On gonads and gadflies: the estrus angle

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Abstract

The first sex steroid to be crystallized was the vertebrate ovarian hormone, estrone – a less potent metabolite of 17βestradiol, which in mammals stimulates the female urge to mate (estrus). The gadfly (Greek *oistros*) lent its name to the process of estrus, as an insect that bites and torments in classical Greek mythology. With the purification and crystallization of a moult-inducing steroid (ecdysone) from insects, an interesting parallel emerged between mating and moulting in lower mammals and arthropods. Ecdysterone (potent ecdysone metabolite) has anabolic effects in mammalian muscle cells that can be blocked by selective estrogen receptor-antagonists. And insects utilise ecdysteroids in similar ways that vertebrates use estrogens, including stimulation of oocyte growth and maturation. Ecdysteroids also modify precopulatory insect mating behaviour, which further reinforces the gonad-gadfly/mate-moult analogy.

Introduction

"In the Lucanian woods among the oaks

Of green Alburnus' slopes there swarms a fly

***(By us called gad-fly, oestrus by the Greeks)**.*

It's fierce and buzzes monstrously: whole herds

In terror of it scatter through the woods,

Until the sky rings with their bellowing…"

Virgil, *The Georgics,* Book III (Mackenzie 1969)

Virgil's description of demented cattle shrouded in clouds of stinging gadflies provides a striking metaphor for hormone-induced sexual arousal and unwittingly links steroid signalling in mammals and insects. The Victorian reproductive biologist Walter Heape (1900) seized upon Virgil's verse to bring forward the concept of the 'estrous' cycle (Box 1) in which a female's period of heightened sexual receptivity to the male is termed estrus (Greek *oistros* "gadfly, breeze, sting, mad impulse"). Crystallization of an estrus-inducing steroid (eventually named estrone) from the urine of pregnant women founded the sex hormone era and effectively launched the clinical specialism of reproductive medicine. Decades on, a moult-inducing steroid that controls ecdysis (Greek *ekduo* "to take off, strip off ") in insects – including gadflies – would be crystallized from silk-moth pupae, and named ecdysone.

This commentary draws attention to the equivalence of estrus and ecdysis as fundamental reproductive events, and celebrates the enduring impact of the sex-steroids that control them. It also marks the centenary of the classic estrogen bioassay, the vaginal smear test for estrus (Stockard & Papanicolaou 1917), which was instrumental to the discovery of estrone.

Essence of estrus

Box 1. The Estrous Cycle of Lower Mammals (Heape 1900)

1. Pro-estrus: The period of "coming on heat" or "coming in season" when the generative organs become hypertrophied and congested

2. Estrus: The period of heightened sexual arousal and activity; desire for coition.

3. Metestrus: When, in the absence of the male, the desire for coition gradually dies away

4. Diestrus or anestrus: The rest interval between recurrent cycles or breeding seasons.

A defining moment in steroid endocrinology was the announcement that surgical removal of the ovaries from adult female rabbits caused uterine atrophy, which could be prevented by auto-transplantation of ovarian fragments to ectopic sites in the abdominal cavity (Knauer 1900). This classic ablation-replacement experiment started the hunt that resulted in the discovery of the first female sex steroid a quarter of a century later.

Allen & Doisy (1924) proved the existence of an estrus-inducing substance in the ovary by injecting fresh porcine follicular fluid or ovarian extracts into ovariectomized rats. Estrus was detected as cornification of the vaginal epithelium, observed by microscopic examination of vaginal smears (Allen & Doisy1924). This simple test for estrus, developed by Stockard & Papanicolaou (1917), was instrumental to the eventual isolation of a pure estrogen: *"The clear-cut nature of this test lessens the confusion of uncertain results. This improvement in testing has enabled us to make more than 600 separate tests of extracts in a little over 1 year…"* (Allen & Doisy 1924).

The first estrus-producing hormone to be crystallized was estrone (Doisy *et al.* 1929,1930; Butenandt 1929), quickly followed by estriol (Marrian 1930) – both from human pregnancy urine. In the event, estrone and estriol proved to be weak metabolites of 17βestradiol (estradiol), which is the major estrogen secreted by the ovary (Simpson & Santen 2015). Estradiol itself was purified (from porcine ovarian follicular fluid) five years later (MacCorquodale *et al.* 1936). However, without question it was the initial isolation of crystalline estrone that launched the sex hormone era.

What's in a name?

The naming of estrone is a story in its own right (Corner 1964). Doisy suggested 'theelin', derived from the Greek *thelys,* meaning "female" Veler *et al.* 1930). Others suggestions included 'folliculine' (Courrier 1924), 'menoformon' (Laquer et al. 1930) and 'progynon' (Butenandt 1930). Parkes & Bellerby (1926), harking back to Virgil, proposed 'oestrin' explicitly to accommodate the compound's estrus-producing property. Oestrin would eventually morph into estrone, providing the etymological stem for a generation of natural and synthetic estrus-producing steroids that were about to be discovered and synthesized (Doisy 1941).

Cholesterol connections

The basic chemistry of the sterol/steroid ring system was solved for cholesterol in 1932 (Bloch 1982). Along the way, Heinrich Wieland was awarded the 1927 Nobel Prize in Chemistry for "…investigations of the constitution of the bile acids and related substances", and the 1928 Nobel Prize in Chemistry was awarded to Adolf Windaus for "research into the constitution of the sterols and their connection with the vitamins." Adolf Butenandt, who had been Windaus' graduate student, was therefore ideally placed to deduce the chemical constitution of the estrus-inducing steroid (Butenandt 1932) (Fig 1).

A consensus nomenclature for steroidal estrogens quickly emerged, based on the carbon atom numbering system already used for C27-cholesterol. In this system, the parent C18 molecule was termed 'estrane', i.e., the core four-ring gonane skeleton with one methyl group and minus the cholesterol side-chain (Adam *et al*. 1933) Fig. 1). Thus when the parent estrus-inducing steroid was eventually obtained (MacCorquodale *et al.* 1936), a systematic nomenclature was already available to accommodate it as estradiol.

Raging hormones

Intense interest in estrogens and their therapeutic potential heralded the ‘decade of the sex hormones’, in which all the major mammalian sex steroids were sourced, purified and crystallized. Adolph Butenandt – arguably the steroid chemist of his century – was involved throughout. On the androgen front, Butenandt & Tschering (1934) isolated 15 mg of a hormonal substance they named androsterone from 15,000 liters of male urine. Androsterone proved to be a weak metabolite of the main testicular androgen, testosterone, which was isolated from bull testes by Ernst Lacquer (David *et al.* 1935) and simultaneously synthesized by Ruzicka & Wettstein (1935). Butenandt (1934) also achieved isolation of the ‘second’ female sex steroid, progesterone – the hormone of pregnancy – from pig ovaries. So in the space of five years Butenandt had contributed to the discovery of all three major sex hormones, for which he and Ruzicka shared the 1939 Nobel Prize in Chemistry. There was more to come.

The gonane guarantee

By1936 the sex steroid pantheon – estradiol, testosterone, progesterone – was complete, and the term ‘steroid’ (sterol-like) was coined: "for the group of compounds comprising the sterols, bile acids, heart poisons, saponins, and sex hormones." All distinguished by the trademark gonane motif (Callow & Young 1936).

But other nuggets remained to be mined. Not least cortisone, which was crystallized independently by Edward Kendal (Mason *et al.* 1936) and Tadeus Reichstein (1936) from bovine adrenal glands. Dubbed ‘the hormone of life’, the anti-inflammatory properties of cortisone made it one of the wonder drugs of the 20th century (Hillier

2007). Aldosterone, the salt-conserving adrenal steroid, would follow nearly two decades later (Simpson *et al.* 1953), almost coincident with the discovery of ecdysone (see below).

After a hiatus during World War II, there was an explosion of interest in steroid chemistry and pharmaceutics. With the discovery of sex steroids and cortisone, the race was on to synthesize more and more steroid analogues with beneficial therapeutic effects. By 1956, the number of known steroidal substances was listed at around 7,000 (Klyne 1957).

Application of X-ray crystallography to resolve steroid structures confirmed the canonical, four-ring nuclear structure of cholesterol that had been deduced by the early steroiders, and underscored the boundless potential of 3D-steroidal signalling (Carlisle & Crowfoot 1945) (Fig. 2).

Butenandt's butterflies

The ubiquity of steroids and their critical importance to plant and animal life were becoming increasingly clear. Not yet appreciated was that members of the most abundant animal phylum on earth – the arthropods – have a customized sex-steroid signalling system, seemingly based on a prototypic sex steroid that acquired control over moulting and metamorphosis during evolution (De Loof *et al.* 2016).

All arthropod species moult (Chang & Mykles 2011). It is a defining feature of animals with a rigid exoskeleton, which is split and shed to accommodate serial phases of growth and development (Nijhout & Callier 2015) (Box 2). The process depends on moult-inducing steroids secreted by the prothoracic gland as metabolites of dietary cholesterol (Schwedes & Carney 2012). The first moult-inducing steroid to

Box 2. The Insect Moult (Nijhout & Callier 2015)

1. Apolysis: Moulting hormones are pulsed into the haemolymph from the prothoracic gland and the old cuticle separates from the underlying epidermal cells.

2. Ecdysis: The old cuticle splits and is shed. The instar larva (progressive moult) or adult form emerges, as its new cuticle is being synthesized.

3. Sclerotisation: The new cuticle hardens. At the final moult, the wings expand as haemolymph is forced into the wing veins.

be identified – ecdysone – was crystallized in 1954 by none other than Adolf Butenandt (Butenandt & Karlson 1954). Butenandt's partner in crystallizing ecdysone, Peter Karslon, recalls: *'We collected 500 kg of male pupae from which 5 kg of a concentrate were extracted. This was then further reduced, and finally we obtained 25 mg of the crystalline hormone, a purification factor of 1:10,000,000… Thus, after 20 years, Butenandt was back to steroids.'* (Fig. 3) (Karlson 1995).

Ecdysone is necessary and sufficient to stimulate all stages of the insect moult (Nijhout & Callier 2015). Considering the evolutionary success of arthropods (>1 million species recorded), extending back to the Cambrian period (i.e. over 500 myr), ecdysone is probably the most widespread and influential steroid hormone on earth (Bathori *et al*. 2008).

Beyond buzzy

It took over a decade before the steroidal structure of ecdysone was deduced (Huber & Hoppe 1965; Karlson *et al.* 1965), when it also emerged that ecdysone is a steroidal prohormone. Hydroxylation at C20 is required to produce the active moult-inducing hormone, 20hydroxyecdysone or ecdysterone in insects (King & Siddall 1966; Kaplanis *et al*. 1969) and crustaceans (Horn *et al.* 1966) (Fig. 3).

Unexpectedly, ecdysterone and related ecdysteroids were also found in various ferns

and evergreen plants (Kaplanis *et al*. 1967). The active compound (named ponosterone A) in the leaves of the conifer *Podocarpus nakaii* Hay was found to be very close in structure and activity to insect ecdysterone (Nakanishi *et al.* 1966). Critically, the recovery of ecdysterone from this plant was orders of magnitude higher than from insect sources, prompting the authors to remark: *"The ready isolation of insect moulting hormones from plants, in contrast to the extremely poor yield from insects…makes it possible to supply large amounts of active substances for biological testing"*. This proved to be the case, as shown below.

Most plant species are now believed to contain ecdysteroids of one form or another, some at concentrations up to 3% of their dry weight (Laekeman & Vlietinck 2013; www.ecdybase.org). They are normally present as a cocktail of substances in which ecdysterone is dominant. At the time of writing, well over 400 ecdysteroidal compounds have been detected in over 100 plant families (annual and perennial): ecdysterone being the most widespread (Tarkowská & Strnad 2016).

Ecdysteroid anabolics

A likely function of ecdysteroids in plants is to defend against insect and nematode infestation, through unbridled activation of ecdysteroid receptor (EcR) signalling (i.e. endocrine disruption) in the invader (Dinan 2009). Testing of plant-derived ecdysteroids in animal and human settings has revealed hepatoprotective, immunoprotective, antioxidant, hypoglycemic and anabolic properties, which have led to their use in dietary supplements and nutraceutical preparations. The anabolic potential of ecdysterone mainly rests on its ability to enhance physical performance associated with increased body mass in mice, as well as stimulation of protein synthesis in mouse and human skeletal muscle cells *in vitro* (Sláma & Lafont 1995;

Gorelick-Feldman *et al.* 2008). The mechanism of action includes binding and activation of estrogen receptor (ER) signalling. Selective ER antagonists block ecdysterone stimulated myotubular growth *in vitro*, and *in silico* molecular docking experiments support an ERβ-mediated mode of ecdysterone action (Parr *et al*. 2014,2015). Conversely, *in silico* studies imply estrogenic activation of EcR in arthropods (Swetha *et al*. 2016). The anabolic potential of ecdysterone as a dietary supplement in humans is such that it could yet be prohibited as a performance-enhancing drug by the World Anti-Doping Agency (Parr *et al*. 2015).

Sex steroid surrogates

Whether vertebrate-type sex steroids have physiological roles in insect reproduction is controversial (Swevers 1991; Das 2016; De Loof *et al.* 2016). On the other hand, insects evidently can use ecdysteroids in similar ways that vertebrates use estrogens, including e.g., stimulation of oocyte growth and maturation (De Loof 2006; De Loof *et al.* 2016). This is consistent with the widespread expression of EcR protein in adult insect tissues – including gonads and CNS (Schwedes & Carney 2012) – and lack of an ER orthologue in the *Drosophila* genome (Boulanger & Dura 2015). Estrogenic stimulation of oocyte growth and vitellogenesis in experimental crabs – also devoid of ER – has been explained by estradiol binding to EcR, based on *in silico* ligand docking experiments (Swetha *et al.* 2016).

Insect precopulatory mating behaviour is also modified by ecdysteroids. The female fruit fly initially rejects potential suitors by various means, including full extrusion of her ovipositor to prevent copulation. But, as courtship proceeds, the ovipositor is partially withdrawn to signal increased receptiveness to the male. Ecdysterone-depleted female flies show reduced rejection behaviour in advance of copulation,

which can be reversed by ecdysone feeding (Ganter *et al*. 2012). This implies a negative action of ecdysterone on female reproductive behaviour, the significance of which remains to be determined

A sting in the tail

"See where she rages in Sila's woods,

A lovely heifer, while the rival bulls

Do mighty battle with alternate charge..."

Virgil, *The Georgics,* Book III (Mackenzie 1969)

Virgil's cattle were tormented by gadflies but might have bellowed for another reason.

The male's ambition and capacity to mate is driven by testicular androgens – principally testosterone. The urine and saliva of bulls and boars also contain high concentrations of odorous 16-androstane testosterone metabolites (Cox & Turner 1984), which are implicated in the 'stink wars' between rutting males that compete for the prize of copulating with females in estrus (Wyatt 2009). A major component of this smelly cocktail is the boar taint steroid, androstenone. To humans this steroidal pheromone "has an unpleasant odour of perspiration" (Patterson 1968). However, to an estrous sow, a mere whiff can induce her to adopt the receptive mating stance, in readiness for copulation (Patterson 1968; Dorries *et al.* 1997).

Summary

1. Estrus describes the vertebrate female state of maximum sexual desire and readiness for copulation.
2. Estrone was the first estrus-inducing steroid to be isolated.
3. Estrus and estrone are named after the Greek word for gadfly *oistros*.
4. The gadfly features in Virgil's verse in *The Georgics*, as a biting, buzzing insect that incites frenzy in grazing livestock.
5. Insects (arthropods) moult: ecdysis (Greek *ekduo* "to take off, strip off").
6. Ecdysis is stimulated by moult-inducing steroids, of which ecdysone was first to be isolated.
7. Ecdysterone (potent ecdysone metabolite) has anabolic effects in vertebrates, mediated by ER.
8. Estradiol (potent estrone congener) has ecdysteroid-like actions in arthropods, mediated by EcR.
9. Ecdysteroids function as vertebrate sex-steroid equivalents in insects.
10. Ecdysteroids modify precopulatory mating behaviour in flies.

Conclusion

The roles of estrone and ecdysone in estrus and ecdysis testify to the ubiquitous impact of sterol/steroid signalling on reproductive fitness in mammals and insects. The mate-moult parallel clearly warrants a more detailed analysis than that drawn here, in the spirit of Medawar 's (1953) dictum that *"...'endocrine evolution' is not an evolution of hormones but an evolution of the use to which they are put."*

Declaration of interest

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Figure 1

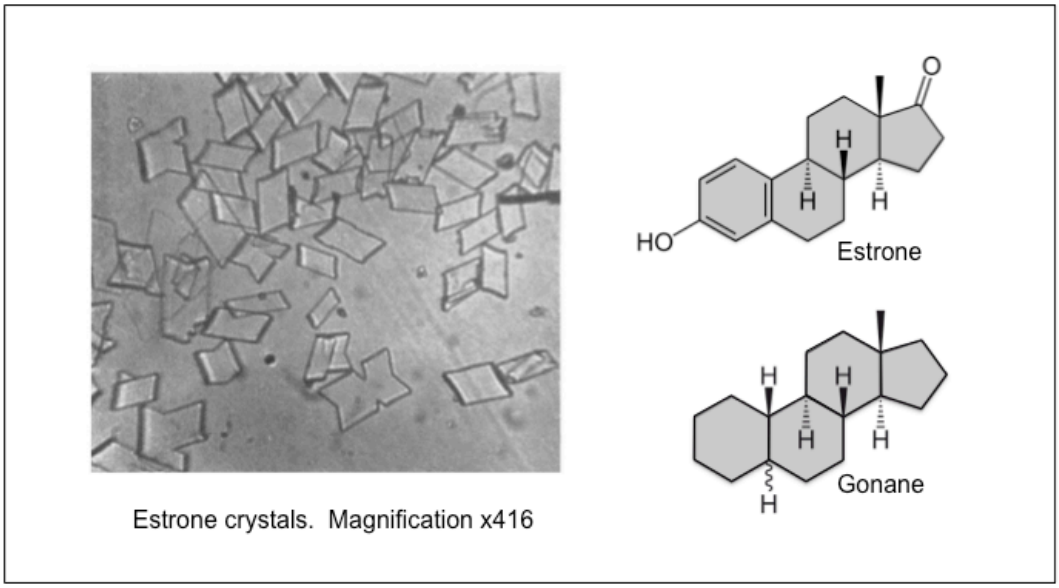

Fig. 1 Pure estrone. *Left* Platelet crystals of estrone. (This research was originally published in the Journal of Biological Chemistry. EA Doisy, CD Veler & S Thayer. The preparation of the crystalline ovarian hormone from the urine of pregnant women. *Journal of Biological Chemistry.* 1930; 86:499–509. © the American Society for Biochemistry and Molecular Biology). *Top right* Estrone steroidal structure. *Bottom right* Gonane, the nuclear hydrocarbon structure upon which the systematic nomenclature of all natural and synthetic steroidal substances is based.

Figure 2

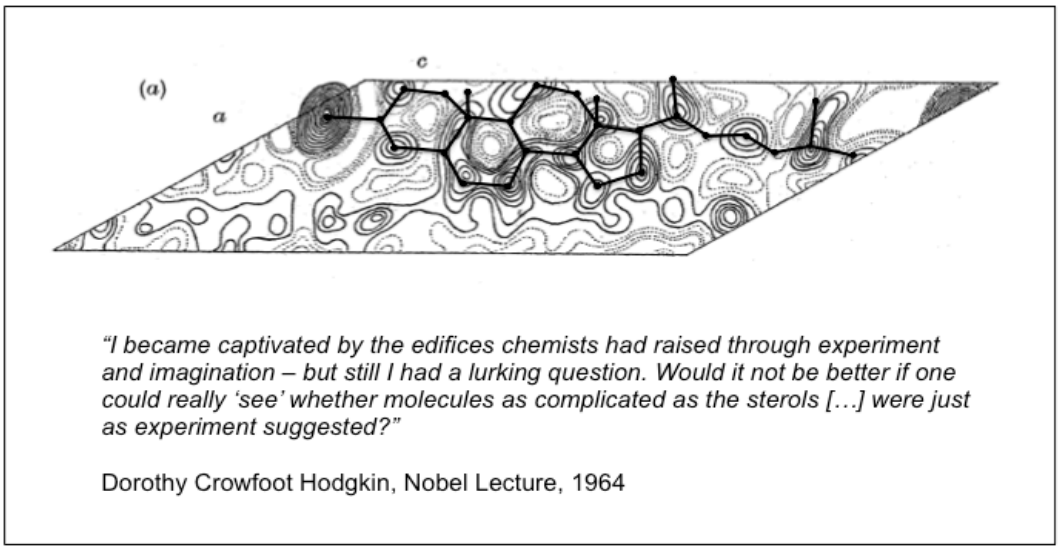

Fig. 2 Crystal structure of cholesteryl iodide. X-ray diffraction analysis, showing an electron-density map with the tetracyclic gonane nucleus and alkyl side-chain contour drawn in. Adapted, with permission, from Carlisle CH & Crowfoot D (1945) The crystal structure of cholesteryl iodide. *Proceedings of the Royal Society of London: Series A, Mathematical and Physical Sciences*, volume **184**, pages 64–83. Quoted text reproduced from Hodgkin DC (1972) The X-ray analysis of complicated molecules. [Nobel Lecture, December 11, 1964]. In *Nobel Lectures, Chemistry 1963–1970*, 71–91. Amsterdam: Elsevier Publishing Co.

Figure 3

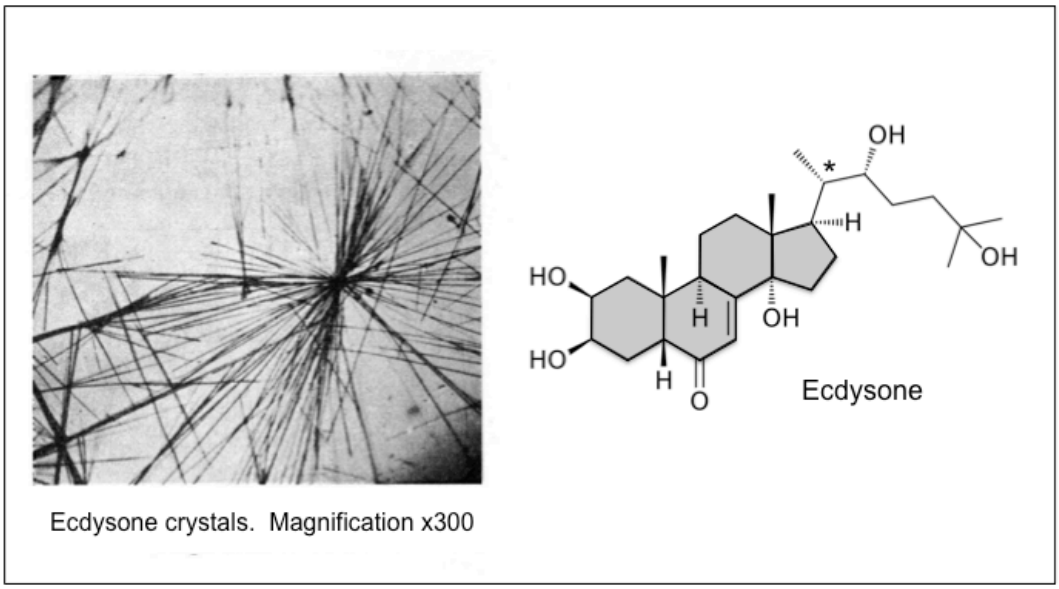

Fig. 3 Moult-inducing steroids. *Left* Crystals of ecdysone isolated from silk-moth pupae. (Reproduced with permission from Butenandt A & Karlson P Über die isolierung eines metamorphose-hormons der insekten in kristallisierter form; in: *Zeitschrift fur Naturforschung B,* vol 9: issue 6, Berlin: De Gruyter, 1954, pp. 389–391, Fig. 3.). *Right* Steroid structure of ecdysone. Ecdysone is a prohormone for the more potent moult-inducing hormone 20-hydroxyecdysone (ecdysterone), which is formed by hydroxylation at C20 (asterisk).

Figure 4

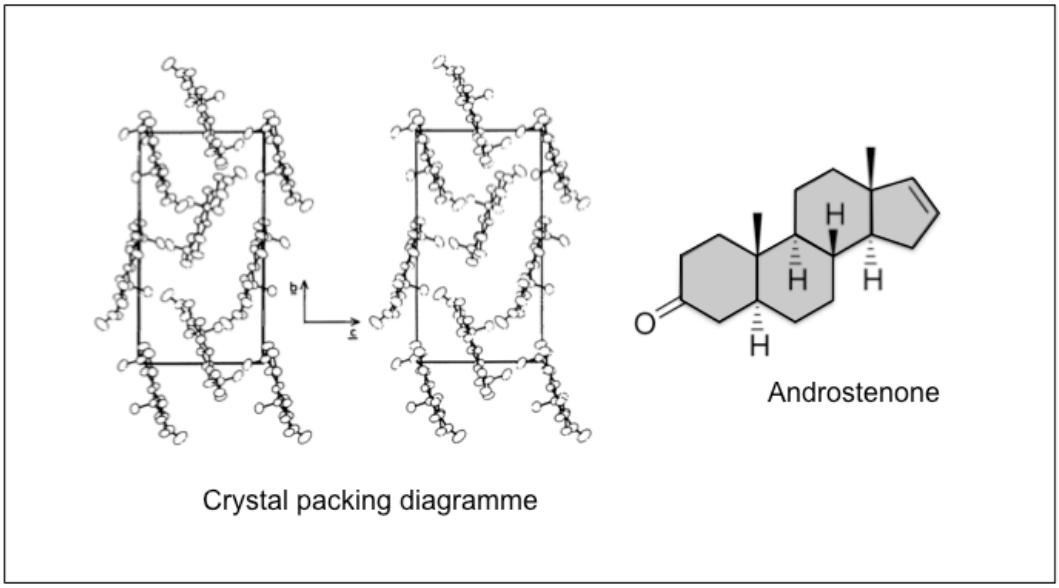

Fig. 4 Structure of the steroidal pheromone, androstenone. *Left* Stereoscopic view of the crystal packing diagramme. (Reprinted from *Tetrahedron*, vol 40, issue 16, PJ Cox & AB Turner, Synthesis, X-ray structure and molecular mechanics studies of the boar taint steroid (5-androst-16-en-3-one), vol 40, issue16, pp. 3135–3138, Copyright (1984), with permission from Elsevier). *Right* Steroidal structure of androstenone deduced by Prelog *et al*. (1944).