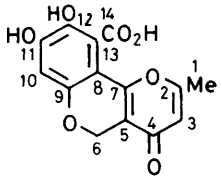
An Investigation of the Biosynthesis of Citromycetin in *Penicillium frequentans* using ^{13}C- and ^{14}C-Labelled Precursors

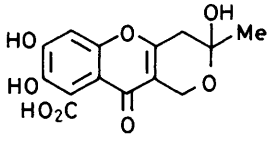
Geoffrey E. Evans and James Staunton *
University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW

Citromycetin (**1**) has been shown by ^{13}C n.m.r. to incorporate seven intact acetate units from [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-acetates in accordance with a polyketide biosynthesis. The distribution of radioactivity following incorporation of [2-^{14}C]malonate was consistent with the utilisation of two starter units. The following possible advanced precursors, labelled with ^{14}C, were not incorporated: 2,4-dihydroxy-6-methylbenzoic acid (**14**), 4,5,7-trihydroxyphthalide (**16**), and 4,7-dihydroxy-5-methylcoumarin (**18**). Two compounds, 2,4,5-trihydroxy-6-methylbenzoic acid (**15**) and triacetic acid lactone (**17**), were degraded to radiolabelled acetate prior to incorporation.

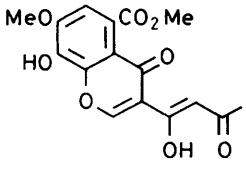
Citromycetin (**1**), a yellow metabolite of *Penicillium frequentans*, was discovered by Hetherington and Raistrick [1] in 1931, and its structure was established 20 years later by a series of degradations performed by Cavill *et al.*[2] The only other structures of this type are fulvic acid (**2**),[3] lapidosin (**3**),[4] and the recently discovered polivione (**4**).[5]



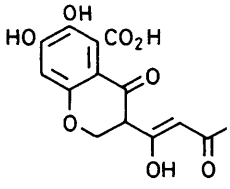
(**1**)



(**2**)

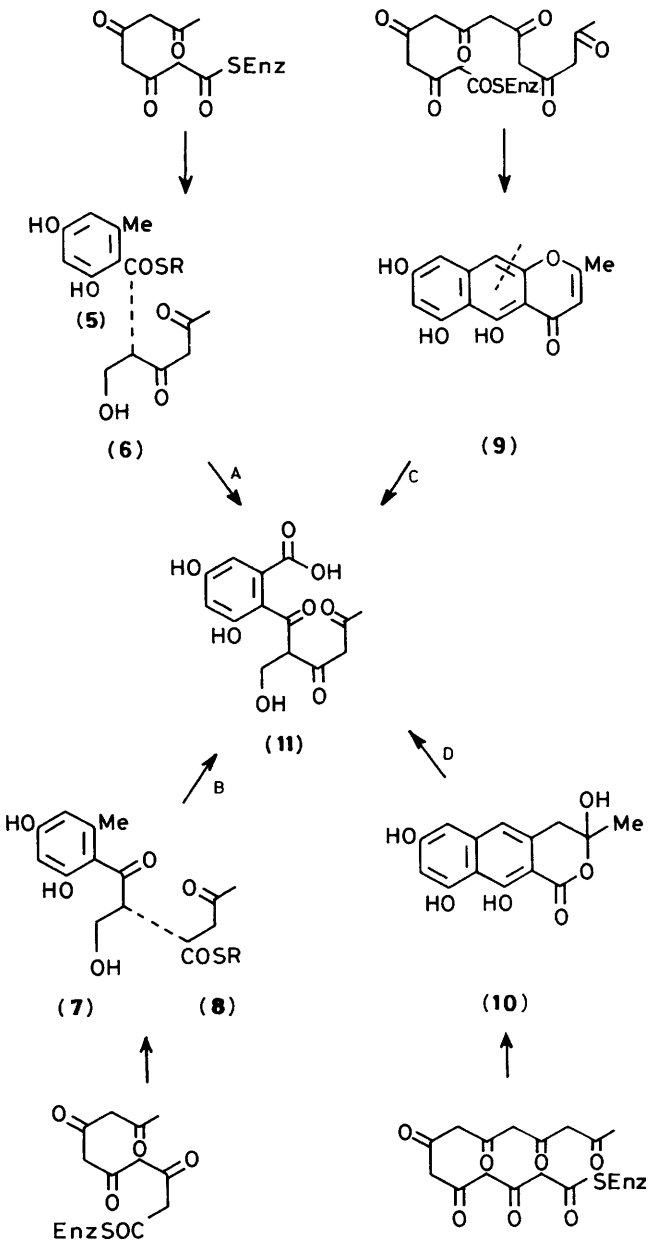


(**3**)



(**4**)

The alternating oxygenation pattern of the carbon skeleton of these metabolites suggests a polyketide biosynthesis but the unusual branched structure cannot be derived straightforwardly from a single unbranched polyketone chain. The biosynthesis of citromycetin (**1**) has therefore been the subject of much speculation. Four representative proposals which have guided our planning in the present investigation are presented in Scheme 1. Pathways A and B are 'two chain pathways,'[6] according to which the citromycetin skeleton might be formed by the combination of two polyketide intermediates, each formed separately from a different polyketide chain. Pathways C[7] and D[8] are of a more conventional type in which the skeleton might arise by modification of an intermediate derived from a single chain. The various structural types represented by intermediates (**5**)—(**10**), are well represented among polyketide natural products, with the notable exception of compound (**7**), which is unusual in having its carboxy terminus as the longer of the two uncyclised residues attached to its aromatic ring.[9] For clarity, it is assumed in Scheme 1 that a compound such as (**11**), an uncyclised analogue of (**1**), is formed as an intermediate. Such a compound could serve as a common intermediate for all four metabolites, (**1**)—(**4**); there is evidence from biosynthetic

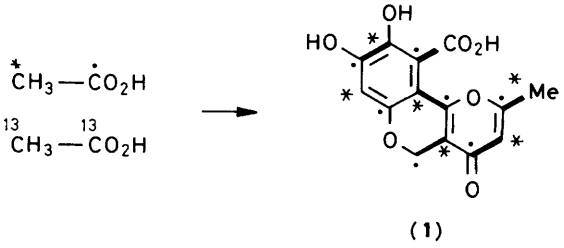


Scheme 1.

studies that the carbon frameworks of both fulvic acid (**2**)[10] and polivione (**4**)[11] arise in the same way as that of citromycetin (**1**).

The four pathways of Scheme 1 have much in common, notably the need to adjust the level of oxidation at several sites. Thus, C-12 becomes hydroxylated, C-6 becomes reduced from a carboxylic acid to alcohol (to an aldehyde in lapidosin), and C-14 becomes oxidised to a carboxylic acid. The latter change is especially interesting because a carboxy group might be expected to be generated at that site as a consequence of the oxidative cleavage step on pathway C or D. In contrast, the oxidation at C-14 would be incidental to the generation of the carbon skeleton in pathway A or B.

Citromycetin (**1**) was one of the first compounds studied by Birch *et al.* in their pioneering studies of polyketide biosynthesis using radiotracer techniques.[12] Following administration of [1-^{14}C]acetate to *P. frequentans*, radioactive citromycetin was isolated. Degradation studies revealed the degree of labelling at several key sites, and all the results were consistent with the uniform incorporation of seven acetate units to produce citromycetin (**1**) labelled as in Scheme 2. In a separate experiment



(**1**)

Scheme 2.

the incorporation of formate was low and non-specific. These results confirm the polyketide origin of citromycetin, but they do not distinguish between the various possible routes shown in Scheme 1.

In attempts to decide whether one or two polyketide chains are involved in building the carbon skeleton, Mosbach and Gatenbeck,[13] and Birch *et al.*[14] studied the incorporation of [2-^{14}C]malonate to determine how many starter units were present. The resulting citromycetin was degraded by Kuhn–Roth oxidation to isolate the C_2-unit, C-1 + C-2, as acetic acid. This residue is required to be the starter unit for all four pathways, and, in accordance with this prediction, its degree of incorporation was found to be lower than would be expected if there were a uniform distribution of activity over the seven methyl-derived sites in the metabolite. The key C_2-unit, however, is the residue C-14 + C-13, which would be expected to be a starter unit, and therefore be lower in activity, according to pathways A and B, but not according to pathways C and D. The relevant carbon, C-14, was isolated by decarboxylation, and found to be lower in activity than the calculated average, and to be comparable in activity with C-1. This result suggests that there are two starter units and, therefore, that pathway A or B is in operation.

In an attempt to discriminate between pathways A and B, Birch *et al.*[14] tested [1-^{14}C]butyrate as a precursor in the hope that it would undergo oxidation *in situ* to a suitable precursor equivalent to compound (**8**). Radioactivity was incorporated, but not selectively into C-4, as was hoped. Instead the activity was distributed uniformly over all seven C_2-units, as would be expected if the precursor had suffered degradation to labelled acetate prior to incorporation. The experiment was, therefore, inconclusive.

Biosynthetic pathways which have been proved to involve two chains are rare. One firmly established example is

Table 1. ^{13}C N.m.r. data for citromycetin (**1**)

Carbon	δ_C (p.p.m.)[a]	$J_{^1H-^{13}C}$ (Hz)	$J_{^{13}C-^{13}C}$ (Hz)[b]
1	19.3	130 (q)	51.3
2	167.3		51.1
3	114.2	168 (d)	56.2
4	178.2		56.4
5	112.6		47.9
6	63.4	151 (t)	48.3
7	158.2		67.4
8	105.2		67.3
9	153.7		72.5
10	105.9	163 (d)	72.7
11	152.9		70.2
12	141.7		70.6
13	117.9		72.0
14	170.0		72.0

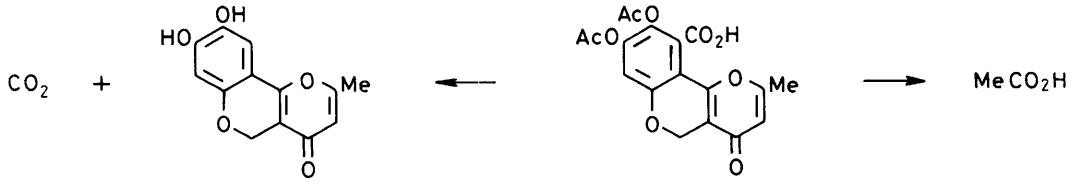
[a] Relative to Me_4Si. [b] Measured for citromycetin (**1**) enriched by incorporation of [1,2-$^{13}C_2$]acetate.

rotiorin.[15] Other related metabolites are known to originate from two chains.[9] Sclerin was initially thought to come into this category,[16] but it was subsequently proved to be derived from a single chain.[17] A two-chain route has been proposed for mollisin[18] but an alternative single chain route can be proposed. A biosynthesis from two chains is therefore an unusual event, and so there is a strong incentive to continue the investigation of citromycetin biosynthesis.

With modern tracer methods based on stable isotopes such as ^{13}C, it is possible to carry out much more thorough investigations of sites of labelling, including sites which are effectively inaccessible to standard degradation techniques. In addition, more searching investigations with multiply labelled precursors are possible. In re-opening the investigation of citromycetin biosynthesis using these techniques, our initial task was to assign the ^{13}C n.m.r. spectrum. The assignments shown in Table 1 were made straightforwardly, on the basis of ^{1}H–^{13}C couplings, and comparison of chemical shifts with calculated values[19] and experimental values measured for suitable model compounds (*e.g.* 2,6-dimethylpyran-4-one).

Incorporation studies with [1-^{13}C]- and [2-^{13}C]-acetates as precursors gave labelling results in complete agreement with those of Birch *et al.*[12] (see Scheme 2); in the first experiment all the even-numbered carbons gave rise to singlets of enhanced intensity, in the second all the odd-numbered ones were similarly found to be enriched. In addition, [1,2-$^{13}C_2$]acetate was used as a precursor. In the resulting citromycetin all 14 carbons showed ^{13}C–^{13}C doublets flanking the natural abundance singlets, thus proving that all seven C_2-units are incorporated intact (as indicated by heavy lines in Scheme 2), and that none of them undergoes an unexpected cleavage or rearrangement in the course of the biosynthesis. All four pathways of Scheme 1 are consistent with these ^{13}C incorporation results.[20]

Our next aim, therefore, was to re-examine the evidence that C-14 + C-13 is a starter C_2-unit. The most direct technique using stable isotopes employs deuterium labelling in combination with ^{13}C as a reporter nucleus to show that three deuteriums can be retained from $^{13}C^2H_3$-labelled acetate.[21] Regrettably this was not suitable for citromycetin because the key site, C-14, undergoes oxidation to a carboxy group with loss of all its attached hydrogens. We, therefore, sought to detect differential incorporation of ^{13}C from [1-^{13}C]acetate. This precursor was administered to the organism in the presence of an excess of unlabelled malonate in the hope that incorporation of the label at the chain-building sites would be suppressed. In the event, labelled citromycetin was obtained but there was no



10 (13); 88 (12); 100 11

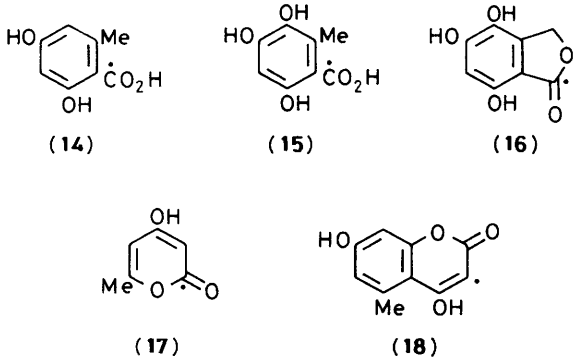
Scheme 3. Incorporation of [2-^{14}C]malonate into citromycetin indicating the relative molar activities of the degradation products; CO_2 was trapped in a scintillator solution containing 2-phenylethylamine and acetic acid was counted as the *p*-bromophenacyl derivative

evidence from intensity measurements in the ^{13}C n.m.r. spectrum for enhanced incorporation at either of the postulated starter C_2-units.

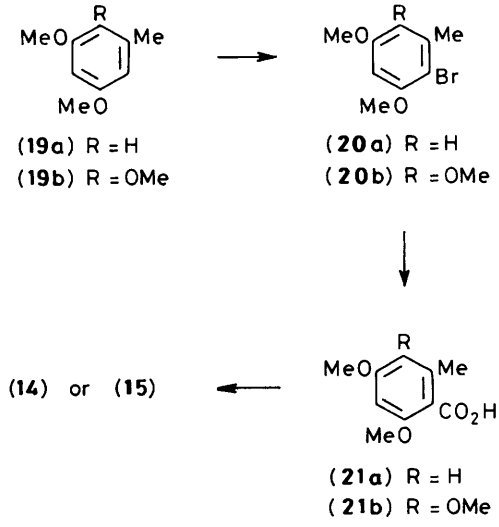
Carbon n.m.r. spectroscopy is unreliable for quantitative measurements of enrichments at different sites, especially when only small variations in enrichment are involved. We, therefore, repeated the earlier work [13,14] in which [2-^{14}C]malonate was used as the precursor. Our procedure for determining the distribution of label in the resulting citromycetin is shown in Scheme 3. The citromycetin was converted into its diacetyl derivative (**12**), which was recrystallised to constant activity. A sample of this was then decarboxylated by warming in sulphuric acid. Both products, citromycin (**13**) and CO_2, were collected and their radioactivities measured. A second sample of derivative (**12**) was subjected to Kuhn–Roth oxidation and the resulting acetic acid was purified as its *p*-bromophenacyl derivative for radioactivity measurement.

From the radioactivities of the degradation products (see Scheme 3), both C-14 and C-1 appear to be lower in radioactivity than would be expected for a uniform distribution of the isotope over seven sites of citromycetin (**1**). The degree of differential labelling between the apparent starter units and chain extension units is low, however, and it is close to the margin allowed by the experimental errors in the radioactive counting. Our results are close to those reported by Birch *et al.*,[14] but a much higher level of differential labelling was found by Mosbach and Gatenbach.[13] Presumably the difference between our experiment and theirs is a consequence of a slight variation in the conditions of the incubation, or perhaps of the use of a different strain of the organism. It therefore seemed prudent at this stage to assume that one of the 'two-chain' pathways, A or B, is in operation.

In order to subject these two pathways to direct experimental test, we next resorted to advanced precursor studies. Given that the many oxidation and reduction processes mentioned earlier could take place in any order, and that some or all of them may take place before the two separately formed intermediates are linked by carbon–carbon bond formation, it seemed wise to begin our tests with relatively unelaborated compounds. Accordingly, to test pathway A, four compounds, (**14**)—(**17**), were synthesized; the first three are analogues of the resorcyclic acid derivative (**5**), the fourth being an analogue of the proposed complementary building block (**6**). To test pathway B, the coumarin (**18**), an analogue of compound (**7**), was synthesized; note that Birch *et al.*[14] had already tested butyric acid as a possible precursor of compound (**8**), with negative results. It was hoped that one or more of these test compounds might either be actual intermediates on the pathway, or that they might be readily converted into true intermediates *in vivo*.



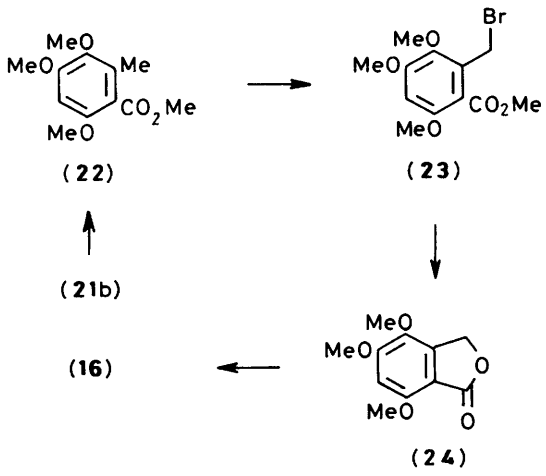
The synthetic routes developed for the preparation of isotopically labelled compounds are summarised in Schemes 4—7.



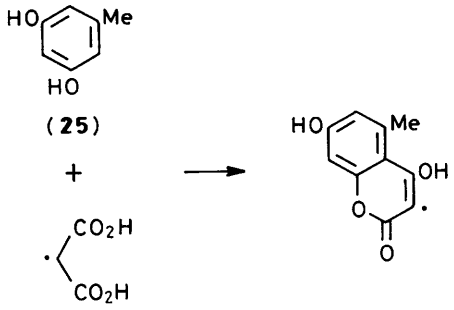
Scheme 4.

Scheme 4 shows the routes to compounds (**14**) and (**15**), starting from the appropriate di- or tri-methoxytoluene. Regioselective bromination was followed by lithiation and treatment with CO_2 to give the substituted benzoic acid; use of radioactive CO_2 gave the required labelled intermediate which was then demethylated. The preparation of the phthalide (**16**) (Scheme 5) starts with compound (**21b**) which is first methylated, and then selectively brominated under radical conditions; phthalide formation and demethylation complete the route. Radiolabelled material was obtained from the radiolabelled starting material (**21b**) prepared according to Scheme 4. The labelled coumarin (**18**) was prepared in one step (Scheme 6) by a Friedel–Crafts reaction between orcinol (**25**) and radiolabelled malonate. The preparation of compound (**17**) proceeded by the two steps shown in Scheme 7: the dianion of acetylacetone was carboxylated with radiolabelled CO_2 to give the diketo acid (**26**), which was cyclised to produce compound (**17**).

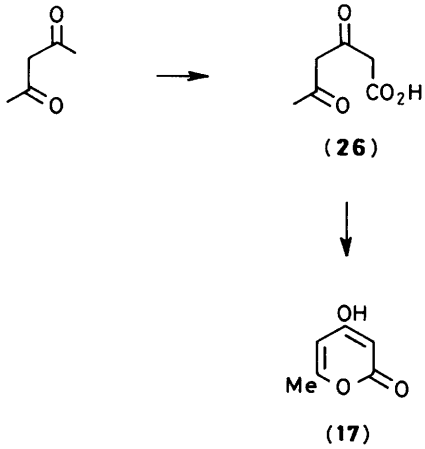
The five labelled test compounds were administered to growing cultures of *P. frequentans* and the citromycetin (**1**)



Scheme 5.



Scheme 6.



Scheme 7.

isolated after a period of further incubation. In each case, the resulting metabolite was purified as its diacetate (**12**) for measurement of incorporation of radioactivity. Three compounds [(**14**), (**16**), and (**18**)] gave negligible incorporations but the remaining two [(**15**) and (**17**)] gave initially promising degrees of incorporation (see Table 2). To test whether these incorporations were specific, each sample of diacetylcitromycetin (**12**) was converted into citromycin (**13**) (see Scheme 3), which was then subjected to Kuhn–Roth oxidation. The resulting acetic acid was isolated in the usual way as the *p*-bromophenacyl derivative for measurement of radioactivity. A specific result would be expected to give non-radioactive acetate; in the event, this fragment of the metabolite was found to retain approximately one seventh of the total activity of the original metabolite for both experiments. From this we conclude that both precursors have not been incorporated intact, but that they have been degraded to acetate *in vivo* prior to incorporation *via* the normal route of polyketide biosynthesis.

Table 2. Incorporation experiments with possible advanced precursors

Test compound[a]	Incorporation (%)[b]	Relative molar activity of acetic acid[c] derived from C-1 + C-2 (%)
(**14**)	<0.01	
(**15**)	0.8	14
(**16**)	<0.01	
(**17**)	1.5	15
(**18**)	<0.01	

[a] Labelled with ^{14}C at the starred site on each structure. [b] Relative to the amount administered (125 Ci). [c] Counted as the *p*-bromophenacyl derivative.

In conclusion, therefore, all the experiments with possible advanced precursors have given negative results in both our own investigation and that of Birch *et al.* Such results are, of course, inconclusive, since a negative outcome is possible even with a true precursor, if it cannot reach the sites of biosynthesis because of permeability problems.

Rather than carry on with further advanced precursor experiments on a hit-or-miss basis, we decided to screen the culture medium for minor metabolites which might provide clues to possible advanced precursors. This new line of attack provided some surprises and proved fruitful, as will be described in full in subsequent papers.[22] Suffice it to say here that of the four pathways shown in Scheme 1, pathway D is the one most likely to be in operation in the organism, despite the evidence for a 'two-chain' pathway. More surprising still is our discovery that citromycetin (**1**) may be a chemical artifact of the isolation procedure rather than a true natural product in its own right.

Experimental

M.p.s were determined with a Kofler hot-stage apparatus and are uncorrected. U.v. spectra were measured on a Unicam SP8000 spectrometer. I.r. spectra were recorded on a Perkin-Elmer 257 i.r. spectrometer. ^1H N.m.r. spectra were obtained on either a Varian HAD 100, a Perkin-Elmer R12, or a Perkin-Elmer R24A spectrometer with Me_4Si as an internal reference. ^{13}C N.m.r. spectra were obtained with a Varian XL100 spectrometer. Chemical shifts are quoted in p.p.m. relative to Me_4Si. Mass spectra were obtained with either an AEI MS9 or MS30. Radioactive counting involved the use of a Packard 3385 liquid scintillation counter; hexadecane was used as the internal standard.

Qualitative thin layer chromatography (t.l.c.) was carried out on commercially prepared plates coated with Merck Kieselgel GF_{254}. Preparative layer chromatography involved the use of plates (20 × 20 cm) coated with the same silica gel to a thickness of 1 mm. Organic solutions were dried over anhydrous sodium sulphate.

Production and Isolation of Citromycetin (**1**).—Suitable strains of the mould *Penicillium frequentans* Westling [International Mycological Institute No. 91914ii(64)] obtained from the Commonwealth Mycological Institute were subcultured on to slopes of Czapek-Dox agar made by using 1 pellet of

Oxoid Czapek-Dox agar and distilled water (4 ml per test-tube) and sterilising these at 120 °C/15 p.s.i. for 15 min. After inoculation, these slopes were incubated at 25 °C for 6 days. They were then stored at 4 °C until required as inocula for large-scale cultures.

For production of citromycetin, *P. frequentans* was grown on a modified liquid Czapek-Dox medium of the following composition: glucose (50 g), sodium nitrate (2 g), potassium dihydrogen phosphate (1 g), potassium chloride (0.5 g), magnesium sulphate heptahydrate (0.5 g), iron(II) sulphate heptahydrate (0.2 g), zinc sulphate heptahydrate (10 mg), copper(II) sulphate pentahydrate (5 mg), and distilled water (1 l). 500-ml Portions of this medium were placed in still-culture flasks and sterilised by autoclaving for 20 min at 120 °C/15 p.s.i. The fungus was introduced as a vegetable inoculum from agar slopes and grown in a stationary culture for 10—14 days, after which the brown medium was decanted off. Sulphuric acid was added (50%; 20 ml) to 1 l of this solution. After 30 min, a brown floccular material was precipitated and was filtered off under suction through a bed of Celite. The resulting clear yellow solution was extracted with ethyl acetate and the organic extracts dried and evaporated to give an orange–brown gum. This material (1 g) was dissolved in hot ethanol (10 ml) and ether (40 ml) added. Further brown floccular material was precipitated. The solution was filtered and evaporated under reduced pressure to 5 ml; hot water (5 ml) was added. Citromycetin crystallised slowly from this solution (typically over a period of 10—14 days) in the form of very small yellow needles. The citromycetin was filtered off, washed with water, and dried *in vacuo*. The yield was variable, but typically in the range 0.4—2.0 g^{-1} of medium. M.p. above 260 °C (decomp.) (lit.,[1] darkens at 163 °C, m.p. 283—285 °C); $\lambda_{max.}$(EtOH) 255, 307, and 376 nm (23 000, 9 000, and 14 000); $\nu_{max.}$(Nujol) 3 600—2 700br, 3 500m, 1 700m, 1 640s, 1 600m, and 1 520m cm^{-1}; $\delta(C_5D_5N)$ 2.05 (3 H, s, pyrone-Me), 5.19 (2 H, s, OCH_2), 6.16 (1 H, s, pyrone-H), and 6.87 (1 H, s, ArH); *m/z* 290 (15%, *M*), 272 (21, $M - H_2O$), 246 (100, $M - CO_2$), 245 (67, $M - CO_2H$), and 217 (67, $M - CO, CO_2H$).

O,O-*Diacetylcitromycetin* (**12**).—A stirred mixture of citromycetin (1 g), anhydrous sodium acetate (1 g), and acetic anhydride (3 ml) was heated under reflux for 1 h. After cooling, the mixture was dissolved in water (10 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 and then dissolved in aqueous sodium hydrogen carbonate and reprecipitated with hydrochloric acid. The diacetyl compound was filtered off, washed with water, and dried *in vacuo* (9.93 g, 72%). A sample crystallised from ethanol in the form of prisms, m.p. 222—223 °C (lit.,[1] 223—224 °C); $\delta(CD_3OD)$ 2.20 (6 H, s, 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).

Citromycin (**13**).—A mixture of diacetylcitromycetin (1 g) and 1M sulphuric acid (50 ml) was heated under reflux. The diacetyl compound rapidly dissolved and hydrolysed to give an orange–yellow solution. The mixture was heated for 14 h and then diluted with water (50 ml), and the hot solution filtered. Citromycetin crystallised out from the filtrate in the form of orange–yellow needles, which were filtered off, washed with water, and dried (0.58 g, 88%). A sample recrystallised from aqueous ethanol in the form of yellow needles, darkening above 250 °C, m.p. 283—290 °C (decomp.) (lit.,[1] darkens 255—260 °C, m.p. 285—290 °C (decomp.); $\lambda_{max.}$(EtOH) 253, 297, and 358 nm; $\delta(C_5D_5N)$ 2.05 (3 H, s, pyrone-Me), 5.29 (2 H, s, OCH_2), 6.16 (1 H, s, pyrone-H), 6.86 (1 H, s, ArH), and 7.41 (1 H, s, ArH); *m/z* 246 (100%, *M*) and 217 (42, $M - CHO$).

Kuhn–Roth Degradation of Citromycetin (**1**) *and Citromycin* (**13**).—A mixture of citromycetin (50 mg) or citromycin (50 mg) with 5M chromic acid (10 ml) and concentrated sulphuric acid (2 ml) was heated at 100 °C for 2 h. After cooling, the mixture was diluted with water (10 ml) steam distilled until 400 ml of distillate had been collected. The distillate was neutralised with 0.05M aqueous sodium hydroxide using phenolphthalein as indicator and then evaporated under reduced pressure.

The residue from the Kuhn–Roth degradation was dissolved in water (1 ml) and ethanol (9 ml). *p*-Bromophenacyl bromide (50 mg) was added and the mixture heated under reflux for 2 h. After cooling, the mixture was evaporated under reduced pressure and the residue dissolved in ether (10 ml) and water (10 ml); the aqueous layer was extracted with ether and the combined ethereal extracts were dried and evaporated under reduced pressure to give a white crystalline residue, which was purified by preparative layer chromatography on silica gel [ethyl acetate–benzene (1:10)]. The phenacyl derivative was recrystallised from light petroleum (b.p. 40—60 °C), m.p. 85—86 °C (lit.,[23] 86 °C); $\delta(CDCl_3)$ 2.20 (3 H, s, Me), 5.26 (2 H, s, CH_2), 7.57 (2 H, s, *J* 10 Hz, ArH), and 7.75 (2 H, d, *J* 10 Hz, ArH).

3,5-*Dimethoxytoluene* (**19a**).—Orcinol was freed from water of crystallisation by heating under reflux with benzene in a Dean–Stark apparatus. A mixture of anhydrous orcinol (6.2 g), dimethyl sulphate (13.8 g), and anhydrous potassium carbonate (14 g) in dry acetone (100 ml) was heated under reflux under nitrogen for 7 h. After cooling, the mixture was filtered, the residue washed with acetone, and the combined filtrates were evaporated under reduced pressure. The residue was dissolved in ether and the ethereal solution washed with 10% aqueous sodium hydroxide and water, dried, and evaporated under reduced pressure. Distillation gave the dimethoxy compound (6.4 g, 82%), b.p. 74 °C/0.2 mmHg (lit.,[24] 240 °C/720 mmHg); $\lambda_{max.}$(EtOH) 223 and 275 nm; $\nu_{max.}$(liquid) 1 600s and 1 480m cm^{-1}; $\delta(CDCl_3)$ 2.30 (3 H, s, ArMe), 3.70 (6 H, s, OMe), and 6.30 (3 H, s, ArH); *m/z* 152 (*M*).

2-*Bromo*-3,5-*dimethoxytoluene* (**20a**).—Copper(II) bromide (6.6 g) was slowly added over a period of 1 h to a vigorously stirred solution of 3,5-dimethoxytoluene (**19a**) (3.04 g) in dry 1,2-dimethoxyethane (40 ml). The resulting green solution was stirred for a further 1 h to precipitate a salt which was filtered off. The solvent was evaporated off and the residue dissolved in a little dichloromethane and filtered through a column of Florisil (30 g) with dichloromethane as eluant. Evaporation of the solvent gave the bromide which recrystallised from ethanol in the form of needles (3.2 g, 68%), m.p. 58 °C (lit.,[24] 57 °C); $\lambda_{max.}$(EtOH) 226 and 285 nm; $\nu_{max.}(CHCl_3)$ 1 600s and 1 500s; $\delta(CDCl_3)$ 2.47 (3 H, s, ArMe), 3.70 (3 H, s, OMe), 3.83 (3 H, s, OMe), and 6.35 (2 H, q, J_{AB}2 Hz, ArH); *m/z* 232 and 230 (*M*).

2,4-*Dimethoxy*-6-*methylbenzoic Acid* (**21a**).—A solution of 2-bromo-3,5-dimethoxytoluene (**20a**) (5 g) in dry ether (40 ml) was flushed with nitrogen and cooled to −78 °C. Butyl-lithium in hexane (15% w/w; 142 ml) was injected, and the mixture stirred at −78 °C for 1 h. Solid CO_2 (10 g) was then added, with stirring, and the mixture left to warm to room temperature. The slurry of the lithium salt was diluted with water (50 ml) and the mixture shaken. The aqueous layer was separated, washed with ether, and acidified with concentrated hydrochloric acid. After 1 h at 0 °C, the resulting acid precipitate was filtered off, washed with water, and dried *in vacuo*. Recrystallisation from aqueous ethanol gave needles (3.5 g, 82%) m.p. 140 °C (decomp.) [lit.,[25] 140 °C (decomp.)], $\lambda_{max.}$(EtOH) 282 nm; $\nu_{max.}(CHCl_3)$ 3 300—2 800br, 1 730s, 1 600s, and 1 580m cm^{-1}; $\delta(CDCl_3)$ 2.56 (3 H, s, ArMe), 3.82 (3 H, s, OMe), 3.92 (3 H, s, OMe), and 6.41 (2 H, s,

ArH); *m/z* 196 (100%, *M*), 179 (76, *M* − OH), and 178 (94, *M* − H_2O).

Orsellinic Acid (**14**).—A stirred mixture of 2,4-dimethoxy-6-methoxybenzoic acid (**21a**) (500 mg), anhydrous aluminium chloride (1 g), and dry chlorobenzene (3 ml) was heated under reflux for 45 min. After cooling, the mixture was poured onto crushed ice (3 g), and the solution was filtered. The aqueous layer was separated, washed with ether, and acidified with conc. HCl. Orsellinic acid separated as fine needles (230 mg, 54%), m.p. 175 °C (decomp.) [lit.,[26] 176 °C (decomp.)]; $\lambda_{max.}$(EtOH) 282 nm; $\nu_{max.}$(Nujol) 3 400br s, 1 660s, and 1 600s cm^{-1}; δ(CD_3OD) 254 (3 H, s, Me) and 6.20 (2 H, br s, ArH); *m/z* 168 (M^+) and 124 (*M* − CO_2).

2,3,5-*Trimethoxytoluene* (**19b**).—A solution of bromo-2,3,5-trimethoxybenzene[27] (2.71 g) in anhydrous ether (20 ml) was cooled to −78 °C and flushed with nitrogen. Butyl-lithium in hexane (15% w/w; 9.75 ml) was injected, and the mixture was stirred for 1 h. Methyl iodide (5 g) was injected at the same temperature with stirring, and the mixture allowed to warm to room temperature. The mixture was then diluted with water and shaken to dissolve the lithium iodide formed; the ethereal layer was separated, washed with water and brine, dried, and evaporated under reduced pressure to give the *trimethoxytoluene* as a light oil (Found: C, 66.0; H, 7.86. $C_{10}H_{14}O_3$ requires C, 65.9; H, 7.74%); $\nu_{max.}$($CHCl_3$) 1 600s and 1 500s cm^{-1}; δ($CDCl_3$) 2.25 (3 H, s, ArMe), 3.75 (6 H, s, OMe), 3.81 (3 H, s, OMe) and 6.32 (2 H, q, J_{AB} 3 Hz); *m/z* 182 (100%, M^+) and 167 (96, *M* − Me).

2-*Bromo*-3,4,6-*trimethoxytoluene* (**20b**).—Copper(II) bromide (6.6 g) was added to a vigorously stirred solution of 2,3,5-trimethoxytoluene (3.6 g) in dry 1,2-dimethoxyethane (40 ml), with exclusion of moisture, over a period of 1 h; the mixture was stirred for a further 1 h to precipitate a salt which was filtered off; the filtrate was then evaporated under reduced pressure. The dark green residue was passed down a column of Florisil (30 g) with dichloromethane as eluant and the solvent was evaporated under reduced pressure. Recrystallisation of the residue from ethanol gave the *bromide* as needles (4.0 g, 76%), m.p. 81 °C (Found: C, 46.1; H, 5.17; Br, 30.3. $C_{19}H_{13}BrO_3$ requires C, 45.9; H, 5.02; Br, 30.6%); $\lambda_{max.}$(EtOH) 287 nm; $\nu_{max.}$($CHCl_3$) 1 600s and 1 500s cm^{-1}; δ($CDCl_3$) 2.34 (3 H, s, ArMe), 3.71 (3 H, s, OMe), 3.84 (6 H, s, OMe), and 6.41 (1 H, s, ArH); *m/z* 262, 260 (100%, M^+), 246, 245 (88, *M* − Me), and 219, 217 (41, *M* − MeCO).

2,4,5-*Trimethoxy*-6-*methylbenzoic Acid* (**21b**).—Butyl-lithium in hexane (15% w/w; 3.25 ml) was injected into a stirred solution of 2-bromo-3,4,6-trimethoxytoluene (**20b**) (1.31 g) in dry ether (20 ml), cooled to −78 °C and flushed with dry nitrogen. The mixture was stirred for 1 h at this temperature after which crushed solid CO_2 (10 g) was added to it with stirring. When the mixture had warmed to room temperature, it was diluted with water, and shaken. The aqueous layer was separated, washed with ether, and acidified with concentrated hydrochloric acid to give the acid as a dense precipitate; this was filtered off, washed with water, and dried *in vacuo* (0.95 g, 84%), m.p. 149 °C [lit.,[28] 147—149 °C (decomp.)]; $\lambda_{max.}$(EtOH) 287 nm; $\nu_{max.}$($CHCl_3$) 3 200—2 700br w, 1 730s, 1 600s, and 1 500s cm^{-1}; δ(CD_3)$_2$CO], 2.22 (3 H, s, ArMe), 3.69 (3 H, s, OMe), 3.82 (3 H, s, OMe), 3.89 (3 H, s, OMe), and 6.64 (1 H, s, ArH); *m/z* 226 (50%, M^+), 211 (40, *M* − Me), 194 (50, *M* − MeOH), and 179 (100, *M* − CO_2H).

2,4,5-*Trihydroxy*-6-*methylbenzoic Acid* (**15**).—Boron tribromide (1 ml) was added to a stirred solution of 2,4,5-trimethoxy-6- methylbenzoic acid (**21b**) (480 mg) in dichloromethane (5 ml), under nitrogen at −78 °C. The stirred mixture was allowed to warm to room temperature, and then cautiously diluted with water to destroy the excess of boron tribromide. Ether and water were added to the mixture, which was shaken to dissolve the inorganic material. The aqueous layer was separated and extracted with ether and the combined organic layers were washed with 5% aqueous glycerol and brine, dried, and evaporated under reduced pressure to give the trihydroxy acid as a pale brown crystalline residue (290 mg, 79%), which recrystallised from aqueous methanol in the form of needles, m.p. 192—193 °C (decomp.) [lit.,[28] 194 °C (decomp.)]; $\lambda_{max.}$(EtOH) 264 and 324 nm; $\nu_{max.}$(Nujol) 3 100—2 600br s, 1 700s, and 1 600s cm^{-1}; δ(CD_3)$_2$CO], 2.49 (3 H, s, ArMe), and 6.32 (1 H, s, ArH); *m/z* 184 (23%, *M*) and 140 (100, *M* − CO_2).

Methyl 2,4,5-*Trimethoxy*-6-*methylbenzoate* (**22**).—Ethereal diazomethane was added to a solution of 2,4,5-trimethoxy-6-methylbenzoic acid (**21b**) (2.0 g) in methanol until the yellow colour persisted. After 1 h, a few drops of glacial acetic acid were added to destroy the excess of diazomethane. The solution was washed with 10% aqueous sodium hydroxide and brine, dried, and evaporated under reduced pressure. The residue was purified by chromatography on silica (Fisons, 80—200 mesh) with ether as eluant (1.87 g, 88%). A sample recrystallised from ether–hexane as plates, m.p. 70 °C (lit.,[28] 69—71 °C); $\lambda_{max.}$(EtOH) 287 nm; $\nu_{max.}$($CHCl_3$) 1 725s, 1 600s, and 1 500s cm^{-1}; δ(CCl_4) 2.12 (3 H, s, ArMe), 3.66 (3 H, s, CO_2Me), 3.75 (3 H, s, OMe), 3.78 (3 H, s, OMe), 3.82 (3 H, s, OMe), and 6.37 (1 H, s, ArH); *m/z* 240 (100%, *M*) 225 (73, *M* − Me), 209 (43, *M* − MeO), and 181 (57, *M* − CO_2Me).

Methyl 2-*Bromomethyl*-3,4,6-*trimethoxybenzoate* (**23**).—A mixture of methyl 2,4,5-trimethoxy-6-methylbenzoate (**22**) (480 mg), *N*-bromosuccinimide (400 mg), azoisobutyronitrile (25 mg), and dry carbon tetrachloride (10 ml) was stirred for 1 h and then placed in a bath preheated to 120 °C and immediately irradiated with a 100 W lamp. After being stirred and irradiated under reflux for 1 h, the mixture was cooled to 0 °C and the precipitate filtered off and washed with carbon tetrachloride. The combined filtrates were evaporated under reduced pressure and the residue recrystallised from hexane to give the *bromide* in the form of needles (490 mg, 77%), m.p. 79 °C; $\lambda_{max.}$(EtOH) 307 nm; $\nu_{max.}$($CHCl_3$) 1 725s, 1 600s, and 1 500m cm^{-1}; δ($CDCl_4$) 3.76 (3 H, s, CO_2Me), 3.83 (9 H, s, OMe), 4.54 (2 H, s, CH_2Br), and 6.40 (1 H, s, ArH) (Found: M^+, 320.0083 and 318.0102. $C_{12}H_{15}BrO$ requires *M*, 320.0083 and 318.0097); *m/z* 320, 318 (100%, M^+) and 305, 303 (12, *M* − MeO).

4,5,7-*Trimethoxyphthalide* (**24**).—A mixture of methyl 2-bromomethyl-3,4,6-trimethoxybenzoate (**23**) (320 mg), dioxane (4 ml), and water (4 ml) was heated under reflux, in a stream of nitrogen, for 24 h. After cooling, the solution was evaporated to about half its volume and left at 0 °C; the *phthalide* crystallised out from the solution and was filtered off, washed, and dried. Recrystallisation from chloroform–hexane gave pale brown needles (188 mg, 34%), m.p. 135—136 °C; $\lambda_{max.}$(EtOH) 258 and 300 nm; $\nu_{max.}$($CHCl_3$) 1 620s and 1 760s cm^{-1}; δ($CDCl_3$) 3.86 (3 H, s, OMe), 3.98 (6 H, s, OMe), 5.25 (2 H, s, $ArCH_2O$), and 6.54 (1 H, s, ArH) (Found: M^+, 224.0679. $C_{11}H_{12}O_5$ requires *M*, 224.0684); *m/z* 224 (*M*).

4,5,7-*Trihydroxyphthalide* (**16**).—4,5,7-Trimethoxyphthalide (**24**) (480 mg) was demethylated following the procedure described for the trihydroxy acid (**15**). The *trihydroxyphthalide* (260 mg, 71%) recrystallised from aqueous methanol, m.p. 174—176 °C (chars above 120 °C); $\lambda_{max.}$(EtOH) 258 and 326

nm; $\nu_{max.}$(Nujol) 2 900—3 300br s, 1 755s, and 1 620s cm^{-1}; δ[$(CD_3)_2CO$] 5.16 (2 H, s, $ArCH_2O$) and 6.40 (1 H, s, ArH) (Found: M^+, 185.1342. $C_8H_6O_5$ requires M, 183.1340); m/z 182 (83%, M) and 137 (100, $M - CO_2$).

4,7-*Dihydroxy*-5-*methylcoumarin* (**18**).—A mixture of anhydrous orcinol (310 mg), malonic acid (260 mg), anhydrous zinc chloride (1.02 g), phosphorus oxychloride (0.69 ml), and chlorobenzene (10 ml) was heated under reflux, with stirring, for 36 h. After cooling, the solvent was decanted from the gummy precipitate, which was stirred with ice–water (2 g in 10 ml). The solid precipitate was filtered off and dissolved in aqueous sodium carbonate. The solution was acidified with concentrated hydrochloric acid to precipitate the coumarin, which was filtered off, washed with water, and dried (3.02 g, 55%). A sample recrystallised from ethanol as pale yellow needles, m.p. 265 °C (lit.,[29] 265—267 °C); $\lambda_{max.}$(EtOH) 318, 308, 290, 234, and 218 nm; $\nu_{max.}$(Nujol) 3 000—3 200s, 1 720s, 1 680s, and 1 600s cm^{-1}; δ[$(CD_3)_2SO$] 2.56 (3 H, s, ArMe), 3.51 (1 H, s, 3-H), 6.48 (2 H, br s, 6-and 8-H) and 11.0—12.0 (2 H, br, OH, exchangeable with D_2O); m/z 192 (100%, M) and 164 (47, $M - CO$).

4-*Hydroxy*-6-*methyl*-2H-*pyran*-2-*one* (*Triacetic Acetic Acid Lactone*) (**17**).—Butyl-lithium in hexane (15% w/w; 13 ml) was injected into a solution of di-isopropylamine (2.02 g) in dry tetrahydrofuran (THF) (15 ml), under nitrogen and the mixture was stirred for 20 min. It was then cooled to −78 °C when, with continued stirring, a solution of acetylacetone (1.0 g) in dry THF (5 ml) was injected into it. The mixture was then stirred at −78 °C for 30 min after which crushed solid CO_2 (5 g) was added to the stirred solution. When the mixture had warmed to room temperature, it was poured into a separating funnel containing ether (50 ml) and 1.5M sulphuric acid (25 ml). After shaking to dissolve the lithium salts, the ethereal layer was separated, washed with brine, dried, and evaporated under reduced pressure to give the dioxo acid as a colourless oil (1.3 g, 90%); δ($CDCl_3$), 2.08 (3 H, s, Me), 3.37 (2 H, s, CH_2CO_2H), 5.63 (1 H, s, C=H) (enol form, 80%); 2.24 (3 H, s, Me), 3.59 (3 H, s, CH_2CO_2H), and 3.78 (2 H, s, $OCHC_2CO$) (keto form, 20%).

3,5-Dioxohexanoic acid (1.3 g) was dissolved in trifluoroacetic acid (5 ml) and trifluoroacetic anhydride (10 ml) and the mixture heated under reflux for 0.5 h. After cooling, the mixture was evaporated under reduced pressure to give the pyrone as a mass of orange needles (1.1 g, 94%). A sample recrystallised from ethyl acetate in the form of needles, m.p. 189—190 °C (lit.,[30] 190—191 °C); $\lambda_{max.}$(EtOH) 282 nm; $\nu_{max.}$(Nujol) 1 720s, 1 660s, 1 630s, and 1 590s cm^{-1}; δ($(CD_3)_2CO$] 2.26 (3 H, br s, Me), 5.35 (1 H, d, J 2 Hz, 3-H), and 6.00 (1 H, m, 5-H); m/z 126 (100%, M^+), 111 (37, M − Me), and 98 (63, $M - CO$).

Acknowledgements

We thank the S.E.R.C. for financial support.

References

1 A. C. Hetherington and H. Raistrick, *Philos. Trans. R. Soc. London, Ser. B*, 1983, **220**, 209.
2 A. Robertson and W. B. Whalley, *J. Chem. Soc.*, 1949, 848; G. W. K. Cavill, A. Robertson, and W. B. Whalley, *J. Chem. Soc.*, 1950, 1031 and 2965; A. Robertson, W. B. Whalley, and J. Yates, *J. Chem. Soc.*, 1951, 2013.
3 A. E. Oxford, H. Raistrick, and P. Simonart, *Biochem. J.*, 1935, **29**, 1102; F. M. Dean, R. A. Eade, R. Moubasher, and A. Robertson, *J. Chem. Soc.*, 1957, 3497.
4 W. B. Turner, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1621.
5 A. K. Demetriadou, E. D. Laue, F. J. Leeper, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1985, 762.
6 Z. Vanek and M. Soucak, *Folia Microbiol.*, 1962, **7**, 262.
7 D. F. Jones, Ph.D. Thesis, University of Liverpool, 1960.
8 F. M. Dean, R. A. Eade, R. A. Moubasher, and A. Robertson, *Nature*, 1957, **179**, 366; W. B. Whalley in 'Recent Developments in the Chemistry of Natural Phenolic Compounds,' ed. W. B. Ollis, Pergamon Press, Oxford, 1961, p. 49; C. E. Stickings and A. Mahmoudian, *Chem. Ind.*, 1962, 1718; T. Money, *Nature*, 1963, **199**, 592.
9 W. B. Turner, 'Fungal Metabolites,' Academic Press, London, 1971, ch. 5.
10 I. Kurobane, C. R. Hutchinson, and L. C. Vining, *Tetrahedron Lett.*, 1981, **22**, 493.
11 A. K. Demetriadou, E. D. Laue, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1985, 764.
12 A. J. Birch, P. Fitten, E. Pride, A. J. Ryan, H. Smith, and W. B. Whalley, *J. Chem. Soc.*, 1958, 4576.
13 S. Gattenbeck and K. Mosbach, *Biochem. Biophys. Res. Commun.*, 1963, **11**, 166.
14 A. J. Birch, S. F. Hussain, and R. W. Rickards, *J. Chem. Soc.*, 1964, 3494.
15 J. S. E. Holker, J. Staunton, and W. B. Whalley, *J. Chem. Soc.*, 1964, 16.
16 T. Kubota, T. Tokoroyama, S. Oi, and Y. Satomuram, *J. Chem. Soc.*, 1971, 2703; J. S. E. Holker and R. F. Cos, *J. Chem. Soc., Perkin Trans. 1*, 1976, 2077; M. Yamasaki, Y. Maebayashi, and T. Tokoroyama, *Tetrahedron Lett.*, 1977, 489.
17 M. J. Garson and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1976, 928 and 1978, 158; J. A. Barber, M. J. Garson, and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2584.
18 H. Seto, L. W. Cary, and M. Tanaba, *J. Chem. Soc., Chem. Commun.*, 1973, 867.
19 G. C. Levy, R. L. Lichter, and G. L. Nelson, 'Carbon-13 Nuclear Magnetic Resonance Spectroscopy,' John Wiley, New York, 1980.
20 G. E. Evans and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1976, 760.
21 M. J. Garson, R. A. Hill, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1977, 921.
22 A. K. Demetriadou, E. D. Laue, F. J. Leeper, and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1987, following paper; A. K. Demetriadou, E. D. Laue, and J. Staunton, *ibid.*, 1987, 7/116.
23 A. I. Vogel, 'A Text Book of Practical Organic Chemistry,' Longmans, London, 1948.
24 G. Buchi, D. H. Klaubert, R. C. Shank, S. M. Weinreb, and G. N. Wogan, *J. Org. Chem.*, 1971, **36**, 1143.
25 A. Robertson and R. J. Stevenson, *J. Chem. Soc.*, 1932, 1388.
26 R. Bentley, *J. Biol. Chem.*, 1963, **238**, 1895.
27 H. W. Dorn, W. H. Warren, and J. L. Bullock, *J. Am. Chem. Soc.*, 1939, **61**, 144.
28 Y. Asahina and T. Kusaka, *Bull. Chem. Soc. Jpn.*, 1942, **17**, 152.
29 P. Venturella, A. Bellino, and F. Piozzi, *Tetrahedron Lett.*, 1974, 97.
30 A. Schloberl and A. Wagner, 'Methoden der Organischen Chemie,' Houben-Weyl, 4th Edition, vol. 9, ch. 23.

Received 23*rd January* 1987; *Paper* 7/114