Sequence Studies in Biosynthesis: Trichothecin

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An investigation of the precursor activity of α-bisabolol, γ-bisabolene and monocyclofarnesol indicates that these compounds are not intermediates in the biosynthesis of trichothecin.

Une étude sur l'activité du précurseur de l'α-bisabolol, γ-bisabolène et monocyclofarnésol montre que ces composés ne sont pas des intermédiaires dans la biosynthèse de la trichotécine.

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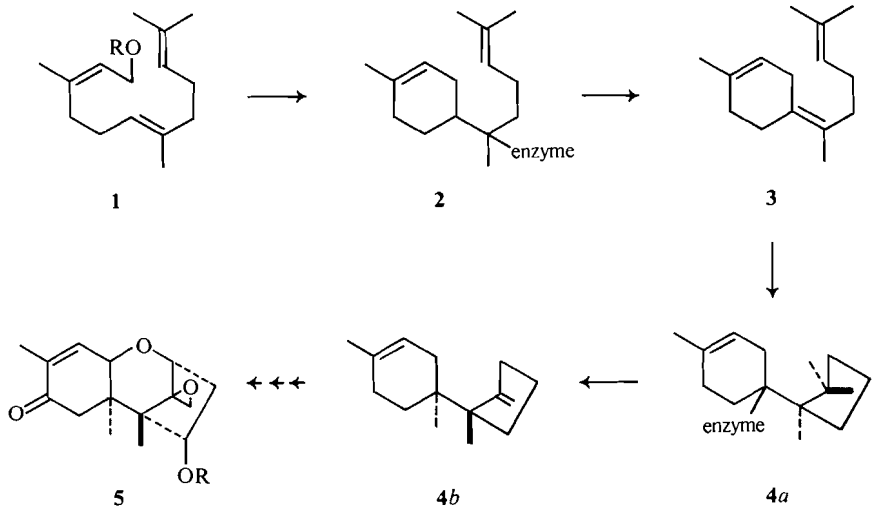
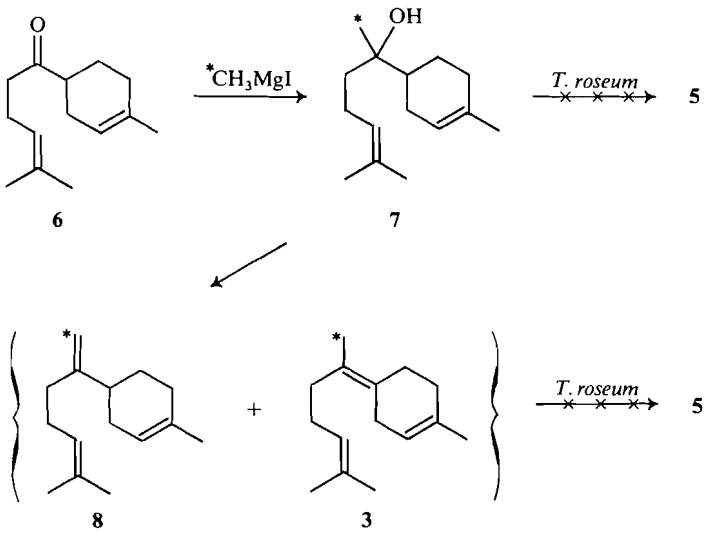
The biosynthesis of the trichothecane antibiotics has been the subject of considerable investigation. Results obtained by various research groups have shown that the complex structures (*cf.* **5**) of the group are derived from three molecules of mevalonic acid (1–4) and that farnesyl pyrophosphate (**1**) (2, 5) is an intermediate in this process (see Scheme 1). Beyond this the biosynthetic sequence has not been elucidated although the proposal has been made that cyclization of **1** could lead to γ-bisabolene (**3**) (3, 6–10) via a monocyclic intermediate **2** of undetermined structure. Subsequent cyclization of γ-bisabolene (**3**) to the trichothecane skeleton (*cf.* **4***b*) in addition to oxidation reactions of an undefined nature (3, 7, 8) could produce representative members of this group *e.g.* trichothecin (**5***a*). We have been concerned with the validity of these proposals and recent results by Adams and Hanson (4) which elegantly preclude the intermediacy of γ-bisabolene (**3**) prompt us to record our own observations in this area.

Our first investigations (1968) involved α-bisabolol (**7**) which we considered to be a possible intermediate in the process (*i.e.* equivalent to **2**) (see Scheme 2). Radioactive α-bisabolol (**7**) was obtained from ketodiene (**6**) (11) by treatment with C^{14}-methylmagnesium iodide. An aqueous solution of 9-C^{14}-α-bisabolol (0.37 mCi/mmol) was solubilized with Tween 20 and added to 5-day-old cultures of *Tricothecium roseum*.



SCHEME 1



SCHEME 2

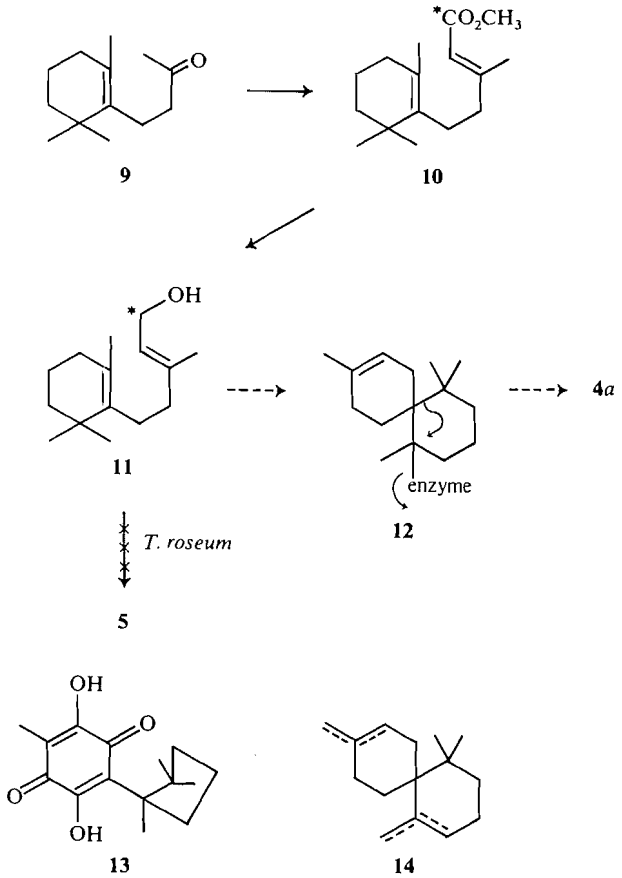
After a period of three weeks trichothecin was isolated (1) and repeatedly crystallized from petroleum ether (60–80°) until constant radioactivity (5.3×10^{-5} mCi/mmol) had been attained (incorporation 0.014%). Alkaline hydrolysis (KOH/MeOH) of tricothecin (**5***a*) yielded trichothecolone (**5***b*) (3.3×10^{-5} mCi/mmol) possessing only 60% of the original activity. Jones and Lowe have shown (1) that the isocrotonate unit in trichothecin is acetate-derived and our results indicate that extensive degradation of α-bisabolol to radioactive units (acetate?) had occurred prior to incorporation.

We then turned our attention to the precursor activity of γ-bisabolene (**3**). Dehydration of 9-C^{14}-α-bisabolol (**7**) with $POCl_3$/pyridine yielded a mixture of β-bisabolene (**8**) and γ-bisabolene (**3**). The presence of at least 50% γ-bisabolene was determined by g.l.c. and in non-radioactive runs the isomeric bisabolenes were isolated and identified by n.m.r. For the biosynthetic experiment an aqueous solution of the mixture[1] of **8** and **3** (solubilized by Tween 20) was administered to *T. roseum*. Trichothecin was isolated after three weeks and was shown to have specific activity of 1.88×10^{-5} mCi/mmol (specific incorporation 0.005%). Alkaline hydrolysis yielded trichothecolone (1.15×10^{-5} mCi/mmol), possessing only 60% of the original activity present in trichothecin.

The low and non-specific incorporation of α-bisabolol, β-bisabolene, and γ-bisabolene seemed to exclude the intermediacy of these compounds in the biosynthetic process and, as noted earlier, this has been decisively shown by Adams and Hanson (4) using specifically labelled mevalonic acid and geranyl pyrophosphate. It is of interest to note that Nozoe and co-workers (12) have come to a similar conclusion with the cuparene-type sesquiterpene helicobasidin (**13**).

An alternative biosynthetic route to the cuparene type intermediate (**4***a*) has been considered (13). This involves cyclization of monocyclofarnesol (**11**) to a spiro bicyclic intermediate (**12**) (see Scheme 3) reminiscent of the type postulated in the biosynthesis of the chamigrenes (**14**). Subsequent ring contraction could then provide **4***a*. We have evaluated the merit of this biosynthetic proposal by examining the precursor activity of monocyclofarnesol (**11**). The latter compound was synthesized in radioactive form by condensing dihydro-β-ionone (**9**) (14), in a modified Wittig reaction (15, 16), with methyl

[1]Although it would have been more meaningful to have proceeded with studies on pure β- and γ-bisabolene, the separation of small quantities of radioactive samples of these compounds would have resulted in considerable loss of valuable material. In addition we considered that if either or both isomers were on the main biosynthetic route a considerable and specific incorporation would be easily discerned.



(* = carbon – 14)

SCHEME 3

1-C^{14}-bromoacetate followed by reduction of the intermediate ester **10**. Administration of **11** to *Trichothecium roseum* in the manner described above yielded trichothecin with specific activity 0.867×10^{-5} mCi/mmol (specific incorporation 0.003%). Conversion to trichothecolone (0.237×10^{-5} mCi/mmol) with substantial decrease in radioactivity indicated that monocyclofarnesol (**11**) had been extensively degraded prior to incorporation.

These results would seem to suggest that the biosynthetic route from farnesyl pyrophosphate to trichothecin proceeds directly to intermediate **4***a* and does not involve intermediates of the type previously suggested.[2]

[2]A recent report (20) describes the incorporation of trichodiene (**4***b*) into trichothecin.

Experimental

All melting points were determined on a Kofler block and are uncorrected. I.r. spectra (ν_{max}) were recorded on a Perkin–Elmer Infracord model 137 spectrophotometer. N.m.r. spectra (τ) were determined in carbon tetrachloride or deuteriochloroform solution and recorded on a Varian Associates model T-60 spectrometer. Signal positions are given in the Tiers τ scale, with tetramethylsilane as an internal standard; the multiplicity, coupling constants (where appropriate), and integrated peak areas are indicated in parentheses; s = singlet, br.s = broad singlet, d = doublet, t = triplet, m = multiplet. Mass spectra (M^+) were determined with an AEI model MS9 spectrometer. Microanalyses were determined by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia. T.l.c. results were obtained using silica gel HF^{254} of 0.5 mm thickness. A Nuclear-Chicago Mark 1 model 6860 Liquid Scintillation counter was used for radioactivity measurements.

α-Bisabolol (7)

To a solution of methyl magnesium iodide, prepared from

methyl iodide (106.5 mg, 0.75 mmol) and magnesium powder (19.8 mg, 0.83 mmol) in ether (5 ml), was added ketodiene (**6**) (11) (51.5 mg, 0.25 mmol) in ether (2 ml). The mixture was allowed to stir at room temperature for 2 h then worked-up by adding saturated aqueous ammonium acetate solution. The ether layer was separated and the aqueous phase extracted twice with ether. The combined ether layers were washed with brine and dried (Na_2SO_4). Evaporation of the ether gave α-bisabolol (**7**) (55 mg, 100%) as a colorless oil; ν_{max}(liquid film) 3435, 2915, 1440, 1370, 1105, 830, 800 cm^{-1}; τ (CCl_4) 4.67 (s, 1H), 4.90 (t, $J = 7$ Hz, 1H), 7.60–8.23 (m, 7H), 8.37 (s, 9H), 8.50–8.87 (m, 5H), 8.93 (d, $J = 2$ Hz, 3H): M^+ *m/e* 222.

9-C^{14}-α-Bisabolol (7)

α-Bisabolol (**7**) was prepared as above using C^{14}-methyl iodide (213.0 mg, 1.5 mmol, 1 mCi, 0.667 mCi/mmol), magnesium powder (39.6 mg, 1.65 mmol) and ketodiene (**6**) (103 mg, 0.5 mmol). The yield of 9-C^{14}-α-bisabolol (**7**) was 110.0 mg (100%), and the specific activity was determined to be 0.33 mCi/mmol.

Isolation of Trichothecin (5a) from Trichothecium roseum

Trichothecium roseum was grown as described in the literature (17, 18) except that the p*H* was not adjusted. Trichothecin (**5***a*) was isolated as described in the literature (18) except that preparative t.l.c. on silica gel was used in the purification rather than column chromatography on alumina. In a typical experiment 10 flasks of *T. roseum* were allowed to grow for 3 weeks. The broth was then filtered, the mycelia washed with a little water, and the combined washings and broth extracted with carbon tetrachloride (2 × 100 ml). Evaporation of the carbon tetrachloride gave a gum (148.6 mg) which was purified by t.l.c. on silica gel, using petroleum ether–ethyl acetate (1:1). Under u.v. radiation the trichothecin (**5***a*) showed up as a dark band approximately half way up the plate. Extraction of this band with ethyl acetate and subsequent removal of solvent gave trichothecin (**5***a*) (94.3 mg). Crystallization from petroleum ether (60–80°) provided pure trichothecin (**5***a*) as white needles, m.p. 116–118 °C (lit. (17), m.p. 118 °C); ν_{max} ($CHCl_3$) 1715, 1685 cm^{-1}.

Administration of 9-C-^{14}-α-Bisabolol (7) to Trichothecium roseum

To 9-C^{14}-α-bisabolol (**7**) (52 mg, 0.087 mCi) was added water (20 ml) and Tween 20 (2 ml). This well shaken mixture was then evenly distributed among 10 flasks of 5-day-old cultures of *T. roseum* which were then left for a further 20 days. The trichothecin was isolated as before and recrystallized to constant specific activity (5.3×10^{-5} mCi/mmol) (specific incorporation, 0.014%).

Trichothecolone (5b)

Trichothecin (50 mg, 0.15 mmol) was stirred overnight at room temperature in 1 *N* methanolic potassium hydroxide solution (2 ml). Water (1.5 ml) was added and the methanol evaporated *in vacuo*. The aqueous layer was then extracted with chloroform, removal of which gave a colorless gum which slowly crystallized. This was recrystallized from benzene–petroleum ether (2:1) to give trichothecolone (39.8 mg, 100%) as white needles, m.p. 182–184 °C (lit. (19), m.p. 183–184 °C); ν_{max}($CHCl_3$) 3560, 1680 cm^{-1}.

Hydrolysis of C^{14}-Labelled Trichothecin (5a)

C^{14}-Labelled trichothecin (**5***a*) (5.3 mg) was diluted with inactive trichothecin (10.6 mg) and this was hydrolyzed as above to give C^{14}-labelled trichothecolone (**5***b*) (9.5 mg) which was recrystallized to a constant specific activity of 1.1×10^{-5} mCi/mmol, representing loss of 40% of the radioactivity.

γ-Bisabolene (3)

To a solution of α-bisabolol (**7**) (75 mg, 0.3 mmol) in pyridine (1 ml) was added a solution of phosphorus oxychloride (0.5 ml) in pyridine (2 ml). The reaction mixture was stirred overnight, added to ice-water and the aqueous mixture extracted with ether. The combined ether extracts were washed with brine, dried ($MgSO_4$), and the ether evaporated *in vacuo* to give a yellow oil (45.6 mg, 75%). Purification of this oil by preparative g.l.c. on a 30% Carbowax column at 180 °C gave two components with very similar retention times. The less volatile peak (~50%) was shown to be γ-bisabolene (**3**); ν_{max}(liquid film) 2935, 1440, 1370, 1145, 1100, 910, 825, 800 cm^{-1}; τ (CCl_4) 4.67 (br.s, 1H), 4.93 (t, $J = 7$ Hz, 1H), 7.37 (m, 2H); 8.07 (m, 8H), 8.38 (m, 12H). The more volatile peak (~50%) was shown to be β-bisabolene (**8**) plus other unidentified isomers(s), ν_{max} (liquid film) 2940, 1650, 1445, 1375, 920, 890, 830, 800 cm^{-1}; τ (CCl_4) very similar to the n.m.r. spectrum of γ-bisabolene (**3**) but showing signal at 5.3 τ attributable to C=CH_2 group in (**8**).

9-C^{14}-γ-Bisabolene (3)/9-C^{14}-β-Bisabolene (8)

C^{14}-Labelled α-bisabolol (55 mg) was dehydrated as above and the crude product purified by chromatography on alumina to yield 9-C^{14}-γ-bisabolene (**3**) and 9-C^{14}-β-bisabolene (**8**) as a colorless oil (13 mg).

Administration of 9-C^{14}-γ-Bisabolene (3)/9-C^{14}-β-Bisabolene (8) to Trichothecium roseum

A well shaken mixture of 9-C^{14}-γ-bisabolene (**3**) (13 mg, 0.024 mCi), water (5 ml), and Tween (20) (0.5 ml) was evenly distributed among five flasks of 1-week-old cultures of *T. roseum*. After 3 weeks trichothecin was isolated as before and crystallized to constant specific activity (1.88×10^{-5} mCi/mmol) (specific incorporation 0.005%). The remaining trichothecin (**5***a*) was hydrolyzed as described above to give trichothecolone (**5***b*) with specific activity of 1.15×10^{-5} mCi/mmol representing a loss of 40% of the radioactivity originally present in trichothecin.

Dihydro-β-ionone (9)

β-Ionone (19.2 g, 0.1 mol) was hydrogenated in ethanol (50 ml) using Raney nickel (~4 g) as catalyst. After uptake of the theoretical volume of hydrogen the catalyst was removed by filtration, the ethanol removed *in vacuo* and the resulting oil distilled to give dihydro-β-ionone (**9**) (14.0 g, 72%) as a colorless oil, b.p. 115–116 °C (9 mm) (lit. (14), b.p. 122–122.5 °C (12 mm)); ν_{max} 2940, 1725, 1360, 1155 cm^{-1}; τ (CCl_4) 7.68 (m, 4H), 7.94 (s, 3H), 8.43 (s, 3H), 9.00 (s, 6H); M^+ *m/e* 194.

Anal. Calcd. for $C_{13}H_{22}O$: C, 80.35; H, 11.41. Found: C, 80.59; H, 11.48.

Methyl Monocyclofarnesate (10)

Ether washed 50% sodium hydride/oil dispersion (0.48 g, 0.01 mol NaH) was added to dry benzene (50 ml), and tri-

methylphosphonoacetate (1.82 g, 0.01 mol) in benzene (5 ml), added at room temperature. After stirring at room temperature for 0.5 h, dihydro-β-ionone (**9**) (1.35 g, 0.007 mol), in benzene (5 ml) was added dropwise and the solution refluxed for 15 h. On cooling, water was added and the resulting oil extracted three times with ether, the ether was dried ($MgSO_4$) and evaporated *in vacuo*. Distillation gave methyl monocyclofarnesate (**10**) (1.0 g, 57%) as a colorless oil, b.p. 81–83 °C (0.02 mm); ν_{max} 2935, 1720, 1645, 1430, 1355, 1220, 1140 cm^{-1}; τ (CCl_4) 4.37 (br.s, 1H), 6.37 (s, 3H), 7.80 (s, 2H), 7.84 (s, 3H), 8.38 (s, 3H), 8.97 (s, 6H); M^+ *m/e* 250.

Anal. Calcd. for $C_{16}H_{26}O_2$: C, 76.76; H, 10.47. Found: C, 77.16; H, 10.59.

Monocyclofarnesol (11)

Lithium aluminum hydride (30 mg, 0.79 mmol) was added to a solution of methyl monocyclofarnesate (**10**) (300 mg, 1.2 mmol) in ether (10 ml). The resulting solution was stirred overnight and worked-up by adding ice–dilute HCl. The ether layer was separated and the aqueous layer was extracted three times with ether. The combined ether extracts were washed thoroughly with brine, dried ($MgSO_4$) and the ether evaporated *in vacuo* to give monocyclofarnesol (**11**), (252 mg, 95%) as a colorless oil, ν_{max} 3350, 2940, 1670, 1460, 1380, 1110, 1000 cm^{-1}; τ (CCl_4) 4.64 (t, $J = 7$ Hz, 1H), 5.98 (d, $J = 7$ Hz, 2H), 8.99 (s, 6H); M^+ *m/e* 222.

1-C^{14}-Monocyclofarnesol (11)

1-C^{14}-Methyl bromoacetate (306 mg, 2 mmol, 1 mCi, 0.5 mCi/mmol), trimethyl phosphite (248 mg, 2 mmol), and benzene (1 ml) were refluxed for 5 h. On cooling, benzene (4 ml) and pentane washed sodium hydride (50% oil dispersion) (240 mg, 5 mmol) were added and the solution allowed to stir at room temperature for 1.5 h. Dihydro β-ionone (**9**) (388 mg, 2 mmol) in benzene (5 ml) was then added and the solution allowed to reflux overnight. The reaction mixture was worked-up by adding water followed by ether extraction. Removal of the solvents gave a yellow oil which was purified by chromatography on silica. The fraction eluting with petroleum ether–benzene (3:1) consisted of methyl monocyclofarnesate (**10**) (171 mg). Methyl monocyclofarnesate (**10**) (171 mg, 0.68 mmol) was stirred in ether (10 ml) with lithium aluminum hydride (17.1 mg, 0.45 mmol) for 10 h. The reaction mixture was worked-up by adding dilute HCl followed by ether extraction and evaporation of the ether to give 1-C^{14}-monocyclofarnesol (**11**) (110 mg). The specific activity was determined to be 0.29 mCi/mmol.

Administration of 1-C^{14}-Monocyclofarnesol (11) to Trichothecium roseum

A well shaken mixture of 1-C^{14}-monocyclofarnesol (**11**) (55.0 mg, 0.071 mCi), water (18 ml), and Tween 20 (2 ml) was evenly distributed among 10 flasks of 7-day-old cultures of *T. roseum*. After a further 24 days the trichothecin (**5***a*) was isolated in the usual manner and recrystallized to constant specific activity (0.867×10^{-5} mCi/mmol), representing an incorporation of 0.003%. The remaining trichothecin was hydrolyzed as above to give trichothecolone (**5***b*) with specific activity of 0.237×10^{-5} mCi/mmol.

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1. E. R. H. Jones and G. Lowe. J. Chem. Soc. 3959 (1960).
2. B. A. Achilladelis, P. M. Adams, and J. R. Hanson. Chem. Commun. 511 (1970), and refs. cited therein.
3. R. Achini, B. Muller, and Ch. Tamm. Chem. Commun. 404 (1971).
4. P. M. Adams and J. R. Hanson. Chem. Commun. 1414 (1971).
5. B. A. Achilladelis and J. R. Hanson. Chem. and Ind. 1643 (1967).
6. L. Ruzicka. Pure Appl. Chem. **6**, 512 (1963).
7. W. Parker, J. S. Roberts, and R. Ramage. Q. Rev. **21**, 331 (1967).
8. P. M. Adams and J. R. Hanson. Chem. Commun. 1569 (1970).
9. W. O. Godtfredson and S. Vangedal. Acta Chem. Scand. **19**, 1068 (1965).
10. W. Zurcher, J. Gutzwiller, and Ch. Tamm. Helv. Chim. Acta, **48**, 839 (1965).
11. A. Manjarrez and A. Guzman. J. Org. Chem. **31**, 348 (1966).
12. S. Nozoe, M. Marisaki, and H. Matsumoto. Chem. Commun. 926 (1970); *cf.* R. Bentley and D. Chen. Phytochem. **8**, 2171 (1969).
13. S. Ito, K. Endo, T. Yoshida, M. Yatagai, and M. Kodama. Chem. Commun. 186 (1967); J. J. Sims, W. Fenical, R. M. Wing, and P. Radlick. J. Am. Chem. Soc. **93**, 3774 (1971).
14. A. Caliezi and H. Schinz. Helv. Chim. Acta, **33**, 1129 (1950).
15. W. S. Wadsworth, Jr. and W. D. Emmons. J. Am. Chem. Soc. **83**, 1733 (1961).
16. T. Kato, S. Kanno, and Y. Kitahara. Tetrahedron, **26**, 4287 (1970).
17. G. G. Freeman and R. I. Morrison. Biochem. J. **44**, 1 (1949).
18. G. G. Freeman. J. Gen. Microbiol. **12**, 213 (1955).
19. G. G. Freeman, J. E. Gill, and W. S. Waring. J. Chem. Soc. 1105 (1959).
20. Y. Machida and S. Nozoe. Tetrahedron Lett. 1969 (1972).