Microbiological Hydroxylation of 17-Norkauran-16-one and *ent*-17-Norkauran-16-one with the Fungus *Rhizopus nigricans*

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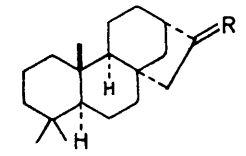
Incubation of 17-norkauran-16-one (1b) and *ent*-17-norkauran-16-one (2b) with *Rhizopus nigricans* gave mixtures of mono- and di-hydroxy-derivatives [(8a—d) and (5a—d), respectively]. A modification of an earlier representation of the relevant enzymic sites is used to explain the patterns of hydroxylation observed.

In attempts to prepare oxygenated derivatives of kaurene (1a), *ent*-kaurene (2a), and phyllocladene (3a), we have incubated [1] the derived 17-nor-16-ketones, (1b)—(3b), with various fungal species. Transformations were observed with several of these, although only in the case of *Aspergillus niger* was there convincing t.l.c. evidence for the production of derivatives from all three ketones. Structures have been assigned [1] to these products. Two other organisms appeared promising: *Calonectria decora*, converting (2b) into a more polar compound; and *Rhizopus nigricans*, producing several polar derivatives from both (1b) and (2b). Unfortunately, the product from *C. decora* is simply the related *endo*-alcohol (4) which was formed in 22% yield during 5 days of incubation. However, the products from *R. nigricans* are of the type sought and we now describe the assignment of their structures and discuss the mode of their formation.

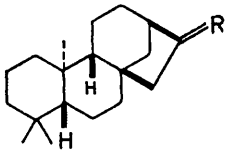
Incubation of ent-17-*Norkauran*-16-*one* (2b).—The ketone [2] (2b) was added in dimethylformamide (DMF) to a submerged culture of *R. nigricans* which had been growing for 4 days in a full nutrient medium [3] under aerobic conditions. After 5 days the mycelium was filtered off and the filtrate extracted with ethyl acetate. T.l.c. comparison of this extract with that of a control culture, to which only DMF had been added, showed the presence in the former of appreciable quantities of four transformation products in addition to substrate (2b). Extraction of the ground mycelium yielded substrate but no transformation products. The extract from the filtrate when chromatographed over silica gel gave (2b) and then, in later fractions, the four trans-

[1] A. B. Anderson, R. McCrindle, and J. K. Turnbull, *Canad. J. Chem.*, in the press.
[2] R. A. Appleton, P. A. Gunn, and R. McCrindle, *J. Chem. Soc.* (*C*), 1970, 1148.
[3] J. C. Galbraith and J. E. Smith, *Brit. Mycol. Soc. Trans.*, 1969, **52**, 237.

formation products. These will be discussed in order of increasing chromatographic polarity on silica gel.



(1) a; R = CH_2
b; R = O



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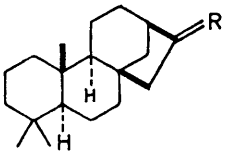

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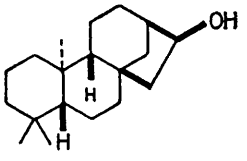
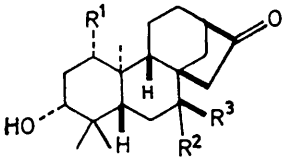
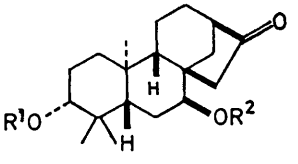


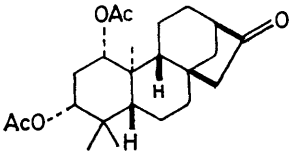
(4)



(5) a; R^1=R^2=R^3=H
b; R^2=OH, R^1=R^3=H
c; R^3=OH, R^1=R^2=H
d; R^1=OH, R^2=R^3=H



(6) a; R^1=R^2=Ac
b; R^1=H, R^2=Ac
c; R^1=Ac, R^2=H



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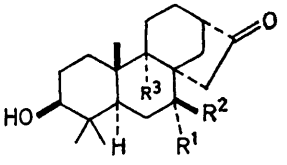
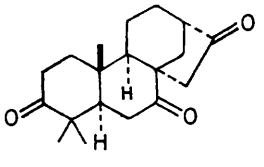
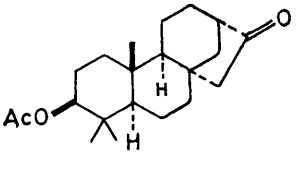


(8) a; R^1=R^2=R^3=H
b; R^2=OH, R^1=R^3=H
c; R^1=OH, R^2=R^3=H
d; R^3=OH, R^1=R^2=H



(9)



(10)

The least polar product (5a) (2% based on substrate not recovered) was readily identified by direct comparison with the known [4] keto-alcohol which we had obtained [1] earlier from incubation of (2b) with *A. niger*. The major transformation product (5b) (15% yield) was eluted next together with a small proportion of the third product (5c). The former was purified by preparative t.l.c. and formulated as a keto-diol on the basis of its t.l.c. polarity and i.r. and mass spectra. Evidence for the location of the hydroxy-groups came from its ^{1}H n.m.r. spectrum. Two broad multiplets (each 1H) represent the X parts of two ABX systems and can be ascribed to two axial carbinol protons each of which has only two neighbouring protons in a vicinal relationship. Thus the hydroxy-groups are equatorial and situated at C-1, C-3, or C-7. Further, the position and multiplicity of the upfield carbinol proton resonance and the chemical shift pattern of the three singlets arising from the tertiary methyl groups are almost identical with those of (5a). This suggests the presence of an equatorial hydroxy-group at C-3 and that the other substituent is positioned in such a way that it does not affect the chemical shifts of the methyl groups. Therefore, the possibility of a C-1 substituent can be discarded and the second hydroxy-group assigned to C-7. Confirmatory evidence for this comes from the location (τ 7·32) of the resonance attributed to H-15α, the 7α-hydroxy-group inducing [5] a pronounced downfield shift from its normal value [*cf.* τ 7·85 for (2b)]. Indeed, when the ^{1}H n.m.r. spectrum of (5b) was run in pyridine this doublet was further shifted downfield [6] by 0·37 p.p.m. Incubation of (5a) with *Rhizopus nigricans* † gives a compound to which structure (5b) has been assigned.[7] Direct comparison of these samples [8] confirmed that they were identical.

The next component (5c) was eluted from the column in fractions which in addition contained either the major product (5b) or the most polar product (5d). Preparative t.l.c. furnished (5c) (7% yield), which has many spectral features in common with (5b). Thus their mass and i.r. spectra are very similar, as are the chemical shift patterns of the resonances attributable to the three tertiary methyl groups. In addition, the ^{1}H n.m.r. spectrum of (5c) contains a multiplet which in shift position and shape is almost identical with that ascribed to the axial carbinol proton at C-3 in (5b). A resonance at slightly lower field is attributable to an equatorial carbinol proton. This evidence was taken to imply that (5c) is an isomer of (5b), an axial hydroxy-group in the former being present in lieu of the 7α-hydroxy-group in the latter. The spectral properties of the di- and mono-acetates (6a—c) derived from (5c) supported this conclusion and allowed assignment

† An Australian group are also studying [7] the hydroxylation of tetracyclic diterpenoids by fungi. We thank Professor P. R. Jefferies for discussions of unpublished results.

[4] P. R. Jefferies and R. W. Retallack, *Austral. J. Chem.*, 1968, **21**, 1311; D. A. H. Taylor, *J. Chem. Soc.* (*C*), 1967, 1360.

[5] L. M. Jackmann and S. Sternhell, 'Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry,' Pergamon, Oxford, 1969, p. 237.

[6] *Cf.* B. P. Hatton, C. C. Howard, and R. A. W. Johnstone, *J.C.S. Chem. Comm.*, 1973, 744.

[7] J. P. Beilby, E. L. Ghisalberti, P. R. Jefferies, M. A. Sefton, and P. N. Sheppard, *Tetrahedron Letters*, 1973, 2589.

[8] P. R. Jefferies, personal communication.

of the axial hydroxy-group to C-7. The shifts induced by $Eu(dpm)_3$ on the methyl resonances in the ^1H n.m.r. spectra of the two monoacetates (6b) and (6c) were particularly valuable. For the less polar monoacetate (6b) the induced shifts were in a normalised ratio [9] which is entirely consistent [1,9] with the presence of an equatorial hydroxy-group at C-3. In the case of the more polar monoacetate (6c) the shifts induced in the resonances of the two C-4 methyl groups were similar in magnitude whereas those of the C-10 and acetyl methyl groups were larger and smaller, respectively. Only a hydroxy-group at C-7 could provide a reasonable explanation for these observations. Since the alternative 3-*ax*,7-*eq*-diol formulation for (5c) is irreconcilable with the spectral data, inter-relation of (5b) and (5c) by oxidation of both to the corresponding trione would establish the structure of (5c) firmly. This has been accomplished for the enantiomeric diols (8b and c) (see below).

That the most polar transformation product is also a keto-diol (5d) (7% yield) was apparent from its spectral characteristics. However, the ill-defined nature of its carbinol proton resonances precluded their use in determining the location of the hydroxy-groups. The ^1H n.m.r. spectrum of the diacetate (7) was more informative. In this the analogous resonances were attributable to the X parts of ABX systems, the observed multiplicities indicating that both carbinol protons are axial and each is coupled to two vicinal protons, one axial and one equatorial. Thus the hydroxy-functions in (5d) are equatorial and at C-1, C-3, or C-7. Since this final transformation product is not identical with (5b) it must be either the 1-*eq*,3-*eq*- or the 1-*eq*,7-*eq*-diol. That it is the former was shown by comparison with data [8] for a sample of (5d) prepared [7] by Jefferies and his co-workers.

Incubation of 17-*Norkauran*-16-*one* (1b).—T.l.c. transformation products obtained similarly from (1b) suggested that appreciable quantities of four diterpenoids were present in the mixture and that three of them, (8a—c), were enantiomeric with products from (2b) while the fourth contained a new substitution pattern. All four were isolated by chromatography, the first (1%), third (21%), and fourth (8%) (order of increasing chromatographic polarity) being identified as (8a), (8b), and (8c), respectively by comparison of their physical and spectral characteristics with those of their enantiomers (5a—c). In addition, (8b) and (8c) were correlated by oxidation of both to the trione (9).

The other component, second in polarity, was the keto-diol (8d) (15%). Its i.r., mass, and ^1H n.m.r. spectra pointed strongly to its formulation as a keto-diol, with one hydroxy-group secondary (equatorial) and the other tertiary. The tertiary nature of one of these groups was confirmed by acetylation, only a monoacetate (10) being formed even under forcing conditions. The ^1H n.m.r. spectrum of (10) was run in the presence of $Eu(dpm)_3$ in the hope that it might indicate the location of the tertiary hydroxy-group. However, the addition of the shift reagent, even in substantial quantities, did not induce shifts large enough to be of any diagnostic value, presumably because the hydroxy-group is too hindered to complex significantly with the reagent. Although no information had been acquired about the location of the tertiary substituent, the $Eu(dpm)_3$-induced shifts in the ^1H n.m.r. spectrum of the parent diol (8d) could now be examined in the knowledge that only complexing with the secondary alcohol would be important in determining their magnitude. Indeed, the normalised ratio of the shifts induced in the methyl resonances agrees closely with that expected [1,9] for an equatorial hydroxy-group at C-3. The ^1H n.m.r. spectra of (8d) and (10) allowed assignment of the other hydroxy-group to C-9α. In both, the resonance of H-15α appears far downfield (about 0·8 p.p.m.) from its position in related compounds which lack oxygenation at C-7 and C-9. The resonance is easily assigned on the basis of its multiplicity. In norkauran-16-ones this proton shows a large geminal coupling, since it is adjacent to a carbonyl group, and in addition a long-range coupling to H-14β. Thus the tertiary hydroxy-group must be at C-9 and have the α-orientation expected on mechanistic grounds, a hydroxy-group normally having [10] the same orientation as the hydrogen atom which suffers microbial displacement. Thus the diol can be formulated as (8d), an assignment which has been substantiated [11] by an *X*-ray structural analysis of (10), by direct methods.

Mechanism.—The outcome of the microbial oxidation reported here for (1b) and (2b) can be readily rationalised on the basis of an extension of a mechanism proposed [12,13] by Jones, Meakins, and their co-workers for the hydroxylation of steroidal substrates by *R. nigricans*. They noted that certain steroidal ketones are dihydroxylated and considered [12] that both hydroxy-groups may be introduced in a concerted manner by a single enzyme–substrate complex. They suggested that the simplest explanation would involve an enzyme which has three active sites, each with binding and hydroxylating capabilities, located in a triangular arrangement, corresponding to C-3, C-11, and C-16 of the steroid nucleus [see (11a)]. This predicts the location of hydroxylation in many, but not all, cases although it fails to explain the orientation (α or β).

If one refines Jones' representation in a three dimensional sense, practically all the relevant results [12-16] in

[9] D. G. Buckley, G. H. Green, E. Ritchie, and W. C. Taylor, *Chem. and Ind.*, 1971, 298.

[10] M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek, and D. H. Peterson, *J. Amer. Chem. Soc.*, 1958, **80**, 2336; E. J. Corey, G. A. Gregoriou, and D. H. Peterson, *ibid.*, p. 2338.

[11] G. Ferguson and W. C. Marsh, unpublished result.

[12] (*a*) J. W. Browne, W. A. Denny, Sir E. R. H. Jones, G. D. Meakins, Y. Morisawa, A. Pendlebury, and J. Pragnell, *J.C.S. Perkin I*, 1973, 1493; (*b*) V. E. M. Chambers, W. A. Denny, J. M. Evans, Sir E. R. H. Jones, A. Kasal, G. D. Meakins, and J. Pragnell, *ibid.*, p. 1500.

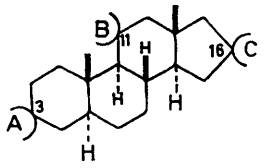
[13] Sir E. R. H. Jones, *Pure Appl. Chem.*, 1973, **33**, 39.

[14] A. M. Bell, V. E. M. Chambers, Sir E. R. H. Jones, G. D. Meakins, W. E. Müller, and J. Pragnell, *J.C.S.Perkin I*, 1974, 312.

[15] Z. Prochazka, *Abh. Deut. Akad.Wiss. Berlin, Kl. Med.*, 1968, no. 2, 131.

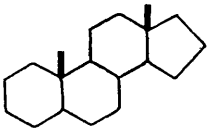
[16] W. Charney and H. L. Herzog, 'Microbial Transformations of Steroids,' Academic Press, New York, 1967.

the steroid field and those reported here are explained. The only refinement necessary is to define a reference

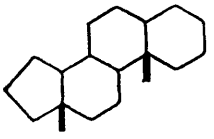


(11a)

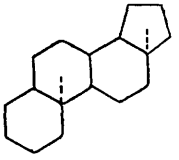
(11b) Aα; Bα or coplanar; Cβ



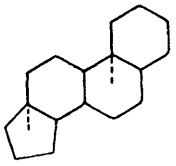
(12) normal (*n*) mode



(13) reversed (*r*) mode



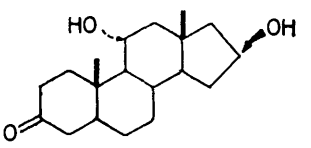
(14) capsized normal (*cn*) mode



(15) capsized reversed (*cr*) mode

plane as that of the steroidal ring system of a substrate and to require that relative to this plane site A is below, site B is below (or coplanar), and site C is above (11b). The four most obvious binding modes [12,13] for a steroid are drawn [(12)—(15)]. When one analyses the results from the steroid field it appears that the uncapsized modes, (12) and (13), are generally preferred for binding. In binding, site A favours oxygen atoms below the plane and hydroxylates from below (α); site B has similar binding requirements but can hydroxylate α (axial or equatorial) or β (equatorial only); site C prefers to bind oxygen functions above the plane and hydroxylates β.

Thus in the case of 5α-androstane-derived substrates [12a] a 3-ketone interacts (16) with site A (*n* mode) and a β-hydroxy-group is inserted at C-16 (site C) and an α-hydroxy-group at C-11 (site B). On the other hand, a 2-ketone interacts (17) with site C (*r* mode) and α-hydroxy-groups are inserted at C-16 (site A) and at C-6 (site B). With appropriately disubstituted substrates,[12b] interactions with the binding sites will be optimised. Thus the 17β-hydroxy-3-ketone (18) suffers [*n* mode; β-ol at site C, C(3)=O at site A] mainly C-11 hydroxylation while its 3β-OH,C(17)=O isomer (19) is hydroxylated [*r* mode; β-ol at site C, C(17)=O at site A] at C-7. In cases where the functionality is so disposed that binding through two sites simultaneously is not possible, then only one will be utilised, the resulting competition among different available modes of binding giving rise to mixtures [12b] of products. Difficulties were experienced in accounting for some of the minor products. Thus the hydroxylation of C-17 derivatives at the 5α- and 6α-positions is not readily rationalised by the model presented here unless one assumes that an adsorptive mode such as (20) * is involved [*cf.* the site A—site C distance in (21) and (22)], hydroxylation proceeding at site A. Further, it seems likely that during insertion of the first hydroxy-group into ring B or ring C steroidal monoketones, modes of interaction other than (12)—(15) are involved in which the ketone complexes with either site A or site C rather than the expected site B. Two final points should be noted. First, in mono-oxygenated substrates the two



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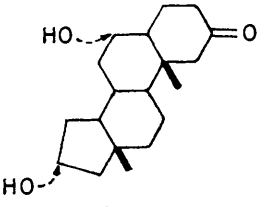

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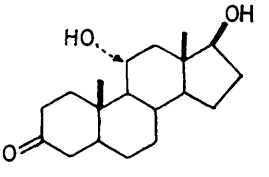

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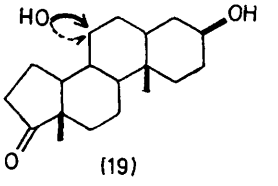

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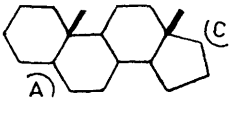

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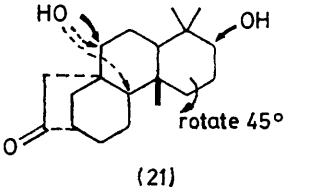

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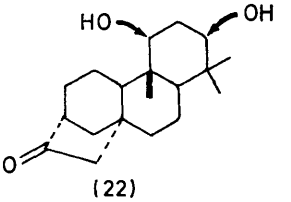

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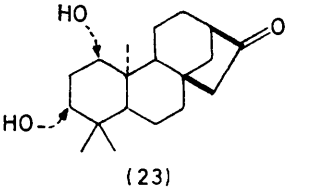

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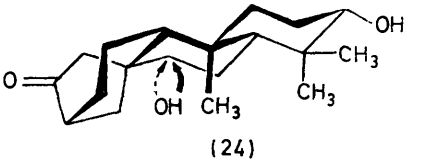

(24)

hydroxy-groups may be introduced by a sequential, rather than a simultaneous, process and second, the model of the binding–hydroxylating sites outlined above

* Such a mode would also explain the considerable amount of 11α-hydroxylation,[12b] at the expense of the more usual C-3 hydroxylation, in 6α-hydroxy-5α-androstan-17-one, the 6α-hydroxy-group being suitably oriented for interaction with site A [C(17)=O at site C and hydroxylation at site B].

may represent the *sum* of the locations and properties of the active sites on two or more enzyme units.

Based on this general mechanism the following enzyme–substrate complexes * would explain the production of (8a—d) from (1b) and (5a—d) from (2b). Interaction (21) (*r* mode; ring C boat?) of the ketone of (1b) with site A would result in insertion of a β-OH at C-3 by the participation of the suitably disposed hydroxylation site C. This accounts for the formation of (8a). The two functional groups in (8a) then cause binding (21) (*r* mode) at the same, or a new, active region and allow hydroxylation (site B) to proceed at the 7β- (equatorial) or 7α-position as observed with steroids. However, with this substrate, a molecular model indicates that more efficient interaction of the C(16)=O with site A could be achieved by starting with this *r* mode, (21) (ring C chair), and rotating it *ca.* 45° around the C-3,C-16 axis in such a way that H-9 rotates downwards and back toward hydroxylation site B. Thus formation of the three products (8b—d) is explained.

In formation of the substrate–enzyme complex with (2b) two possible modes of interaction, *r* mode (22) (ring C boat?) and *cn* mode (23) (ring C chair) present themselves, *both* of which predict formation of (5a) and by further attack (5d). Hydroxylation at C-7 in the formation of (5b and c) requires absorption of the substrate in a tilted *cr* mode (24), an interaction which appears acceptable on the basis of the active site representation outlined above when one compares (24) with (19) using space-filling molecular models.

EXPERIMENTAL

For general experimental directions see ref. 1. Molecular rotations were measured for solutions in methanol, unless otherwise stated. Coupling constants reported are observed values.

Incubation of ent-17-*Norkauran*-16-*one with* Calonectria decora.—Cultures (2 × 2·25 l) of *C. decora* were grown in Medium B [17] lacking beef extract (but containing all essential nutrients) for 5 days. *ent*-17-Norkauran-16-one [2] (2b) (707 mg) in DMF (50 ml) was added and the fermentation was stirred vigorously for 7 days. The mycelium was filtered off and the filtrate extracted with ethyl acetate (2 × 1·2 l per 1·5 l of filtrate) after the addition of sodium chloride (500 g). Evaporation of the combined ethyl acetate fractions gave an oil (1·4 g) which was chromatographed on silica gel (60 g). [A control culture (2·5 l), containing DMF (25 ml) alone, was treated similarly.] Elution with ether–light petroleum (1 : 4) gave first the starting ketone (2b) and in later fractions *ent*-17-norkauran-16α-ol (4) (27 mg, 22%), m.p. 160—162° (from methanol–water) (lit.,[2] 162—163°); ν_{OH} 3630 cm^{-1}; τ 9·17, 9·14, and 8·95 (all s, quaternary CMe) and 5·70 (1H, m, $W_{\frac{1}{2}}$ 30 Hz, H-16). Elution with more polar solvents, including ethyl acetate–methanol mixtures, gave only compounds which were shown to be present in the control extract by analytical t.l.c. and n.m.r.

The mycelium was extracted with ethyl acetate and the resulting extract chromatographed on silica gel (30 g) to give the starting ketone (2b). All fractions containing (2b) were combined (total 584 mg).

Incubation of ent-17-*Norkauran*-16-*one with* Rhizopus nigricans.—After 4 days of growth, cultures (4 × 2·5 l) of *Rhizopus nigricans* in a full nutrient medium [3] were inoculated with *ent*-17-norkauran-16-one (2b) (2·0 g total) in DMF (50 ml). The fermentation was continued for 5 days and then the mycelium was filtered off. After addition of sodium chloride (1 kg) the filtrate was extracted with ethyl acetate (2 × 1·5 l per 2·5 l of filtrate). Evaporation of the extracts gave an oil (8·3 g) which was chromatographed over silica gel (300 g). [A control fermentation (2·5 l) containing DMF (7 ml) was treated in the same way.] Elution of the column with ether–light petroleum (1 : 4 and 1 : 1) gave fractions that contained the starting nor-ketone (2b) (600 mg), along with non-diterpenoid compounds. Later fractions contained the following transformation products.

ent-3β-*Hydroxy*-17-*norkauran*-16-*one* (5a).—Elution with pure ether gave a fraction (150 mg) containing a compound not present in the control extract. The major component of this fraction, a non-terpenoid fungal metabolite, was removed by distillation under vacuum at 50° to leave a solid (75 mg) which was subjected to careful preparative t.l.c. (pure chloroform, then ethyl acetate–light petroleum, 1 : 2). The resulting keto-alcohol (5a) (17 mg, 1·7%), on crystallisation from methanol–water and then light petroleum had m.p. 179—183° (lit.,[1] 179—181°); mixed m.p. 180—183°.

ent-3β,7β-*Dihydroxy*-17-*norkauran*-16-*one* (5b).—Elution of the column with pure ether gave, in later fractions, a white solid (175 mg) which showed one major and one minor component on t.l.c., both of which were not present in the control extract. Preparative t.l.c. (methanol–ethyl acetate, 1 : 19) separated these compounds to give the keto-diol (5b) and a small amount of the keto-diol (5c) (see below). The keto-diol (5b) (175 mg, 15%) on crystallisation from methanol–water had m.p. 219—221° (reported,[8] 223—224°); $[\alpha]_D$ −32°; ν_{OH} 3630, ν_{CO} 1740 cm^{-1}; τ ($CDCl_3$) 9·20, 8·99, and 8·89 (all s, quaternary CMe), 7·32 [1H, dd, H-15α(*exo*), J_{gem} 18, $J_{15\alpha.13}$ 2 Hz], and 6·22—6·90 (2H, complex m, H-3 and -7); τ (pyridine) 9·00, 8·92, and 8·83 (all s, quaternary CMe), 6·95 (1H, dd, H-15α), and 6·3—6·8 (2H, complex m, H-3 and -7); *m/e* 306 (90%, M^+), 291 [10, $(M - 15)^+$], 288 [25, $(M - 18)^+$], 273 (30), and 121 (100); mixed m.p.[8] 221—223°.

ent-3β,7α-*Dihydroxy*-17-*norkauran*-16-*one* (5c).—The initial fractions eluted with ether–ethyl acetate (1 : 1) contained (5c). This was separated from the major contaminant (5b) by preparative t.l.c. (methanol–ethyl acetate, 1 : 19). On crystallisation from methanol–water and then ether, the *keto-diol* (5c) (75 mg, 7%) had m.p. 208—210°, $[\alpha]_D$ −8°; ν_{OH} 3620, ν_{CO} 1747 cm^{-1}; τ ($CDCl_3$) 9·20, 9·01, and 8·90 (all s, quaternary CMe), 7·85 (d, part of H-15α signal, $J_{15\alpha.13}$ 1·5 Hz), 6·70 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz), 6·30 (1H, m, H-7, $W_{\frac{1}{2}}$ 8 Hz); τ (pyridine) 8·94, 8·92, and 8·74 (all s, quaternary CMe), 7·55 (d, downfield part of H-15α signal), 6·48 (1H, m, H-3), and 6·10 (1H, m, H-7); *m/e* 306 (10%, M^+), 291 [5, $(M - 15)^+$], 288 [15, $(M - 18)^+$], 274 (13), 255 (11), and 121 (100) (Found: C, 74·2; H, 9·8. $C_{19}H_{30}O_3$ requires C, 74·45; H, 9·85%).

ent-1β,3β-*Dihydroxy*-17-*norkauran*-16-*one* (5d).—Later

* A referee has suggested that in some of these (see text) interaction of the substrate with the enzyme sites could be optimised if ring C were to adopt a boat conformation.

[17] J. W. Blunt, I. M. Clark, J. M. Evans, Sir E. R. H. Jones, G. D. Meakins, and J. T. Pinhey, *J. Chem. Soc.* (*C*), 1971, 1136.

fractions eluted with ether–ethyl acetate (1 : 1) contained the keto-diol (5d) along with some (5c). These were separated by preparative t.l.c. (methanol–ethyl acetate, 1 : 19). On crystallisation from methanol–water the keto-diol (5d) (70 mg, 7%) had m.p. 246—251° (reported,[8] 245—246°); $[\alpha]_D$ −37°; ν_{OH} 3630 cm^{-1}; τ ($CDCl_3$) 9·22, 9·05, and 8·84 (all s, quaternary CMe), 6·4—6·9 (2H, complex m, H-1 and -3); τ (pyridine) 8·99, 8·85, and 8·67 (all s, quaternary CMe), 6·4—6·8 (2H, complex m, H-1 and -3); *m/e* 306 (17%, M^+), 288 [31, $(M - 18)^+$], 273 (22), 270 (39), 255 (44), 234 (70), 233 (100), 232 (90), 191 (70), 177 (63), 174 (60), and 163 (47).

Extraction of the mycelium with ethyl acetate gave an oil (1·2 g) which was combined with the impure (2b) previously isolated. Chromatography over silica gel and elution with ether–light petroleum (1 : 8) gave the starting ketone (2b) (965 mg).

Acetylation of the Keto-diol (5c).—The keto-diol (5c) (65 mg) was treated with acetic anhydride (1 ml) and pyridine (4 ml) for 1 h at 0 °C. Methanol (2 ml) was then added and the solvent evaporated off under vacuum to give an oil, which was subjected to preparative t.l.c. (chloroform). The top band, consisting of diacetate, and the middle band, two monoacetates, were retained, and the large quantity of starting material in the bottom band was eluted and acetylated as before. After four of these cycles and a fifth cycle of 2 h reaction time, the products were isolated by preparative t.l.c.

The least polar band contained ent-3β,7α-*diacetoxy*-17-*norkauran*-16-*one* (6a) (18 mg), which on crystallisation from ether–light petroleum and then ether had m.p. 175—177°; $[\alpha]_D$ −6°; ν_{CO} 1740 cm^{-1}; τ 9·23, 9·15, and 8·88 (all s, quaternary CMe), 7·97 (6H, s, 2 × OAc), 5·47 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz), and 5·10 (1H, m, H-7, $W_{\frac{1}{2}}$ 8 Hz) (Found: M^+, 390·2410. $C_{23}H_{34}O_5$ requires *M*, 390·2406).

The band of intermediate polarity gave a solid (35 mg) consisting of two compounds which were separated by preparative t.l.c. (ethanol–chloroform 1 : 100, run three times). The less polar was ent-7α-*acetoxy*-3β-*hydroxy*-17-*norkauran*-16-*one* (6b) (13 mg), which on crystallisation from ether had m.p. 216—217°; ν_{OH} 3620, ν_{CO} 1738 cm^{-1}; τ 9·18 (s, 19-H_3), 9·07 (s, 18-H_3), 8·87 (s, 20-H_3), 7·93 (s, OAc), 6·71 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz), and 5·07 (1H, m, H-7, $W_{\frac{1}{2}}$ 8 Hz) (Found: M^+, 348·2303. $C_{21}H_{32}O_4$ requires *M*, 348·2301). In the presence of Eu(dpm)$_3$ (28 mg), (6b) (10 mg) has τ 6·50 (s, OAc), 4·25 (s, 20-H_3), −0·70 (s, 18-H_3), and −1·25 (s, 19-H_3). The induced shifts are in the ratio, 19-H_3 : 18-H_3 : 20-H_3 : OAc, of 10 : 9·4 : 4·4 : 1·4.

The more polar ent-3β-*acetoxy*-7α-*hydroxy*-17-*norkauran*-16-*one* (6c) (15 mg), on crystallisation from ether, had m.p. 203—205°; ν_{OH} 3620, ν_{CO} 1740 cm^{-1}; τ 9·12 (s, 19- and 18-H_3), 8·87 (s, 20-H_3), 7·95 (s, OAc), 6·27 (1H, m, H-7, $W_{\frac{1}{2}}$ 8 Hz), and 5·45 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz) (Found: M^+, 348·2303. $C_{21}H_{32}O_4$ requires *M*, 348·2301). In the presence of Eu(dpm)$_3$ (27 mg), (6c) (15 mg) has τ 7·20 and 7·07 (both s, quaternary 4-Me_2), 6·30 (OAc), and 5·88 (s, 20-H_3). The induced shifts are in the ratio 20-H_3 : 4-Me : 4-Me : OAc, of 10 : 6·9 : 6·5 : 5·6.

The most polar band of the original t.l.c. plates consisted of the starting keto-diol (5c) (5 mg).

Acetylation of the Keto-diol (5d).—The keto-diol (5d) (60 mg) was treated with acetic anhydride–pyridine (1 : 1; 5 ml) overnight. Removal of solvent and then preparative t.l.c. (ethyl acetate–light petroleum, 1 : 2) gave ent-1β,3β-*diacetoxy*-17-*norkauran*-16-*one* (7) (70 mg), m.p. 202—204° (from ether); ν_{CO} 1740s cm^{-1}; τ 9·12 (s, 19- and 18-H_3), 8·67 (s, 20-H_3), 8·00 and 7·97 (both s, 2 × OAc), and 5·32 and 5·21 (both m, H-1 and -3, $W_{\frac{1}{2}}$ 16 Hz) (Found: M^+, 390·2410. $C_{23}H_{34}O_5$ requires *M*, 390·2406).

Incubation of 17-*Norkauran*-16-*one* (1b) *with* Rhizopus nigricans.—A culture (2 × 2·5 l) of *R. nigricans* in the usual medium [3] was grown for 3 days and then inoculated with 17-norkauran-16-one (1b) (843 mg) in DMF (20 ml). The fermentation was continued with vigorous aeration for 5 days. The mycelium was then filtered off and after the addition of sodium chloride (500 g) the filtrate was extracted with ethyl acetate (2 × 1·5 l per 2·5 l). The extracts were combined and the solvent removed to give an oil. A control culture (2·5 l) containing DMF (10 ml) only was treated similarly. The extracts were compared by t.l.c. which showed the presence of at least four additional compounds in the fermentation extract.

The fermentation extract (2·7 g) was chromatographed over silica gel (70 g). Elution with ether–light petroleum (1 : 4) gave in early fractions the starting ketone (1b) contaminated with fungal metabolites. Later fractions of this eluent gave an oil (100 mg) which contained a further compound not present in the control.

3β-*Hydroxy*-17-*norkauran*-16-*one* (8a).—Preparative t.l.c. (chloroform, run twice), separated the keto-alcohol (8a) (10 mg, 1·3%) from this oil. On crystallisation from methanol–water the *keto-alcohol* had m.p. 181—183°; $[\alpha]_D$ +33° {*cf.* enantiomer (5a), m.p. 180—183°; $[\alpha]_D$ −45°}; ν_{OH} 3620, ν_{CO} 1740 cm^{-1}; n.m.r. and mass spectra as (5a) (Found: M^+, 290·2247. $C_{19}H_{30}O_2$ requires *M*, 290·2246).

Elution with pure ether gave a solid consisting of four components, three of which were not present in the control extract. These were separated by repeated preparative t.l.c. (light petroleum–ethyl acetate, 1 : 3) to give four bands. The top band gave a fungal metabolite (30 mg) which was also present in the control extract.

3β,9α-*Dihydroxy*-17-*norkauran*-16-*one* (8d).—Band two overlapped partially with band three but the top portion gave pure keto-diol (8d). The bottom portion was re-chromatographed. The *keto-diol* (8d) (130 mg, 15%) from all fractions was combined and on crystallisation from first ether–methanol and then methanol–water had m.p. 224—226°; $[\alpha]_D$ +17°; ν_{OH} 3618, ν_{CO} 1742 cm^{-1}; τ ($CDCl_3$) 9·17 (s, 19-H_3), 8·98 (s, 18-H_3), 8·87 (s, 20-H_3), 7·24 (1H, dd, H-15α, J_{gem} 19, $J_{15\alpha.14\beta}$ 2·5 Hz), and 6·80 (1H, m, H-3, $W_{\frac{1}{2}}$ 16 Hz); τ (pyridine) 8·91 and 8·75 (6H) (both s, quaternary CMe), 7·52 (1H, dd, H-14β, J_{gem} 13, $J_{14\beta.15\alpha}$ 2·5 Hz), and 6·80 (1H, dd, H-15α); *m/e* 306 (27%, M^+), 288 [100, $(M - 18)^+$], 270 [30, $(M - 36)^+$], 255 (25), 245 (60), 227 (67), 165 (33), and 123 (100) (Found: C, 74·3; H, 10·0. $C_{19}H_{30}O_3$ requires C, 74·45; H, 9·85%). In the presence of Eu(dpm)$_3$ (28 mg), (8d) (30 mg) had τ 7·40 (s, 20-H_3), 6·00 (s, 18-H_3), and 5·89 (s, 19-H_3). The induced shifts are in the ratio 10 : 9·1 : 4·2 for 19-H_3 : 18-H_3 : 20-H_3.

3β,7β-*Dihydroxy*-17-*norkauran*-16-*one* (8b).—The bottom portion of band three gave pure keto-diol (8b). The top portion, which contained (8d) as well, was rechromatographed. The *keto-diol* (8b) (180 mg, 21%), on crystallisation from methanol–water and then methanol, had m.p. 225—226°; $[\alpha]_D$ +34·5° {*cf.* enantiomer (5b), m.p. 219—221, 223—224°, $[\alpha]_D$ −32°}; ν_{OH} 3630, ν_{CO} 1740 cm^{-1}; τ (pyridine) 9·00, 8·92, and 8·83 (all s, quaternary CMe), 6·95 (1H, dd, H-15β, J_{gem} 18, $J_{15\beta.13}$ 1·5 Hz), and 6·3—6·8 (2H, complex m, H-3 and -7); *m/e* 306 (80%, M^+), 291 [10, $(M - 15)^+$]. 288 [25, $(M - 18)^+$], 273 (35), and 121

(100) (Found: C, 74·25; H, 9·95. $C_{19}H_{30}O_3$ requires C, 74·45; H, 9·85%).

3β,7α-Dihydroxy-17-norkauran-18-one (8c).—Band four contained a small amount of impure material that showed the same chromatographic behaviour as the keto-diol (5c). Elution of the chromatography column with ether–ethyl acetate (1 : 1) gave more of this material. This fraction was combined with band four and subjected to preparative t.l.c. (light petroleum–ethyl acetate, 1 : 3) to give an oil (100 mg) which was homogeneous by t.l.c. Vacuum sublimation of this separated a fungal metabolite from less volatile diterpenoids (80 mg). Preparative t.l.c. (methanol–chloroform, 1 : 19) of the latter gave a glass which could not be induced to crystallise. This material, consisting mainly (*ca.* 85%) of the keto-diol (8c) together with other diterpenoids (*ca.* 15%), had ν_{OH} 3620, ν_{CO} 1747 cm^{-1}; τ ($CDCl_3$) 9·20, 9·01, and 8·90 (all s, quaternary CMe), 7·85 (d, part of H-15β signal, $J_{15\beta,13}$ 1·5 Hz), 6·70 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz), and 6·30 (1H, m, H-7, $W_{\frac{1}{2}}$ 8 Hz) attributable to (8c), and τ 8·75 and 8·70 due to impurities [*cf.* n.m.r. of (5c)]. The yield of (8c) was 8%. This impure diol (23 mg) was treated with acetic anhydride–pyridine (1 : 1·5 ml) overnight. Removal of the solvent followed by preparative t.l.c. (ethyl acetate–light petroleum, 1 : 2, run twice) gave the *3β,7α-diacetate* (25 mg), which on crystallisation from ether–light petroleum (twice) had m.p. 175—176°; $[\alpha]_D$ +7° ($CHCl_3$) {*cf.* enantiomer (6c), m.p. 175—177°; $[\alpha]_D$ −6°}; i.r., n.m.r., and mass spectra identical with those of (6c) (Found: M^+, 390·2410. $C_{23}H_{34}O_5$ requires *M*, 390·2406).

The mycelium was dried and extracted with ether to give an oil (1·3 g) which was combined with the impure starting material obtained from the filtrate (above). This material was then chromatographed over silica gel (50 g). Elution with ether–light petroleum (1 : 4), followed by preparative t.l.c. (ethyl acetate–light petroleum, 1 : 12), gave the starting norketone (1b) (72 mg).

Acetylation of the Keto-diol (8d).—The keto-diol (8d) (46 mg) was treated with acetic anhydride–pyridine (5 ml; 1 : 1) for 3 h. Evaporation of solvent followed by preparative t.l.c. (methanol–chloroform, 1 : 30) gave *3β-acetoxy-9α-hydroxy-17-norkauran-16-one* (10) (41 mg), m.p. 291—295° (from methanol); $[\alpha]_D$ +17·5° ($CHCl_3$); ν_{OH} 3620, ν_{CO} 1737 cm^{-1}; τ 9·11 (s, 19- and 18-H_3), 8·75 (s, 20-H_3), 7·80 (s, OAc), 7·48 (1H, dd, H-14β, J_{gem} 13, $J_{14\beta,15\alpha}$ 2·5 Hz), 7·20 (1H, dd, H-15α, J_{gem} 19, $J_{15\alpha,14\beta}$ 2·5 Hz), and 5·50 (1H, m, H-3, $W_{\frac{1}{2}}$ 17 Hz); *m/e* 348 (4%, M^+), 330 [3, $(M - 18)^+$], 289 (52), 288 [100, $(M - 60)^+$], 270 (32), 255 (48), 245 (70), 227 (90), and 165 (53) (Found: C, 72·1; H, 9·4. $C_{21}H_{32}O_4$ requires C, 72·4; H, 9·25%).

Oxidation of the Keto-diol (8b).—The keto-diol (8b) (52 mg) in acetone (15 ml) was treated with Jones reagent (0·2 ml) for 15 min. Addition of water followed by extraction with ether gave the crude trione. On crystallisation from ether *17-norkaurane-3,7,16-trione* (9) (46 mg), had m.p. 140—142°; $[\alpha]_D$ +6° ($CHCl_3$); ν_{CO} 1745 and 1710 cm^{-1}; τ 8·89 (6H) and 8·63 (both s, quaternary CMe) and 6·88 (1H, dd, H-15β, J_{gem} 18, $J_{15\beta,13}$ 1·5 Hz); *m/e* 302 (54%, M^+), 260 (75), 217 (45), 164 (70), and 136 (100) (Found: C, 75·2; H, 8·8. $C_{19}H_{26}O_3$ requires C, 75·45; H, 8·65%).

Oxidation of the Keto-diol (8c).—Impure keto-diol (8c) (31 mg) was oxidised with Jones reagent as for (8b). The crude product was subjected to preparative t.l.c. (ethyl acetate–light petroleum, 2 : 3) to give the trione (9) (19 mg), m.p. 140—142° (from ether), identical (n.m.r. and i.r. and mass spectra) with a sample of (9) prepared from (8b); mixed m.p. 140—142°.

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