Table 1. Effects of juvenile hormone on *Sarcophaga bullata* pupae. The juvenile hormone and methyl ester were diluted to 1 μl in olive oil and applied topically to the pupal abdomen. Control animals received 1 μl of olive oil, and all emerged as normal adults. The age of the pupa is determined from the onset of tanning of the puparium.

Dose (Tenebrio Units)	Pupal age (hr)	Animals (No.)	Pupal-adult intermediates (No.)
	dl-juvenile hormones		
500	50–55	25	0
1000	50–55	10	0
2000	53–58	20	16
2500	46–52	10	10
2500	70–75	15	0
5000	90–95	10	0
	C_{17} methyl ester		
500	50–55	30	1
1000	48–52	20	0
2500	46–50	10	9
2500	50–55	32	27
2500	65–70	12	6
2500	72–76	12	0

mented hairs and setae. However, the abdomen is typically pupal, with a smooth, transparent cuticle devoid of hairs and pigmentation. The external genitalia are undeveloped or incompletely developed, and the fat body can be seen clearly through the cuticle. Histological sections of eight of these intermediates showed that the head, thorax, and occasionally part of the abdomen were covered by typical adult cuticle. However, in the "pupal" region of the abdomen there are two layers of cuticle (including exo- and endocuticle), one being the original pupal cuticle which had not been digested away and a new pupal cuticle interior to it (Fig. 2). Even the fat body of the pupal region is composed of spherical masses typical of the early pupal stage, whereas in the adult region of the fat body cells have separated normally. Thus, the application of *dl*-juvenile hormone or C_{17} methyl ester induced the epidermal cells of the abdomen to lay down a second pupal cuticle rather than adult cuticle, and this is without doubt a true juvenile hormone effect.

In virtually all corresponding experiments on pupae older than 46 hours in which the hormone was applied to the head or thorax, these parts developed normally, although the abdomen was sometimes affected. In six of ten young pupae (40 to 44 hours after onset of tanning of puparium) receiving 2500 TU of the C_{17} methyl ester, portions of the head and thorax were induced to lay down a second pupal cuticle. It thus appears that the epidermal cells of the head and thorax are determined earlier than those of the abdomen, and, if the technical difficulties involved in applying the hormone at an earlier stage (before the pupal cuticle separates from the puparium) are overcome, we should be able to induce the formation of complete second pupae. It is likely that the imaginal disks of the head and thorax are refractory to the juvenile hormone by 40 hours after initiation of tanning of the puparium.

Using the crude synthetic mixture discussed above (*4*), we conducted two series of experiments. One-microliter samples containing 0.02 μl of the mixture and 0.98 μl of peanut oil were applied to the abdomens of 20 pupae (48 to 56 hours old). Nineteen of the twenty developed into pupal-adult intermediates indistinguishable from those treated with *dl*-juvenile hormone except for a paucity of pigmentation on the head and thoracic hairs. We have not identified the active principle in the mixture, although the major component is the methyl ester of 3,7,11-trimethyl-7,11-dichloro-2-dodecenic acid which exhibits potent juvenile hormone activity when tested on *Pyrrhocoris apterus* (*7*).

Our data demonstrate that the higher Diptera do respond to juvenile hormone when it is applied to the organism at the correct stage and suggest that the natural dipteran juvenile hormone is the same or very similar to that obtained from the *H. cecropia* moth. Further, a unique chemical tool is now available to the developmental geneticist and molecular biologist interested in studying animal differentiation.

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References and Notes

1. C. M. Williams, *Nature* **178**, 212 (1956); L. I. Gilbert, in *Physiology of Insecta*, M. Rockstein, Ed. (Academic Press, New York, 1964), p. 149; H. A. Schneiderman and L. I. Gilbert, *Science* **143**, 325 (1964); F. Sehnal and A. Meyer, *ibid.* **159**, 981 (1968); H. Röller, personal communication.
2. C. M. Williams, *Sci. Amer.* **217**, 13 (1967).
3. H. Röller, K. H. Dahm, C. C. Sweeley, B. M. Trost, *Angew. Chem.* **6**, 179 (1967).
4. J. H. Law, C. Yuan, C. M. Williams, *Proc. Nat. Acad. Sci. U.S.* **55**, 576 (1966).
5. K. H. Dahm, B. M. Trost, H. Röller, *J. Amer. Chem. Soc.* **89**, 5292 (1967); We are grateful to Prof. Röller for samples of the *dl*-juvenile hormone and C_{17} methyl ester.
6. A. Spielman and C. M. Williams, *Science* **154**, 1043 (1966); A. Spielman and V. Skaff, *J. Insect Physiol.* **13**, 1087 (1967).
7. M. Romaňuk, K. Sláma, F. Šorm, *Proc. Nat. Acad. Sci. U.S.* **57**, 349 (1967).
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5-Diazo-4-Oxo-L-Norvaline: Reactive Asparagine Analog with Biological Specificity

Abstract. *The* L-*asparagine analog, 5-diazo-4-oxo-*L-*norvaline, specifically inactivates* L-*asparaginase and inhibits the growth of* L-*asparagine-dependent or* L-*asparaginase-sensitive tumor cells in culture. With 5-^{14}C-labeled compound, a biphasic incorporation into sensitive cells occurs, but the inhibition of cell multiplication is manifest much later than the rapid phase of incorporation of the analog.*

The inhibition of the growth of certain transplantable and spontaneous tumors by the enzyme L-asparaginase [L-asparagine amidohydrolase (E.C. 3.5.1.1)] has been briefly reviewed (*1*) and extended to the treatment of human disease with the enzyme from *Escherichia coli* (*2*). An alternate approach in the treatment of these selected neoplasms would be the development of L-asparagine analogs that might act as antagonists of the synthesis or utilization of L-asparagine. Such an analog is 5-diazo-4-oxo-L-norvaline (DONV). An improved preparation and some biological properties of this compound are now reported.

The synthesis of DONV was first reported by Liwschitz *et al.* (*3*). The difficult stage in the synthesis was the removal of the protecting trifluoroacetyl and methyl ester groups from

Table 1. Specificity of action of diazo-oxo-norvaline. Asparaginase (0.5 ml of guinea pig serum in a total volume of 1 ml of solution) and glutaminase (2.5 mg in a total of 0.5 ml solution) were first incubated at *p*H 8.5 with the inhibitors and protecting agents as indicated for 30 minutes. Enzymic activity was then determined after gel filtration to remove small molecules. Activity is expressed as the number of micromoles of NH_3 liberated per hour per milligram of protein.

Prior treatment		Activity (μmole $hr^{-1}mg^{-1}$)
Compound	Concentration	
	Asparaginase	
None		2.3
DONValine	$6 \times 10^{-4}M$	0.6
DONLeucine	$6 \times 10^{-4}M$	2.3
DONValine	$6 \times 10^{-4}M$	
+ Asparagine	$5 \times 10^{-2}M$	1.8
+ Glutamine	$5 \times 10^{-2}M$	0.7
DONValine	$1 \times 10^{-4}M$	
+ aspartic acid (*p*H 4.5)	1×10^{-2}M	2.1
+ aspartic acid (*p*H 8.5)	$1 \times 10^{-2}M$	0.8
	Glutaminase	
None		18.5
DONValine	$6 \times 10^{-4}M$	18.6
DONLeucine	$6 \times 10^{-5}M$	0.4

5-diazo-4-oxo-*N*-trifluoroacetyl-L-norvaline methyl ester to give DONV. Liwschitz reported that hydrolysis with 0.1*N* tetramethylammonium hydroxide at room temperature for 30 minutes gave a 20 percent yield of a product having maximums in the ultraviolet absorption spectrum at 274 nm ($E_{1\ cm}^{1\%} = 600$) and at 244 nm ($E_{1\ cm}^{1\%} = 310$). In the improved synthesis, this final hydrolysis was achieved by adding cold 0.2*N* NaOH (100 ml) to a solution of 5-diazo-4-oxo-*N*-trifluoroacetyl-L-norvaline methyl ester in methanol (1.35 g in 30 ml) at −16°C. The mixture was allowed to stand at −16°C for 48 hours. The orange-red solution was adjusted at 4°C to *p*H 6.1 with 1*N* HCl (about 10 ml) and lyophilized. The dark-red residue was dissolved in water (10 ml), and the solution was passed through a column (2 by 35 cm) of charcoal (Darco G-60, Atlas Chemical Industries) and Celite 535 (1:1, by weight). The column was eluted with 1 percent aqueous acetone at 4°C, and the eluate was lyophilized to give a colorless or faintly yellow product (0.7 g; yield 90 percent) of high analytical purity. Ultraviolet absorption maximums in aqueous solution were at 274 nm ($E_{1\ cm}^{1\%} = 700$) and 244 nm ($E_{1\ cm}^{1\%} = 380$). The calculated values for $C_5H_7N_3O_3$ were C, 38.22; H, 4.49; N, 26.74. Those found were C, 38.20; H, 4.65; N, 26.54. Radioactive preparations of DONV have been made by the use of ^{14}C-diazomethane in the next to the last step of the synthesis.

To assess the specificity of DONV as an analog of L-asparagine, we determined the effect of this compound on L-asparaginase from guinea pig serum and L-glutaminase from *Escherichia coli* (Table 1). An experimental decline in asparaginase activity occurs via a saturable reaction which causes irreversible inhibition. The L-asparaginase (EC-2) from *E. coli* (*4*) is similarly inactivated by DONV.

The effects of DONV on cell cultures of L5178Y lymphoblastic leukemia and the P815Y mast-cell leukemia were assayed because these represent L-asparagine-dependent and nondependent tumor lines, respectively. The rate of killing of cells in logarithmic growth was determined by incubation with DONV and subsequent cloning to determine cell viability. At a DONV concentration of 6×10^{-4} mole/liter, 80 percent of the L-asparagine-requiring L5178Y cells in culture were killed in 4 hours, whereas the P815Y

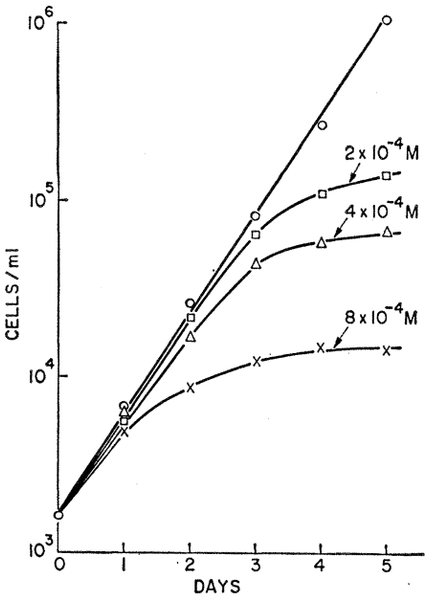

Fig. 1. Growth of L5178Y cells in culture in the presence of various concentrations of DONV. The analog was added to cultures in the logarithmic phase of growth in Fischer's medium (*5*), and the cell number was determined with a Coulter counter at the indicated times.

line, which does not require exogenous asparagine, sustained only 25 percent loss in viability.

Continuous exposure of growing cultures of L5178Y cells to different concentrations of DONV produces essentially complete inhibition of growth at a time which is dependent upon dose (Fig. 1).

The incorporation of DONV labeled with ^{14}C at position 5 into L5178Y cells in culture was followed (Fig. 2). Despite a relatively delayed effect on cell replication, the fixation of isotope into an acid-insoluble form occurs primarily within the first 4 to 5 hours. The incorporation after this period is

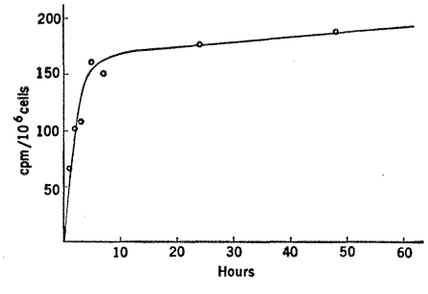

Fig. 2. Uptake of 5-^{14}C-DONV by L5178Y cells in culture. Cells (2.5×10^5 per milliliter) in the logarithmic phase of growth were incubated with 5-^{14}C-DONV ($5 \times 10^{-5}M$; 1.1 μc/μmole) for the indicated times. Incorporation into acid-insoluble material was determined by membrane filtration.

proportional to further growth of the cells and may represent further synthesis of target sites or nonspecific alkylation by this reactive compound. Under these conditions of incubation, active concentration of free DONV into the cells from the medium occurred within 5 minutes.

In other experiments, the uptake of radioactive thymidine into DNA, or uridine into RNA and DNA and of L-asparagine or L-leucine into total protein was not inhibited specifically by DONV ($7 \times 10^{-4}M$) at a time (22 hours) when marked inhibition of cell growth was becoming apparent (*6*). In mice, DONV (50 mg/kg; intraperitoneally, twice daily) inhibits the total growth of ascitic L5178Y leukemia (76 percent) and subcutaneous 6C3HED lymphoma (44 percent), with less than 5 percent weight loss. The effects on the 6C3HED cell line, however, are not as dramatic as those observed with L-asparaginase. It is interesting that DONV caused a 49.7 percent inhibition in 48 hours of the growth of a line of the Jensen sarcoma dependent on L-asparagine, but did not inhibit the growth of a subline (JA 1) of this cell type that was not dependent (*7*).

The usefulness of DONV in the investigation of receptor sites for L-asparagine, an amino acid essential to the growth of certain tumor cells, is apparent. The exploration of possible therapeutic applications of this type of compound also warrants serious consideration.

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References and Notes

1. Annotations, *Lancet* **1967-I**, 431 (1967).
2. J. M. Hill, J. Roberts, E. Loeb, A. Kahn, A. MacLellan, R. W. Hill, *J. Amer. Med. Ass.* **202**, 116 (1967).
3. Y. Liwschitz, R. D. Irsay, A. I. Vincze, *J. Chem. Soc.* **1959**, 1308 (1959).
4. H. A. Campbell, L. T. Mashburn, E. A. Boyse, L. J. Old, *Biochemistry* **6**, 721 (1967).
5. G. A. Fischer and A. C. Sartorelli, *Meth. Med. Res.* **10**, 247 (1964).
6. R. E. Handschumacher, C. J. Bates, P. K. Chang, A. T. Andrews, G. A. Fischer, *Proc. Amer. Ass. Cancer Res.* **8**, 25 (1967).
7. M. K. Patterson, pesonnel communication.
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