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THE MAJOR CONJUGATES OF ECDYSTEROIDS IN YOUNG EGGS AND IN EMBRYOS OF LOCUSTA MIGRATORIA.

G. Tsoupras, C. Hetru, B. Luu*, M. Lagueux, E. Constantin and J.A. Hoffmann

Laboratoire de Chimie Organique des Substances Naturelles (LA CNRS 31) et
Laboratoire de Biologie Générale (ERA CNRS 118), Université Louis Pasteur,
67084 Strasbourg, France

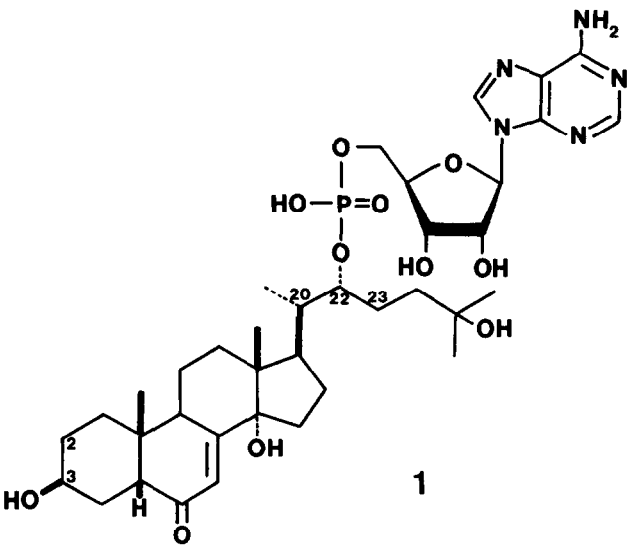
Abstract. Newly-laid eggs of migratory locust contain as the major ecdysteroid conjugate donated by the female to its offspring the 22-adenosinemonophosphoric ester of 2-deoxyecdysone, in 8 days old embryos, the major ecdysteroid is the 3-phosphate of 3-epi 2-deoxyecdysone.

In the migratory locust, *Locusta migratoria*, the females produce large quantities of ecdysteroids near the end of the maturation of the oocytes (1,2). These maternal ecdysteroids are mostly conjugates and can be isolated together with the major yolk protein, vitellin, to which they are bound (3). The presence of ecdysteroid conjugatès in ovaries and/or eggs has also been reported in other insects (4-7). During the maturation of embryos, free ecdysteroids (ecdysone and 2-deoxyecdysone) exhibit several concentration peaks, two of which occur at such early stages that they must result from hydrolysis of the maternal conjugates : the embryonic endocrine glands are not yet differentiated (8). During later stages of embryonic development, the maternal ecdysteroids are to a large extent transformed into an unidentified metabolite, called NI_2, which is also predominantly present in a conjugated form (2).

We have now identified the first major maternal ecdysteroid conjugate as the 22-adenosinemonophosphate of 2-deoxyecdysone (1), and the major embryonic conjugate NI_2 as the 3-phosphate of 3-epi 2-deoxyecdysone (4).

The maternal ecdysteroid conjugate.From about 1 million of newly-laid eggs, we have extracted the major yolk protein, vitellin, by known procedures (9,10). The ecdysteroids carried over by this protein were extracted with 50% aqueous methanol, and the extract was partitioned between hexane and 50% aqueous methanol. The methanol phase was subjected to liquid chromatography on reverse phase C8, the elution of ecdysteroids being monitored by their UV absorption at 254 nm and by radioimmunoassays (11). The ecdysteroid-containing fractions were rechromatographed in the same conditions, to give 25 mg of a predominant ecdysteroid conjugate, and lower amounts of a second conjugate which will be described latter.

The major conjugate was identified as 1 by the following data. Enzymatic hydrolysis (*Helix pomatia* gastric enzymes) gave 2-deoxyecdysone (2), identified by TLC and C18 reversed phase HPLC, by GC/MS after trimethylsilylation (12), by ^{1}H NMR in d_5-pyridine (13), and by ^{13}C NMR (Table 1). The conjugate itself showed by ^{31}P NMR a signal at δ = 1 ppm relative to phosphoric acid, showing the presence of a phosphate group microanalysis confirmed the presence of one phosphorus atom per molecule. In ^{13}C NMR spectrum of the conjugate, all



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the signals of 2-deoxyecdysone (2) are present and unchanged, except those of C-22 now a doublet (J = 4 Hz) when proton decoupled and shifted downfield by 5 ppm, and therefore attached to a phosphate group (14). The signals of C-20 and C-23 are shifted upfield by 2 ppm (cf. 15).

The other moiety linked to phosphate was identified by partial hydrolysis (HCl, pH 2), as adenosine identified by UV,reversed phase HPLC and direct introduction mass spectrometry on a gold support (16) both with electron impact and chemical ionization (NH_3). The presence of adenosine in the conjugate was furthermore confirmed by mass spectrometry (direct introduction on a gold support), which gave all the fragments of 2-deoxyecdysone and of adenosine (though not the molecular ion) and ^{13}C NMR, which confirms in particular the

Table 1. ^{13}C NMR data compounds (1) and (4) in D_2O and (2) and (3) in d_5-pyridine, δ in ppm from methanol or TMS as internal standard, multiplicity of signals indicated as d, doublet, t, triplet.

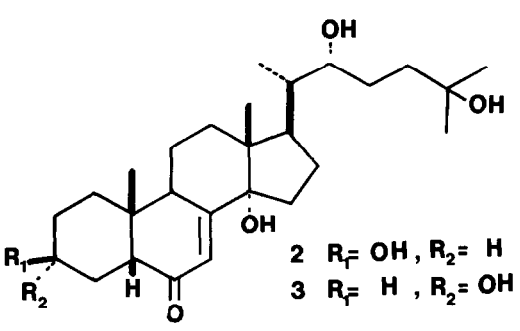
Number of carbon atom	(1)	(2)	(3)	(4)
2	27,9 (t)	29,07 (t)	32,10 (t)	29,70 (t)
3	65,5 (d)	64,06 (d)	69,90 (d)	74,60* (d)
4	32,4 (t)	33,07 (t)	35,90 (t)	33,60 (t)
20	40,2 (d)	42,99 (d)	43,20 (d)	42,70 (d)
22	78,5* (d)	73,9 (d)	74,20 (d)	75,50 (d)
23	34,4 (t)	26,70 (t)	26,80 (t)	26,60 (t)

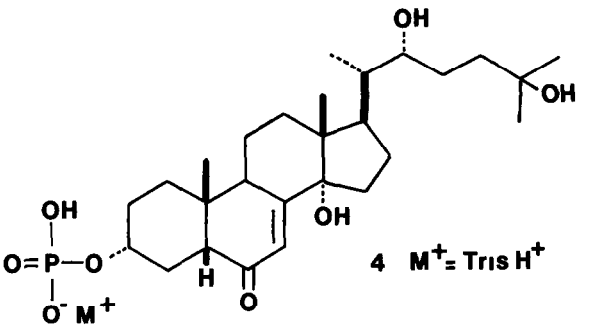
* In proton-noise decoupled spectra this signal appears as a doublet.

C-5'/phosphate link. The structure of 1 is thus established.

As a phosphate ester, this conjugate is unstable in the presence of amines (17), this implies that, in the isolation procedure, care must be taken to avoid solvolysis, which happens for instance easily and completely during heating in the presence of a Tris-buffer.

As explained above, the conjugates of newly-laid eggs undergo enzymatic hydrolysis, and further metabolism, during embryonic maturation.





The major embryonic ecdysteroid conjugate. *Locusta* eggs were incubated at 33°C for 8 days, and lyophilized. From about 1,5 kg, extracts were prepared after homogenization, and monitored for ecdysteroids by radioimmunoassay after *Helix pomatia* hydrolysis (11). The hexane and chloroform extracts contained little or no ecdysteroid and were discarded. The methanol and aqueous methanol (60%) extracts were chromatographed over silicagel, then on a reversed phase C8 packing, and rechromatographed on the latter material. Two substances were isolated 2 mg of an ecdysteroid 3, and 25 mg of a highly polar conjugate 4.

Substance 3 was deduced to be 3-epi 2-deoxyecdysone by an analysis of its mass spectrum, and mostly of its ^{1}H and ^{13}C NMR spectra, showing the identity of the whole molecule with that of 2-deoxyecdysone, except for ring A, which must bear a 3-equatorial hydroxy group. These ^{1}H and ^{13}C NMR data are indeed identical with those of 3-epi 2-deoxyecdysone (3) isolated in 1981 from the fern *Blechnum vulcanicum* (18) and from developing *Schistocerca gregaria* eggs (19)

The highly polar substance 4 was found to be the Tris [tris(hydroxymethyl)-aminomethane]- salt of the 3-phosphate of 3, from the following data its ^{31}P NMR spectrum and microanalysis indicate the presence of a phosphate group. The attachment of this group to one of the hydroxyl groups of a substance like 3 would be expected to shift the ^{13}C NMR peak of the corresponding carbon downfield by 5 ppm, and to transform it into a doublet in proton-noise decoupled spectra, the ^{13}C peaks of the immediate neighbouring atoms would be expected to be shifted upfield. This is exactly what is observed in the ^{13}C NMR spectrum of 4, which shows the 27 peaks of 3 at identical places, except for C-3, now a proton-decoupled doublet (J_{P-O-C} = 6 Hz) and shifted by + 5 ppm, and those of C-2 (-2,4 ppm) and C-4 (-2,3 ppm).

The cation associated with 3-epi 2-deoxyecdysone 3-phosphate was identified as Tris (from the buffer system in the extraction procedure) by its ^{13}C NMR spectra, and by GC-MS after trifluoroacetylation (20).

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