20,26-Dihydroxyecdysone, a New Steroid with Moulting Hormone Activity from the Tobacco Hornworm, *Manduca sexta* (Johannson)

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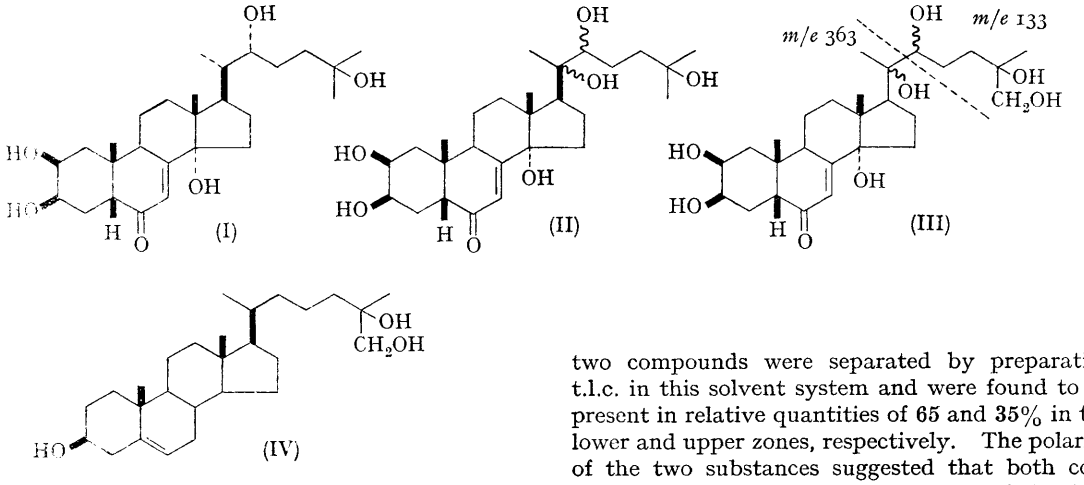
IN a previous paper,[1] we reported on the isolation and identification of α-ecdysone (I) and 20-hydroxyecdysone (II) from 7-day-old pupae of the tobacco hornworm, *Manduca sexta* (Johannson). We have now isolated a third tobacco hornworm ecdysone (THE-III) and suggest structure (III) (2β,3β,14α,20ξ,22ξ,25,26-heptahydroxy-5β-cholest-7-en-6-one) for this new steroid. This ecdysone was detected by the house-fly assay[2] in tubes 2—6 that remained following the complete separation of α- and 20-hydroxy-ecdysone[1] by countercurrent distribution after 66 transfers in cyclohexane–butanol–water (5 : 5 : 10). In processing a large group of 7-day-old hornworm pupae (40·2 kg.) by methods similar to those previously described,[1] this more polar hormone was again found to be concentrated mainly in the earlier countercurrent distribution tubes 2—7. The pooled fractions (19 mg.) were further purified by repeated partitioning between equal volumes of water and cyclohexane–butanol (2 : 8), and the concentrate from the upper phase was chromatographed on silicic acid‡ as reported[1] for the purification of α- and 20-hydroxy-ecdysone. Monitoring of the column fractions by u.v analyses also showed the material to be more

‡ Unisil, 100—200 mesh, Clarkson Chemical Company, Williamsport, Pa.



polar than 20-hydroxyecdysone. Attempted recrystallization from ethyl acetate–methanol or benzene–methanol afforded 3 mg. of amorphous material which, when heated, consistently became transparent at 149—153°, λ_{max} at 245 mμ in methanol (ϵ 10,400).

The new hormone had an R_F value of 0·08 (*cf.* 0·37 for α-ecdysone and 0·24 for 20-hydroxyecdysone) on an activated silica gel G (Merck AG) thin-layer plate with chloroform–95% ethanol (80 : 20) as the solvent system. It was similar to α-ecdysone in that it gave a turquoise spot-sprayed with vanillin–sulphuric acid reagent;[3] 20-hydroxyecdysone developed as a yellow-green spot. The hormone, when subjected to thin-layer chromatography (t.l.c.) with the more polar solvent system chloroform–95% ethanol (65 : 35) at 20°, separated into two zones (R_F 0·36 and 0·45). The two compounds were separated by preparative t.l.c. in this solvent system and were found to be present in relative quantities of 65 and 35% in the lower and upper zones, respectively. The polarity of the two substances suggested that both contained one more hydroxy-group than (II). The n.m.r. spectra of the compounds from both zones§ closely resembled the n.m.r. spectrum of (II) (Table) and permitted the assignment of the additional hydroxy-group at the C-26 position and the formation of structure (III) for the compound of the lower zone. Tobacco hornworm ecdysone-III ("THE-III") refers to the compound from the lower zone only. By the house-fly assay[2] one house-fly unit of THE-III was found to be 0·05—0·075 μg., that is, about 1/10 to 1/15 the biological activity of α-ecdysone.¶

Whereas THE-III exhibits four methyl resonance peaks of similar magnitude (Table) and also shows a methylene envelope[4] at 132 c./sec., α- and 20-hydroxy-ecdysone exhibit their strongest peaks at 139 and 136 c./sec., respectively [methyl protons C-OH(CH_3)$_2$]. The n.m.r. spectrum of model compound (IV), which shows the C-27

TABLE

Methyl resonances (c./sec.) (deuteriopyridine solution)

	C-18	C-19	C-21	C-27 and/or 26
20-Hydroxyecdysone (II)	121	107	158	136
THE-III	122	108	158	148
Upper Zone	121*	104*	156*	147*
Model compound (IV)	67*	107*	95*, 103*	149*

* Values obtained on 60 Mc./sec. instrument and converted to c./sec. at 100 Mc./sec.

§ We thank Dr. E. L. Gooden of this Division for this spectrum. The n.m.r. spectrum was determined in deuteriopyridine using a Varian HR-100 spectrometer equipped with a C-1024 time-averaging computer, and the signal was locked down-field on benzene.

¶ We acknowledge the assistance of Mr. B. M. Bryce, Mrs. E. M. Jensen, Miss P. Sterling, and Mr. L. Tabor of this laboratory.

methyl resonance at 149 c./sec., further supports the suggested structure. Both THE-III and 20-hydroxyecdysone exhibit their C-19, C-18, and C-21 methyl resonances in similar regions. Since the resonance frequency of angular methyl protons is dependent on both the nature and orientation of substituent groups in the steroid skeleton, it is most likely that THE-III and 20-hydroxyecdysone have a similar steroid nucleus with similarly oriented substituents.

The infrared spectrum showed the hydroxyl absorption band at 3430 cm.$^{-1}$ and the $\alpha\beta$-unsaturated ketone band at 1655 cm.$^{-1}$ THE-III and the compound from the upper zone, after treatment with hot methanolic hydrochloric acid solution, showed peaks at 300 and 244 mμ. α-Ecdysone and 20-hydroxyecdysone underwent a similar change to the elimination of the 14-hydroxy-group which resulted in the formation of products having λ_{max} at 293 and 244 mμ ($\Delta^{7,14}$-6-one and $\Delta^{8(9),14}$-6-one chromophores, respectively[5]). Thus, it is most probable that THE-III and its faster moving companion both have the 7-en-6-one chromophore and the 14-hydroxy-group.

Although comparison of the n.m.r. and chemical data of THE-III and of the material from the upper t.l.c. zone with those of α- and 20-hydroxy-ecdysone permits the assignment of the Δ^7-6-one and 14-hydroxy-groups and the 20-, 22-, 25-, and 26-hydroxy-groups, the mass-spectral data†† of THE-III fully supports the assigned structure (III). Because of the tendency of these highly hydroxylated compounds to eliminate water, the highest mass number obtained by mass-spectroscopic analyses for THE-III (M 496·62) and the less polar companion was 442, indicating that three molecules of water already had been eliminated. The peak at m/e 427 ($M - 54 - 15$) showed loss of methyl, and major peaks at m/e 409 and 391 showed that two additional molecules of water could still be eliminated. The major peaks at m/e 133 and 115 are expected side-chain fragments for structure (III) and disclose the C-20–C-22 bond cleavage without rearrangement that occurs in 20-hydroxyecdysone.[1,6] The fragmentation pattern also places the additional hydroxy-group in the side-chain and not in the steroid nucleus. This is further demonstrated by major peaks at m/e 363 ($M - 133$) and 345 ($M - 133 - 18$). These peaks at m/e 363 and 345 are also present in the spectra of (II) and indicate that the fragments remaining after cleavage of the side-chain are similar in both compounds. The intense peak at mass 31 (CH_2OH^+) agrees with the 1,2-glycol substituent[7] shown in structure (III). The prominent peak at m/e 427 ($M - 54 - 15$) showing the loss of a methyl in THE-III that is typical of terminal methyl 1,2-glycols[7] also favours the assigned structure (III) since loss of this ion is not so discernible in the mass spectra of (II). The mass-spectral data of THE-III and the less polar substance are quite similar for the major fragments, but they differ considerably in their overall fragmentation patterns. The chemical and spectral data presented above clearly demonstrate the structural similarity of 20-hydroxyecdysone and THE-III, except that THE-III has an additional hydroxy-group at C-26 as shown in structure (III).

All the accumulated data, then, indicate that THE-III is a 20,26-dihydroxyecdysone. The less polar material that accompanies it and is only separated from it by t.l.c. is most likely a stereoisomer of (III). The n.m.r. spectrum of this less polar material shows a difference of 4 c./sec. for the C-19 methyl when compared with THE-III. Thus, one is tempted to speculate that the differences in the two compounds lie within the C-19 environment and suggest that it is a 5α-isomer. Its t.l.c. behaviour is also consistent with our finding that the 5α-isomer of 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one is the faster moving component. However, additional data are required before we can definitely state that the compound from the upper zone is a 5α-isomer of THE-III.

Studies to date have shown that both the α- and 20-hydroxy-ecdysone exist in the same insect.[1,8,9] We have found 20-hydroxyecdysone to be the major moulting hormone in 7-day-old hornworm pupae, and biological tests indicate that this compound is equally active to, or somewhat more active than, α-ecdysone.[1,9,10] The fact that 20,26-dihydroxyecdysone is less active than either α-ecdysone or 20-hydroxyecdysone indicates that increased hydroxylation is a mechanism in insects for the deactivation of the ecdysones. If the 20,26-dihydroxyecdysone is a metabolic product of the more active ecdysones, then we could expect an increase in titre of this compound in the later period of pupal development, and this is currently being investigated.

Three different ecdysones have been isolated and identified from tobacco hornworm pupae examined at their maximum titre of moulting hormone activity. Although both the structures and biological activity indicate that these three

†† We thank Dr. H. Fales of the National Institutes of Health for the mass spectra.

hormones—α-ecdysone (I), 20-hydroxyecdysone (II), and 20,26-dihydroxyecdysone (III)—are intermediates in a biosynthetic scheme, each could have a specific function(s).

(*Received, April 10th*, 1967; *Com.* 340.)

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