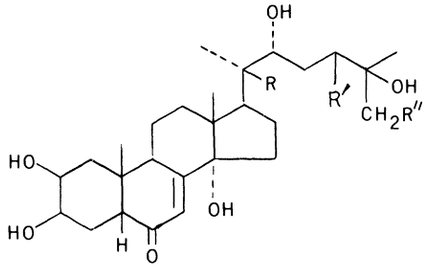
# Makisterone A: A 28-Carbon Hexahydroxy Molting Hormone from the Embryo of the Milkweed Bug

Abstract. *Makisterone A is the predominant ecdysone in the 96 ± 4-hour-old embryo of the large milkweed bug and it is the first molting hormone with a C-24 alkyl substituent on the side chain to be isolated and identified from an insect. In addition, unknown compounds that may represent other $C_{28}$ ecdysones were detected in very low concentrations. The milkweed bug could well possess a biosynthetic-metabolic pathway for $C_{28}$ molting hormones instead of or in addition to known pathways for the $C_{27}$ ecdysones.*

The five known naturally occurring insect ecdysones [α-ecdysone (**1**), 20-hydroxyecdysone (**2**), 3-epi-20-hydroxyecdysone (**3**), 26-hydroxyecdysone (**4**), and 20,26-dihydroxyecdysone (**5**)] are all $C_{27}$ steroids (*1, 2*). By contrast, certain plants possess a variety of $C_{28}$ and $C_{29}$ steroids with molting hormone activity as well as certain of the $C_{27}$ molting hormones isolated and identified from insects (*3*). Among the invertebrates, only in a species of crustacean (*4*) is there evidence for the presence of a $C_{28}$ molting hormone, an indication that steroids similar to the $C_{28}$ photoecdysones also occur in this group of animals. We now report on the occurrence of makisterone A (*5*), the $C_{28}$ hexahydroxy ecdysone 2β,3β,14α,20,22,25-hexahydroxy-24ξ-methyl-5β-cholest-7-ene-6-one (**6**), as a major molting hormone from an insect, the embryo of the large milkweed bug *Oncopeltus fasciatus* (Dallas). Extracts of milkweed bug eggs less than 1 day old contain less than 20 housefly units (based on α-ecdysone) per gram equivalent of eggs (wet weight), whereas extracts from 96- and 120-hour-old embryos exhibit 150 to 250 housefly units per gram (*6*). The increase in activity indicates that the developing embryo of the milkweed bug has the capacity to produce ecdysones with molting hormone activity as does the embryo of the tobacco hornworm [*Manduca sexta* (L.)] (*7*).




**1** R=R′=R″=H
**2** R=OH; R′=R″=H
**3** R=OH; R′=R″=H (3α-OH)
**4** R=R′=H; R″=OH
**5** R=R″=OH; R′=H
**6** R=OH; R′=$CH_3$; R″=H

Our starting material was 0.5 kg of embryos that were 96 ± 4 hours old (*6*). The eggs were collected from adults maintained on sunflower seeds and were held at 27° ± 1°C. At this temperature hatching occurs shortly after 6 days, and the average hatch for samples taken from the eggs used in our study was 91 percent.

The procedures for extracting, fractionating, isolating, and monitoring by bioassay the molting hormones were as described (*7, 8*) except that: (i) the volume of methanol and methanol solution for blending the eggs was increased to 0.6 ml/g and (ii) the crude methanol extract, after removal of a 10 percent sample to determine whether ecdysone conjugates were present (*9*), was first subjected to column chromatography on alumina and eluted with methanol (*10*) to facilitate further handling.

When the biologically active methanol eluate from the alumina column was fractionated on silicic acid (*11*) with increasing concentrations of methanol in benzene (*7*), the activity was eluted with a mixture of benzene and methanol (90 : 10). This column fraction is known to elute α-ecdysone, 20-hydroxy-ecdysone, 3-epi-20-hydroxyecdysone, and a small amount (10 percent) of the 26-hydroxyecdysone. No significant biological activity was detected in the earlier column fraction of a mixture of benzene and methanol (95 : 5) or in the later column fraction of a mixture of benzene and methanol (75 : 25) that elutes the 26-hydroxyecdysone and the 20,26-dihydroxyecdysone. Thus, the embryo of the milkweed bug differs from the embryo of the tobacco hornworm in which the major hormone was eluted in the latter fraction and characterized as 26-hydroxyecdysone (*7*).

When the active eluate from the mixture of benzene and methanol (90 : 10) fraction from the milkweed bug embryo was subjected to a 50-tube countercurrent distribution system (*7*), about 96 percent of the biological activity was concentrated in tubes 23 to 31 which had not previously been observed to contain any of the known naturally occurring insect ecdysones. After exhaustive purification of the material in these tubes by column chromatography on silicic acid and by countercurrent distribution, we isolated about 200 μg of the substantially pure but noncrystalline ecdysone. This hormone gave a distribution coefficient (*K*) and an $R_F$ value by thin-layer chromatography (*2, 7*) clearly different from any of the known naturally occurring insect ecdysones. However, these values were identical to the *K* and $R_F$ value of our authentic standards of the $C_{28}$ phytoecdysone, makisterone A (Table 1) (*12*). The nuclear magnetic resonance and mass spectra of the major molting hormone isolated from the embryo of the milkweed bug and of published data for makisterone A (*5*) as well as the spectra of our authentic standards were also identical. The biological activity of makisterone A from the embryo of the milkweed bug and that of authentic standards of makisterone A were in agreement; they were one-fourth to one-third as active as α-ecdysone in the housefly assay (*8*).

In addition to the biological activity present in the countercurrent tubes for makisterone A, detectable concentrations of active substances were also present in tubes prior to and after those from which we isolated makisterone A. The concentrations (2 to 10 microgram-equivalents) of these hormones were too small to permit further fractionation and analyses to determine whether they were also $C_{28}$ ecdysones.

In conclusion, we propose the existence of biosynthetic and metabolic pathways for $C_{28}$ and $C_{29}$ ecdysones analogous to those now known for the $C_{27}$ ecdysones in certain species of insects. Some insects then could utilize certain $C_{28}$ and $C_{29}$ plant sterols directly, without dealkylation to $C_{27}$ sterols (*1, 15*), as precursors for their molting hormones.

Table 1. Distribution coefficients (*K* values) by countercurrent distribution and $R_F$ values by thin-layer chromatography of the known naturally occurring $C_{27}$ insect ecdysones and of authentic makisterone A and makisterone A from the milkweed bug embryo.

| Molting hormone | *K** | $R_F$ † |
|---|---|---|
| α-Ecdysone | 3.54 (*13*) | 0.23 (*14*) |
| 20-Hydroxyecdysone | 0.52 (*13*) | 0.15 (*7*) |
| 3-Epi-20-hydroxyecdysone | 0.52 (*14*) | 0.17 (*2*) |
| 26-Hydroxyecdysone | 0.39 (*7*) | 0.08 (*14*) |
| 20,26-Dihydroxyecdysone | 0.06 (*14*) | 0.05 (*14*) |
| Makisterone A‡ | 1.27 | 0.20 |
| Makisterone A‡ | 1.27 | 0.20 |

*Countercurrent distribution; solvent system, cyclohexane, butanol, and water (5 : 5 : 10); 24° to 26°C. †Preparative silica gel G plates; solvent system, chloroform and ethanol (8 : 2) with wick. ‡Values for known standard makisterone A and active substance from milkweed bug embryos were identical.

J. N. KAPLANIS, S. R. DUTKY
W. E. ROBBINS
M. J. THOMPSON, E. L. LINDQUIST
*Insect Physiology Laboratory, Plant Protection Institute, U.S.D.A. Agricultural Research Service, Beltsville, Maryland 20705*
D. H. S. HORN
M. N. GALBRAITH
*Division of Applied Chemistry, C. S. I. R. O., Box 4331, G.P.O., Melbourne, Victoria 3001, Australia*

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6. Extracts from sunflower and milkweed seeds were inactive in the housefly assay when tested at a sensitivity of 10 housefly units per gram-equivalent (wet weight). The housefly unit in our assay system (*8*) is now 3 ng based on α-ecdysone.
7. J. N. Kaplanis, W. E. Robbins, M. J. Thompson, S. R. Dutky, *Science* **180**, 307 (1973).
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9. Because ecdysone conjugates are retained on alumina and conjugates in crude extracts are not readily hydrolyzed by enzymes, the sample was first fractionated on silicic acid and the polar fraction or fractions from the column known to contain conjugates were subjected to enzymic hydrolysis with a mixture of sulfatase and glucosidase. Conditions for hydrolysis, extractions, and fractionations were as described by J. N. Kaplanis, S. R. Dutky, W. E. Robbins, M. J. Thompson in *Invertebrate Endocrinology and Hormonal Heterophylly*, W. J. Burdette, Ed. (Springer-Verlag, New York, 1974), chap. 14, p. 161. No biological activity was detected in the extracts after enzymic hydrolysis, indicating again that, in the embryo (*7*), conjugation does not occur to an appreciable extent in this period of development.
10. Woelm neutral grade I alumina deactivated with 20 percent water. Column, 9.5 cm (inside diameter) by 2.7 cm high; 225 g of adsorbent. The crude extract was eluted with methanol (2 liters) then with 75 percent methanol (1 liter). Only the methanol fraction was biologically active.
11. Unisil, 100 to 200 mesh (Clarkson Chemical Co., Williamsport, Pa.)
12. Two authentic standards of makisterone A were available for these studies. One of the samples was from *Podocarpus macrophyllus* and the other was from *Podocarpus elatus*.
13. J. N. Kaplanis, M. J. Thompson, R. T. Yamamoto, W. E. Robbins, S. J. Louloudes, *Steroids* **8**, 605 (1966).
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## In vitro Demonstration of an Endothelial Proliferative Factor Produced by Neural Cell Lines

Abstract. *Cultured endothelial cells exhibit a six- to tenfold increase in thymidine labeling index in response to a soluble factor elaborated by clonal cell lines of neural origin. This factor, endothelial proliferation factor, appears to be a unique property of tumor cells and may mediate the vascularization of these neoplasms.*

Neovascularization has long been recognized as necessary for tumor growth and maintenance. Algire (*1*) suggested that an attribute of tumor cells is their capacity to elicit the continual production of capillary endothelium in vivo. This concept was strengthened and extended by the work of Tannock *et al.* (*2*) who showed that the rate of tumor growth could be directly related to vascular supply, and of Gimbrone *et al.* (*3*) who showed that in the absence of neovascularization a tumor becomes restricted in size to a spheroid about 2 to 3 mm in diameter. Folkman and his coworkers (*4*) have suggested that tumor cells elaborate a factor known as tumor angiogenesis factor (TAF), which stimulates the proliferation of endothelial cells and thereby the neovascularization of the tumor. Endothelial cells in the adult have an extremely low proliferation rate (*5*), which suggests the existence of a stimulatory factor associated with neoplasms.

Folkman *et al.* (*6*), using the in vivo dorsal air sac and other in vivo assay techniques, have demonstrated an increase in vascularity in response to a soluble factor isolated from animal and human neoplasms. The proliferative response is not, however, restricted to the capillary endothelium but also involves pericytes and surrounding connective tissues. Cavallo *et al.* (*7*) have speculated that this broad mitogenic response reflects an impure TAF fraction containing several mitogenic factors or, alternatively, is a manifestation of the wound-healing process. The wound-healing response is difficult to distinguish from a specific tumor angiogenic response in the in vivo assay. These considerations prompted us to develop an in vitro assay with cultured human endothelial cells as the target of the trophic factor or factors produced by clonal cell lines of tumor origin. We now describe the development of an in vitro tissue culture assay, the use of which clearly demonstrates a stimulation of endothelial proliferation in response to a soluble factor produced by tumor cells. We have called this factor endothelial proliferation factor (EPF).

Table 1. Thymidine labeling index of human endothelial cells cultured with medium conditioned by tumor cell lines. Proliferation response is given as percentage.

| Conditioning medium | Response |
|---|---|
| *Controls* | |
| Fresh medium | 2.1 |
| Amniotic fluid | 1.1 |
| Fibroblast conditioned medium | 4.5 |
| *Glia tumor cells* | |
| C6 (A) | 98.3 |
| C6 (A) | 97.6 |
| C6JDV (B) | 92.6 |
| C6JDV (B) | 83.3 |
| C6-CRL-107 (C) | 25.7 |
| C6-CCL-107 (C) | 36.7 |
| C6-CCL-107 Co-culture | 28.7 |
| Human astrocytoma primary | 30.1 |
| *Neuroblastoma tumor cells* | |
| NB-41 | 90.0 |
| IMR-32 | 78.7 |

Endothelial cells were obtained from the umbilical veins of human umbilical cords at term by the method of Jaffe *et al.* (*8*) with some modification. We cannulated the umbilical vein using a 16- or 18-gage trochar needle. After removal of blood from the vein by flushing with Hanks solution (Ca and Mg free), collagenase (2 mg/ml) (Sigma) in Hanks solution was introduced into the vein. The endothelial cells were dissociated from the wall of the vein by digestion with collagenase for 20 minutes and flushed free by rinsing with Hanks solution. The Hanks solution containing the endothelial cells and remaining red blood cells was centrifuged for 3 to 5 minutes and resuspended in a small volume of F10 medium plus 20 percent fetal calf serum.

The cells were then plated on glass cover slips in six well culture dishes (Linbro). After 12 to 18 hours the red blood cells were washed free of the cover slips with Hanks solution and then 2.0 ml of growth medium, either medium 199 or F10 supplemented with 20 percent fetal calf serum and containing penicillin (50 unit/ml) and streptomycin (50 μg/ml) (all from Gibco), was added to each well. These primary cultures of human endothelial cells were the vehicle for our in vitro assay. Six uniform cultures from a single umbilical cord allowed each sample to have a control and five experimental cultures.

Tumor-conditioned medium was tested for the presence of EPF as follows. After 1 to 3 days in culture, an additional 1.0 ml of test medium was added to each culture well. Twenty-four hours later, the cultures were labeled with [$^3$H]thymidine (0.5 μc/ml; specific activity > 10 mc/mmole; New England Nuclear) for 72 hours. After fixation in either acetic acid and ethanol (1 : 3) or 10 percent buffered formalin, the cover slips were coated with NTB-2 nuclear tracking emulsion (Kodak) and exposed for 5 days. The exposed cover slips were developed and stained with toulidine blue or Giemsa.

Tumor-conditioned media have been obtained from actively growing cultures of the following cell types:

*C6 rat astrocytoma.* A clonal cell line derived from a tumor chemically induced with *N*-nitrosomethylurea (*9*). Three subclones of this tumor have been assayed for production of EPF: (A) a subclone obtained from J. de Vellis; (B) a subclone obtained from Stephen Pfieffer; (C) a sub-