Shaw, in *Symposium on Human Cytogenetics*, C. B. Lozzio and A. I. Chernoff, Ed. (Birth Defects Evaluation Center, University of Tennessee, Knoxville, 1968).
4. Lymphocyte chromosomes were cultured and prepared by the standard method [P. S. Moorhead, P. C. Nowell, W. J. Mellman, D. M. Battips, D. A. Hungerford, *Exp. Cell Res.* **20**, 613 (1960)].
5. W. Schmid, in *Human Chromosome Methodology*, J. J. Yunis, Ed. (Academic Press, New York, 1965), p. 91.
6. J. H. Tjio and J. Whang, in *ibid.*, p. 51; T. Kajii, T. Imai, T. Homma, K. Oikawa, *Lancet* **1966-II**, 1323 (1966).
7. Fibroblasts were grown from a skin explant and harvested after 52 and 106 days in culture [T. Kajii, R. L. Neu, L. I. Gardner, *Lancet* **1967-I**, 897 (1967)].
8. The criteria for determining the minimum number of cells to be scored were derived from sequential analysis with α and $\beta = 0.05$. Previously, we had found that only 2 of 2800 metaphases from 100 persons unrelated to the present family had breaks in the long arm of chromosome 16. Thus, if 2 of 60 metaphases from a person showed isochromatid breaks at the fragile site, the score was sufficient to reject the null hypothesis that the person did not have the fragile site.
9. Y. Matsuya, H. Green, C. Basilico, *Nature* **220**, 1199 (1968).
10. E. B. Robson, P. E. Polani, S. J. Dart, P. A. Jacobs, J. H. Renwick, *ibid.* **223**, 1163 (1969).
11. R. P. Donahue, W. B. Bias, J. H. Renwick, V. A. McKusick, *Proc. Nat. Acad. Sci. U.S.* **61**, 949 (1968). The metacentric nature of chromosome 1 makes it somewhat uncertain as to which arm bears the paracentromeric uncoiled region and the Duffy locus. The special nature of the X chromosome has complicated attempts to localize linkage groups to a particular arm of the X.
12. E. R. Giblett, *Genetic Markers in Human Blood* (Davis, Philadelphia, 1969), p. 64.
13. The Hp phenotypes in this family are Hp 1-1, Hp 2-1, Hp 2-2, and Hp 2-1 Carlberg. Detailed linkage results including lod scores for Hp as well as other serum, red cell, and enzyme markers will be presented when family studies are complete.
14. J. H. Renwick, *Brit. Med. Bull.* **25**, 65 (1969).
15. B. Amos, G. Cabrera, W. B. Bias, J. M. McQueen, S. L. Lancaster, J. G. Southworth, F. E. Ward, in *Histocompatibility Testing 1970*, P. Terasaki, Ed. (Munksgaard, Copenhagen, 1970), p. 259.
16. W. B. Bias, D. B. Amos, F. E. Ward, O. C. Yoder, J. H. Renwick, V. A. McKusick, personal communication.
17. The word syntenic is defined in (*14*): two genes on the same chromosome.
18. We thank Dr. K. M. Taylor for lymphocyte cultures of several family members living near San Diego; Dr. E. Bardana for referring the patient; Dr. W. Kimberling for statistical consultation; Dr. J. Higgins, S. Underwood, and P. Evans for help with family studies; S. Ditewig, C. MacDonald, B. McCaw, T. Ryan, M. Case, D. Worthington, C. Trombly, and H. Wyandt, for helping R.E.M. and F.H. with chromosome studies; and S. Rowe, J. Beck, and S. Hazard for helping E.W.L. with gene marker studies. We especially thank the family for their interest and cooperation. R.E.M. is a postdoctoral fellow on NIH genetics training grant 5-T01-HD00165. Work was supported by NIH grant AM-13173 and by a grant from the Children's Bureau for a Genetics Program.

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Potent Sex Attractant of the Gypsy Moth: Its Isolation, Identification, and Synthesis

Abstract. *The sex attractant emitted by the female gypsy moth has been identified as* cis-*7,8-epoxy-2-methyloctadecane. The structure was verified by spectral, gas chromatographic, and biological comparisons with the synthesized compound. Nine closely related isomers were considerably less effective.*

The gypsy moth *Porthetria dispar* (L.) is a serious despoiler of forest and shade trees in the northeastern part of the United States. Crude extracts of the abdominal tips of virgin females containing the sex attractant are used in traps to determine the occurrence and abundance of the moth (*1*); control measures are then applied to prevent excessive defoliation and the migration of the moth into new territory. Since the application of insecticide is made only where needed, residues are held to a minimum.

Jacobson and co-workers (*2*) identified the gypsy moth sex attractant as *cis*-7-hexadecene-1,10-diol 10-acetate (gyptol) and reported that its homolog, gyplure (*cis*-9-octadecene-1,12-diol 12-acetate), was also a highly active gypsy moth sex attractant. Preparations of these compounds by other researchers were reported as inactive (*3*); a reinvestigation by Jacobson *et al.* (*4*) confirmed that the compounds were inactive and indicated that the original gyptol preparation was active because of the presence of traces of another substance with "extraordinarily high biological activity."

We have isolated the gypsy moth sex attractant, and it has been identified as *cis*-7,8-epoxy-2-methyloctadecane, for which the name disparlure is proposed. The synthesized compound was active in laboratory and field tests at concentrations of 2×10^{-12} and 1×10^{-9} g.

The sex attractant was extracted from 78,000 tips (last two abdominal segments of female moths) collected in Spain and concentrated essentially as described previously (*5*). The concentrate was refluxed under nitrogen with ethanolic potassium hydroxide to saponify fats (*6*), and the neutral fraction, which contained the activity (*7*), was chromatographed on Florisil (*8*). The hydrocarbons were eluted with hexane and the active material with 2 to 6 percent ether in hexane. The active material was chromatographed again on Florisil and purified further by silica-gel thin-layer chromatography (TLC) with a double development with 20 percent CH_2Cl_2 in cyclohexane and another double development of the eluted active portion with 40 percent CH_2Cl_2 in cyclohexane (R_F, 0.50).

A portion of the partially purified material was analyzed to obtain information on the nature of the active substance; the activity was determined by laboratory bioassay (*7*). Chromatographic mobility (R_F) on silica-gel TLC plates was consistent with an aliphatic ketone (but not a methyl ketone), aliphatic epoxide, methyl alkyl ether, or an alkyl ester other than an acetate, an indication that the compound was monofunctional. Gas-chromatographic retention indices (*9*) indicated that the compound contained 18 to 20 carbon atoms (*10*). Because ozonolysis of the compound did not diminish activity or affect the R_F value on silica-gel TLC, the presence of a double bond was excluded. The material on a TLC plate was overspotted with aqueous semicarbazide hydrochloride solution and the plate developed; elution of the area active with an untreated sample indicated that the active component was not an aldehyde or ketone. Similar spotting of the sample with phosphoric acid, which reacts with epoxides and retains them at the origin, eliminated activity. Both powdered lithium aluminum hydride and dry, powdered periodic acid destroyed the activity.

Reaction gas-chromatographic trials with subtraction loops (*11*) mounted after the column were also used, and male moths were used to monitor the effluent to determine whether the active compound survived reaction. Boric acid (subtracts alcohols) and *o*-dianisidine (subtracts aldehydes) loops did not affect activity. A benzidine loop (subtracts aldehydes, ketones, and some epoxides) diminished activity, while a loop containing phosphoric acid (subtracts epoxdes) totally eliminated activity.

These results led us to postulate that the natural attractant is a C_{18} to C_{20} alkyl epoxide. Unfortunately the amount of pure attractant in the 78,000 tips was considered insufficient for adequate characterization of the attractant.

We speculated that the insect might contain an olefin precursor from which the attractive epoxide could be formed, and that this olefin might be used to generate more of the sex attractant. Treatment of the original neutral fraction with *m*-chloroperbenzoic acid, which epoxidizes olefins, caused a tenfold enhancement of the activity of the

fraction. Similar treatment of the hydrocarbon fraction to the exclusion of all other fractions, likewise generated considerable activity, indicating that the original extract contained much more olefin precursor than active attractant.

To isolate and identify the olefin precursor, we chromatographed the hydrocarbon fraction on a silica-gel column containing 25 percent silver nitrate (*12*) to separate the paraffins, monoolefins, and polyolefins. The monoolefin fraction, which became biologically active on epoxidation, was further separated by gas chromatography (*13*). Only one of the monoolefins trapped, which gave a single peak of about 10 to 15 μg, could be epoxidized to active material, and this material was active in laboratory and field tests. Its retention times (t_R) on diethyleneglycolsuccinate (DEGS) and OV-17 columns were consistent with a branched C_{19} olefin; the epoxidized olefin had the same t_R on an SE-30 column as the natural attractant in the laboratory bioassay (*7*).

The position of the double bond was determined by ozonolysis (*14*) of a 1-μg portion of the trapped monoolefin. Two major fragments were obtained. The t_R of one was identical to that of undecanal, and the t_R of the other was slightly less than that of *n*-octanal, which by Kovats Retention Indices (*9*) is consistent with a branched C_8 aldehyde (*15*). The olefin is therefore $CH_3(CH_2)_9CH{=}CH{-}R$ where R is a branched C_7 alkyl group. The double bond was established as *cis* by TLC on a plate impregnated with silver nitrate (*16*).

The position of the methyl group was determined by instantaneous hydrogenation of 1 to 2 μg of the olefin in the gas chromatographic system (*17*), by trapping the product in hexane, and by determining its mass spectrum by combined gas chromatography–mass spectrometry (*18*). The spectrum showed a molecular ion in 5 percent abundance at *m/e* (mass to charge) 268 (corresponding to $C_{19}H_{40}$) and large peaks at *m/e* 253 (P-15) and 225 (P-43), consistent with a single methyl branch in the 2-position of the hydrocarbon (that is, a terminal isopropyl group).

The data indicated that the sex attractant is *cis*-7,8-epoxy-2-methyloctadecane, and this compound was synthesized as follows. Hydrogen bromide was added to 6-methyl-1-heptene (Chemical Samples Co., Columbus, Ohio) in the presence of benzoyl peroxide (reverse Markownikoff addition) to form 1-bromo-6-methylheptane (66 percent yield). The compound was refluxed overnight with triphenylphosphine in acetonitrile to form the phosphonium salt. *n*-Butyllithium was added to the salt in dry dimethylsulfoxide, and undecanal was then added. Workup of the product gave 2-methyl-7-octadecene in an overall yield of 35 percent. The compound was 98.5 percent pure by gas chromatography and had a nuclear magnetic resonance spectrum consistent with the expected structure (*19*): (CCl_4, τ) three methyl groups as doublet plus unresolved multiplet at 9.14; eleven CH_2 groups as singlet at 8.75; single C–H as multiplet about 8.4; two CH_2 groups adjacent to double bond as multiplet at 8.02; and $-CH{=}CH-$ as multiplet at 4.74. The calculated values for $C_{19}H_{38}$ were C, 85.63 percent and H, 14.37 percent; the values found were C, 85.89 percent and H, 14.35 percent. Treatment of the synthetic olefin with *m*-chloroperbenzoic acid gave the epoxide. The calculated values for $C_{19}H_{38}O$ were C, 80.78 percent and H, 13.56 percent; the values found were C, 80.73 percent and H, 13.56 percent. The synthetic olefin was separated by column chromatography on silica gel–silver nitrate (*12*) into its *cis* (85 percent) and *trans* (15 percent) isomers; from these, the two isomeric epoxides were prepared and bioassayed. Each gave a single spot by TLC. As little as 2 pg of the synthetic *cis* epoxide was active in the laboratory bioassay, and the *cis* compound was about ten times as active as its *trans* analog. No reduction in activity (masking) of the *cis* compound was observed when the *trans* isomer was added. Removal of the *trans* isomer from synthetic preparations therefore does not appear to be necessary.

Table 1. Relative activity of compounds related to gypsy moth sex lure. Epoxides are estimated to be 85 percent *cis*. Activity was set at 100 percent for epoxidized natural olefin and, except as noted, was determined by laboratory bioassay.

Synthetic compound	Activity (%)
8,9-Epoxynonadecane	0.02
7,8-Epoxy-2-methyloctadecane	100
7,8-Epoxy-3-methyloctadecane	5
7,8-Epoxy-4-methyloctadecane	0.5
3,4-Epoxy-2-methyloctadecane	0.1
3,4-Epoxy-3-methyloctadecane	0.001
3,4-Dimethyl-6,7-epoxyheptadecane	0.1
9,10-Epoxy-2-methyloctadecane	*
10,11-Epoxy-2-methyloctadecane	*

* Inactive at 20 μg when compared to 1 μg of disparlure in field tests.

Confirmation of identity of the synthetic sex lure with the epoxidized natural olefin was obtained by several routes. The synthesized olefin, 2-methyl-*cis*-7-octadecene, was hydrogenated in the gas-chromatographic pathway (*17*) and trapped. The mass spectrum of the resulting paraffin (*18*) was virtually identical to that of the hydrogenated natural olefin precursor of the sex attractant. The mass spectra of the two olefins were also the same. Ozonolysis of the synthetic olefin also produced aldehyde fragments having the same t_R values as those produced from the natural olefin. Retention indices (*9*) of the *cis* and *trans* isomers of the synthetic epoxide, 7,8-epoxy-2-methyloctadecane, on Carbowax 20M (*20*) were significantly different, 2205 and 2189, respectively. The retention index of the epoxide of the natural olefin was 2205, providing further confirmation of its *cis* configuration.

The identity of the natural sex lure with the synthetic remained to be established. A portion of the purest TLC zone of the natural active attractant was purified further by gas chromatography on a DEGS column; the mass spectrum and t_R of the active fraction were determined by combined gas chromatography–mass spectrometry (*18*). Although the gas chromatogram showed the active material was still a two-component mixture, one of the peaks coincided with the synthesized epoxide in t_R, and its mass spectrum was the same as that of the synthetic epoxide. In bioassays of several active fractions, activity varied directly with the area of the peak corresponding to that of the synthesized epoxide. Response of the moths exposed to equivalent concentrations of the natural and synthesized sex lure was about equal. In this way, we were able to identify the attractant using 10 to 15 μg of the olefin and only a few micrograms of the natural sex lure.

Activity of the synthetic was confirmed in a simulated field test early in May 1970 in Massachusetts. Male moths reared in the laboratory were released periodically in the vicinity of eight traps; four were baited with 1 μg each of the synthetic sex attractant and four with a potent extract of natural gypsy moth sex attractant equivalent to ten abdominal tips, the amount used in survey traps. In the 10-day test, the synthetic attractant caught 110 moths, the natural attractant caught 3.

A number of related compounds were synthesized (Table 1) to determine the effect of structural variations, but none approached the activity of *cis*-7,8-

epoxy-2-methyloctadecane. The high specificity of the sex lure is evident from the great differences in activity encountered with slight variations in molecular structure.

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References and Notes

1. R. F. Holbrook, M. Beroza, E. D. Burgess, *J. Econ. Entomol.* **53**, 751 (1960).
2. M. Jacobson, M. Beroza, W. A. Jones, *Science* **132**, 1011 (1960); *J. Amer. Chem. Soc.* **83**, 4819 (1961); M. Jacobson, *J. Org. Chem.* **25**, 2074 (1960); *ibid.* **27**, 2760 (1962); U.S. Patents 3,018,219 and 3,050,551; M. Jacobson and W. A. Jones, *J. Org. Chem.* **27**, 2523 (1962); R. M. Waters and M. Jacobson, *J. Econ. Entomol.* **58**, 370 (1965).
3. D. K. Sefanovic, B. Grujic-Inac, D. Micic, *Zastita Bilja* **73**, 235 (1963) [CA **61**, 1199d (1964)]; K. Eiter, E. Truscheit, M. Boness, *Ann. Chem.* **709**, 29 (1967).
4. M. Jacobson, R. M. Waters, M. Schwarz, *J. Econ. Entomol.* **63**, 943 (1970).
5. F. Acree, Jr., M. Beroza, R. F. Holbrook, H. L. Haller, *ibid.* **52**, 82 (1959).
6. H. L. Haller, F. Acree, Jr., S. F. Potts, *J. Amer. Chem. Soc.* **66**, 1659 (1944).
7. Activity of fractions was determined by laboratory or field bioassay. In the unpublished laboratory procedure devised by one of us (C.W.C.), the sample is injected into a gas chromotograph, and the number of moths [clipped by their wings to a rack as described by B. C. Block, *J. Econ. Entomol.* **53**, 172 (1960)] activated by the effluent is compared to the number activated by an arbitrary standard. The laboratory bioassay, which registers excitement and possible attraction, was used when field testing was not possible. The field bioassay, available only a few weeks of the year, was the ultimate test of activity. Field bioassays were carried out as described in (*1*). We thank Dan Cook and Robert Tardiff of the Plant Protection Division, Otis Air Force Base, Mass., for conducting these bioassays.
8. Florisil, 60/100 mesh, activated overnight at 110°C. Fisher Scientific Co., No. F-100.
9. E. Kovats, *Advan. Chromatogr.* **1**, 229 (1966).
10. Retention times of the active material were equivalent to a C_{20} *n*-alkane on an SE-30 packing and to a C_{22} to C_{23} *n*-alkane on the more polar Carbowax 20M and DEGS packings.
11. B. A. Bierl, M. Beroza, W. T. Ashton, *Mikrochim. Acta* **1969**, 637 (1969).
12. Adsorbosil CABN, 60/100 mesh, 25 percent $AgNO_3$ on silica gel (Applied Science Laboratories, State College, Pa.).
13. A copper column (0.9 m by 0.9 cm, outer diameter) containing 10 percent OV-17 on 70/80 mesh Anakrom ABS (Analabs, Hamden, Conn.) at 180°C was used for the first two purifications and a copper column (1.2 m by 0.45 cm, outer diameter) containing 5 percent DEGS on base-washed, 60/80 mesh Chromosorb W at 110°C for the final purification.
14. M. Beroza and B. A. Bierl, *Anal. Chem.* **39**, 1131 (1967).
15. A copper column (3.6 m by 0.6 cm, outer diameter) containing 5 percent Carbowax 20M on 60/80 mesh, acid-washed GasChrom P was used. Small peaks with t_R values the same as those of C_9 and C_{10} *n*-aldehydes were found, indicating the presence of olefin impurities differing in double-bond position. Epoxides of the olefins that would give the C_9 and C_{10} aldehydes were synthesized and were inactive in field tests (see last two compounds of Table 1). We thank R. Sarmiento of our Beltsville laboratory for helping prepare these compounds.
16. Three developments with hexane gave zones at R_F 0.32 and 0.51 for *cis*- and *trans*-9-octadecene (supplied by John E. Russell, of the Eastern Regional Research and Development, ARS, USDA, Philadelphia, Pa.), respectively. All of the natural olefin coincided with the *cis* zone.
17. M. Beroza and R. Sarmiento, *Anal. Chem.* **38**, 1042 (1966).
18. An LKB 9000 instrument equipped with a glass column (2.7 m by 0.6 cm, outer diameter) containing 2 percent SE-30 on 80/100 mesh GasChrom Q at 180°C was used. We thank G. Bagley and W. Reichel of the U.S. Department of the Interior, Patuxent, Md., and D. P. Schwartz and C. R. Brewington of the ARS, USDA, Washington, D.C., for determining these and other spectra.
19. The NMR spectra were determined on a Varian HA-100 instrument.
20. Same column as in (*15*).

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Biochemically Marked Lymphocytoid Lines: Establishment of Lesch-Nyhan Cells

Abstract. *Two lymphocytoid cell lines have been established from a patient with the Lesch-Nyhan syndrome. The cells are deficient in hypoxanthine-guanine phosphoribosyltransferase, as demonstrated by their failure to incorporate [H^3]hypoxanthine and by their inability to grow in medium in which they were nutritionally dependent upon exogenous hypoxanthine. This represents the first establishment of presumptively permanent human lymphocytoid cell lines that are deficient in a specific enzyme.*

Several hundred apparently permanent lymphocytoid cell lines have now been established from patients with various diseases (*1*) as well as from numerous healthy individuals (*2*). A few of these cell lines, derived from patients with sex chromosome aneuploidy, are chromosomally marked (*3*). However, there has been no report thus far of the establishment of biochemically marked lymphocytoid cell lines derived from patients with inborn metabolic errors. We have recently established two lymphocytoid cell lines from a patient with the Lesch-Nyhan syndrome and have found that both cell lines are deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity.

The patient was a 2-year-old white male with mental retardation, compulsive self-mutilation, and hyperuricemia. Our method of establishing cell lines has been published elsewhere (*4*). To establish the lymphocytes of this patient, 10 ml of heparinized venous blood was mixed with 0.1 ml of phytohemagglutinin (Burroughs Wellcome) and allowed to stand at 5°C for 45 minutes, after which time the leukocytes were separated off in a plasma suspension by centrifugation at 1000 rev/min for 1 minute. Approximately 3×10^6 to 5×10^6 leukocytes were divided equally and then transferred into four 30-ml plastic tissue culture flasks (Falcon), each of which contained 10 ml of RPMI (Roswell Park Memorial Institute) medium 1640 (Grand Island Biological) supplemented with 20 percent fetal calf serum (Baltimore Biological Laboratories), 100 units of penicillin G per milliliter, and 100 μg of streptomycin sulfate per milliliter. At the time of initiation of the culture, 0.5 ml of a cell lysate from the UM-3 line (University of Michigan, third lymphocytoid line) was added to three of the four culture flasks. The lysate was obtained by freezing and thawing 1×10^7 to 5×10^7 cells suspended in 2 ml of fresh medium. The UM-3 line is a recently established lymphocytoid cell line, derived from a female patient with mental retardation of undetermined etiology. The same technique was used in establishing that line as was used for the present one, except that for the UM-3 line intact cells, rather than a lysate, of the UM-1 cell line were added to the initial cultures. The karotypes of the UM-3 and UM-1 lines are normal 46,-XX, and 46,XY, respectively.

Cultures were then incubated at 37°C, 25 percent of the medium being replaced with fresh medium every 3 to 5 days. After 7 weeks of incubation, two of the three cultures to which lysates had been added began to grow very rapidly. The fourth culture, with no lysate added, failed to show any growth. The two successful cultures are now maintained as the UM-10 and UM-11 lines. Cytogenetic studies on these two cell lines revealed a normal 46,XY chromosome constitution in each. Immunodiffusion and immunoelectrophoresis on the concentrated, spent culture mediums showed that the UM-10 cells produce immunoglobulin G (IgG) and immunoglobulin M (IgM) of type K, and that the UM-11 cells produce IgG and IgM of type L. This suggested that although both lines were established from the same venous blood