. However, due to the complexity of the structures and the lack of suitable models, further evidence was required to assign each resonance to an individual carbon atom, the main problems being the differentiation of the six carbonyl resonances, and those of the quarternary carbons.

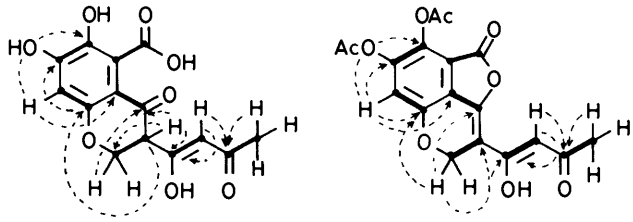


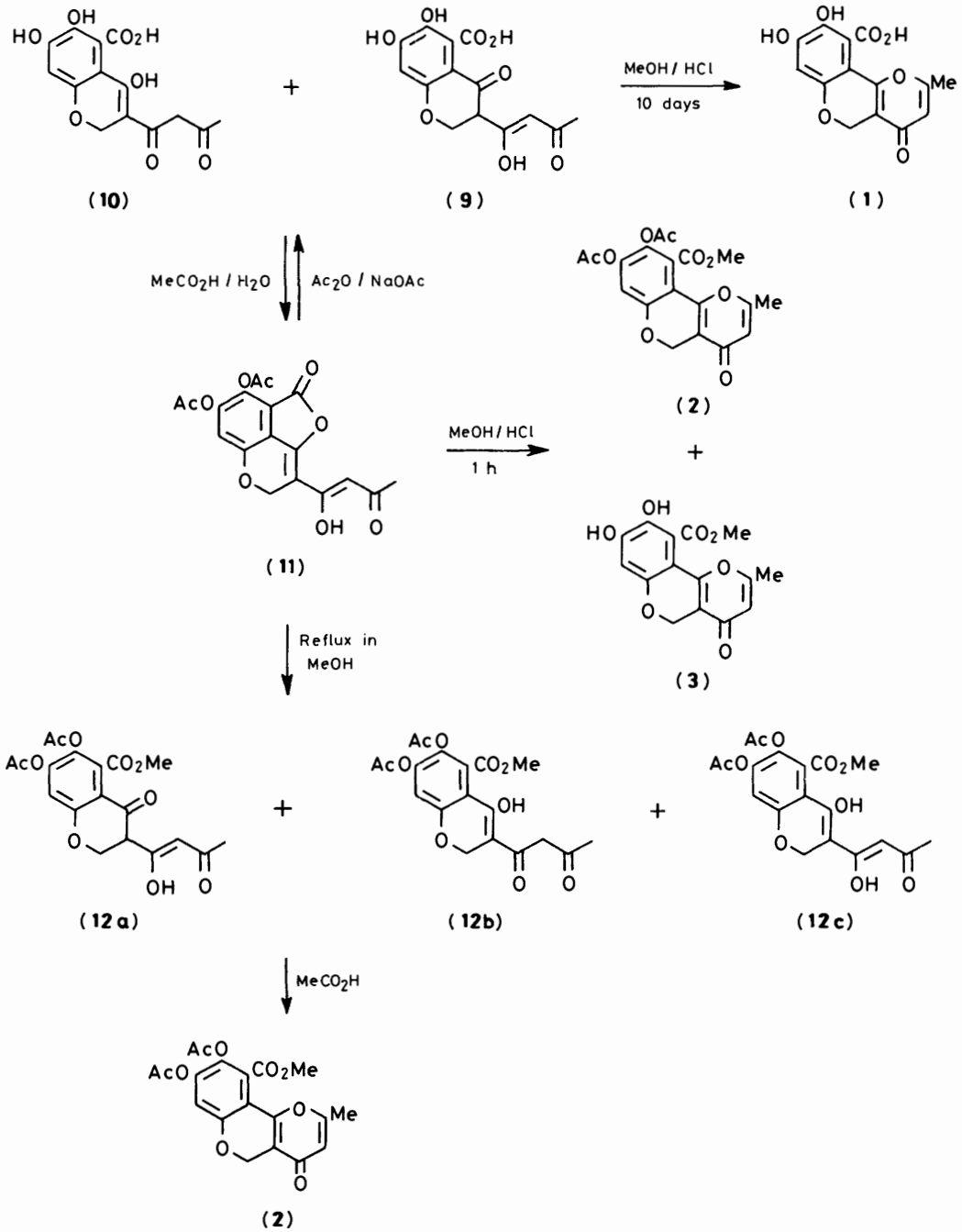
Figure. Sites of spin–spin couplings in the ^{13}C n.m.r. spectra of polivione (**9**) and diacetylanhydropolivione (**11**); heavy lines denote C_2-units showing strong ^{13}C–^{13}C coupling in compounds (**9**) and (**11**) derived from [1,2-$^{13}C_2$]acetate; dotted arrows denote ^1H-^{13}C long-range couplings detected by the 2D ^1H–^{13}C correlation experiment

The problem was solved by a combination of two-dimensional correlation experiments, which allowed us to map the complete skeleton of C–C and C–H bonds for both tautomers (**9**) and (**10**). Thus a carbon–carbon correlation spectrum[10] of polivione enriched by biosynthesis from [1,2-$^{13}C_2$]acetate, showed ^{13}C–^{13}C couplings which established for tautomer (**9**) the connectivities indicated by heavy lines in the Figure. The p.n.d. ^{13}C n.m.r. spectrum of the same material showed for each peak, in addition to the normal uncoupled middle singlet, a doublet due to coupling to the neighbouring ^{13}C-enriched carbon atom of the biosynthetically derived C_2-units: the doublets could be paired unambiguously by coupling constant (Table 2), thus confirming the incorporation of seven intact acetate residues. The enrichment level was not sufficiently high for the detection of the ^{13}C–^{13}C couplings between adjacent C-2 units. These gaps were bridged, however, by a ^1H–^{13}C two-dimensional correlation experiment[11] optimised to detect long range proton-carbon couplings. The pattern obtained is indicated by arrows in the Figure. The long range proton-carbon connectivities of polivione were also checked by a series of heteronuclear decoupling experiments. This technique involves irradiation of an individual proton signal and observation of the effect in the ^{1}H-coupled ^{13}C spectrum. The most important result of these experiments was the irradiation of one of the methylene protons at 6-H (δ 4.53 p.p.m.) which caused the ^{13}C multiplet centred at δ 162.7 p.p.m. to collapse to a triplet (2 overlapping doublets), the remaining couplings being to the aromatic proton (10-H) and the non-irradiated methylene proton at 6-H. The coupling detected between 6-H and C-9 establishes the presence of the ether link in compound (**9**). Furthermore, irradiation of the 3-H vinyl proton of compound (**9**) at δ 5.5 p.p.m. resulted in sharpening of the diffuse ^{13}C multiplets at δ 189.54 and δ 189.56 p.p.m., whereas irradiation of the methyl protons at δ 2.03 p.p.m. caused the ^{13}C multiplet at δ 189.56 p.p.m. to collapse to a doublet, confirming its assignment to C-2 of compound (**9**). The multiplet at δ 189.54 p.p.m. showed a strong intensity increase on irradiation of the methine proton at δ 3.65 p.p.m. and must therefore be C-4 of compound (**9**). Equivalent evidence was obtained to support compound (**10**) as the structure of the minor tautomer. These experiments allowed the unambiguous assignment of the ^{13}C n.m.r. spectra of compounds (**9**) and (**10**) with the exception of the enolised diketone residue of tautomer (**9**), C-2, C-3, and C-4. This could be enolised equally well in the opposite direction to that shown; indeed from the closeness of the chemical shifts of C-2 and C-4, it is suspected that the two possible enol forms may be in rapid equilibrium on the n.m.r. time-scale. Similarly there is a possibility that compound (**10**) may exist as two rapidly interconverting tautomers of the enolised diketone residue.

The chemical properties of polivione were investigated both to confirm its structure and to investigate its relationship to citromycetin (**1**). Firstly, it was converted into citromycetin in high yield when treated with hydrochloric acid in aqueous

methanol. This facile transformation strongly supports the proposed structure, but it frustrated many attempts to prepare derivatives; polivione is very unstable, possibly due to the presence of minute quantities of residual phosphoric acid which, like hydrochloric acid, may catalyse its conversion into citromycetin. On acetylation however, polivione afforded a crystalline anhydro derivative ($C_{18}H_{14}O_9$) whose i.r. spectrum showed strong absorption bands at 1 800 and 1 790 cm^{-1} (in $CHCl_3$) supporting the presence of an enol lactone. The structure of the anhydro diacetate (**11**) is based mainly on the analysis of the high field ^1H and ^{13}C n.m.r. spectra. Extensive homonuclear decoupling experiments, n.O.e., and D_2O-exchange experiments enabled us to assign the ^1H n.m.r. spectrum. The ^{13}C n.m.r. data for diacetylanhydropolivione, as collated in Table 2, were assigned by analysing the long-range ^1H–^{13}C couplings in the fully ^1H-coupled ^{13}C n.m.r. spectrum using specific proton irradiations. In the assignment of the different ^{13}C n.m.r. resonances use was also made of the two- and three-bond (C,H) connectivity pattern as determined by a ^1H–^{13}C two-dimensional correlation experiment (Figure). The final unambiguous assignment of the carbon resonances in the ^{13}C n.m.r. spectrum of diacetylanhydropolivione came from the ^{13}C–^{13}C couplings observed in the proton-noise decoupled ^{13}C n.m.r. spectrum of the diacetate (**11**) formed from polivione enriched biosynthetically by incorporation of sodium [1,2-$^{13}C_2$]acetate; the ^{13}C–^{13}C coupling constants measured from the spectrum were unambigiously paired as shown in Table 2.

Chemical support for the structure of diacetylanhydro



Scheme.

polivione (**11**) came from the chemical conversions summarised in Scheme 1. Firstly, it was hydrolysed back to polivione [(**9**) and (**10**)] by treatment with weak aqueous acid. Secondly, on treatment with hydrochloric acid in aqueous methanol, it was converted into a mixture of diacetylcitromycetin methyl ester (**2**) and citromycetin methyl ester (**3**) (1:3). The structures of these compounds were confirmed by spectroscopic comparison with authentic samples prepared as follows from citromycetin (**1**). Diacetylcitromycetin (**4**) was prepared according to the method of Hetherington and Raistrick[12] by heating citromycetin with anhydrous sodium acetate and acetic anhydride. Further treatment of a methanolic solution of diacetylcitromycetin (**4**) with ethereal diazomethane afforded the diacetylcitromycetin methyl ester (**2**) as a white crystalline solid after chromatography. Citromycetin methyl ester (**3**) was obtained by treating compound (**2**) with hydrochloric acid in aqueous MeOH.

A third informative transformation of the diacetate (**11**) occurred when its solution in methanol was heated. The product was an unstable yellow oil which appeared to be a single compound by t.l.c. analysis. The product was identified spectroscopically as a mixture of three slowly interconverting tautomers (a, b, and c) of diacetylpolivione methyl ester (**12**). Treatment of compound (**12**) with glacial acetic acid afforded diacetylcitromycetin methyl ester (**2**).

Our twin discoveries that polivione is the major component of extracts of *P. frequentans* and that it is readily chemically converted into citromycetin (**1**) raise doubts concerning the status of the latter compound as a natural product. Whatever the truth, it seems likely that polivione is the chemical or biochemical precursor of the small amount of citromycetin detected in the crude extracts by our procedure, and therefore that ring A of citromycetin (**1**) is formed *in vivo* before ring B. In a recent supposedly biomimetic synthesis of the citromycetin system the opposite strategy was employed.[5] This in no way detracts from the merits of the synthesis as such, but it does mean that the task of devising a close chemical model for the biosynthesis remains open. More detailed comment on the biosynthetic implications of our discovery of polivione will be presented in a following paper.

Experimental

M.p.s were determined using a Reichert hot-stage apparatus and are uncorrected. I.r. spectra were recorded on a Perkin-Elmer 297 spectrometer, and are in chloroform solution unless noted otherwise. U.v. spectra were recorded on a Pye-Unicam SP8-100 or SP8-400 spectrometer. ^{1}H N.m.r. spectra were recorded on a Bruker WM-250 or WP-80SY spectrometer. Chemical shifts are given as p.p.m. downfield of tetramethylsilane. ^{13}C N.m.r. spectra were obtained on a Bruker WM-250 or WH-400 spectrometer. Mass spectra were obtained on an AEI MS 30 or MS 50 spectrometer.

Qualitative t.l.c. was ordinarily carried out on commercially prepared plates coated with Merck Kieselgel 60 GF_{254}; preparative t.l.c. was performed using plates coated with the same silica gel (20 × 20 × 0.1 cm). Materials for chromatography on acidified silica gel were prepared as follows: analytical t.l.c. plates by dipping precoated commercial plates in MeOH containing anhydrous H_3PO_4 (0.1M) followed by drying at 100 °C for 5 min; for p.l.c., plates were prepared using Merck Kieselgel 60 GF_{254} slurried with aqueous phosphoric acid (2%); silica gel for column chromatography by adding conc. H_3PO_4 to a slurry of the gel in water until the pH reached 2, followed by filtration and drying at 100 °C for 15 min. Solvents were distilled before use and organic solutions were dried over anhydrous sodium sulphate.

Growth of P. frequentans *and Isolation of Citromycetin* (**1**) *and Polivione* (**9**) *and* (**10**).—*Penicillium frequentans* Westling (CMI 91914) was grown on agar slopes at 28 °C for 10 days during which extensive mycelial growth and sporulation took place. They were then stored at 4 °C and used weekly to inoculate large-scale cultures of sterile Czapek-Dox[12] medium (500 ml). The fungus was introduced into the flasks by shaking the agar slopes with a few ml of distilled water. The cultures were grown at 28 °C for 7—10 days. The resulting deep brown solution was decanted and then acidified to pH 1 with 50% sulphuric acid. The brown flocculant material formed was filtered off under suction through a bed of Celite. The resulting clear yellow solution was extracted with ethyl acetate (6 × 200 ml), the combined extracts dried ($MgSO_4$), and evaporated under reduced pressure to give an orange-brown gum. Initial purification was carried out by flash chromatography on acidified silica gel (150 g), eluting with ethyl acetate–hexane (1:1). The fraction showing a bright green fluorescence was concentrated to give a gum which was further purified by p.l.c. on plates of acidified silica gel, eluting with ethyl acetate–hexane (7:3). Extraction of the green band at R_F 0.5 gave polivione as a yellow glass which failed to crystallise from normal solvents. The total yield of *polivione* was typically 1.4—1.8 g l^{-1} of culture medium (Found: C, 54.2; H. 3.89. $C_{14}H_{12}O_8$ requires C, 54.5; H, 3.92%); R_F [ethyl acetate–hexane (7:3)] 0.5; $\nu_{max.}$($CHCl_3$) 3 600—3 000 (OH), 1 726 (ketone C=O), 1 668 (carboxy C=O), and 1 609 cm^{-1}; $\lambda_{max.}$($CHCl_3$) 390, 310, and 275 nm; δ_H($CDCl_3$) [isomer (**9**)] 2.05 (3 H, s, Me), 3.65 (1 H, t, *J* 6 Hz, C*H*CH_2), 4.53 (1 H, dd, *J* 6, 11 Hz, CHC*H*$_2$), 4.85 (1 H, dd, *J* 6, 11 Hz, CHC*H*$_2$), 5.5 (1 H, s, vinyl-H), and 6.7 (1 H, s, ArH); [isomer (**10**)] 2.3 (3 H, s, Me), 3.6 (2 H, s, CH_2), 4.8 (2 H, s, OCH_2), 6.65 (1 H, s, ArH); hydroxy resonances for both isomers at 14.0, 14.5, and 15.0 p.p.m. (Found: M^+, 308.0504. $C_{14}H_{12}O_8$ requires *M*, 308.0532); *m/z* 308 (15%, M^+), 290 (18, $M - H_2O$), and 206 (100).

The crude extract of the culture can be converted into citromycetin (**1**) by the standard procedure for crystallising citromycetin:[13] the gum (0.5 g) is dissolved in hot ethanol (5 ml) and diethyl ether (20 ml) is added. The solution is filtered to remove any flocculant material present and the solvent is concentrated to 5 ml; hot water (2 ml) is then added. Citromycetin crystallises slowly from this solution (typically over a period of 10—15 days) in the form of yellow needles. Citromycetin (**1**) is filtered off, washed with water, and dried *in vacuo*, above 240 °C (decomp.) (lit.,[12] darkens at 163 °C, m.p. 283—285 °C) (Found: C, 57.8; H, 3.40. $C_{14}H_{10}O_7$ requires C, 57.9; H, 3.47%); R_F [ethyl acetate–hexane (7:3)] 0.2; $\nu_{max.}$(Nujol) 3 500—3 000 (OH), 1 700 (ketone C=O), 1 650 (carboxyl C=O), 1 600 and 1 540 cm^{-1}; $\lambda_{max.}$(MeOH) 370, 300, and 257 nm; δ_H(CD_3OD) 2.3 (3 H, s, pyrone-Me), 4.95 (2 H, s, OCH_2), 6.20 (1 H, s, pyrone-H), 6.5 (1 H, s, ArH) (Found: M^+, 290.0447. $C_{14}H_{10}O_7$ requires *M*, 290.0426); *m/z* 290 (20%, M^+), 272 (10, $M - H_2O$), 246 (100, $M - CO_2$), 245 (43, CO_2H), and 217 (62, CO, CO_2H).

Conversion of Polivione (**9**) *and* (**10**) *into Citromycetin* (**1**).—Polivione (300 mg, 0.97 mmol) was dissolved in methanol (10 ml) and concentrated hydrochloric acid was added. After 10 days at room temperature the conversion of polivione into citromycetin (monitored by t.l.c.) was complete. The solvent was evaporated under reduced pressure, the residue dissolved in water (5 ml), and then extracted with ethyl acetate (4 × 15 ml). The combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was recrystallised from methanol to give citromycetin (**1**) (182 mg, 64%) as a microcrystalline solid, m.p. above 240 °C (decomp.) (lit.,[12] darkens at 163 °C, m.p. 283—285 °C); R_F [ethyl acetate–hexane (7:3)] 0.2. This compound was identified by comparison with

the i.r. and ^{1}H n.m.r. spectra of an authentic sample of citromycetin.

Diacetylanhydropolivione (**11**).—A mixture of polivione (0.5 g, 1.6 mmol), anhydrous sodium acetate (0.5 g, 5 mmol), and acetic anhydride (10 ml) was stirred at room temperature for 3 h. The mixture was diluted with water (20 ml) and extracted with ethyl acetate (4 × 30 ml). The combined extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by p.l.c. on acidified silica gel eluting with ethyl acetate–hexane (7:3), and was recrystallised from dichloromethane–light petroleum (b.p. 60—80 °C) to give the *title compound* (**11**) (0.49 g, 82%) as yellow needles, m.p. 185—187 °C (Found: C, 57.6; H, 3.73. $C_{18}H_{14}O_9$ requires C, 57.7; H, 3.76%); R_F [ethyl acetate–hexane (7:3)] 0.72; $\nu_{max.}$($CHCl_3$) 3 000 (OH), 1 800 (acetate C=O), 1 790 (lactone C=O), 1 680 (ketone C=O), 1 600, and 1 500 cm^{-1}; $\lambda_{max.}$($CHCl_3$) 265 and 415 nm; δ_H($CDCl_3$) 2.15 (3 H, s, Me), 2.31 (3 H, s, COMe), 2.35 (3 H, s, COMe), 5.46 (2 H, s, OCH_2), 6.26 (1 H, s, vinyl-H), 6.82 (1 H, s, ArH), and 15.5 (1 H, br s, OH) (Found: M^+, 374.0636. $C_{18}H_{14}O_9$ requires M, 374.0638); m/z 374 (22%, M^+), 326 (87), 295 (65), and 262 (100).

Conversion of Diacetylanhydropolivione (**11**) *into Diacetylcitromycetin Methyl Ester* (**2**) *and Citromycetin Methyl Ester* (**3**).—Diacetylanhydropolivione (**11**) (0.5 g, 1.33 mmol) was dissolved in methanol (50 ml) and concentrated hydrochloric acid (5 ml) was added. The mixture was stirred at room temperature for 1 h, then poured into water (20 ml), and extracted with ethyl acetate (4 × 25 ml). The combined extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by p.l.c. on acidified silica gel, eluting with ethyl acetate, to give two products: *diacetylcitromycetin methyl ester* (**2**) (90 mg, 22%) as a microcrystalline solid, m.p. 190—192 °C (from chloroform–hexane) (Found: C, 58.3; H, 4.07. $C_{19}H_{16}O_9$ requires C, 58.7; H, 4.15%); R_F (ethyl acetate) 0.44; $\nu_{max.}$($CHCl_3$) 1 780 (acetate C=O), 1 730 (ester C=O), 1 660 (ketone C=O), and 1 600 cm^{-1}; $\lambda_{max.}$($CHCl_3$) 280 and 342 nm; δ_H($CDCl_3$) 2.23 (3 H, s, Me), 2.25 (3 H, s, COMe), 2.26 (3 H, s, COMe), 3.89 (3 H, s, OMe), 5.18 (2 H, s, OCH_2), 6.15 (1 H, s, vinyl-H), and 6.95 (1 H, s, ArH) (Found: M^+, 388.0779. $C_{19}H_{16}O_9$ requires M, 388.0795); m/z 388 (12%, M^+), 304 (38, 2 × M − CH_2O), and 272 (10, M − MeOH, 2 × CH_2CO).

The second product was *citromycetin methyl ester* (**3**) (260 mg, 65%) as yellow needles, m.p. 152—154 °C (from methanol) (Found: C, 51.7; H, 3.94. $C_{15}H_{12}O_7$ requires C, 51.21; H, 3.97%); R_F (ethyl acetate) 0.35; $\nu_{max.}$(Nujol) 2 950 (CH), 1 730 (ester C=O), 1 660 (ketone C=O), 1 600, and 1 580 cm^{-1}; $\lambda_{max.}$(MeOH), 256, 300, and 370 nm; δ_H(CD_3OD) 2.4 (3 H, s, pyrone-Me), 3.97 (3 H, s, OMe), 5.1 (2 H, s, OCH_2), 6.36 (1 H, s, pyrone-H), and 6.56 (1 H, s, ArH) (Found: M^+, 304.0586. $C_{15}H_{12}O_7$ requires M, 304.0583); m/z 304 (52%, M^+) and 272 (100, M − MeOH).

Diacetylcitromycetin (**4**).—A stirred mixture of citromycetin (**1**) (1 g, 3.4 mmol), anhydrous sodium acetate (1 g, 12.1 mmol), and acetic anhydride (5 ml) was heated under reflux for 3 h. After cooling, the mixture was dissolved in water (25 ml) and the solution filtered. Acidification of the filtrate with concentrated hydrochloric acid gave a thick cream precipitate, which was filtered off, washed with water (20 ml), and then dissolved in aqueous sodium hydrogen carbonate, and reprecipitated with hydrochloric acid. The product was filtered off, washed with water, and dried *in vacuo*. Recrystallisation from ethanol gave diacetylcitromycetin[12] (**4**) (0.9 g, 70%) as prisms, m.p. 221—223 °C (lit.,[12] 223—224 °C) (Found; C, 57.6; H, 3.74. $C_{18}H_{14}O_9$ requires C, 57.8; H, 3.76%); R_F (ethyl acetate) 0.38; $\nu_{max.}$(Nujol) 1 780 (acetate C=O), 1 700 (ketone C=O), 1 650 (carboxy C=O), 1 600, and 1 570 cm^{-1}; $\lambda_{max.}$(MeOH) 290 and 340 nm; δ_H(CD_3OD) 2.20 (6 H, s, 2 × COMe), 2.27 (3 H, s, pyrone-Me), 5.10 (2 H, s, OCH_2), 6.17 (1 H, s, pyrone-H), and 6.94 (1 H, s, ArH) (Found: M^+, 374.0644. $C_{18}H_{14}O_9$ requires M, 374.0650); m/z 374 (11%, M^+), 290 (20, 2 × M − $COCH_2$), and 272 (100, H_2O, 2 × $COCH_2$).

Conversion of Diacetylcitromycetin (**4**) *into Diacetylcitromycetin Methyl Ester* (**2**).—An ethereal solution of diazomethane [prepared from Diazald (5 g)] was added to a suspension of diacetylcitromycetin (**4**) (0.2 g, 0.53 mmol) in methanol (100 ml) and left at room temperature in the dark for 10 min. Glacial acetic acid was then added to destroy the excess of diazomethane and the solvent evaporated under reduced pressure to give a cream residue. Recrystallisation from chloroform–hexane yielded *diacetylcitromycetin methyl ester* (**2**) (0.15 g, 85%) as a white crystalline solid, m.p. 190—192 °C; R_F (ethyl acetate) 0.44, with spectral properties identical with those obtained before.

Conversion of Diacetylcitromycetin Methyl Ester (**2**) *into Citromycetin Methyl Ester* (**3**).—Diacetylcitromycetin methyl ester (**2**) (50 mg, 0.13 mmol) was dissolved in methanol (20 ml) and concentrated hydrochloric acid (2 ml) was added. The mixture was stirred at room temperature for 3 days. The solvent was evaporated under reduced pressure and the residue purified by p.l.c. on acidified silica gel eluting with ethyl acetate–hexane (1:1, 2 elutions) to give *citromycetin methyl ester* (**3**) (25 mg, 64%), as a yellow solid, m.p. 152—153 °C; R_F (ethyl acetate) 0.35; $\nu_{max.}$(Nujol) 2 950 (CH), 1 730 (ester C=O), 1 660 (ketone C=O), and 1 600 cm^{-1}; $\lambda_{max.}$(MeOH) 256, 300, and 370 nm; δ_H(CD_3OD) 2.39 (3 H, s, Me), 3.98 (3 H, s, OMe), 5.1 (2 H, s, OCH_2), 6.36 (1 H, s, pyrone-H), and 6.56 (1 H, s, ArH) (Found: M^+, 304.0576. $C_{15}H_{12}O_7$ requires M, 304.0583); m/z 304 (45%, M^+) and 272 (100).

Conversion of Diacetylanhydropolivione (**11**) *into Polivione* (**9**) *and* (**10**).—A stirred mixture of diacetylanhydropolivione (**11**) (1 g, 2.67 mmol), water (100 ml), and glacial acetic acid (50 ml) was heated under reflux for 1 h. After cooling, the mixture was extracted with ethyl acetate (5 × 50 ml) and the combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was subjected to column chromatography on acidified silica gel eluting with ethyl acetate–hexane (1:1). It was further purified by p.l.c., eluting with ether (2 elutions) to give *polivione* (**9**) and (**10**) (0.1 g, 12%) as a yellow glass. This compound was identified by comparison with the i.r. and ^{1}H n.m.r. spectra of an authentic sample of polivione.

Diacetylpolivione Methyl Ester (**12**).—A solution of diacetylanhydropolivione (**11**) (0.1 g, 0.27 mmol) in methanol (50 ml) was heated under reflux with stirring. After 30 min t.l.c. indicated an almost complete reaction. The solution was evaporated under reduced pressure and the residue was purified by p.l.c. eluting with ethyl acetate–hexane (7:3), to give the *ester* (**12**) (87 mg, 80%) as an oil, exhibiting green fluorescence, R_F [ethyl acetate–hexane (7:3)] 0.78; $\nu_{max.}$($CHCl_3$) 2 950 (CH), 1 780 (acetate C=O), 1 720 (ester C=O), 1 660, 1 600, and 1 580 cm^{-1}; $\lambda_{max.}$($CHCl_3$) 262, 314, and 376 nm. Although t.l.c. indicated one very pure product, the ^{1}H n.m.r. spectrum showed a mixture of three tautomers: δ_H($CDCl_3$) [isomer (**12a**)] 2.04 (3 H, s, Me), 3.58 (1 H, dd, J 4.44 and 2.83 Hz, CH_2CH), 4.6 (1 H, dd, J 4.44 and 7.28 Hz, CH_2CH), 4.86 (1 H, dd, J 4.44 and 7.28 Hz, CH_2CH), 5.6 (1 H, s, vinyl-H), 7.0 (1 H, s, ArH); [isomer (**12b**)] 2.04 (3 H, s, Me), 3.56 (2 H, s, CH_2), 4.8 (2 H, br s, OCH_2), 6.9 (1 H, s, ArH); [isomer (**12c**)] 2.04 (3 H, s, Me), 4.8 (2 H, br s, OCH_2), 5.28 (1 H, s, vinyl-H), 6.8 (1 H, s, ArH); the acetate resonances of all three tautomers were superimposed at δ 2.22,

2.23, and 2.24 (s), similarly the hydroxy resonances at δ 14.5, 15.0, and 15.2 (br s), and the methoxy resonances at δ 3.86, 3.88, and 3.89 (s) (Found: M^+, 406.0907. $C_{19}H_{18}O_{10}$ requires M, 406.0900); m/z 406 (15%, M^+), 364 (25, $M - CH_2CO$), 322 (46, $2 \times M - CH_2CO$), and 290 (100, M − MeOH, $2 \times CH_2CO$).

Conversion of Diacetylpolivione Methyl Ester (**12**) *into Diacetylcitromycetin Methyl Ester* (**2**).—A solution of diacetylpolivione methyl ester (**12**) (0.1 g, 0.2 mmol) in glacial acetic acid (10 ml) was heated under reflux for 2 h, and then stirred without heat until the conversion of the green fluorescent starting material into the blue fluorescent product was complete (4 days). The solvent was then evaporated under reduced pressure, and the residue washed with toluene to remove any residual acetic acid. Recrystallisation from $CHCl_3$–hexane (9:1) gave the methyl ester (**2**) (85 mg, 89%) as needles, m.p. 191—192 °C. The structure of this compound was confirmed by spectroscopic comparison with an authentic sample prepared from citromycetin.

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