# THE ECDYSONES

KOJI NAKANISHI

*Department of Chemistry, Columbia University, New York, N.Y. 10027, USA.*

## ABSTRACT

The discovery of ecdysones, moulting hormones of insects and crustaceans, from plants in late 1966 has resulted in subsequent isolations of a total of about 40 new phytoecdysones. This has contributed a great deal to the understanding of the chemistry and physicochemical data of ecdysones. A general account of these aspects is given, together with results of preliminary bio-organic studies concerning the metabolic fate of exogenous ecdysones and the site of biosynthesis of ecdysones in the silkworm. Finally, very recently several plants have been discovered to contain substances which inhibit the moulting as bioassayed by the topical dipping method. The structure of the first to be elucidated, ajugalactone, is discussed. The second inhibitor to be isolated is interesting because it is a non-steroid. Another intriguing aspect of these inhibitors is their specificity towards the ecdysone structure.

## INTRODUCTION

The Chinese antitumour remedy 'Pai-ju-chin' or leaves of *Podocarpus nakaii* HAY, was one of the herbs we were investigating in 1964 at Tohoku University, Sendai, in collaboration with Dr H. Y. Hsu and co-workers (Taipei). Several closely related constituents were isolated but, as is frequently the case, none suppressed tumour growth. They were not pleasant compounds to work with because of poor crystalline properties. Nevertheless, we undertook structural studies because they seemed to be new $C_{29}$ nortriterpenoids. The molecular ions could not be measured with the earlier inlet system, and it was not until mid-1966 that the molecular formula of the major compound was established as $C_{27}H_{44}O_6$.

While attending Professor P. Karlson's lecture on ecdysones and other insect hormones[1] at the Fourth IUPAC International Symposium on the Chemistry of Natural Products, 1966, Stockholm, I had no idea that, within a month, we would become involved in this fascinating but unfamiliar field. However, upon my return to Sendai, the $C_{29}$ formulae were revised to $C_{27}$, and then it was a relatively simple matter to deduce the structure which today represents ponasterone A. The structural similarity with α-ecdysone was obvious, and the structure was reported in August 1966[2] with a comment that it might be ecdysone active. Later, we received exciting reports from Drs T. Okauchi (Takeda Chemical Industries, Osaka) and D. H. S. Horn (CSIRO, Melbourne) who had kindly carried out bioassays on *Samia cynthia* and *Calliphora*, respectively. Clearly, if the structure of α-ecdysone had not been elucidated the preceding year[4], the *P. Nakaii* constituent would have been reported as merely

being a new polyhydroxy steroid. Independently, Professor T. Takemoto and co-workers (Department of Pharmacy, Tohoku University, Sendai) were investigating the crude drug *Achyranthes fauriei* and characterized β-ecdysone and inokosterone. These were the first demonstrations of the occurrence of ecdysones in plants[3, 5], which resulted in the discovery of nearly 30 active compounds (*Table 1*) during the ensuing three years. Most of the plants from which ecdysones were isolated have already been investigated in the past, but these polyhydroxy steroids presumably eluded detection because of their relatively high solubility in water. In any case, the plants have now made available large quantities of various ecdysones for basic and practical studies.

Very recently, M. Goto *et al.* disovered that some plants contain substances which antagonize the action of ecdysones, as assayed by the Chilo dipping method. Further studies are obviously required, but this new class of compounds may contribute to our understanding of ecdysone action, and suggest an approach for new means of pest control.

A general account of the structural aspects of ecdysones, preliminary results on bio-organic studies of ecdysones and structural studies of the first natural ecdysone inhibitor is presented in the following.

## THE VARIOUS ECDYSONES

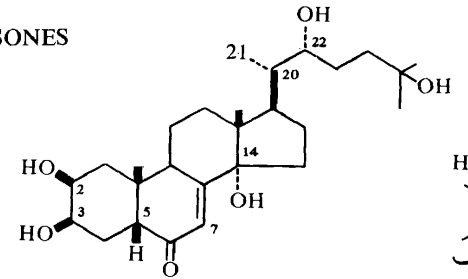
*Figure 1* depicts all known ecdysones to date, while representative members of zooecdysones* and phytoecdysones* characterized in the earlier stage are shown in *Figure 2*.

The first moulting hormone, α-ecdysone[6–8] was isolated in 1954 by Butenandt and Karlson; the structure determination was hampered by the limited amount but was elucidated in 1965[9] by means of x-ray crystallography[4] without usage of heavy atom derivatives. This is still the sole ecdysone for which the entire stereochemistry, including C-20 and C-22, has been elucidated. In the initial isolation, 500 kg of silkworm (*Bombyx mori*) pupae gave 25 mg of crystalline α-ecdysone[6], which corresponds to about 50 per cent of the original content[1]. Shortly after structure determination, the Schering/Hoffmann–La Roche[10] and Syntex[11] groups succeeded in its synthesis and, more recently, the Teikoku Hormone group[12] announced its synthesis. From plants, it was first isolated from *Polypodium vulgare* L.[13a] and from *Pteridinium aquilinum* (bracken fern)[13b], but its distribution in plants is not as widespread as β-ecdysone or ponasterone A; also, it has not so far been isolated from Crustacea.

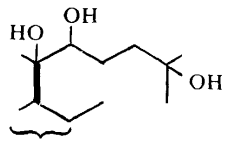
The second zooecdysone isolated from *Bombyx* and designated β-ecdysone was not initially available in large enough quantities for structure studies (0.33 mg)[14], but subsequently several groups isolated it from various sources and determined its structure at about the same time: crustecdysone[15], 2 mg from 1 ton of *Jasus lalandei* (crayfish) waste (this was the first isolation of a crustacean moulting hormone); 20-hydroxyecdysone from *Bombyx*[16]; ecdysterone from *Bombyx*[17]; and β-ecdysone from *Manduca sexta* (tobacco hornworm) [18]. The terminology is confusing but the hormones from different sources have been

* Since the discovery of ecdysone-active hydroxy steroids from plants, it is convenient, in some cases, to differentiate those with the prefix phyto- from those isolated from insects ;and Crustacea (zooecdysones). However, most zooecdysones are present in plants as well.

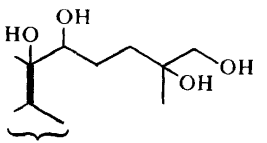
## ZOOECDYSONES



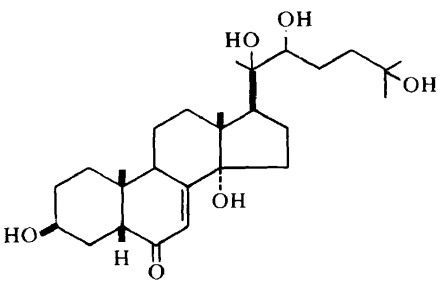

α-ecdysone
*Bombyx mori* (silkworm) (1954)[a]
structure, 1964[b]



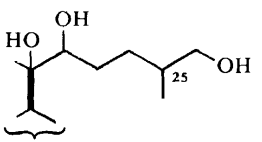

β-ecdysone
*B.mori* (1956)[c]
*Jasus lalandei* (1966)[d]
(sea-water crayfish)
*B.mori*(1966)[e, f]



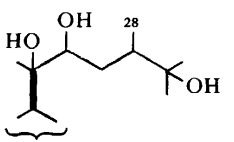

20,26-dihydroxy-α-ecdysone
*Manduca sexta* (1967)[g]
(tobacco hornworm)




2-deoxy-β-ecdysone
*J. lalandei* (1968)[h]




callinecdysone A
(≃inokosterone)

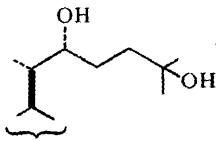



callinecdysone B
(≃makisterone A)

*Callinectes sapidus* (1969)[i]
(marine crab)

PHYTOECDYSONES—I

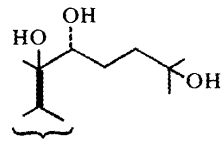
$C_{27}$-analogues



α-ecdysone

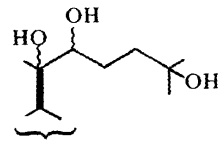
*Pteridium aquilinum* (1967)[j]

*Polypodium vulgare* (1967)[k]



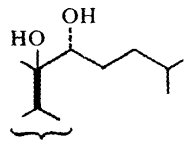
β-ecdysone

*Achyranthes fauriei* (1967)[l]



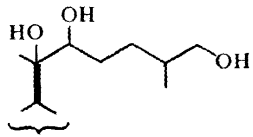
shidasterone

*Blechnum niponicum* (1968)[m]



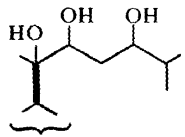
ponasterone A

*Podocarpus nakaii* (1966)[n]



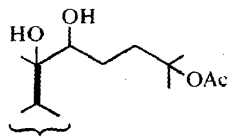
inokosterone

*Achyranthes fauriei* (1967)[l]



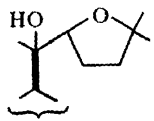
pterosterone

*Lastrea thelpteris* (1968)[o]

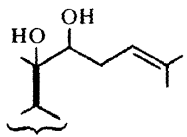


viticosterone E

*Vitex megapotamica* (1969)[p]



stachysterone D



stachysterone C

*Stachyurus praecox* (1970)[q]

PHYTOECDYSONES—II



ponasterone B

*Podocarpus nakaii* (1968)[r]



ponasterone C

*P. nakaii* (1970)[s]



polypodine B

*Polypodium vulgare* (1967)[t]



ajugasterone C

*Ajuga japonica* (1969)[u]



β-D-glu

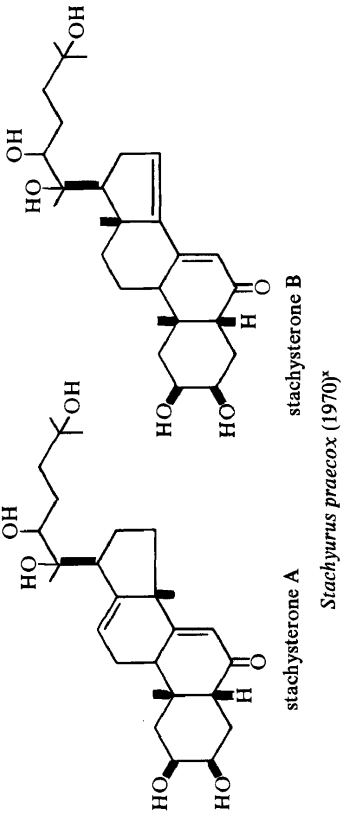
ponasteroside A

*Pteridium aquilium* var *latiusculum* (1968)[v]



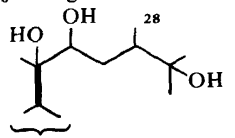
podecdysone B

*Podocarpus elatus* (1969)[w]



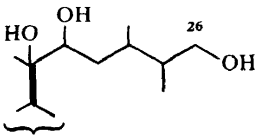

*Stachyurus praecox* (1970)[8]

$C_{28}$-analogues




makisterone A

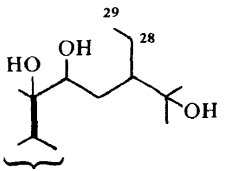
*Podocarpus macrophyllus* (1968)[y]




makisterone B

*P. macrophyllus* (1968)[z]

$C_{29}$-analogues




podecdysone A (1968)

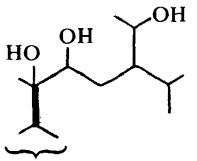
*Podocarpus elatus*[aa]

makisterone C (1968)

*P. macrophyllus*[z]

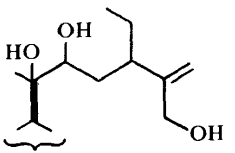
lemmasterone

*Lemmaphyllum microphyllum*[11]



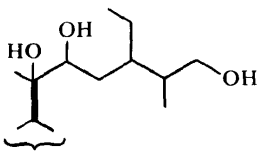

makisterone D

*P. macrophyllus* (1968)[z]



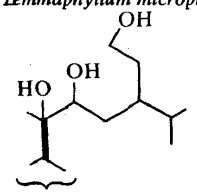

ajugasterone B

*Ajuga incisa* (1969)[bb]



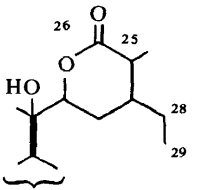

amarasterone A

*Cyathula capitata* (1968)[cc]



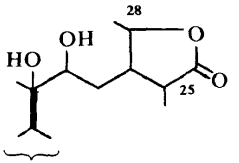

amarasterone B

*C. capitata* (1968)[cc]




capitasterone

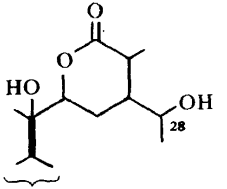
*C. capitata* (1968)[dd]




cyasterone (1967)[ee]

25-epicyasterone (1970)[ff]

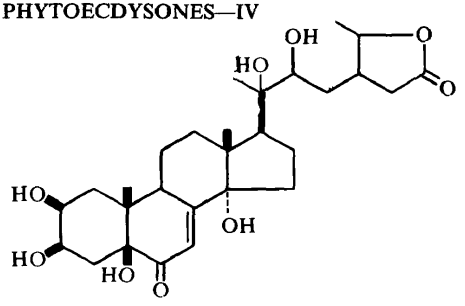
*C. capitata*




precyasterone

*C. capitata* (1970)[gg]

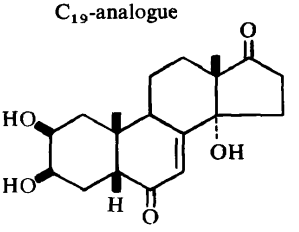
PHYTOECDYSONES—IV



sengosterone

*Cyathula capitata* (1969)[hh]

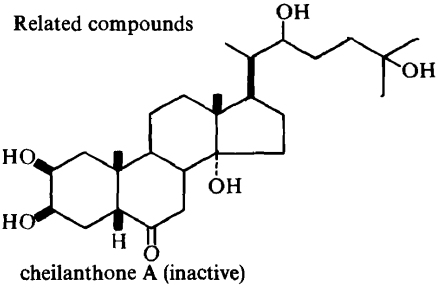
$C_{19}$-analogue



rubrosterone

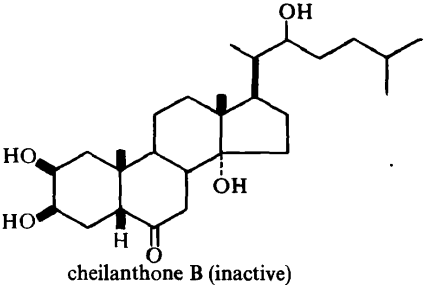
*Achyranthes rubrofusca* (1968)[ii]
(very weak activity)

Related compounds



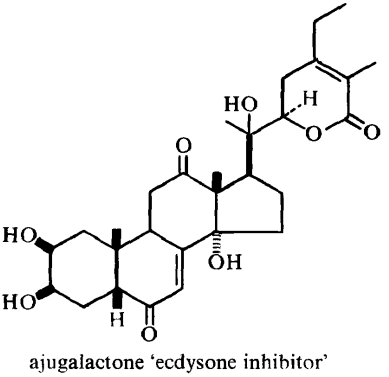
cheilanthone A (inactive)

*Cheilanthes tenuifolia*[jj]



cheilanthone B (inactive)

*C. tenuifolia* (1970)[jj]



ajugalactone 'ecdysone inhibitor'

*Ajuga decumbens* (1970)[kk]

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[ll] T. Takemoto, Y. Hikino, T. Arai and H. Hikino, *Tetrahedron Letters*, 4061 (1968).

*Figure 1.* Zooecdysones, phytoecdysones and related compounds. Generally only the first isolation or structure determination is quoted.

found to be identical[19], and in the following we shall call it β-ecdysone, a name which has been used for many years by insect physiologists. Several groups have reported its synthesis[20, 21]. Together with inokosterone, β-ecdysone was the first phytoecdysone isolated by Takemoto[5]. Isolated also from *P. elatus* in an early stage[22], β-ecdysone is the most widely distributed ecdysone in plants and insects.

Ponasterone A which we had initially obtained from *P. Nakaii*[2, 3] together with ponasterones B and C[3, 23, 24], is a phytoecdysone with a wide distribution in plants. From 5 kg of *P. Nakaii* leaves, about 6 g of a mixture of ponasterones A, B and C can be isolated. The moulting activity of ponasterones[25] is dependent on the test organisms but is generally comparable to or more potent than β-ecdysone, which in turn is roughly ten times more active than α-ecdysone[26]. Ponasterone C is one of the ecdysones with a 5β-hydroxyl group, which was

first encountered in polypodine B (see *Table 1*) by Jizba *et al.*[27] (isolated in the high yield of one per cent from *Polypodium vulgare* L.).

Inokosterone[5] appears to be an epimeric mixture at C-25[28]. It has also been extracted from the crab, *Callinectus sapidus*[29]. As depicted in *Figure 1*, the majority of ecdysones have the 2β, 3β, 14α, 20, 22-pentahydroxy-7-en-6-one

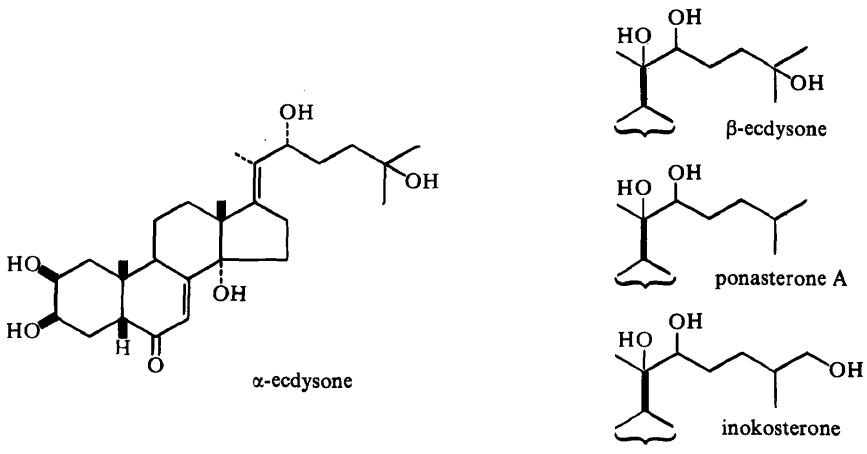



*Figure 2.* Typical zoo- and phyto-ecdysones.

system. Four of them have a 5β-OH group, exemplified by polypodine B. The variation is mainly in the sidechain but none is substituted at C-23. Nuclei of phytoecdysones range from $C_{27}$ to $C_{29}$ (excepting rubrosterone[30] lacking the sidechain—this is a biogenetically interesting compound but is almost devoid of activity), but it is interesting that the $C_{28}$ callinecdysone B (identical with makisterone A[31] or its C-24 epimer)[29] is present in the crab, *C. sapidus*. Stachysterone A is the first ecdysone having a rearranged steroid nucleus[32].

## SCREENING AND ISOLATION OF PHYTOECDYSONES

When ponasterones, β-ecdysone and inokosterone were discovered, it was suspected that ecdysones might be widely present in other plants. We theretore devised a general extraction procedure for these rather water-soluble compounds[33]. Takemoto *et al.* had carried out the screening of about 180 crude drugs and plants[34]. However, a most extensive screening was undertaken by the Takeda group[35], in which 1056 species (1845 samples) of plants, carefully selected from 738 genera of 186 families (total number of families in Japan is about 190), and an additional 350 crude drugs were screened for their activity. This disclosed 56 active species and has resulted in many of the new phytoecdysones shown in *Figure 1*. There appears to be, however, no clear correlation between the distribution of active plants and chemotaxonomy. Two new techniques were essential for the accomplishment of this extensive screening, namely, the *Chilo* dipping test[36] and automatic high-pressure liquid chromatography[37]. Bioassay for moulting hormone is carried out by injection

into *Calliphora, Bombyx mori,* and other insects, but they are unsuited for rapid large-scale screening tests. In this first dipping method developed for bioassay, the fifth instar *Chilo suppressalis* (rice-stem borer, a 2 cm long larva weighing about 70 mg) is ligated and dipped for 10 sec in a methanol test solution; an active solution causes sclerotization and tanning of the abdomen after 24–48 hours. A dose of 0.5 to 1 μg ponasterone A per individual provoked 100 per cent pupation. The topical method has since been applied to *Galleria*

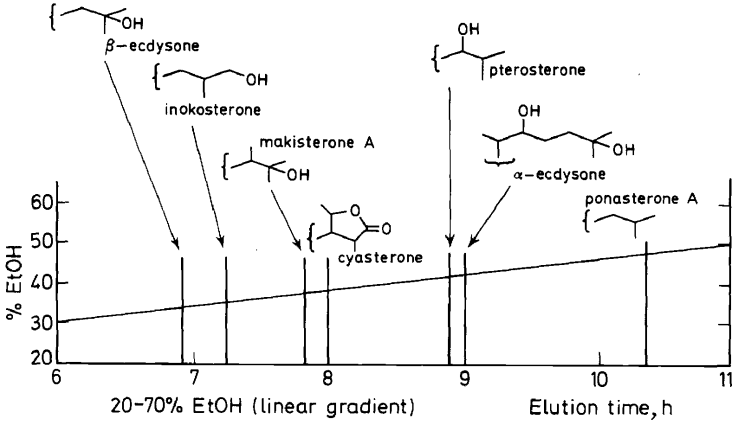



*Figure 3.* Automatic liquid chromatography (high-pressure) of ecdysones. Column: Amberlite XAD-2, 9 × 1500 mm (200–400 mesh); temp. 20°C; flowrate: 60 ml/h [adapted from ref. 37].

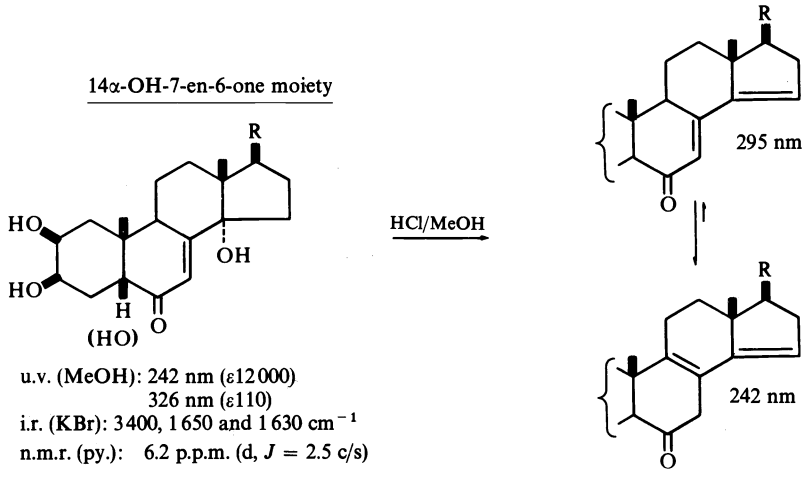
*mellonela*[38]. The pressurized liquid chromatography[37] is ideal for analytical and preparative work of ecdysones as the peaks are separated over a wide range (*Figure 3*) and retention times are highly reproducible. The peaks are detected by u.v. light and the chromatogram is developed by single solvents or linear or sigmoid gradient solvent mixtures. In favourable cases, a crude methanol plant extract can be submitted directly to this method[37]. Possibly the elution could be shortened greatly by recent improved instrumentation and packing materials.

## STRUCTURE ELUCIDATION OF ECDYSONES

The structures of 30 ecdysones have been determined to date, and the information accumulated during these studies now makes it possible to determine a new ecdysone structure with a few milligrammes (or less) of sample.

### The 14α-hydroxy-7-en-6-one moiety (*Figure 4*)

The u.v. extinction of the 242 nm band is useful for gaining information on sample purity because ecdysones have poor crystalline properties and in many cases the amount is too minute for analytical purification. The i.r. band at 1 650 $cm^{-1}$ is low for a conjugated enone (in KBr disc), which is presumably due to intermolecular hydrogen-bonding with a hydroxyl group in the solid phase. Because of solubility problems, the n.m.r. of free ecdysones are measured in




u.v. (MeOH): 242 nm (ε12000)
326 nm (ε110)
i.r. (KBr): 3400, 1650 and 1630 $cm^{-1}$
n.m.r. (py.): 6.2 p.p.m. (d, $J$ = 2.5 c/s)

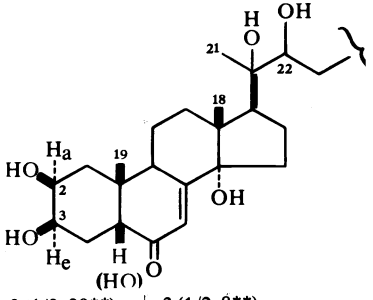
*Figure 4.* The 14α-hydroxy-7-en-6-one moiety.

pyridine. The 7–H appears constantly at 6.2 p.p.m. The n.m.r. peaks of carbinyl protons overlap and are of no diagnostic value.

The 14–OH can be characterized readily as treatment of a minute amount in MeOH/HCl at 80° gives rise to the dienone and deconjugated diene mixture[9] (checked by u.v.) with the equilibrium in favour of the latter presumably due to less strain; ring C of the dienone has two exocyclic double bonds. The 242 nm diene maximum is close to the maximum of the starting material but the two differ in polarity and are easily distinguishable by thin-layer chromatography (TLC). Several solvent systems for TLC of ecdysones have been reported[33]. The acid equilibrium reaction is applicable to 5β-hydroxyecdysones as they also give rise to two products having maxima at 294 and 242 nm.

**The n.m.r. data** [*Figure* 5(a), 5(b), 5(c)]

Free ecdysones are measured in pyridine [*Figure* 5(b)] while acetates are




General n.m.r. data in p.p.m. (and Hz)

free (in py.)

| | 18 | 19 | 21 | 22 (4, 8) |
|---|---|---|---|---|
| 5β-H | 1.19 | 1.06 | 1.54 | 3.80 |
| 5β-OH | 1.16 | 1.11 | 1.54 | 3.80 |

acetate (in chf.) (2, 3, 22-OAc)

| | 18 | 19 | 21 | 22 (4, 8)* | 2 (1/2:20**) | 3 (1/2:8**) |
|---|---|---|---|---|---|---|
| 5β-H | 0.85 | 1.02 | 1.24 | 4.8–4.9 | 5.05 | 5.31 |
| 5β-OH | 0.86 | 0.93 | 1.24 | 4.8–4.9 | 5.18 | 5.25 |

* lower field with C-24 subst. ** half-band width

*Figure* 5(a). General n.m.r. data of methyl and carbinyl protons.

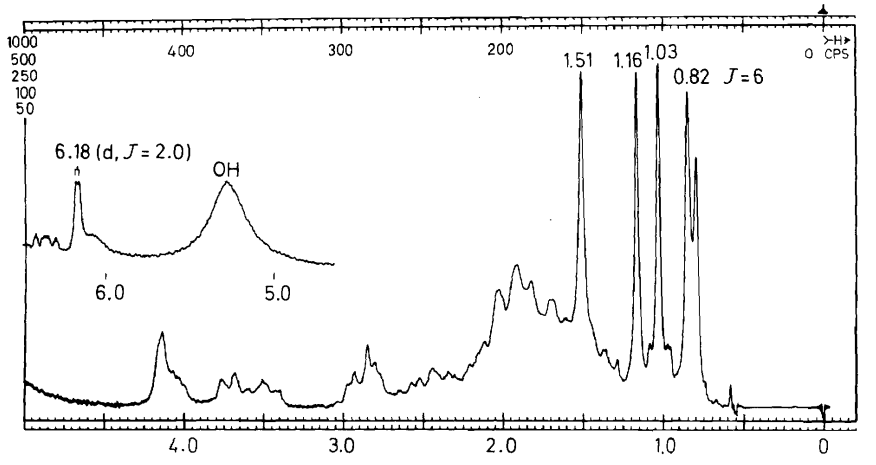



*Figure 5*(b). The n.m.r. spectrum of ponasterone A (in pyridine).

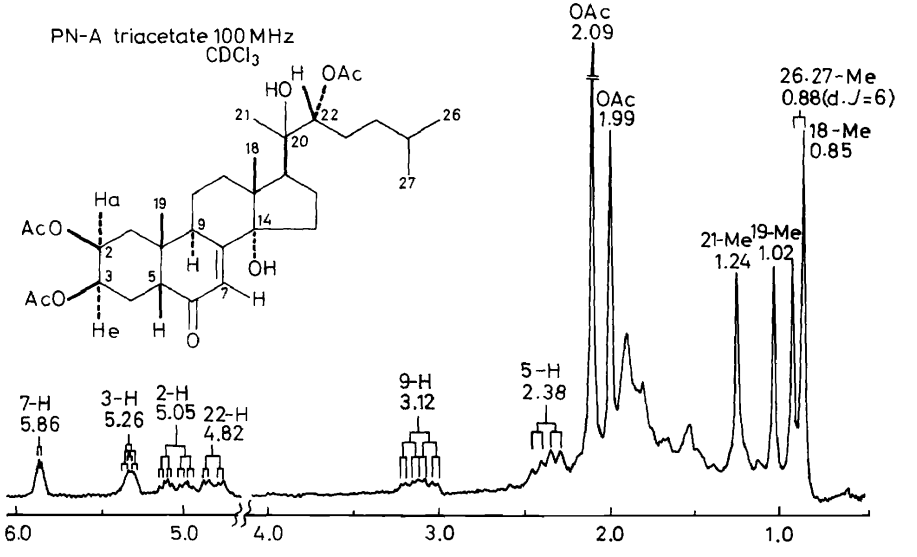



*Figure 5*(c). The n.m.r. spectrum of ponasterone A triacetate (in $CDCl_3$).

measured in deuteriochloroform [*Figure 5*(c)]. The chemical shifts tabulated in *Figure 5*(a) vary little, and are of diagnostic value for recognition of ecdysone structures. In the acetates of 5β-H ecdysone, the broad 2–H signal (half-band width 20 Hz) and narrow 3–H signal (half-band width 8 Hz) are well separated, but in 5β-OH ecdysones the two bands characteristically overlap. Five *J* values involving 1-H, 2-H, 3-H and 4-H were measured for ponasterone A triacetate using deuterio-acetone as solvent. The values were indispensable for structural elucidation of ring A because they indicated that 2-H and 3-H were adjacent,

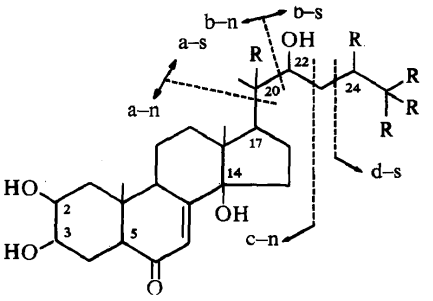
one being axial and the other equatorial, and that both had neighbouring methylene groups[3]. The 22-H, which is shifted to lower fields when alkyl or hydroxyl groups are attached to C-24, appears as a clear doublet of doublets (*J*, 4 and 8 Hz).

**The m.s. data—I, Generalization** (*Figure 6*)

Mass spectroscopic data understandably played a particularly significant role in structural studies of the zooecdysones which were available in very limited amounts (see *Figure 1* for references).

The $M^+$ peaks of ecdysones are measurable without difficulty with modern direct inlet systems, but previously this was not so because of ease of dehydration under electron impact. All data discussed in this section are based on high-resolution measurements of free ecdysones[39], their acetates[40] and acetonides[40]. The acetonide fragmentation can be corroborated by use of deuterio-acetonides[3].

General m.s. fragmentation patterns




(i) *nucleus*
a–n, b–n: presence/absence of additional groups

(ii) *sidechain*
a–s, b–s
d–s: branching at C-24

(iii) *acetonide*
characteristic fragmentations
m★

*Figure 6.* General m.s. fragmentation patterns.

The main fragmentations occur at C-17/20 (fisson a) and, to a greater extent, at 20/22 (fission b). Both fissions give rise to two series of peaks designated 'n' (nuclear) and 's' (sidechain). Fission c between 22/23 is minor. An additional fission d occurs if branching is present at C-24.

Similar fragmentation processes take place with the acetates as well[40]. The 2,3,20,22-diacetonide and 20,22-mono-acetonide spectra[3, 40] are characterized by strong peaks above *m/e* 300, and many metastable peaks, the main fission occurring at 17/20.

**The m.s. data—II, Detection of extra skeletal hydroxyl group** (*Figure 7*)

Mass spectral behaviour of an extra skeletal hydroxyl group is exemplified

by ajugasterone C[41] in *Figure 7*. Parenthesized and bold type numerals in the figure denote weak and very conspicuous peaks, respectively.

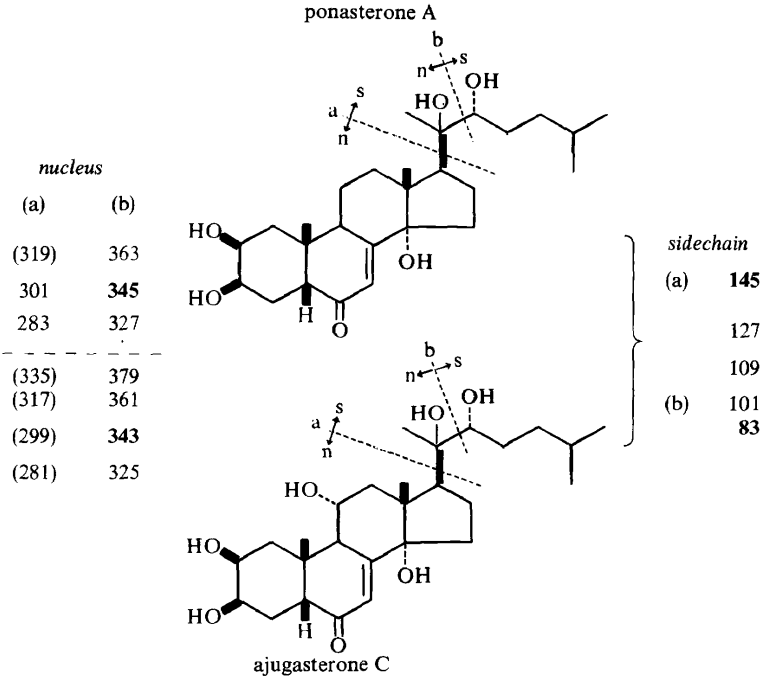



*Figure 7*. Comparison of fissions of ponasterone A and ajugasterone C.

(a) Fission-a and -b peaks originating from the nucleus of ponasterone A are followed by losses of two HOH units. The *m/e* 345 constitutes the base peak. The two series of peaks, 319/301/283 and 363/345/327 are typical for all ecdysones containing no extra OH (at C-5 or C-11).
(b) The extra 11α-OH in ajugasterone C increases the nuclear peak series by 16 units, and also each series consists of four instead of three peaks. The pattern is identical in the 5β-OH ecdysones, e.g. ponasterone C[42].
(c) The sidechain peaks in the two ecdysones give rise to the same two series, a–s and b–s, the former and latter consisting of three and two peaks, respectively, which is a reflection of the number of hydroxyl groups in the sidechain fragments.

**The m.s. data—III, Information on sidechain** (*Figure 8*)

The leaves of *Podocarpus macrophyllus* D.DON. afforded two $C_{27}$ ecdysones (β-ecdysone, ponasterone A), two $C_{28}$ ecdysones (makisterones A, B) and two $C_{29}$ ecdysones (makisterones C, D)[31]. The four makisterones provide a typical example of the straightforward application of mass spectroscopy for sidechain structural studies[43].

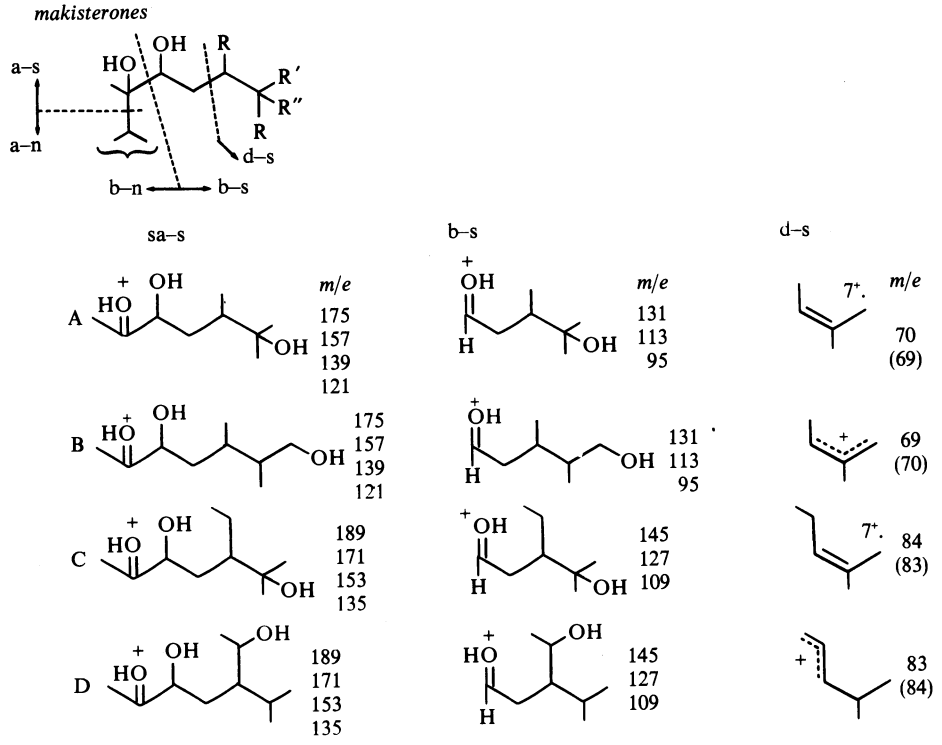



*Figure 8.* Comparison of fissions of makisterones A, B, C and D.

(a) All have branching at C-24 and hence in addition to a–s and b–s peaks, an additional d–s peak is present.
(b) Again, each series consists of a group of peaks differing by 18 mass units. Also, the number of peaks in a series reflects the number of hydroxyl functions in that fragment.

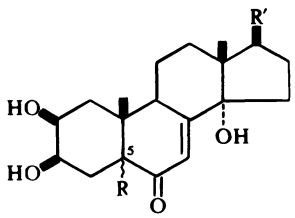
**General data on optical rotation** (*Figure 9*)

The enone system in ring B shows two Cotton effects in the RD or CD curves (*Figure 9*), the signs, amplitudes and wavelengths of which are subject to factors such as the following:

(a) A/B ring juncture—Amplitudes of the *trans* ring system are larger than those of the *cis* system. (The 5α-OH datum[42] is for an ergostenone with 14α-H.)
(b) The 5β-OH ecdysones can be characterized by the longer and shorter locations, respectively, of the $\pi,\pi^*$ and $n,\pi^*$ bands as compared with the 5β-H ecdysones.
(c) Signs of the $\pi,\pi^*$ (negative) and $n,\pi^*$ (positive) Cotton effects of the 5β-H and 5β-OH ecdysones can be correlated with the chirality of the enone group. Namely, the negative $\pi,\pi^*$ band and positive $n,\pi^*$ band suggest the

ring B enone groups to be twisted in the direction depicted in *Figure 11*[44, 45].

The Cotton effect signs and amplitudes are clearly under the influence of subtle structural variations. This is demonstrated by the change in sign of the $\pi,\pi^*$ Cotton effect in ajugalactone (*Figure 17*).




Amplitudes of Cotton effects (in dioxane)

| R | rings A/B | $\pi \rightarrow \pi^*$ ($\Delta\varepsilon$) | $n \rightarrow \pi^*$ ($\Delta\varepsilon$) |
|---|---|---|---|
| 5β-H | *cis* | 242 (−3.5) | 340 (+1.6) |
| 5α-H | *trans* | 240 (−7.1) | 340 (+3.3) |
| 5β-OH | *cis* | 251 (−5.5) | 325 (+2.8) |
| 5α-OH (14α-H) | *trans* | 245 (−12.7) | 349 (+3.6) |

*Figure 9.* CD Cotton effects of ring B enone.

The $\pi,\pi^*$ Cotton effect signs are more sensitive to unclarified changes in environmental structure[46]; accordingly, when predictions based on the $\pi,\pi^*$ and $n,\pi^*$ Cotton effects are in conflict, signs of the $n,\pi^*$ Cotton effects should be employed for deducing the chirality. In the case of ajugalactone, the $n,\pi^*$ Cotton effect sign is positive, but that of the $\pi,\pi^*$ Cotton effect is also positive, the amplitude of which is too large to be accounted for solely by the unsaturated lactone[78].

In summary, the enone CD data reflect subtle changes in stereostructures and thus afford important structural information[46a], especially with respect to the aspects mentioned in a–c of this section.

**The dibenzoate chirality method**[47, 48] (*Figure 10*)

An isolated benzoate group attached to an asymmetric carbon gives rise to a Cotton effect with an amplitude of about 3.5 ($\Delta\varepsilon$) centred around 225 nm. This formed the basis of the benzoate sector method which allows one to deduce the absolute configuration of secondary alcohol groups[49]. It was extended to the dibenzoate chirality method[47, 48], which has the potentialities of being extendable to other aromatic systems[50, 51].

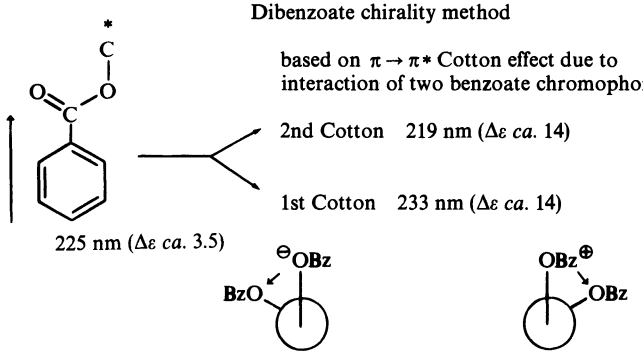



*Figure 10.* The dibenzoate chirality method.

When two benzoate groups are situated close together in space, the long axis charge transfer transitions interact and induce a Davydov splitting in the excited electronic states. As a result, two extremely strong Cotton effects of opposite sign appear at 233 nm (first Cotton effect) and 219 nm (second Cotton effect). If we define the chirality of two benzoate groups forming left-handed and right-handed screws as negative and positive, respectively, it was found that the sign of the first Cotton effect coincided with the sense of handedness. The Cotton effect signs are in agreement with non-empirical calculations[47, 48], and application of the method is also very simple and straightforward.

**Application of the dibenzoate chirality method to ponasterone A** (*Figure 11*)

The CD curve of ponasterone A is shown by the shaded area. As mentioned earlier, the Cotton effects at 327 nm (positive) and 248 nm (negative) define the shape of the ring B enone, i.e. whether the 6-one is up as depicted or down. The enone Cotton effects themselves are quite strong for a common organic molecule, but the 2,3-dibenzoate Cotton effects at 235 and 218 nm (Davydov splitting) are much stronger. The negative first Cotton effect clearly shows that the two benzoates constitute a left-handed screw, and therefore ring A of ponasterone A dibenzoate adopts a chair conformation.

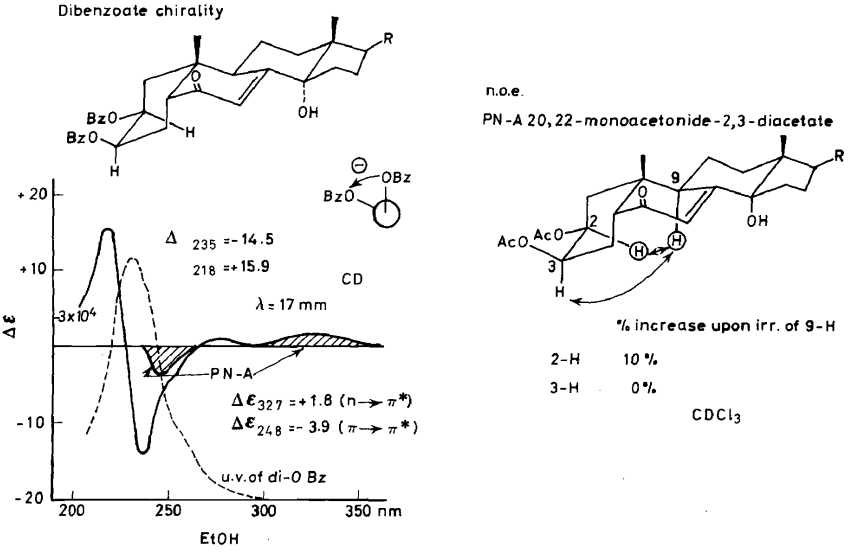



*Figure 11.* Ponasterone A—application of dibenzoate chirality method and n.o.e.

Another technique applicable to conformational studies of ecdysones is measurement of the intramolecular nuclear Overhauser effect[52]. In an A/B *cis* steroid having a chair-shaped ring A, the 9-H and 2-H are close to each other (*Figure 11*). In support of this conformation, irradiation of the 9-H n.m.r. signal results in a ten per cent increase in the integrated area of the 2-H n.m.r. signal [see *Figure 5*(c) for 9-H, 3-H and 2-H signals]; as expected no n.o.e. is observed on 3-H.

A combination of these two methods is extremely useful for collectively establishing configurations at C-2, 3, 5, and the conformation of ring A[52].

**Application of dibenzoate chirality method to ajugasterone C**[52] (*Figure 12*)

The dibenzoate chirality method can be extended to non-1.2–glycols[48](e.g. 3β,6β-di-*p*-chlorobenzoyloxy-5α-cholestane[47]), and also to triols[53]. Without exception, so far, CD measurements of tribenzoates of various sugars show that, depending on mutual spatial relations of the three benzoate groups, the chirality effects mutually augment to give Davydov-split Cotton effects of great

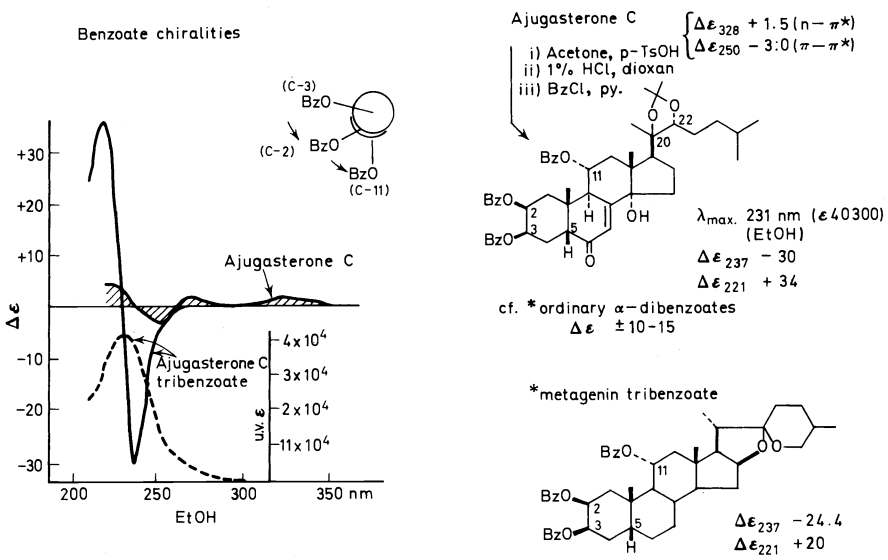



*Figure 12.* Application of the dibenzoate chirality method to determination of 11-hydroxyl in ajugasterone C.

amplitude or cancel out to give ill-defined Cotton effects of very low amplitude[53]. Ajugasterone C furnishes an interesting example where a non-adjacent triol group is present at C-3, C-2 and C-11. Ease of acetylation of the 11-hydroxyl group permitted the assignment of an equatorial and α-configuration[41]. This conclusion was corroborated as follows[52]. Ajugasterone C, the CD of which shows a positive and negative Cotton effect at 328 nm and 250 nm, respectively (shaded curve), was converted into the 20,22-acetonide-2,3,11-tribenzoate through the steps shown in *Figure 12*. The CD curve had Cotton effects with amplitudes of −30 and +34, which is twice as strong as the values for 1,2-dibenzoates ($\Delta\varepsilon \pm 10$–15). This indicated that the chirality effects were augmenting each other and, therefore, 11-OH should be α-oriented. This is supported by the similar data of metagenin tribenzoate having an established structure[54].

**Configurations at C-20 and C-22** (*Figure 13*)

The sidechain configurations of α-ecdysone are established on the grounds of

x-ray studies[4], but those of all other ecdysones remain to be clarified. Unfortunately, no crystals suitable for x-ray crystallography seem to have been prepared to date. Of the thirty ecdysones known, α-ecdysone is the only one lacking a hydroxyl group at C-20, and, consequently, n.m.r. data of 18-, 21- and 22-protons in α-ecdysone, which would subtly reflect configurations at C-21

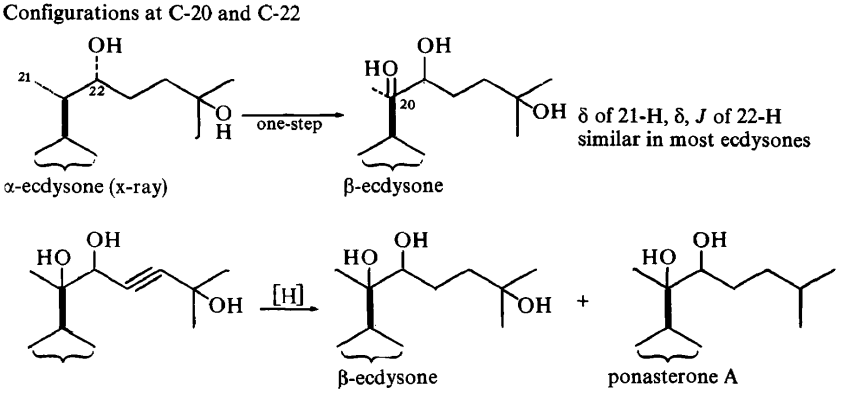



*Figure 13.* Configurations at C-20 and C-22.

and C-22, cannot be utilized for derivation of configurations at these centres in other ecdysones. On the other hand, chemical shifts of 18-, 21- and 22-H and *J* values of 22-H for the ecdysones other than α-ecdysone are all very similar (with the exception of shidasterone, which is some sidechain isomer of β-ecdysone[55]), thus indicating that they belong to the same stereochemical series. Determination of configurations at C-20 and C-22 is important as it is known that, in spite of the occurrence of various hydroxylated ecdysones in nature and their same activity levels, the activity of synthetic C-20 and C-22 epimers is much lower.

The rapid conversion of α-ecdysone to β-ecdysone in biological systems has been reported by several workers (see below). The fact that no other intermediate seems to be involved in this conversion (see below) suggests a one-step reaction and, hence, the 22-OH configuration should be as in α-ecdysone. A direct proof of the identity of 22-OH configurations in β-ecdysone and ponasterone A was obtained when hydrogenation of the triple bond in a synthetic intermediate yielded the two ecdysones[56].

**Configuration of 22-hydroxyl group**[57] (*Figure 14*)

L-(−)-Leucine was converted into 3-acetoxy-5-methylhexan-2-one of well defined absolute configuration through the steps shown. The replacement of $NH_2$ by OH proceeds through configuration retention due to participation of the neighbouring carboxyl group[58]. On the other hand, acetylation of ponasterone A under vigorous conditions gave the 17-ene (configuration of double bond is unknown) which was cleaved with ozone to give a homologous methyl ketone. The RD curves of the two ketones had Cotton effects of opposite signs,

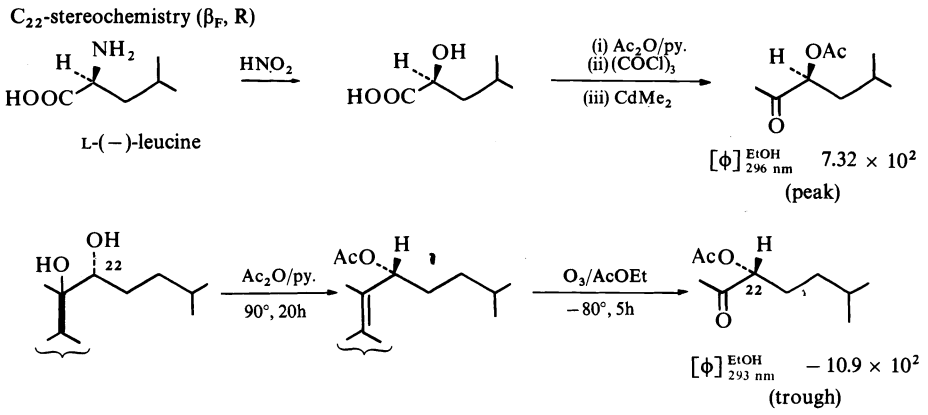



*Figure 14.* Determination of absolute configuration at C-22.

and this establishes the 22-OH configuration in ponasterone A and other ecdysones as being $\beta_F$ or R.

The C-20 configuration remains to be clarified.

## METABOLIC STUDIES OF ECDYSONES AND RELATED COMPOUNDS

*(Table 1)*

Alkylation[59] of $C_{27}$ steroids yields the $C_{28}$ and $C_{29}$ steroids[60], which in insects are reconverted to the $C_{27}$ cholesterol[61], and elaborated further into ecdysone[62]. The ecdysones then perform their biological functions[63, 64, 65], and are degraded to metabolic products. Details of these aspects related to ecdysones are still far from clear, and provide challenging problems for future investigations.

The availability of α-ecdysone and related compounds specifically labelled with $^3H$ has made it possible to investigate the metabolic fate of these compounds in insects and crustaceans. Such recent results are summarized in *Table 1* and in the following:

(a) α-Ecdysone is biosynthesized from cholesterol[62, 65a, 66].
(b) The 'triol' cholest-7-en-2β,3β,14α-triol-6-one is converted into α- and β-ecdysones[67].
(c) α-Ecdysone is a precursor of β-ecdysone[68, 71, 72].
(d) 25-Deoxy-α-ecdysone is probably not a normal intermediate in ecdysone biosynthesis because ponasterone A and inokosterone have not yet been detected in insects[69]. However, inokosterone (or an epimer) has recently been isolated from a crab[29].
(e) Conversion of ponasterone A into β-ecdysone and inokosterone[73] may account for the moulting activity of ponasterone A and other phytoecdysones, and seems to indicate that the enzymes involved are relatively nonspecific with respect to variations in sidechain structures.
(f) Substances A to D[71] and metabolites I to IV[72] are treated in the following section.

*Table 1.* Metabolism and catabolism of ecdysone and related compounds

| *Compound* | *Animal* | *Product* | *Ref.* |
|---|---|---|---|
| [1-$^3$H]-cholesterol | *Calliphora stygia* (blowfly) | β-ecdysone | 65a |
| [1-$^3$H]-7-dehydrocholesterol | | | |
| [4-$^{14}$C]-cholesterol | *Bombyx mori* (silkworm) | α- and β-ecdysone | 66 |
| [1-$^3$H]-5β-cholest-7-en-2β,3β,14α-triol-6-one ('triol') | *Manduca sexta* (tobacco hornworm) | α- and β-ecdysone<br>20,26-dihydroxyecdysone | 67 |
| [23,24-$^3H_4$]-α-ecdysone | *Crangon nigricauda* (shrimp)<br>*Uca pugilator* (crab)<br>*Calliphora vicina* (blowfly) | β-ecdysone | 68 |
| [23,24,25-$^3H_5$]-25-deoxy-α-ecdysone | *Calliphora stygia* (blowfly) | β-ecdysone<br>ponasterone A<br>inokosterone | 69 |
| [23,24-$^3H_4$]-β-ecdysone | *Calliphora stygia* (blowfly) | 4-hydroxy-4-methylpentanoic acid | 70 |
| [23,24-$^3H_4$]-α-ecdysone | *Bombyx mori* (silkworm) | β-ecdysone<br>substances A, B, C, D | 71 |
| [23,24-$^3H_4$]-α-ecdysone | *Antherea polyphemus* (silkworm) | β-ecdysone, metabolites I, II, III, IV | 72 |
| [24,25-$^3H_2$]-ponasterone A | *Bombyx mori* (silkworm) | β-ecdysone<br>inokosterone | 73 |

## METABOLIC FATE OF α-ECDYSONE[71] (*Figure 15*)

Very little is known about ecdysone metabolism or about the target tissues of ecdysone. Furthermore, the wide occurrence of phytoecdysones, e.g. of α-ecdysone in mulberry leaves[74] (diet of silkworm larvae), raises the interesting question as to whether ingested phytoecdysones are assimilated or not.

Fifth instar *Bombyx mori* (silkworm) larvae were injected or fed with [23,24-$^3H_4$]-α-ecdysone and, after suitable intervals, the animal body was separated into various fractions. The fractions were homogenized, extracted four times with hot ethanol for 24 hours, and the extracts were submitted to radio–liquid chromatography[37]. The chromatographic fractions were monitored with u.v.

An example of the radio-chromatogram is shown in *Figure 15*, which was obtained by injecting larvae with α-ecdysone on day 6 (just before spinning), and sacrificing them after 15 minutes.

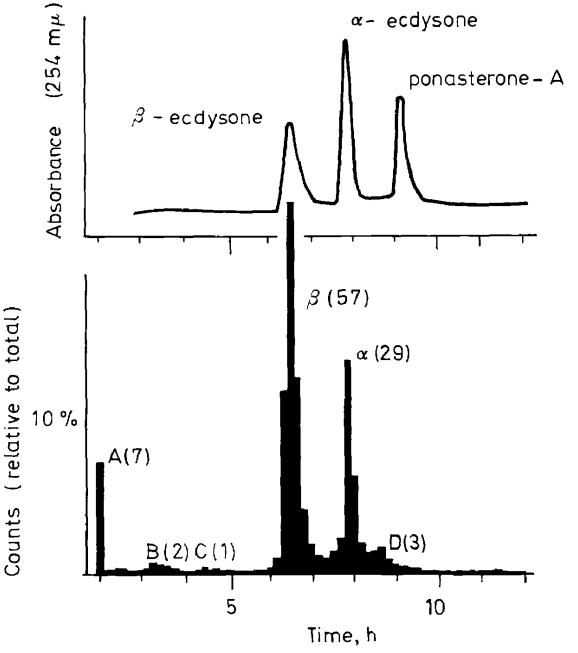



*Figure 15.* Upper figure: Liquid chromatogram[37] of three ecdysones, as monitored by u.v. Lower figure: Radio–liquid chromatogram[37] obtained upon injection of $^3H$-α-ecdysone to day 6 of fifth instar *B. mori*, and extracting the body liquid after 15 min. Numerals in parentheses are relative radioactivity counts of respective peaks. Abscissae represent elution time in hours.

Results of this detailed study clarified the following points. Injected or ingested α-ecdysone is rapidly metabolized to β-ecdysone and four compounds A, B, C and D. Very similar results have been obtained by Cherbas and Cherbas[72] with *Antherea polyphemus*(silkworm) pupae. They also detected four metabolites I, II, III and IV, which correspond to the presentB, C, A and D, respectively[72]. Metabolite A was too polar to be checked for its purity but could be a carboxylic acid salt derived from the sidechain and containing C-23 and C-

24. Only substance A was excreted into the gut, which showed the presence of it already after 15 minutes from injection. Ohtaki *et al.* have demonstrated with *Sarcophaga peregrina* (fleshfly)[75] and *Samia cynthia*[76] that injected α-ecdysone is inactivated rapidly, and that since the inactivation is blocked by low temperatures or anaerobic conditions, the possibility of an oxidative chemical reaction is suggested. Such an observation may be related to the formation of substance A (or metabolite III).

Compound C could be hydrolysed to β-ecdysone and may be an ester of the latter with a polar acid such as a phosphate, sulphate or glucuronate. The nature of substances B and D is unknown.

The conversion of α- to β-ecdysone occurs throughout the fifth instar period; the rate of α- to β-conversion increases but the rate of further metabolism of formed β to substances A,B and C decreases markedly towards time of pupation (day 9).

The conversion of α-ecdysone to β-ecdysone and metabolites A, B, C and D occurs efficiently in isolated abdomens, thus indicating that the prothoracic gland is not the site of α to β conversion. The extraglandular α to β conversion was observed with the isolated abdomens of *Calliphora* as well.

Ingested $^{3}$H-α-ecdysone was assimilated and distributed throughout the body, and was metabolized in a manner virtually identical to the metabolism of injected ecdysone.

## BIOSYNTHESIS OF ECDYSONES OUTSIDE THE PROTHORACIC GLANDS[66] [*Figures 16*(a) and *16*(b)]

It is generally understood that the moulting hormone or ecdysones are secreted from the two prothoracic glands (PTG) located close to the brain. In the case of the last (fifth) instar of *B. mori*, the glands are in a restive stage during days 0 to 2 but became active after day 3 and secreted ecdysones. However, there has been no experimental evidence pertaining to the role of PTG in ecdysone biosynthesis. In the following we describe observations which provide the first chemical evidence that α-ecdysone is biosynthesized *outside* the PTG.

Fifty larvae (day 6, fifth instar) were ligated at the first abdominal segment and the anterior parts were cut off. These isolated abdomens were each injected with 5μCi of $^{14}C_4$-cholesterol. After incubation, homogenization, extraction with 80 per cent aqueous ethanol and addition of 1 mg each of α-and β-ecdysone, the aqueous ethanol extracts were concentrated to dryness, chromatographed through silica gel, and the eluate containing ecdysones was subjected to liquid chromatography upon which 0·07μCi and 0·08μCi, respectively, of α- and β-ecdysone were obtained [*Figure 16*(a)]. The yield corresponds to about 0·006 per cent conversion of cholesterol into α- and β-ecdysone (total conversion, about 0·012 per cent).

In contrast, injection of labelled cholesterol on larvae of day 2 and similar work-up produced no labelled ecdysones. This leads us to the following conclusions.

(i) The ecdysones are biosynthesized outside the PTG during the PTG-active stage, but no biosynthesis occurs in abdomens during earlier stages.

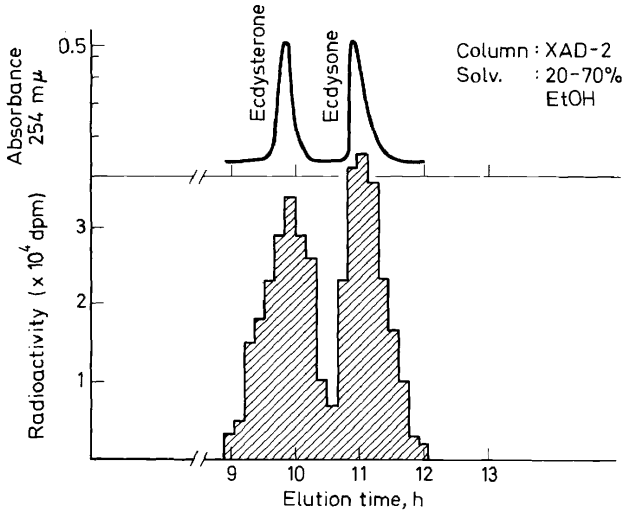



*Figure 16*(a). Liquid chromatogram (upper trace) and radio–liquid chromatogram of ecdysones biosynthesized in isolated posterior halves of *Bombyx* larvae.

(ii) It would be difficult to disprove in a convincing manner that no ecdysones are secreted from the PTG. However, above mentioned results may be interpreted by assuming the gland to secrete an enzyme which is involved in the cholesterol to ecdysone biosynthesis, and that this enzyme is absent in the body fluid in early days.

(iii) Although the majority of α-ecdysone is converted into β-ecdysone within 15 minutes when administered with exogenous material (*Figure 15*), the relative contents are reversed in biosynthesized ecdysones [*Figure 16*(a)]. This then suggests that biosynthesized α-ecdysone is in a bound form (presumably bound to a biopolymer), the bonding of which is loose so that α-ecdysone is liberated upon extraction of abdomens with 80 per cent aqueous ethanol [*Figure 16*(b)].

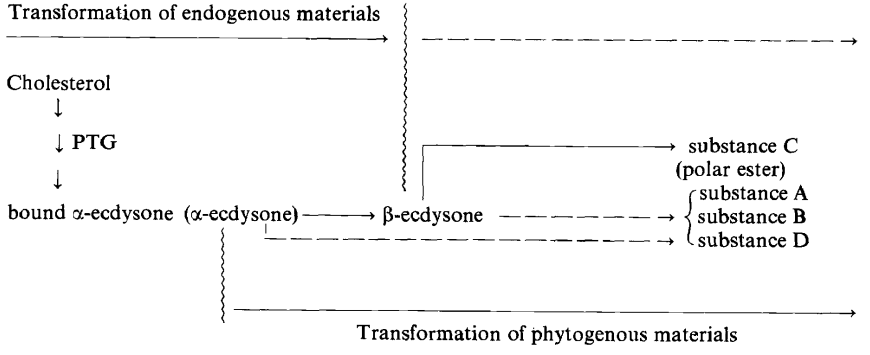


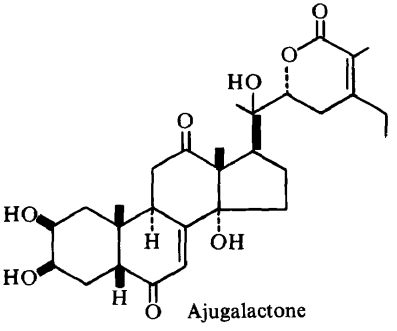

*Figure 16*(b). Biosynthesis and metabolism of ecdysones. Solid arrows denote routes verified by experiments. Broken arrows denote routes which may or may not exist.

(v) Results from metabolic studies of exogenous ecdysones and biosynthesis of ecdysones from cholesterol leads to a scheme shown in *Figure 16[a]*. Clearly this scheme is merely a starting point for many detailed studies on a variety of extremely intriguing problems.

## NATURAL PRODUCTS EXHIBITING ANTIECDYSONE ACTIVITY IN THE CHILO DIPPING METHOD (*Figure 17*)

An extensive effort to search for natural antiecdysones has resulted in the identification of several plants exhibiting moulting inhibitory activity[77] as assayed by the *Chilo* dipping method[36].

The search for antiecdysones in plants was complicated by the fact that the antagonistic activity of some plant extracts was overshadowed by the presence of phytoecdysones. In such cases, the ecdysones first had to be separated, and the residual fraction was then submitted to a competitive bioassay. For example, *Ajuga decumbens* THUNB. (2 kg) gave 2.5 g of β-ecdysone, 1.2 g of cyasterone, 30 mg of ajugasterone C, 30 mg of ajugasterone B and 200 mg of an inhibitor designated ajugalactone.




Ajugalactone

*Figure 17.* Structure of ajugalactone.

The preliminary bioassays seemed to indicate a remarkable specificity towards ecdysone structure. Thus, ajugalactone suppressed the activity of ponasterone A in a weight ratio of 1 : 5 (hormone versus antihormone) but its suppression of the moulting activity of β-ecdysone or cyasterone was very weak. The structure of ajugalactone has been deduced as indicated in *Figure 17*[78]. On the other hand, another antihormone isolated in amorphous form from *Cinnamonium laureirii* NEES antagonizes the moulting action of β-ecdysone and cyasterone, also in a weight ratio of about 1 : 5, but the ponasterone A was only weakly suppressed. The structure of this second antihormone is being investigated, but it appears to be a complex catechin derivative. This structural diversity, i.e. a steroid and a polyphenol, of the two antihormones so far studied is another noteworthy aspect.

However, as the present results are only based on the Chilo dipping technique, other bioassays are necessary to clarify the scope of antagonistic activity[79].

## ACKNOWLEDGEMENTS

I am most grateful to my able collaborators who carried out the ecdysone studies: Dr M. Koreeda was engaged in the problem throughout, Dr H. Moriyama started the metabolic studies, Dr N. Harada developed the dibenzoate chirality method, Professor S. Sasaki made indispensable contributions in the early stage, Mr T. Kurokawa established a general extraction procedure, and Mr H. Hagiwara studied the sidechain stereochemistry.

It has been a great pleasure to work with Dr H. Y. Hsu (Chinese Drug Research Institute, Taipei) and co-workers on medicinal plants which introduced us to a new field.

Part of the work was carried out in collaboration with the Takeda Chemical Industries, Osaka, and I express my gratitude to Dr S. Tatsuoka, Director, and Dr Y. Abe, K. Tanaka, M. Goto, K. Morita, and their colleagues, for their very pleasant cooperation.

I thank my colleagues for discussions, especially Professors T. Takemoto and H. Hikino, Tohoku University.

The work was supported in part by the Ministry of Education, Japan, and an NIH PHS Grant.

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