Microbiological Transformations of 17-Norkauran-16-one, *ent*-17-Norkauran-16-one, and 17-Norphyllocladan-16-one by *Aspergillus niger*[1]

ALLAN B. ANDERSON
Department of Botany and Genetics, University of Guelph, Guelph, Ontario N1G 2W1
AND
ROBERT MCCRINDLE[2] AND JAMES K. TURNBULL
Chemistry Department, University of Guelph, Guelph, Ontario N1G 2W1
Received October 28, 1974

ALLAN B. ANDERSON, ROBERT MCCRINDLE, and JAMES K. TURNBULL. Can. J. Chem. **53**, 1181 (1975).

Incubation of 17-norkauran-16-one (**3***b*) and *ent*-17-norkauran-16-one (**2***b*) with *Aspergillus niger* gives the related C-3 equatorial alcohols **3***c* and **2***c*, respectively, whereas 17-norphyllocladan-16-one (**4***b*) gives the 3β-alcohol **4***c* and the corresponding 3-ketone **4***g*. Yields of all of these products are low. Diketone **4***g* is identical with the corresponding compound derived from calliterpenone, thus confirming that calliterpenone should be formulated as **8** rather than **7**.

ALLAN B. ANDERSON, ROBERT MCCRINDLE et JAMES K. TURNBULL. Can. J. Chem. **53**, 1181 (1975).

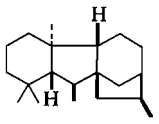
Les incubations de la nor-17 kauranone-16 (**3***b*) et de l'*ent*-nor-17 kauranone-16 (**2***b*) avec l'*Aspergillus niger* conduisent respectivement aux alcools équatoriaux en position 3 (**3***c* et **2***c*) alors que la nor-17 phyllocladanone-16 (**4***b*) conduit à l'alcool 3β (**4***c*) et au dérivé céto-3 correspondant (**4***g*). Les rendements de tous ces produits sont bas. La dicétone **4***g* est identique avec le composé correspondant obtenu à partir de la calliterpénone; ceci confirme donc que la calliterpénone doit être représentée par la formule **8** plutôt que **7**. [Traduit par le journal]

The gibberellins (2) have attracted a large portion of diterpenoid research mainly because of their influence on the growth and development of plants. All known gibberellins are thought to have the gibbane skeleton (**1**) and to be biosynthesized in plants via *ent*-16-kaurene (**2***a*) or a derivative of this diterpene. Other tetracyclic diterpenes, such as kaurene (**3***a*) and phyllocladene (13β-kaurene; **4***a*) occur (3) naturally and we have been seeking evidence for the occurrence of gibberellin-like compounds derived from these. A study of the gibberellins in plants which produce **3***a* or **4***a* would be an obvious approach in this quest. However, since fresh tissue of such plants has not been obtainable by us[3] in sufficient quantity we have undertaken a less direct attack.

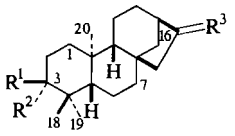
This approach was suggested by observations (6) that although *ent*-kaurene itself has little gibberellin activity, various oxygenated derivatives, all of which contain the ring D *exo*-methylene group, do exhibit significant physiological effects. We planned to submit analogous kaurene and phyllocladene derivatives for testing in bioassays that are specific for gibberellins but since such compounds were not available, synthetic routes from the parent hydrocarbons (**3***a* and **4***a*) were sought. The present paper deals with an investigation of microbial oxygenation as a means of producing the desired derivatives.

Initially, microbial hydroxylation of the more available *ent*-kaurene system was studied as a model. The norketone **2***b* was chosen as a suitable substrate for two reasons. First, carbonyl containing compounds have been successfully used (7, 8) in related steroidal work and second, the C-17 methylene group could be readily reattached by the Wittig reaction. Thus, the norketone **2***b* was incubated with submerged cultures of 13 fungal species (see Experimental) in small scale trial experiments. Transformations of the substrate were detected by t.l.c. com-

[1]For a preliminary account of part of this work see ref. 1.
[2]To whom correspondence should be addressed.
[3]Another group has failed (4) in an attempt to isolate the gibberellins from a variety of *Cryptomeria japonica* which contains (5) **4***a*.

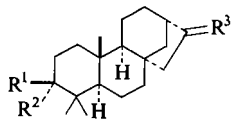


1



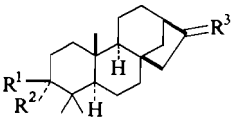
2

a $R^1 = R^2 = H; R^3 = CH_2$
b $R^1 = R^2 = H; R^3 = O$
c $R^1 = H; R^2 = OH; R^3 = O$
d $R^1 = H; R^2 = OH; R^3 = CH_2$
e $R^1 = H; R^2 = OAc; R^3 = O$
f $R^1 = H; R^2 = OAc; R^3 = CH_2$
g $R^1 + R^2 = O; R^3 = CH_2$



3

a $R^1 = R^2 = H; R^3 = CH_2$
b $R^1 = R^2 = H; R^3 = O$
c $R^1 = OH; R^2 = H; R^3 = O$
d $R^1 = OAc; R^2 = H; R^3 = O$
e $R^1 = OH; R^2 = H; R^3 = CH_2$
f $R^1 + R^2 = O; R^3 = O$



4

a $R^1 = R^2 = H; R^3 = CH_2$
b $R^1 = R^2 = H; R^3 = O$
c $R^1 = OH; R^2 = H; R^3 = O$
d $R^1 = OAc; R^2 = H; R^3 = O$
e $R^1 = OH; R^2 = H; R^3 = CH_2$
f $R^1 = OAc; R^2 = H; R^3 = CH_2$
g $R^1 + R^2 = O; R^3 = O$

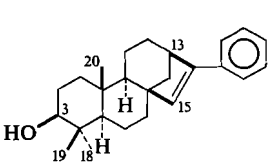
parison of the culture extracts of each organism grown in the presence and absence of **2***b*. Five of these fungi, *Calonectria decora*, *Rhyzopus nigricans*, *Fusarium moniliforme*, *Coprinus lagopus*, and *Aspergillus niger*,[4] appeared to effect transformations of **2***b*. Further small scale screening incubations were then carried out with these five fungi using **3***b* and **4***b* as substrates. *Aspergillus niger* was the only organism for which there was clear t.l.c. evidence for the production of derivatives from both of these norketones. On this basis *A. niger* was selected for large scale investigation with **2***b*, **3***b*, and **4***b*.

ent-17-Norkauran-16-one

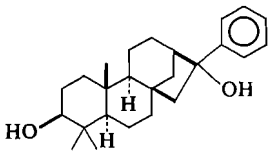
The ketone (**2***b*, ref. 10) was added in DMF to a submerged culture of *A. niger* (ATCC 26693[5]) which had been growing for 3 days in a full nutrient medium (11) under aerobic conditions. After 5 days the mycelia and aqueous medium were extracted with ethyl acetate. Column chromatography of the extract over silica gel afforded a large fraction of the substrate (**2***b*) and a minor amount of a more polar compound, m.p. 179–181°, which was recognized as a norkauranone from its n.m.r., i.r., and mass spectra. In addition, the n.m.r. spectrum indicated that this transformation product (**2***c*) had resulted from the introduction of a secondary hydroxyl group into **2***b*. The multiplicity of the resonance arising from the carbinol proton (dd, $W_{1/2}$ = 16 Hz) revealed the equatorial nature of the hydroxyl group and the presence of only two vicinal protons. This eliminated all but three possible structures for the compound, namely, an *ent*-17-norkauran-16-one with an equatorial (α) hydroxyl group substituted at C-1, C-3, or C-7. A decision in favor of the second of these possibilities was readily made as follows. The hydroxy-*ent*-16-kaurene **2***d*, which was required for biological testing in any case, was prepared from the norketone **2***c* via the acetates **2***e* and **2***f* and its n.m.r. spectrum was recorded in the presence of $Eu(dpm)_3$. The induced shifts of the methyl resonances were in the normalized (12) ratio of 10.0:9.4:4.1 (3 H-19, 3 H-18, and 3 H-20, respectively) which is consistent (13) only with a 3α-hydroxy group. Further, oxidation of **2***d* afforded the corresponding ketone (**2***g*) which gave benzene-induced methyl shifts of the magnitude predicted (14) for a C-3 ketone with ring A in the expected (15) flattened chair conformation. Final confirmation of these structural assignments came from the fact that **2***c*, **2***d*, and **2***e* have identical physical and spectral characteristics with those prepared (16, 17) from diterpenoids of natural provenance.

[4]A group at the University of Western Australia are also studying (9) the metabolism of *ent*-kauranoids by fungi. We thank Professor P. R. Jefferies for discussions of unpublished results.

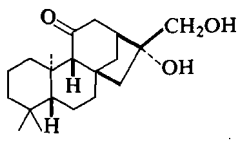
[5]ATCC 26693 refers to the American Type Culture Collection strain No. 26693.



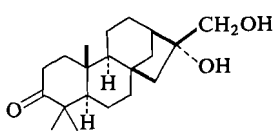
5



6



7



8

17-Norkauran-16-one

When the ketone **3***b* was incubated with *A. niger* (ATCC 26693) in a manner similar to that described above for its enantiomer an analogous product mixture was obtained. Much substrate was again recovered along with a small quantity of the related C-3 equatorial alcohol (**3***c*). The latter was characterized as the derived acetate **3***d* and alcohol **3***e* both of which are identical, except in sign of optical rotation, with the corresponding compounds in the *ent*-series (see above).

17-Norphyllocladan-16-one

When norphyllocladanone (**4***b*, ref. 10) was incubated as above with *A. niger*, (ATCC 26693) small amounts of two transformation products were isolated. The major, more polar, product (**4***c*), m.p. 158–160°, was readily formulated as a keto alcohol on the basis of its spectral properties and those of the corresponding acetate (**4***d*), m.p. 156–158°. The multiplicity of the carbinol proton again indicated that it is axial and only coupled to two protons and thus the hydroxyl group must be β-orientated and located at C-1, C-3, or C-7. The eventual assignment of structure **4***c* to the compound rests on the magnitude of the shifts induced by $Eu(dpm)_3$ on the methyl resonances of **5** and **4***e*. The former was produced in our initial attempt to prepare the latter. In this case solutions of phenyl lithium and methyltriphenylphosphine bromide in ether were mixed and then after 10 min at 20° the keto acetate **4***d* was added in the same solvent and the resulting mixture heated to reflux for 2 days. Work-up gave, rather than the desired acetoxy olefin (**4***f*), the unstable diol **6** which was smoothly transformed into the styrene **5** by treatment with toluene-*p*-sulfonic acid in benzene. When the Wittig reaction was repeated, but this time the phenyl lithium – methyltriphenylphosphine bromide solution was heated for 3 h before addition of **4***d*, the expected product **4***f* was formed. The $Eu(dpm)_3$ induced shifts of the methyl resonances in **5** and **4***e* are in the normalized ratios of 10.0:9.3:3.4 and 10.0:9.4:3.9, respectively, values entirely consistent for 3 H-19, 3 H-18, and 3 H-20 in the presence of a 3β-hydroxyl function.

The less polar transformation product, **4***g*, m.p. 187–189°, shows no absorption in the i.r. attributable to OH stretching but gives two strong peaks in the carbonyl region at 1712 and 1749 cm^{-1}. $Eu(dpm)_3$ shifted n.m.r. spectra of this compound suggested the structure **4***g*, a formulation which was readily confirmed by its preparation from **4***c* by oxidation with Jones' reagent.

This investigation provided us not only with **4***e* for biological testing but also with the means of resolving the controversy concerning the structure of calliterpenone. This constituent of *Callicarpa macrophylla* was originally formulated (18) as a substituted kaurane (**7**), an assignment which was subsequently challenged (19). This second group proposed, on much firmer evidence, an alternative structure **8**, based on the phyllocladane skeleton. That the latter assignment is indeed correct was shown by a direct comparison of the diketone **4***g*, prepared in this study, with the corresponding compound[6] derived (19) from calliterpenone. The two samples are identical.

Mechanism

Recently there has been considerable interest in the nature of the interaction between hydroxylating organisms and their substrates during microbial oxidation. Several groups (for leading examples see ref. 8) have pointed out that a knowledge of such interactions for a given organism might allow prediction of the position(s) of oxygenation in any substrate. The oxidations reported in this paper have several notable features relevant to such studies.

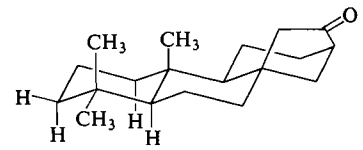
It is striking that *A. niger* inserts oxygen only equatorially at C-3 in the three norditerpenoids tested even although these include a pair of enantiomers. Previous studies (20), using other organisms, have shown that the pattern of oxi-

[6]A generous sample of this compound was supplied by Dr. Asif Zaman, Aligarh Muslim University, Aligarh, India.

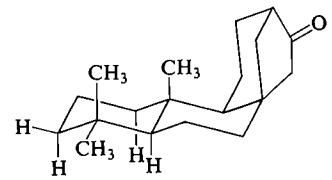
dation of enantiomeric substrates is often different. It has been proposed (8) that polar functional groups such as carbonyls can interact with hydroxylating enzymes in such a way that the site of oxygenation of the substrate bears a fixed geometrical relationship to the carbonyl or other directing group. Certainly the presence of the C-16 carbonyl group in **2***b*, **3***b*, and **4***b* does facilitate hydroxylation with *A. niger*, **2***a* and **4***a* giving no detectable transformation products (see Experimental). Since in each case a reasonable amount of unreacted substrate was recovered from the *filtered* medium it seems likely that **2***a* and **4***a* were present in a physical state suitable for utilization by the organism. It is possible that substrates must contain a polar function to allow their transport through the cellular membrane to a site for hydroxylation within the cell. However, the allylic hydroxylation by *A. niger* of a wide variety of nonoxygenated olefins (21) has been reported and this presumably requires that they diffuse into the cell. Thus, it would appear that the most likely explanation for the absence of oxidation of **2***a* and **4***a* is that the enzymes responsible for remote hydroxylation can react only with substrates which contain a ketone[7] or other polar function.

If the same *A. niger* enzyme is involved in the hydroxylation of **2***b*, **3***b*, and **4***b* then the following model for the role played by the enzyme is attractive in terms of both simplicity and plausibility. First, the hydroxylase "locks on" to the oxygen function at C-16 and then interacts with the flat underside (as drawn in Scheme 1) of **2***b*, **3***b*, and **4***b*. The equatorial proton at C-3 is then singled out for attack. Possibly the enzyme "recognizes" the three axial hydrogens on the underside (Scheme 1) of ring A and allows oxidative displacement of the geminal partner of the one which is most remote from the primary binding site.

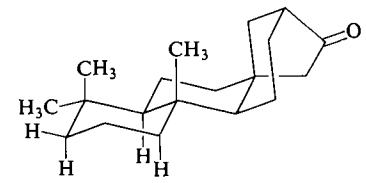
One final aspect of the oxidations of **2***b*, **3***b*, and **4***b* warrants comment, namely that only in the case of norphyllocladanone (**4***b*) was a 3-keto derivative isolated. It has been reported (22) that a strain of *A. niger* oxidizes 3β-hydroxy-5-pregnen-20-one to the 3-ketone while its enantiomer is unaffected. Other 3β-hydroxy steroids are also oxidized (23) to ketones by *A. niger*. Thus it would appear that oxidation of 3β-hydroxy decalin systems which have the same A/B ring junction configuration as that of the naturally occurring steroids is a fairly general property of some strains of this organism. Our failure to detect **3***f* from incubation of the norkauranone **3***b* might have been anticipated in view of the small amount of product **3***c* present and thus available for further transformation. Finally, it appears possible that some strains of *A. niger* may be ineffective for this oxidation of a 3β-hydroxy group to a ketone. In an experiment designed to determine if strains of *A. niger* other than ATCC 26693 can effect hydroxylation of these diterpenoid norketones, **4***b* was incubated with a strain (ATCC 10577) which is well known (7) as an efficient 11α-hydroxylator of steroids. In this case **4***c* was obtained in a yield similar to that from ATCC 26693 but notably no **4***g* could be isolated.



4*b*



3*b*



2*b*

SCHEME I

[7]Some preliminary results involving the interaction of *A. niger* with 15-deuterio derivatives of **2***b* and **2***c* may be taken to indicate the intervention of an enolate, or related, derivative at some stage in the hydroxylation process. For example, **2***b* with an *exo*-deuterium at C-15, after hydroxylation has lost an appreciable proportion of its label while recovered substrate has not. When similarly labelled, **2***c* retains almost all of its deuterium upon exposure for several days to a medium in which *A. niger* has been growing actively.

Experimental

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. For analytical and preparative t.l.c.. chromatoplates were spread with Kieselgel G. (Merck). Silica gel (Baker) used for column chromatography was 40–140 mesh. Gas–liquid chromatography was carried out with a Varian Aerograph 1200 gas chromatograph using a stainless steel column (1/8 in. × 10 ft packed with 5% SE-30) and nitrogen as carrier gas with a flow rate of 25 ml/min. Light petroleum was of b.p. 30–75°. Infrared solution spectra were recorded in carbon tetrachloride on a Beckman I.R. 12 spectrophotometer. Proton magnetic resonance spectra were run on a Varian Associates A-60A or HA 100 spectrometer, in deuteriochloroform unless otherwise stated, using tetramethylsilane as internal standard. Mass spectra were run on a Varian Associates CH7 instrument. Specific rotations were measured in chloroform unless otherwise stated using a Bellingham and Stanley polarimeter or a Bendix automatic polarimeter. *Aspergillus niger* cultures were maintained on potato dextrose agar slopes at 25°. Submerged cultures of *A. niger* for diterpenoid transformation were grown in presterilized Erlenmeyer flasks each of which was plugged with nonabsorbent cotton wool and contained a full nutrient growth medium (11) and a magnetic stirring bar. The medium was inoculated with *A. niger* under sterile conditions and the cultures were aerated by vigorous magnetic stirring.

Incubations of ent-17-Norkauran-16-one (2b) with Various Fungal Species

The ketone (**2***b*, 20mg) was incubated with 13 fungal species in essentially the fashion described below for one of them, *Aspergillus niger*, transformation being detected by comparison of the culture extract with that of a control on t.l.c. *Calonectria decora, Rhyzopus nigricans, Fusarium moniliforme, Coprinus lagopus*, in addition to *A. niger*, appeared to effect transformations, while *A. nidulans, A. ochraceous*, a *Hypomycetes* species, an *Ostracoderma* species, *Penicillium expansum*, a *Trichoderma* species, *Trichothecium roseum*, and a *Zygorhynchus* species did not.

Incubations of ent-17-Norkauran-16-one (2b) with A. niger (ATCC 26693)

An initial screening incubation was performed as follows. A 3-day-old submerged culture of *A. niger* (100 ml nutrient medium) in a 250-ml Erlenmeyer flask was inoculated with *ent*-17-norkauranone (**2***b*, 20 mg) in DMF (1 ml) and stirred vigorously for 7 days. The culture was then sonicated and the mycelium and medium were extracted with ethyl acetate (3 × 100 ml). Comparison by t.l.c. (ethyl acetate – light petroleum, 1:4) of this extract with one from a control culture inoculated with DMF (1 ml) only, showed a polar spot, in addition to that of substrate (**2***b*), not present in the control extract. Large scale incubations were carried out as follows. Vigorously stirred 3-day-old cultures of *A. niger* (2.5 l nutrient medium) in 4-l Erlenmeyer flasks were incubated with the norketone for 5 days. In a typical run, **2***b* (1.0 g) in DMF (50 ml) was added to the growing culture (5 l). After 5 days the mycelia were removed by filtration and the filtrate was then saturated with NaCl and extracted with ethyl acetate (3 × 1.5 l/2.5 l filtrate). The combined ethyl acetate extracts were evaporated to give an oil (2.8 g). The mycelia were killed by treatment with acetone, which was then allowed to evaporate at ambient temperature and pressure. The resulting tissue was ground with sand and extracted with ethyl acetate. This gave an oil (1.8 g). The two oily residues were then chromatographed separately over silica gel (100 g). The material (2.8 g) from the filtrate on elution with ether – light petroleum (1:4) gave substrate **2***b* contaminated with nonditerpenoid compounds (n.m.r. evidence). Elution with ether – light petroleum (1:1) gave an oil (0.4 g) which consisted of several compounds separable by t.l.c. (acetic acid – ethyl acetate – light petroleum, 2:5:25). One of these was not present in the corresponding fraction from a control culture. Preparative t.l.c. (same solvent, run twice) afforded this component, 3α-hydroxy-*ent*-17-norkauran-16-one (**2***c*, 45 mg). Repeated crystallization from methanol gave pure **2***c* (28 mg), m.p. 179–181° (lit. (16) 179–181°, (17) 192–193°). The material (1.8 g) from the mycelia on chromatography yielded small amounts of pure **2***c* (2 mg) and substrate (**2***b*). All fractions from both columns which contained substrate were combined and crystallized from methanol giving pure **2***b* (0.7 g). Based on substrate not recovered the yield of **2***c* was 9%.

3α-Acetoxy-ent-17-norkauran-16-one (2e)

In later incubations of **2***b* with *A. niger* it was found that **2***c* was more readily separated from column chromatography fractions (see above) as the derived acetate (**2***e*). These fractions were treated with acetic anhydride – pyridine (1:1) at 20° for 12 h and then submitted to preparative t.l.c. (chloroform). This gave the keto acetate **2***e* which on crystallization from methanol had m.p. 194–196° (lit. (17) 186°).

3α-Acetoxy-ent-16-kaurene (2f)

A solution of phenyl lithium was prepared by reacting lithium (160 mg) with bromobenzene (800 mg) in ether (20 ml). A portion (1.8 ml) of the resulting solution was added at 20° to methyltriphenylphosphine bromide (12 mg) in ether (30 ml) under nitrogen and followed 15 min later by **2***e* (45 mg) in ether (10 ml). The mixture was heated to reflux for 65 h and then worked up by treatment with water (50 ml) and extraction into ether (2 × 50 ml). Preparative t.l.c. (ethyl acetate – light petroleum, 3:17) of the product furnished 3α-acetoxy-*ent*-16-kaurene (**2***f*, 40 mg) which crystallized from light petroleum and had m.p. 151–154°; τ 5.20 (br s, 2 H-17), 5.49 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 7.96 (s, 3H, $-O_2C-CH_3$), 8.95 (s, 3 H-20), and 9.15 (s, 3 H-18 + 3 H-19); $\nu_{C=O}$ 1730 cm^{-1}.

Mol. Wt. Calcd. for $C_{22}H_{34}O_2$: 330.2559. Found (high resolution mass spectrometry): 330.2561.

3α-Hydroxy-ent-16-kaurene (2d)

The acetate (**2***f*, 53 mg) was heated with excess (200 mg) lithium aluminum hydride in refluxing dry ether for 90 min. The reaction was worked up by addition of saturated aqueous sodium sulfate. The crude product was purified by preparative t.l.c. (ethyl acetate – light petroleum, 1:4) and then crystallization from light petroleum. This gave 3α-hydroxy-*ent*-16-kaurene (**2***d*, 38 mg), m.p. 168–169° (lit. (16) 172–173°); τ 5.20 (br s, 2 H-17), 6.78 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 8.97 (s, 3 H-20), 9.02 (s, 3 H-18), and 9.22 (s, 3 H-19); τ with 0.78 molar

ratio of $Eu(dpm)_3$ to **2***d* 1.25 (s, 3 H-19), 1.73 (s, 3 H-18), 4.80 (br s, 2 H-17), and 5.75 (s, 3 H-20); ν_{OH} 3630 cm^{-1}.

ent-16-Kauren-3-one (2g)

A pyridine (2 ml) solution of alcohol **2***d* (20 mg) was added to a solution of chromium trioxide (0.5 g) in the same solvent (5 ml). After 2 h at 20°, water (80 ml) and ethyl acetate (80 ml) were added and the mixture was stirred for 4 h and then filtered. Evaporation of the ethyl acetate layer gave an oil, the major component of which was separated and purified by preparative t.l.c. (ethyl acetate – light petroleum, 1:9) and crystallization. This yielded *ent*-16-kauren-3-one (**2***g*, 8 mg), m.p. 84–85° (lit. (16) 86–87°); τ 5.20 (br s, 2 H-17), 8.75 (s, 3H, C_4—CH_3), 8.93 (s, 3 H-20), and 8.96 (s, 3H, C_4—CH_3); τ (C_6D_6) 8.80 (s, 3H, C_4—CH_3), 8.99 (s, 3H, C_4—CH_3) and 9.20 (s, 3 H-20); $\nu_{C=O}$ 1710 cm^{-1}.

17-Norkauran-16-one (3b)

(+)-Kaurene (**3***a*, 5.0 g from a *Podocarpus* species[8]) was oxidized with osmium tetroxide – sodium periodate as described previously (10) for its enantiomer. The dark product (4.7 g) was chromatographed over silica gel (200 g) and the fractions eluted with ether – light petroleum (1:8) combined and crystallized from moist methanol. The 17-norkauranone (**3***b*, 2.1 g) obtained had m.p. 116–118° [*cf.* enantiomer, **2***b*, 116–119° (10)], $[\alpha]_D$ +56° (*c*, 1.0); τ 8.91, 9.13, and 9.17 (all s, 3H, quaternary C—CH_3's); $\nu_{C=O}$ 1742 cm^{-1}.

Anal. Calcd. for $C_{19}H_{30}O$: C, 83.15; H, 11.02. Found: C, 83.11; H, 11.00.

Incubations of 17-Norkauran-16-one (3b) with A. niger (ATCC 26693)

Procedures similar to those for *ent*-17-norkauranone were used. In a typical incubation, *A. niger* cultures (4 × 2.5 l) were inoculated with **3***b* (2.0 g) in DMF (50 ml). After 5 days an oil (2.9 g) was recovered from the filtered medium by extraction with ethyl acetate. Chromatography of this oil over a silica gel column (120 g) and elution with ether – light petroleum (1:2) gave fractions containing substrate (see below). Elution with ether gave a complex mixture which contained (t.l.c.) the only component, other than **3***b*, present in the extract from the incubation but absent from that of a control culture. This compound, which had an identical mobility on t.l.c. with that of 3α-hydroxy-*ent*-17-norkauran-16-one (**2***c*) was isolated by repeated preparative t.l.c. (acetic acid – ethyl acetate – light petroleum, 2:25:25 and 2:10:25). The resulting oily 3β-hydroxy-17-norkauran-16-one (**3***c*) (4 mg) could not be induced to crystallize but had $[\alpha]_D$ +30° (lit. (9) enantiomer −45°); τ 6.78 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 8.89, 8.99, and 9.18 (all s, 3H, quaternary C—CH_3's). The compound was characterized as its acetate (see below).

An extract of the mycelia on chromatography afforded fractions containing substrate but no isolable amounts of **3***c*. All fractions containing substrate from the extracts of the medium and the mycelia were combined and crystallized from methanol. This gave pure **3***b* (1.06 g).

[8]We thank Dr. G. B. Sweet, New Zealand Forest Service, for this material.

3β-Acetoxy-17-norkauran-16-one (3d)

In later incubations of **3***b* with *A. niger*, fractions containing **3***c*, which were obtained by column chromatography over silica gel, were combined and treated with acetic anhydride – pyridine (1:1) at 20° for 14 h. Evaporation of reagents *in vacuo* and purification of the product by preparative t.l.c. (chloroform) and crystallization from light petroleum (b.p. 62–67°) gave the keto acetate **3***d*, m.p. 193–196° (*cf.* **2***e*, m.p. 194–196°), $[\alpha]_D$ +18° (*c*, 0.6); τ 5.50 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 7.98 (s, 3H, —O_2C—CH_3), 8.90 (s, 3 H-20), and 9.15 (s, 6H, C_4—CH_3's); $\nu_{C=O}$ 1735 cm^{-1}; *m/e* 332 (14%, M^+), 317 (1%), 288 (2%), 273 (26%), 272 (100%), 256 (60%), 228 (26%), and 216 (39%).

Mol. Wt. Calcd. for $C_{21}H_{32}O_3$: 332.2352. Found (high resolution mass spectrometry): 332.2350.

3β-Hydroxy-16-kaurene (3e)

A portion (2.8 ml) of a phenyl lithium solution, prepared from lithium (100 mg) and bromobenzene (800 mg) in ether (20 ml), was added to methyltriphenylphosphine bromide in ether (30 ml) under dry nitrogen. After 2 h at 20° 3β-acetoxy-17-norkauran-16-one (**3***d*, 9 mg) was added in ether (5 ml) and the mixture heated to reflux for 56 h. Decomposition with water, extraction with ether, and evaporation gave an oil (129 mg) which on t.l.c. analysis (ethyl acetate – light petroleum, 1:7) did not contain a compound of the mobility of 3α-acetoxy-*ent*-16-kaurene (**2***f*). It did contain a component of identical R_f with that of 3α-hydroxy-*ent*-16-kaurene (**2***d*). This component, 3β-hydroxy-16-kaurene (**3***e*, 5.3 mg) was recovered by repeated preparative t.l.c. (ethyl acetate – light petroleum, 1:8; chloroform) and had m.p. 168–170° (from methanol; *cf.* **2***d*, m.p. 168–169°), $[\alpha]_D$ +60° (*c*, 0.5); τ 5.20 (br s, 2 H-17), 6.78 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 8.97 (s, 3 H-20), 9.02 (s, 3 H-18), and 9.22 (s, 3 H-19); ν_{O-H} 3630 cm^{-1}; *m/e* 288 (58%, M^+), 273 (18%), 270 (22%), 255 (52%), 245 (35%), 229 (32%), 227 (53%), 159 (22%), and 91 (100%).

Mol. Wt. Calcd. for $C_{20}H_{32}O$: 288.2453. Found (high resolution mass spectrometry): 288.2454.

Incubations of 17-Norphyllocladan-16-one (4b) with A. niger (ATCC 26693)

The conditions and procedures established for both norkauranone enantiomers (**2***b* and **3***b*) were used for the incubation of **4***b* with *A. niger*. In a typical incubation, cultures (5 × 2.5 l) of *A. niger* were inoculated with the norketone (**4***b*, 2.0 g) in DMF (50 ml). The cultures were harvested after 5 days and gave an oily extract (1.25 g, total from medium and mycelia) which was chromatographed over silica gel (70 g). Elution with ether – light petroleum (1:1) gave substrate (1.36 g), while elution with pure ether gave fractions containing two compounds which were absent from the extract of a control culture. These were isolated by preparative t.l.c. (acetic acid – ethyl acetate – light petroleum, 2:5:25). The less polar transformation product, the diketone (**4***g*, 17 mg, 2.5%) on crystallization from light petroleum had m.p. 187–189°, $[\alpha]_D$ +13° (*c*, 0.8); τ 8.90, 8.95 (each s, 3H, C_4—CH_3's), and 8.99 (s, 3 H-20); τ with a 1.0 molar ratio of $Eu(dpm)_3$ to **4***g* 6.80, 7.08 (each s, 3H, C_4—CH_3's), and 7.84 (s, 3 H-20); $\nu_{C=O}$ 1749 and 1712 cm^{-1}; *m/e* 288 (100%, M^+), 273 (10%), 243 (24%), 232 (40%), 230 (23%), 202 (80%), and 201 (95%).

Mol. Wt. Calcd. for $C_{19}H_{28}O_2$:288.2089. Found (high resolution mass spectrometry):288.2086.

This sample was identical (m.p., mixture m.p., t.l.c., n.m.r., and mass spectra) with **4***g* prepared from calliterpenone (**8**) by Dr. Zaman (19). As obtained from Dr. Zaman **4***g* had m.p. 179–182°, raised to 185–188° by preparative t.l.c. (chloroform) and crystallization from light petroleum.

The more polar transformation product 3β-hydroxy-17-norphyllocladan-16-one (**4***c*, 35 mg, 5.0%), on crystallization from light petroleum had m.p. 158–160°, $[\alpha]_D$ +16° (*c*, 0.7); τ 6.75 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 7.3 (dd, 1 H-15, J_{obs}, 15α-15β = 19 Hz and 14α-15β = 3 Hz), 9.02, 9.13, and 9.24 (all s, 3H, quaternary C—CH_3's) $\nu_{C=O}$ 1750 cm^{-1}, and ν_{O-H} 3640 cm^{-1}; *m/e* 290 (100%, M^+), 275 (18%), 272 (65%), 258 (62%), 234 (29%), and 230 (24%).

Mol. Wt. Calcd. for $C_{19}H_{30}O_2$:290.2276. Found (high resolution mass spectrometry): 290.2272.

Incubation of 17-Norphyllocladan-16-one (4b) with A. niger (ATCC 10577)

Inoculation of cultures (3 × 2.5 l) of *A. niger* (ATCC 10577) with **4***b* (1.35 g) in DMF (50 ml) and then extraction of the mycelia and medium, as described above, afforded an oil (1.82 g). Chromatography of this oil over silica gel (80 g) and preparative t.l.c. of the resulting fractions gave substrate (**4***b*, 0.81 g) and the keto alcohol **4***c* (27 mg, 5%). Only a trace (< 1 mg) of material with the same R_f on t.l.c. (acetic acid – ethyl acetate – light petroleum, 2:10:25 and 2:6:15) as that of the diketone **4***g* was obtained.

Oxidation of 3β-Hydroxy-17-norphyllocladan-16-one (4c)

The keto alcohol (**4***c*, 4 mg) in acetone (5 ml) was treated with Jones' reagent (0.3 ml). After 30 min at 20° the solution was diluted with water (50 ml) and extracted with ether (2 × 50 ml) to give a compound (3 mg) which was identical by t.l.c., g.l.c. (retention time 10.3 min, 195°), i.r. and mass spectra, with the diketone (**4***g*).

3β-Acetoxy-17-norphyllocladan-16-one (4d)

Treatment of the keto alcohol (**4***c*, 35 mg) with acetic anhydride – pyridine (1:1, 5 ml) for 12 h and then evaporation of the reagents *in vacuo* gave the keto acetate (**4***d*). After preparative t.l.c. (chloroform) and crystallization from light petroleum it (28 mg) had m.p. 156–158°, $[\alpha]_D$ +27° (*c*, 1.0); τ 5.45 (dd, 1 H-3, $w_{1/2}$ = 17 Hz), 7.95 (s, 3H, —O_2C—CH_3), 9.08 (s, 3H, quaternary C—CH_3), and 9.10 (s, 6H, quaternary C—CH_3's); $\nu_{C=O}$ 1735 cm^{-1}.

Mol. Wt. Calcd. for $C_{21}H_{32}O_3$:332.2352. Found (high resolution mass spectrometry):332.2350.

3β-Acetoxy-16-phyllocladene (4f)

A portion (2 ml) of a phenyl lithium solution, prepared from lithium (160 mg) and bromobenzene (800 mg) in ether (20 ml), was added to methyltriphenylphosphine bromide (180 mg) in ether (30 ml) under dry nitrogen and the resulting yellow solution heated to reflux for 3 h. The ketoacetate (**4***d*, 28 mg) in ether (5 ml) was then added and the heating continued for 3 days. Decompositon with water (20 ml), extraction with ether (2 × 50 ml) and evaporation gave an oil (220 mg). This was subjected to preparative t.l.c. (ethyl acetate – light petroleum, 3:17) and gave the acetoxy olefin (**4***f*, 19 mg) which on crystallization from methanol had m.p. 121–123°, $[\alpha]_D$ +41° (*c*, 1.1); τ 5.29 (br s, 2 H-17), 5.52 (dd, 1 H-3, $w_{1/2}$ = 16 Hz), 8.00 (s, 3H, —O_2C—CH_3), 9.05 (s, 3H, quaternary C—CH_3), and 9.17 (s, 6H, quaternary C—CH_3's); $\nu_{C=O}$ 1735 cm^{-1}.

Mol. Wt. Calcd. for $C_{22}H_{34}O_2$:330.2559. Found (high resolution mass spectrometry):330.2560.

Preparation of Diol 6

Phenyl lithium solution (1.6 ml, from 80 mg lithium and 400 mg bromobenzene in 10 ml dry ether) was reacted with methyltriphenylphosphine bromide (120 mg) in ether (30 ml) under dry nitrogen at 20°. After 10 min the keto acetate (**4***d*, 43 mg) was added and the mixture heated to reflux for 48 h. Work-up (as above) gave an oil which on t.l.c. (ethyl acetate – light petroleum, 3:17) showed no trace of the acetoxy olefin **4***f* but only a spot of much lower R_f. The component responsible for this was isolated by preparative t.l.c. (ethyl acetate – light petroleum, 2:3 run twice). This oily diol (**6**, 20 mg) had τ 2.4–2.9 (5H, aromatic protons), 6.80 (dd, 1 H-3, $w_{1/2}$ = 15 Hz), 9.02 (s, 6H, quaternary C—CH_3's), and 9.20 (s, 3H, quaternary C—CH_3); ν_{O-H} 3633 and 3610 cm^{-1}.

Rapid decomposition of **6** to the styrene **5** was taking place and further investigations were limited to **5** (see below).

Dehydration of Diol 6

The diol (**6**, 20 mg) in benzene (20 ml) was treated with toluene-*p*-sulfonic acid (15 mg) in the presence of anhydrous calcium chloride (0.5 g) for 100 min. The drying agent and solvent were removed and the oily residue submitted to preparative t.l.c. (ethyl acetate – light petroleum, 1:4). This gave the styrene **5** (13 mg), which on crystallization from ether had m.p. 177–178°; τ 2.4–2.9 (5H, aromatic protons), 3.75 (s, 1 H-15), 6.75 (dd, 1 H-3, $w_{1/2}$ = 16 Hz), 7.1 (m, 1 H-13), 8.98, 9.20, and 9.22 (all s, 3H, quaternary C—CH_3's); τ with 1.23 molar ratio of Eu(dpm)$_3$ to **5** −0.70 (s, 3 H-19), 0.00 (s, 3 H-18), and 5.90 (s, 3 H-20); λ_{max} 264 (ε 14 600); ν_{O-H} 3650 cm^{-1}.

Mol. Wt. Calcd. for $C_{25}H_{34}O$: 350.2610. Found (high resolution mass spectrometry): 350.2612.

3β-Hydroxy-16-phyllocladene (4e)

A suspension of lithium aluminum hydride (110 mg) in ether (10 ml) was stirred under dry nitrogen at 20° for 1 h. The acetate **4***f* (20 mg) was then added in ether (6 ml) and the reaction heated to reflux for 5 h. Addition, in succession, of water (0.1 ml), aqueous sodium hydroxide (15%, 0.1 ml), and water (0.4 ml) followed by removal of the resulting precipitate and the solvent gave an oil. Preparative t.l.c. (chloroform) of this residue furnished 3β-hydroxy-16-phyllocladene (**4***e*, 18 mg) which crystallized from light petroleum and had m.p. 157–158°, $[\alpha]_D$ +3° (*c*, 0.9); τ 5.30 (br s, 2 H-17), 6.85 (dd, 1 H-3, $w_{1/2}$ = 16 Hz), 9.09 (s, 3 H-18), 9.15 (s, 3 H-20), and 9.29 (s, 3 H-19); τ with 0.4 molar ratio of Eu(dpm)$_3$ to **4***e* 4.25 (s, 3 H-19), 4.49 (s, 3 H-18), 4.95 (br s, 2 H-17), and 7.12 (s, 3 H-20); ν_{O-H} 3638 cm^{-1}.

Mol. Wt. Calcd. for $C_{20}H_{32}O$:288.2453. Found (high resolution mass spectrometry):288.2454.

Incubation of ent-Kaurene (2a) and Phyllocladene (4a) with A. niger (ATCC 26693)

Three cultures (100 ml each) of *A. niger* were grown

for 4 days and then one was inoculated with *ent*-kaurene (**2***a*, 20 mg) in DMF (1.5 ml), another with phyllocladene (**4***a*, 27 mg) in DMF (1.5 ml), and the third with DMF (1.5 ml) only. After incubation for 6 days the cultures were extracted with ethyl acetate as before (see above). The three extracts were compared by analytical t.l.c. (ethyl acetate – light petroleum, 1:9 and 1:2). No trace of products (**2***d*, **4***e*) of axial hydroxylation at C-3 in **2***a* or **4***a* was detected in the appropriate extracts, the three extracts appearing identical except for the presence of unreacted **2***a* and **4***a*. *ent*-Kaurene (**2***a*, 18 mg) and phyllocladene (**4***a*, 24 mg) were recovered by preparative t.l.c. (light petroleum).

We thank the National Research Council of Canada for an Operating Grant (to R.McC.) and a Scholarship (to J.K.T.). We are also indebted to Dr. T. Anthonsen, Trondheim, for the high resolution mass spectra and to Dr. G. L. Barron, University of Guelph for gifts of fungal cultures.

1. A. B. ANDERSON, R. MCCRINDLE, and J. K. TURNBULL. Chem. Commun. 143 (1973).
2. A. LANG. Ann. Rev. Plant Physiol. **21**, 53F (1970).
3. J. R. HANSON. The tetracyclic diterpenes. Pergamon Press, Oxford. 1968.
4. J. MACMILLAN and E. R. H. WALKER. J. Chem. Soc. Perkin Trans. I, 981 (1972).
5. R. A. APPLETON, R. MCCRINDLE, and K. H. OVERTON. Tetrahedron, **9**, 581 (1970).
6. P. W. BRIAN, J. F. GROVE, and T. P. C. MULHOLLAND. Phytochemistry, 1475 (1967).
7. W. CHARNEY and H. L. HERZOG. Microbial transformations of steroids. Academic Press, New York. 1967.
8. E. R. H. JONES. Pure Appl. Chem. **33**, 39 (1973). A. M. BELL, P. C. CHERRY, I. M. CLARK, W. A. DENNY, E. R. H. JONES, G. D. MEAKINS, and P. D. WOODGATE. J. Chem. Soc. Perkin Trans. 1. 2081 (1972).
9. J. P. BEILBY, E. L. GHISALBERTI, P. R. JEFFERIES, M. A. SEFTON, and P. N. SHEPPARD. Tetrahedron Lett. 2589 (1973).
10. R. A. APPLETON, P. A. GUNN, and R. MCCRINDLE. J. Chem. Soc. C, 1148 (1970).
11. J. C. GALBRAITH and J. E. SMITH. Brit. Mycol. Soc. Trans. **52**, 237 (1969).
12. D. G. BUCKLEY, G. H. GREEN, E. RITCHIE, and W. C. TAYLOR. Chem. Ind. 298 (1971).
13. P. V. DEMARCO, T. K. ELZEY, R. B. LEWIS, and E. WENKERT. J. Am. Chem. Soc. **92**, 2537 (1970).
14. J. D. CONNOLLY and R. MCCRINDLE. Chem. Ind. 379 (1965). D. H. WILLIAMS and N. S. BHACCA. Tetrahedron, **21**, 2021 (1965).
15. P. WITZ, H. HERRMANN, J.-M. LEHN, and G. OURISSON. Bull. Soc. Chim. Fr. 1101 (1963).
16. P. R. JEFFERIES and R. W. RETALLACK. Aust. J. Chem. **21**, 1311 (1968).
17. D. A. H. TAYLOR. J. Chem. Soc. C, 1360 (1967).
18. A. CHATTERJEE, S. K. DESMUKH, and S. CHANDRASEKHAREN. Tetrahedron, **28**, 4319 (1972).
19. S. A. AHMAD and A. ZAMAN. Tetrahedron Lett. 2179 (1973).
20. L. L. SMITH, G. GREENSPAR, R. REES, and T. FOELL. J. Am. Chem. Soc. **88**, 3120 (1966). Y. Y. LIN, M. SHIBAHARA, and L. L. SMITH. J. Org. Chem. **34**, 3530 (1969).
21. B. R. PREMA and P. K. BHATTACHARYYA. Appl. Microbiol. **10**, 529 (1962).
22. A. WETTSTEIN, F. VISCHER, and C. MEYSTRE. U.S. Patent No. 2. 844, 513 July 22, 1958; Chem. Abstr. **53**, 1629B (1968).
23. J. FRIED, R. W. THOMA, D. PERLMAN, J. E. HERZ and A. BORMAN. Recent Prog. Hormone Res. **11**, 149 (1955).