Tetrahedron. Vol. 25, pp. 1241 to 1248. Pergamon Press 1969. Printed in Great Britain

RUBROSTERONE, A METABOLITE OF INSECT METAMORPHOSING SUBSTANCE FROM *ACHYRANTHES RUBROFUSCA*: STRUCTURE AND ABSOLUTE CONFIGURATION†

T. TAKEMOTO, Y. HIKINO and H. HIKINO

Pharmaceutical Institute, School of Medicine, Tohoku University, Aoba-yama, Sendai, Japan

and

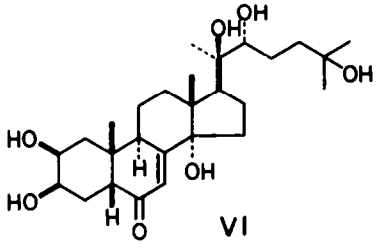
S. OGAWA and N. NISHIMOTO

Research Laboratories, Rohto Pharmaceutical Co. Ltd., Ikuno, Osaka, Japan

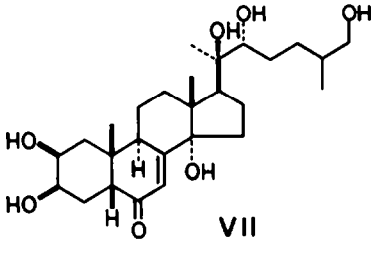
(*Received in Japan* 21 *September* 1968; *Received in the UK for publication* 15 *October* 1968)

Abstract—A novel C_{19} steroid, rubrosterone, isolated first from *Achyranthes rubrofusca* and later from *A. fauriei* (Amaranthaceae), has the stereostructure I on the basis of the chemical and physico-chemical properties of the steroid and its diacetate.

RECENTLY the insect-metamorphosing steroids, ecdysterone (VI) and inokosterone (VII), were isolated from the methanol extract of *Achyranthes rubrofusca* Wight

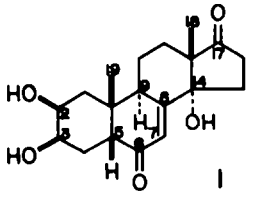


VI



VII

(Amaranthaceae).[1,2] Further detailed survey of the polar fraction of the extract resulted in the isolation of a minor component, a novel C_{19} steroid, which is less polar than ecdysterone and inokosterone, and shows positive color tests for steroids. The steroid is now termed rubrosterone. Later rubrosterone was isolated also from the methanol extract of *A. fauriei* Léveillé et Vaniot,[2] the first plant source of ecdysterone and inokosterone.[3] In the present communication we wish to provide evidence that rubrosterone is represented by stereoformula I.†



I

† This paper constitutes Part IV in the series on Steroids. Part III, H. Hikino, S. Nabetani, K. Nomoto, T. Arai, T. Takemoto, T. Otaka and M. Uchiyama, *Yakugaku Zusshi* in press.

† Part of the material presented herein formed a preliminary communication, *Tetrahedron Letters* 3053 (1968).

Rubrosterone analysed for $C_{19}H_{26}O_5$ and this was confirmed by the molecular weight, determined mass spectroscopically. The IR spectrum (Fig. 1) is similar to the

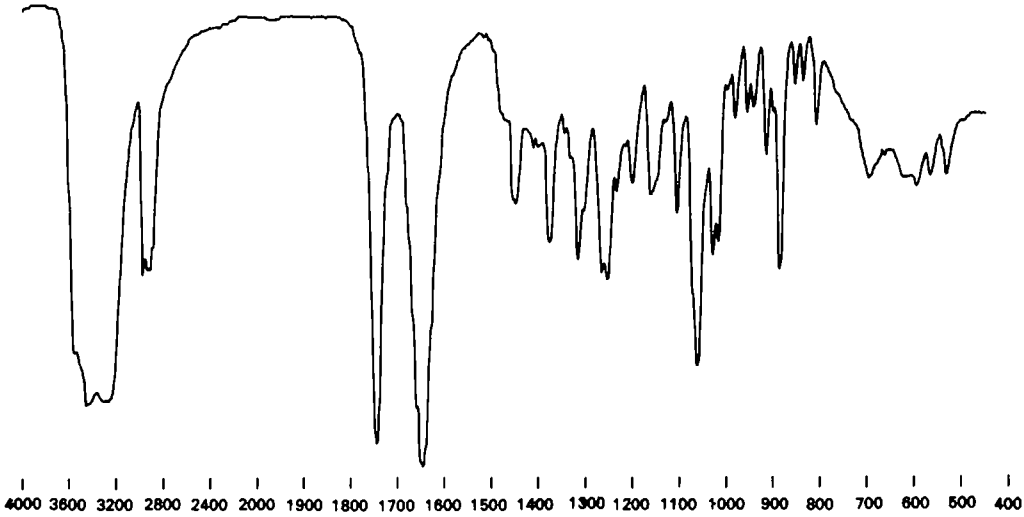
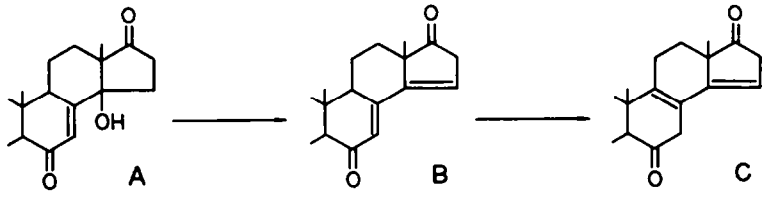


FIG. 1 IR spectrum of rubrosterone (KBr).

spectra of the common ecdysterols, e.g. ecdysone (IV). Indeed, a strong band at 3410 cm^{-1} (OH) and a characteristic band at 1646 cm^{-1} (cyclohexenone) are visible. The most significant feature in the IR spectrum is a band at 1741 cm^{-1} (cyclopentanone). In accord with the last function rubrosterone gave a 2,4-dinitrophenylhydrazone. An absorption maximum at 240 mμ in the UV spectrum, and a vinyl proton signal at 6·23 ppm in the NMR spectrum, only long-range coupled to an allylic proton, as well as the enone band in the IR spectrum demonstrate the presence of a β,β-disubstituted α,β-unsaturated ketone moiety which is ascribable to a 7-en-6-one system in the steroid nucleus. The CD curve of rubrosterone shows a positive Cotton effect centered at 342 mμ (Fig. 2). The similarity of the curve in the region 325–400 mμ to that of ecdysone (IV) is indicative of a like environment for the enone system in rubrosterone, the β-orientation of the C-5 hydrogen being deduced. Further, on acid treatment, rubrosterone gave two products which exhibited UV maxima at 295 and 241 mμ. The sequence of reactions can be represented as in the 14-hydroxy-7-en-6-one (A) → the 7,14-dien-6-one (B) → the 8,14-dien-6-one chromophore (C). Accepting these conclusions the part-structure can be expanded to a 14-hydroxy-7-en-6-one system in the 5β-steroid nucleus.



The significant difference between rubrosterone and the other common ecdysterols is that rubrosterone possesses only two Me groups (both tertiary) as evidenced by two Me singlets at 0·85 and 1·02 ppm (Fig. 3), and has only two secondary OH groups

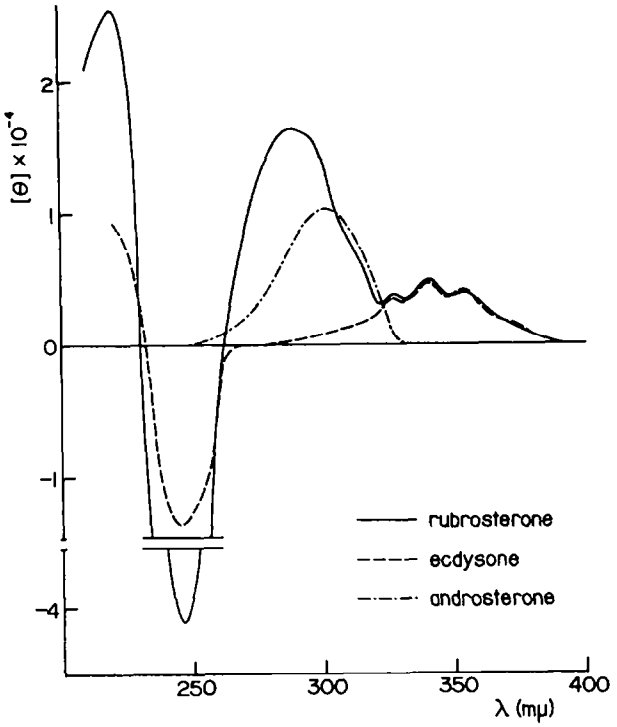


FIG. 2 CD curves of rubrosterone, ecdysone and androsterone (dioxan).

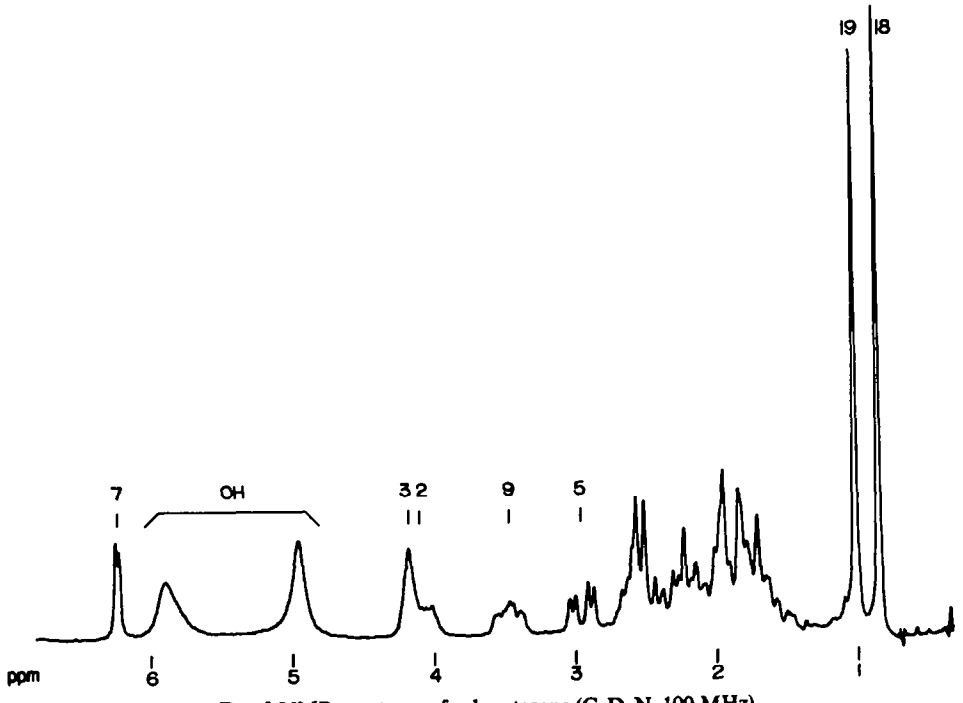
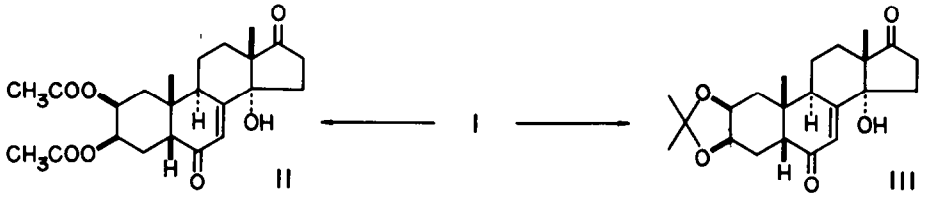


FIG. 3 NMR spectrum of rubrosterone (C_5D_5N, 100 MHz).

as shown by formation of the diacetate (II) which exhibits in the NMR spectrum two carbinyl hydrogen signals. Since the IR spectrum of the diacetate (II) still shows an absorption at 3430 cm^{-1} attributable to an OH group which, therefore, must be tertiary and as previously suggested should be located at C-14. Consequently, the five O atoms can be satisfactorily accounted for.



The environment of the two secondary OH's was clarified by NMR analysis. Thus double resonance experiments showed that both carbinyl hydrogens occurring at 4·94 and 5·32 ppm in the spectrum of the diacetate (II) are spin-coupled to each other (J = 3 Hz) and each hydrogen is further coupled to adjacent methylene hydrogens (partly shown in Fig. 4), indicating the presence of a —CH_2—CH(OH)—CH(OH)—CH_2— system which can only be placed at C-1–C-4 in the steroid skeleton.

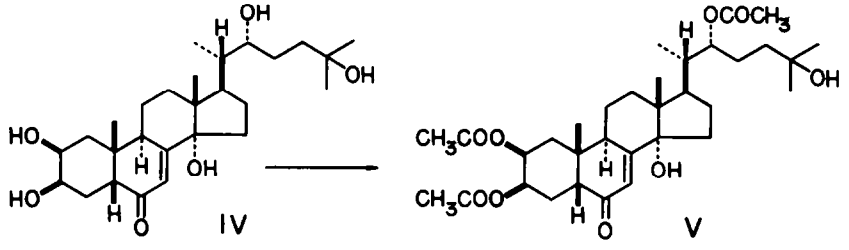


TABLE 1. PROTON SIGNALS ($CDCl_3$, 100 MHz)

	C-2α	C-3α	C-7	C-9	C-18	C-19	C-21	C-22	C-26	C-27
Ecdysone 2,3,22-triacetate	5·05 ddd	5·34 ddd	5·87 d	3·11 ddd	0·67 s	1·02 s	0·94 d	~4·9 ddd?	1·23 s	1·23 s
Rubrosterone 2,3-diacetate	4·94 ddd	5·32 ddd	5·94 d	3·11 ddd	0·85 s	1·04 s	—	—	—	—

Further, the line positions and the splitting patterns of the two carbinyl hydrogen signals coincide with those of ecdysone triacetate (V; Fig. 4 and Table 1), demonstrating that the two OH's at C-2 and C-3 are both β-oriented. This assignment was further supported by the following NMR evidence. Thus the C-19 Me hydrogens of ecdysone (IV) and its triacetate (V) appear at 1·07 and 1·02 ppm, respectively. The shift values on the C-19 Me hydrogens due to C-17β cholestane side-chains and C-17 oxo groups were derived from the C-19 Me signals of androstan-3α-ol (X), cholestan-3α-ol (XI), and androsterone (XII). The calculated chemical shifts of the C-19 Me hydrogens of compounds I and II thus deduced are in good accordance with the observed values for those of rubrosterone and its diacetate (Table 2). The presence of a single *cis*-1,2-glycol in rubrosterone was confirmed chemically by periodate

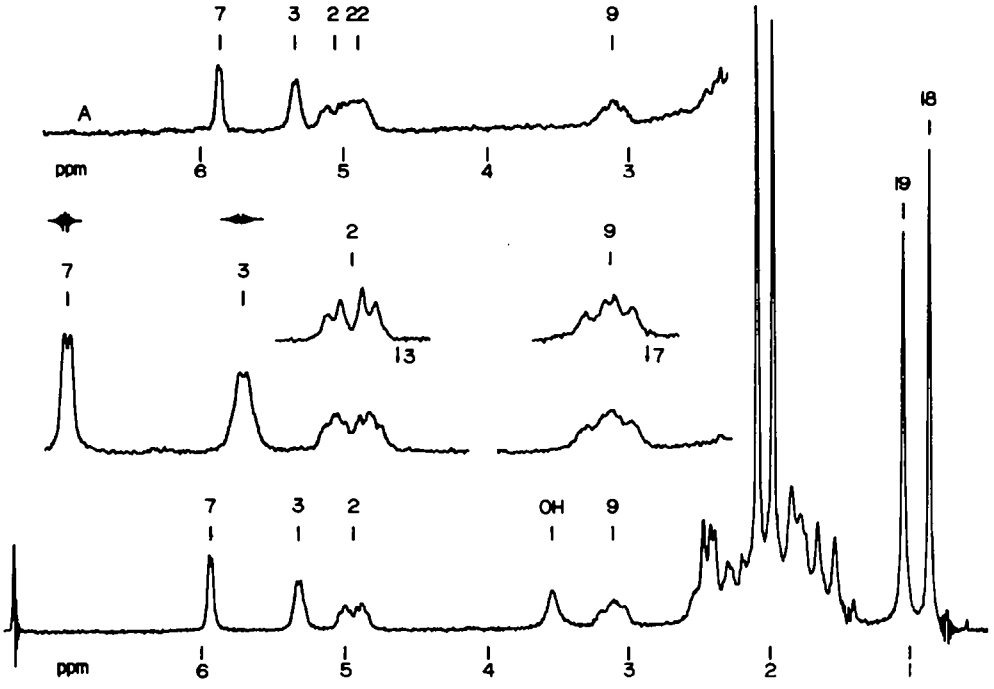
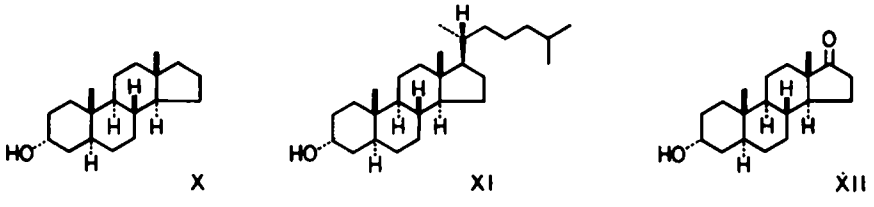


FIG. 4 NMR spectrum of rubrosterone diacetate ($CDCl_3$, 100 MHz). (A: that of ecdysone triacetate).

TABLE 2. METHYL CHEMICAL SHIFTS

	Observed δ for ecdysone		Shift values[a] due to 17β-R[b]		Shift values[a] due to 17-oxo		Calculated δ for I		Observed δ for rubrosterone	
	C-19	C-18	C-19	C-18	C-19	C-18	C-19	C-18	C-19	C-18
Alcohol (pyridine)	1·07	0·74	−0·03	−0·01	+0·02	−0·10	1·02	0·83	1·02	0·85
Acetate (chloroform)	1·02	0·67	+0·01	+0·05	−0·02	−0·15	1·05	0·87	1·04	0·85

[a] ppm, plus sign represents an upfield shift.
[b] R = cholestane side-chain.



oxidation which resulted in the rapid consumption of 1 molecule of the reagent, and by formation of the monoacetonide (III) on treatment with acetone in the presence of *p*-toluenesulfonic acid.

It is now required to prove the location of the remaining carbonyl group which must be situated in ring D since it is in a 5-membered ring. The previously observed

change in the UV spectrum of rubrosterone on acid treatment excludes from consideration the positions C-15 and C-16 for the carbonyl group. Thus, had it been present at C-15, the C-14 OH group would not have been eliminated to give a double bond at C-14:C-15, and had it been present at C-16, the UV absorption of the dehydration product would have occurred at longer wavelengths due to conjugation of the carbonyl. Therefore, the only position C-17 remained. The situation of the carbonyl group together with the α-orientation of the C-14 OH group were deduced by the following NMR evidence. The C-18 Me proton signals of ecdysone (IV) and its triacetate (V) appear at 0·74 and 0·67 ppm, respectively. The shift values for the C-18 Me resonances due to C-17β cholestane side-chains and C-17 oxo groupings in the C/D *trans*-steroids were derived as previously mentioned. The calculated chemical shifts of the C-18 Me protons of compounds I and II also agree well with the observed values for those of rubrosterone and its diacetate, respectively (Table 2). The partial structure of ring D was further examined by a CD analysis. Thus the CD curve of rubrosterone exhibited a positive Cotton effect ($[\theta]^{max}_{287}$ +16,400, dioxan) due to the n → π* transition of the C-17 carbonyl group, which is corresponding to that of a 17-oxo-steroid in the *trans* fused C/D system, e.g. androsterone (XII; $[\theta]^{max}_{303}$ +10,300, dioxan; Fig. 2).[4] It is worth noting that the CD maximum of rubrosterone showed a shift to shorter wavelength of 16 mμ in comparison with the corresponding maximum of androsterone (XII). This hypochromic shift of the CD maximum in question was again observed in methanol, i.e. rubrosterone showed $[\theta]^{max}_{287}$ +19,800 (methanol), while androsterone (XII) exhibited $[\theta]^{max}_{296}$ +11,300 (methanol). This anomaly of the CD curve of the C-17 oxo-steroid, rubrosterone, must be ascribable to the other part of the molecule. This phenomenon will provide an interesting subject in the field of CD study.

All the above observations allow the assignment of stereostructure I to rubrosterone which, then, is the first substance possessing the etiocholane skeleton isolated from plant sources. The stereostructure (I) has recently been confirmed by synthesis.[5]

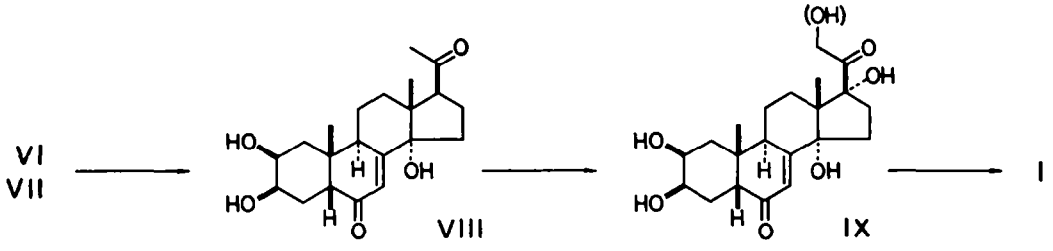
After the deduction of the stereostructure of rubrosterone, we were naturally interested in its biological activities. Rubrosterone was assayed for its activity upon the induction of puparium formation of isolated larval abdomens of the blow-fly (*Sarcophage peregrina*). As a result, it was found that rubrosterone showed only very weak activity (Table 3). It was reported that the methyl ketone (VIII), another possible metabolite of the insect-metamorphosing steroids, was inactive in the *Calliphora* test, but gave positive responses in induction of adult development of the brainless

TABLE 3. ASSAY OF RUBROSTERONE ON ISOLATED ABDOMENS OF THE BLOW-FLY, *Sarcophaga peregrina*

Dose (μg in 10% ethanol (5 μl)/animal)	Number of test animals	Number of pupation
10	15	5
1	15	2
0·1	15	0
0·01	15	0
0	15	0

pupae of the silk moth (*Samia cynthia*).[6] Therefore, in order to determine whether rubrosterone is able to induce imaginal development of dauer pupae (artificially made brainless pupae), 50 or 100 μg was injected into each of 10 dauerpupae (6♂ and 6♀) of the silk worm (*Bombyx mori*) in two groups, but no adult development was observed in 20 days following the injection. On the other hand, we have found that the ecdysterols show high stimulating effect on protein synthesis in mouse liver.[7] Consequently rubrosterone was assayed and found to show high anabolic activity.[8] It appears from the above observations that for metamorphosis of insects a certain side-chain structure as well as the nucleus structure is contributory, but for stimulation of protein synthesis in mouse a certain nucleus structure is essential and no side-chain is indispensable. A detailed study on the structure-activity relationship of ecdysone analogues is now in progress.

Rubrosterone is probably biosynthesized from the insect-metamorphosing steroids, ecdysterone (VI) and inokosterone (VII), *via* the methyl ketone (VIII) and



the hydroxymethyl ketone (IX). When Horn *et al.* isolated crustecdysone (ecdysterone (VI)?) from the extract of crayfish (*Jasus lalandii*), they suggested that it may be metabolized to the methyl ketone (VIII),[9] but later they concluded that this was unlikely since no methyl ketone (VIII) could be detected in the crayfish extract.[6] It is unreasonable, however, that a metabolic pathway is universally ruled out only because of an intermediate being not detected in one species, since the intermediate may be further metabolized at a rapid rate and not be accumulated, or a pathway absent in one species may be present in the other species. In fact, the present isolation of rubrosterone suggests the presence of the degradation sequence analogous to that operating in the case of cholesterol (from 20(*R*),22(*R*)-dihydroxycholesterol to dehydroepiandrosterone) at least in the plant kingdom. It was recently observed that the insect metamorphosing hormone, ecdysone (IV), is rapidly deactivated in insect larvae and pupae.[10] The metamorphosing hormones are very likely degraded through this pathway to give the less active metabolites in animals.

EXPERIMENTAL

M.ps are uncorrected. NMR spectra of the hydroxylated steroids and their acetates were recorded on a Varian HA-100 or a Hitachi H-60 spectrometer in C_5D_5N (C_5H_5N) and $CDCl_3$ ($CHCl_3$) soln, respectively. The chemical shifts are given in ppm from TMS as an internal reference and coupling constants (J) in Hz. Abbreviations: s = singlet. d = doublet, and dd = doublet of doublets.

Rubrosterone. Colorless prisms (from MeOH), m.p. 244–245 (dec), $[\alpha]_D$ +119 (*c* 0·86, MeOH), CD (*c* 0·0430, dioxan): $[\theta]_{400}$ 0, $[\theta]_{355}$ +3950, $[\theta]_{342}$ +4830, $[\theta]_{327}$ +3800, $[\theta]_{296}^{sh}$ +15,700, $[\theta]_{287}$ +16,400, $[\theta]_{262}$ 0, $[\theta]_{246}$ −41,100, $[\theta]_{230}$ 0, $[\theta]_{220}$ +25,400, $[\theta]_{210}$ +21,000, CD (*c* 0·0714, MeOH): $[\theta]_{400}$ 0, $[\theta]_{331}$ +6220, $[\theta]_{287}$ +19,800, $[\theta]_{266}$ 0, $[\theta]_{240}$ −34,000, $[\theta]_{232}$ 0, $[\theta]_{220}$ +27,000, $[\theta]_{210}$ + 20,100, UV λ_{max}^{EtOH} mμ

(log ε): 240 (4·07); IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 1741 (cyclopentanone), 1646 (cyclohexenone); NMR: 3H s at 0·85 ($C_{(18)}\underline{H}_3$), 3H s at 1·02 ($C_{(19)}\underline{H}_3$), 1H ddd at 3·47 (J = 2, 7, 10, $C_{(9)}\underline{H}$), 1H m at 4·10 ($C_{(2)}\underline{H}$), 1H broad s at 4·17 ($C_{(3)}\underline{H}$), 1H d at 6·23 (J = 2, $C_{(7)}\underline{H}$). MS *m/e*: 334 (molecular ion). (Found: C, 68·40; H, 7·96. $C_{19}H_{26}O_5$ requires: C, 68·24; H, 7·84%). Liebermann-Burchard reaction: positive (red), Salkowski reaction: positive (red), Tschugaeff reaction: positive (orange).

Rubrosterone 2,4-dinitrophenylhydrazone, prepared in the customary manner ($(NO_2)_2C_6H_3NHNH_2$–H_2SO_4–EtOH, room temp) and purified by chromatography over silica gel, crystallized from AcOEt–hexane to give orange needles, m.p. 310° (dec); IR ν_{max}^{KBr} cm^{-1}: 3580–3330 (OH, NH), 1661, 1617, 1593, 1514, 1333 (phenylhydrazone). (Found: C, 58·85; H, 5·98; N, 11·23. $C_{25}H_{30}O_8N_4$ requires: C, 58·36; H, 5·88; N, 10·89%).

Acetylation of rubrosterone. Rubrosterone (100 mg) in Ac_2O (1 ml) and pyridine (1 ml) was set aside at room temp overnight. The mixture was diluted with water and the deposited crystals were collected by filtration and crystallized from AcOEt–hexane to afford *rubrosterone diacetate* (II) as colorless plates, m.p. 203–204°. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1740 (cyclopentanone, acetoxyl), 1656 (cyclohexenone), 1242 (acetoxyl); NMR: 3H s at 0·85 ($C_{(18)}\underline{H}_3$), 3H s at 1·04 ($C_{(19)}\underline{H}_3$), 1H ddd at 3·11 (J = 2, 7, 10, $C_{(9)}\underline{H}$), 1H ddd at 4·94 (J = 5, 12, 3, $C_{(2)}\underline{H}$), 1H ddd at 5·32 (J = 3, 4, 4, $C_{(3)}\underline{H}$), 1H d at 5·94 (J = 2, $C_{(7)}\underline{H}$). (Found: C, 65·69; H, 7·03. $C_{23}H_{30}O_7$ requires: C, 66·01; H, 7·23%).

Periodate oxidation of rubrosterone. Rubrosterone (4 mg), dissolved in $NaIO_4$ soln (31·8 mg/ml), was left standing at room temp and titrated with N/50 $Na_2S_2O_3$ in the usual way. After 1 hr the uptake of oxidant ceased with consumption of 1 molecule (0·966 and 1·03 mle in two runs).

Acetonide formation of rubrosterone. Rubrosterone (70 mg) in acetone (10 ml) in the presence of *p*-toluenesulfonic acid (120 mg) was kept at room temp for 52 hr. The mixture was diluted with water and neutralized with Na_2CO_3. The organic solvent was removed by distillation under reduced press and the residue was chromatographed on silica gel (10 g). Elution with AcOEt and crystallized from MeOH–AcOEt to yield *rubrosterone acetonide* (III) as colorless prisms, m.p. 247–248·5, IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1731 (cyclopentanone), 1677 (cyclohexenone). (Found: C, 70·58; H, 7·96. $C_{22}H_{30}O_5$ requires: C, 70·56; H, 8·08%).

Acid treatment of rubrosterone. Rubrosterone (0·2 mg) in dil HCl (conc HCl–EtOH = 1:100, 1 ml) was heated under reflux for 1 hr giving the mixture of the 7,14-dien-6-one and the 8,14-dien-6-one, UV $\lambda_{max}^{EtOH(HCl)}$ mμ: 295, 241.

Ecdysone. CD (*c* 0·0392, dioxan): $[\theta]_{400}$ 0, $[\theta]_{367}$ +1760, $[\theta]_{354}$ +4110, $[\theta]_{340}$ +4730, $[\theta]_{326}$ +3520, $[\theta]_{280}$ 0, $[\theta]_{245}$ −13,700, $[\theta]_{231}$ 0, $[\theta]_{220}$ +9940, $[\theta]_{213}$ +7660, CD (*c* 0·0462, MeOH): $[\theta]_{400}$ 0, $[\theta]_{330}$ +5570, $[\theta]_{285}$ 0, $[\theta]_{251}$ −20,600, $[\theta]_{228}$ 0.

Androsterone. CD (*c* 0·0864, dioxan): $[\theta]_{330}$ 0, $[\theta]_{303}$ +10,300, $[\theta]_{250}$ 0, CD (*c* 0·0881, MeOH): $[\theta]_{340}$ 0, $[\theta]_{296}$ +11,300, $[\theta]_{240}$ 0.

Acknowledgements—We are indebted to Dr. M. C. Woods, Varian Associates, and Miss Y. Tadano, this Institute, for the NMR spectra; to Dr. K. Kuriyama, Research Laboratory, Shionogi & Co. Ltd., for the CD curves; to Research Laboratories, Takeda Chemical Industries Ltd., for the mass spectrum; and to Dr. M. Kobayashi, Sericultural Experiment Station, for the biological test (silk worm).

REFERENCES

[1] T. Takemoto, S. Ogawa, N. Nishimoto and S. Taniguchi, *Yakugaku Zasshi* **87**, 1478 (1967).
[2] T. Takemoto, S. Ogawa, N. Nishimoto, H. Hirayama and S. Taniguchi, unpublished data.
[3] T. Takemoto, S. Ogawa and N. Nishimoto, *Yakugaku Zasshi* **87**, 325 (1967); **87**, 1469 (1967); **87**, 1474 (1967).
[4] cf. also M. Fetizon and M. Golfier, *Bull. Soc. Chim. Fr.* 859 (1966).
[5] H. Hikino, Y. Hikino and T. Takemoto, *Tetrahedron Letters* 4255 (1968).
[6] J. B. Siddall, D. H. S. Horn and E. J. Middleton, *Chem. Comm.* 889 (1967).
[7] S. Okui, T. Otaka, M. Uchiyama, T. Takemoto, H. Hikino, S. Ogawa and N. Nishimoto, *Chem. Pharm. Bull. (Tokyo)* **16**, 384 (1968).
[8] T. Otaka, M. Uchiyama, S. Okui, T. Takemoto, H. Hikino, S. Ogawa and N. Nishimoto, *Ibid.* **16**, 2426 (1968).
[9] D. H. S. Horn, E. J. Middleton, J. A. Wunderlich and F. Hamphshire, *Chem. Comm.* 339 (1966).
[10] T. Ohtaki and C. M. Williams, unpublished data.