(21) P. N. Magee and J. M. Barnes, *Adv. Cancer Res.*, **10**, 163 (1967).
(22) P. D. Lawley and S. A. Shah, *Biochem. J.*, **128**, 117 (1972).
(23) P. S. Schein and S. Loftus, *Cancer Res.*, **28**, 1501 (1968).
(24) P. S. Schein, D. A. Cooney, M. G. McMenamin, and T. Anderson, *Biochem. Pharmacol.*, **22**, 2625 (1973).
(25) S. S. Lazarus and S. H. Shapiro, *Diabetes*, **22**, 499 (1973).
(26) A. Junod, A. E. Lambert, W. Stauffacher, and A. E. Renold, *J. Clin. Invest.*, **48**, 2129 (1969).
(27) C. K. Ho and S. A. Hashim, *Diabetes*, **21**, 789 (1972).
(28) P. D. Lawley, *Nature*, **218**, 580 (1968).

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# Antihyperlipidemic Activity of Sesquiterpene Lactones and Related Compounds

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**Abstract** □ Some naturally occurring pseudoguaianolides and germacranolides as well as synthetic related compounds were observed to be antihyperlipidemic agents in mice. Several of these compounds at a dose of 20 mg/kg/day resulted in lowering of serum cholesterol by ~30% and of serum triglycerides by ~25%. Thiol-bearing enzymes of lipid synthesis, *i.e.*, acetyl-CoA, citrate-lyase, acetyl-CoA synthetase, and β-hydroxy-β-methylglutaryl-CoA reductase, were inhibited by these agents *in vitro*, supporting the premise that these agents alkylate thiol nucleophiles by a Michael-type addition. The α-methylene-γ-lactone moiety, the β-unsubstituted cyclopentenone ring, and the α-epoxycyclopentanone system of these compounds appeared to be responsible for the lowering of serum lipids.

**Keyphrases** □ Lactones, sesquiterpene—antihyperlipidemic activity, structure–activity relationships, serum levels of cholesterol and triglycerides, mice □ Structure–activity relationships—sesquiterpene lactones, antihyperlipidemic activity, effect on serum levels of cholesterol and triglycerides, mice □ Antihyperlipidemic activity—sesquiterpene lactones, structure–activity relationships, serum levels of cholesterol and triglycerides, mice

Pseudoguaianolide (I, III, IV, V, X, and XI) and germacranolide (XII and XIII) sesquiterpene lactones and related compounds (XIV and XV) are potent antineoplastic agents, effective against Walker 256 carcinosarcoma, Lewis lung carcinoma, P-388 lymphocytic leukemia, and Ehrlich ascites carcinoma in rodents (1). Furthermore, these agents significantly inhibited induced inflammation and arthritic states in rats (2).

In a study of the effects of sesquiterpene lactones on cellular metabolism as antineoplastics, these agents inhibited β-hydroxy-β-methylglutaryl-CoA reductase activity of 10-day-old Ehrlich ascites tumor cells. For example, helenalin (I) at 7 μmoles caused 67% inhibition. Compound I also lowered the elevated serum cholesterol level of tumor-bearing mice by 39% (3). These observations suggested that the sesquiterpene lactones should be tested as antihyperlipidemic agents.

## EXPERIMENTAL

**Source of Test Compounds**—Some of the test compounds are natural products and were isolated from plant species by literature techniques (4). Helenalin (I) was isolated from *Balduina angustifolia*[1] (5); tenulin (V) (6) and aromaticin (XI) (6, 7) were isolated from *Helenium amarum*[2]; deoxyelephantopin (XII) was obtained from *Elephantopus carolinianus*[3] (8); and eupahyssopin (XIII) was isolated from *Eupatorium hyssopifolium*[4] (9).

Plenolin (III) was obtained by hydrogenation of helenalin to give the identical compound as the naturally occurring product (10). 2,3-Dihydrohelenalin (II) and 2,3-epoxyhelenalin (IV) were chemically modified from helenalin (11). Dihydrotenulin (VI), 2,3-epoxytenulin (VII), isotenulin (VIII), 2,3-epoxyisotenulin (X), and dihydroisotenulin (IX) were prepared by the methods of Waddell *et al.* (11). Thymine α-methylene-γ-lactone (XIV) (12), α-methylene-γ-lactone (XVIII) (13), and 2,3-epoxycyclopentan-1-one (XVI) (14) were prepared by literature methods. 2-Cyclopentenone[5] (XV), maleic anhydride[5] (XVII), clofibrate[6] (XIX), and acetazolamide[7] (XX) were obtained commercially.

**Serum Hypolipidemic Activity**—$CF_1$ male mice (~30 g) were fed food[8] with water *ad libitum* for the duration of the experiment. The drugs were suspended in 1% carboxymethylcellulose–water and homogenized. The doses were based on the weekly weights of the mice (15). The compounds were tested at 20 mg/kg/day ip.

On Days 9 and 16, blood was collected by tail vein bleeding in alkali-free nonheparinized microcapillary tubes, which then were centrifuged 3 min to obtain the serum. Duplicate 30-μl samples of nonhemolyzed serum were used to determine the serum cholesterol levels in milligram percent by a modification of the Liebermann–Burchard reaction (16). A separate group of mice were bled on Day 14, and their serum triglyceride levels (milligram percent) were determined using duplicate 25-μl samples (17).

**Enzymatic Assays**—Compounds were tested *in vitro* at 1 μmole for their effects on the enzymatic activity of a 10% liver homogenate prepared in 0.25 *M* sucrose and 0.001 *M* ethylenediaminetetraacetic acid at pH 7.2. Adenosine triphosphate citrate-lyase activity was measured by the method of Hoffmann *et al.* (18). Acetyl-CoA synthetase activity was determined by the method of Goodridge (19). The acetyl-CoA formed

[1] Obtained from Dr. M. E. Wall and H. L. Taylor, Research Triangle Institute, N.C. (No. R.T. 4147); Dr. R. E. Perdue, Jr., U.S. Department of Agriculture, Beltsville, Md., provided the identification number NO PR8878.
[2] Identified by Dr. G. S. Van Horn, Department of Biology, University of Tennessee at Chattanooga, Chattanooga, Tenn. Voucher specimen CAM-81775-FAR-TW is available from Dr. T. G. Waddell for inspection.
[3] Identified by S. W. Leonard, Coastal Zone Resources Corp., Wilmington, N.C. A voucher specimen has been placed in the herbarium of the Department of Botany, University of North Carolina at Chapel Hill, Chapel Hill, N.C.
[4] Obtained from Dr. M. E. Wall and H. L. Taylor, Research Triangle Institute, N.C. Voucher specimen NO PR21478 is available.
[5] Aldrich Chemical Co.
[6] Ayerst Laboratories.
[7] Sigma Chemical Co.
[8] Rodent lab chow, Wayne Blox.



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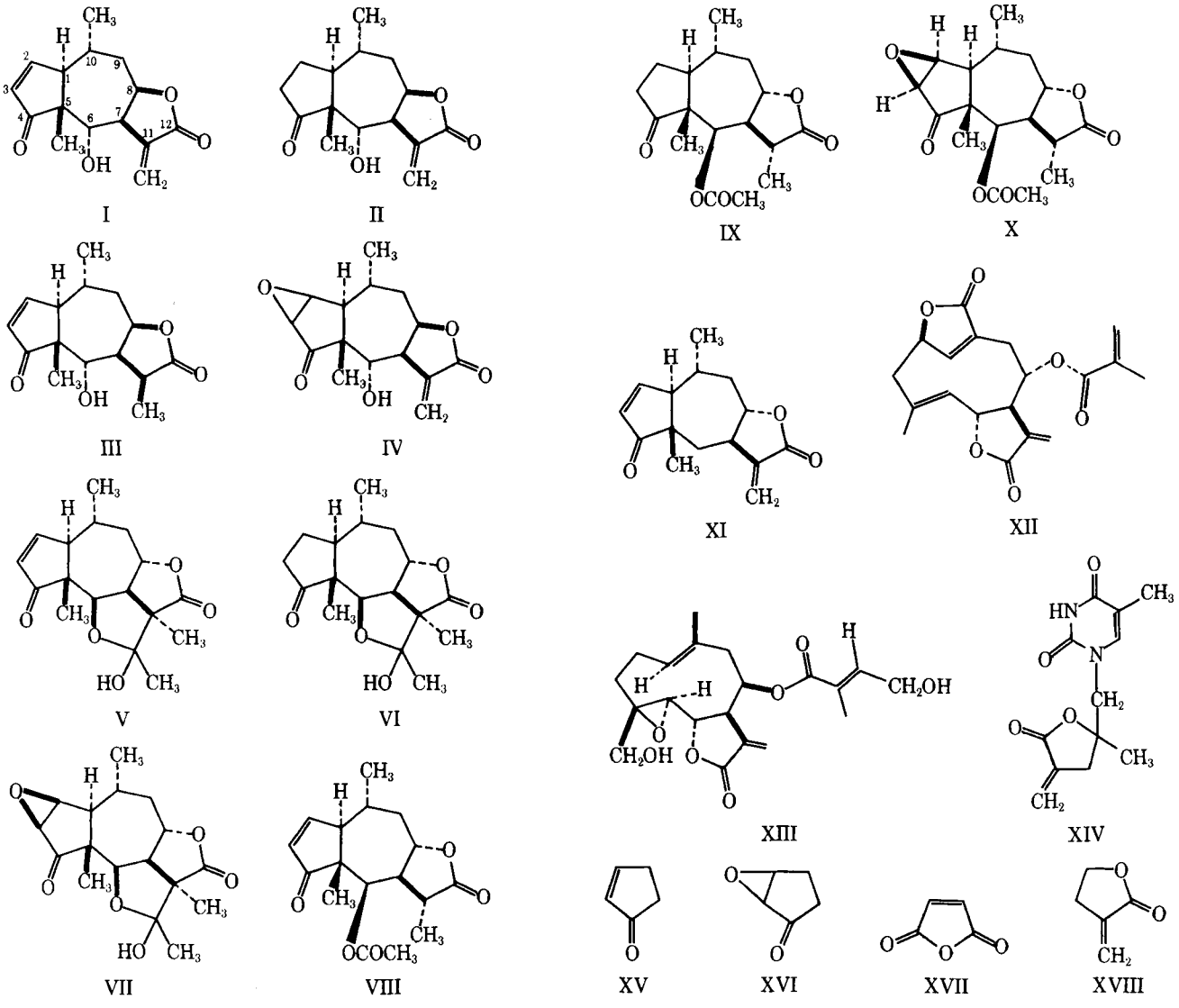


from both enzymatic assays was coupled with hydroxylamine to produce acetyl hydroxylmate, which was measured at 540 nm.

Acetyl-CoA carboxylase activity was measured by a method (20) utilizing sodium [$^3$H]bicarbonate (6.2 mCi/mmole) after 30 min of incubation at 37° for enzyme polymerization (21). Fatty acid synthetase was determined by the method of Brady *et al.* (22) utilizing incorporation of [2′-$^{14}$C]malonyl-CoA (37.5 mCi/mmole) into fatty acids. [$^{14}$C]Acetate (57.8 mCi/mmole) incorporated into cholesterol (β-hydroxy-β-methylglutaryl-CoA reductase activity) was extracted by the method of Wada *et al.* (23) and measured as described by Haven *et al.* (24).

## RESULTS

The sesquiterpene lactones and germacranolides significantly lowered serum cholesterol levels in mice (Table I). Helenalin (I), tenulin (V), 2,3-epoxytenulin (VII), isotenulin (VIII), 2,3-epoxyisotenulin (X), deoxyelephantopin (XII), eupahyssopin (XIII), and thymine lactone (XIV) treatment *in vivo* resulted in >30% reduction in the serum cholesterol levels of mice after 16 days.

Helenalin at 6 mg/kg/day and tenulin at 20 mg/kg/day reduced serum triglyceride levels by 24 and 28%, respectively, after 2 weeks of dosing. 2-Cyclopentenone (XV) and eupahyssopin were essentially inactive, causing 2 and 6% reduction of serum triglyceride levels, respectively. Helenalin, tenulin, deoxyelephantopin, eupahyssopin, and 2-cyclopentenone *in vitro* at 1 μmole suppressed the enzymes that regulate acetyl-CoA availability for triglyceride and cholesterol synthesis, *i.e.*, acetyl-CoA synthetase and citrate-lyase activity. The sesquiterpene lactones tested suppressed acetyl-CoA synthetase activity by 41–49% and citrate-lyase activity by 25–39%. The regulatory enzyme of fatty acid synthesis, acetyl-CoA carboxylase, was inhibited 66–84% in the presence of sesquiterpene lactones. Fatty acid synthetase activity also was inhibited 40–52% by these agents. β-Hydroxy-β-methylglutaryl-CoA reductase activity of the liver was inhibited 21–28% by the sesquiterpene lactones tested.

**Table I—Serum Cholesterol Levels in CF$_1$ Male Mice after Intraperitoneal Dosing at 20 mg/kg/day**

| Compound | Percent of Control Day 9 | Percent of Control Day 16 |
|---|---|---|
| I Helenalin (6 mg/kg/day) | 60 ± 3[a] | 63 ± 4[a] |
| II 2,3-Dihydrohelenalin | 99 ± 5 | 77 ± 7[a] |
| III Plenolin | 89 ± 6[b] | 85 ± 5[c] |
| IV 2,3-Epoxyhelenalin | 85 ± 7[c] | 72 ± 3[a] |
| V Tenulin | 76 ± 5[a] | 65 ± 4[a] |
| VI 2,3-Dihydrotenulin | 79 ± 9 | 89 ± 7[d] |
| VII 2,3-Epoxytenulin | 86 ± 5[c] | 65 ± 4[a] |
| VIII Isotenulin | 81 ± 6[a] | 65 ± 5[a] |
| IX 2,3-Dihydroisotenulin | 79 ± 4[a] | 79 ± 4[a] |
| X 2,3-Epoxyisotenulin | 101 ± 7[a] | 69 ± 6[a] |
| XI Aromaticin | 82 ± 6[a] | 71 ± 5[a] |
| XII Deoxyelephantopin | 83 ± 7[c] | 68 ± 4[a] |
| XIII Eupahyssopin | 65 ± 5[a] | 64 ± 4[a] |
| XIV Thymine lactone | 93 ± 5 | 66 ± 3[a] |
| XV 2-Cyclopentenone | 72 ± 4[a] | 73 ± 6[a] |
| XVI 2,3-Epoxycyclopentan-1-one | 77 ± 8[a] | 74 ± 6[a] |
| XVII Maleic anhydride | 84 ± 7[c] | 78 ± 7[a] |
| XVIII α-Methylene-γ-lactone | 87 ± 5[c] | 73 ± 4[a] |
| XIX Clofibrate | 98 ± 7 | 96 ± 5 |
| 1% Carboxymethylcellulose | 100 ± 5[e] | 100 ± 6[f] |

[a] $p = 0.001$. [b] $p = 0.010$. [c] $p = 0.005$. [d] $p = 0.025$. [e] 115 mg %. [f] 119 mg %.

**Table II—Effects of Sesquiterpene Lactones on Liver Regulatory Enzymes for Cholesterol and Triglyceride Synthesis**

| | Percent of Control, $\bar{x} \pm SD$ | | | | |
|---|---|---|---|---|---|
| | Acetyl-CoA Synthetase Activity | Citrate-Lyase Activity | Acetyl-CoA Carboxylase Activity | Fatty Acid Synthetase Activity | β-Hydroxy-β-Methyl-glutaryl-CoA Reductase Activity |
| I (1 μmole) | 57 ± 3[a] | 75 ± 3[a] | 16 ± 2[a] | 53 ± 4[a] | 72 ± 3 |
| V (1 μmole) | 59 ± 4[a] | 68 ± 3[a] | 26 ± 4[a] | 49 ± 6[a] | 72 ± 4 |
| XII (1 μmole) | 51 ± 2[a] | 66 ± 4[a] | 28 ± 6[a] | 60 ± 3[a] | 79 ± 7 |
| XIII (1 μmole) | 52 ± 6[a] | 61 ± 2[a] | 34 ± 3[a] | 48 ± 5[a] | 77 ± 5 |
| XV (1 μmole) | 51 ± 3[a] | 63 ± 3[a] | 17 ± 4[a] | 49 ± 3[a] | 73 ± 4 |
| XIX (41 μmoles) | — | — | — | — | 77 ± 5 |
| XX | 66 ± 4[a] | 66 ± 7[a] | 13 ± 2[a] | 82 ± 6 | — |
| 1% Carboxymethylcellulose | 100 ± 5[b] | 100 ± 4[c] | 100 ± 7[d] | 100 ± 7[e] | 100 ± 4[f] |

[a] $p = 0.001$. [b] 28.5 mg of acetyl-CoA formed/g of wet tissue/30 min. [c] 30.5 mg of citrate hydrolyzed/g of wet tissue/30 min. [d] 32,010 dpm/g of wet tissue/30 min. [e] 37,656 dpm/g of wet tissue/30 min. [f] 1,142,065 dpm/g of wet tissue/60 min.

## DISCUSSION

The sesquiterpene lactones were effective antihyperlipidemic agents in rodents. The α-methylene-γ-lactone moiety, the β-unsubstituted cyclopentenone ring, and the α-epoxycyclopentanone system contributed to the antihyperlipidemic activity of the sesquiterpene lactones and related compounds. Saturation of the 2,3-double bond of the cyclopentenone ring led to a loss of antihyperlipidemic activity, as was seen with II, VI, and IX. The α-epoxycyclopentanone ring system retained the antihyperlipidemic activity regardless of whether the α-methylene group of the γ-lactone ring (11,13-double bond) was reduced (*i.e.*, IV, VII, and X). Compounds containing the α-methyl-γ-lactone group (V and VIII) as opposed to the α-methylene-γ-lactone moiety (I) were equally active, because in both cases the essential cyclopentenone ring was retained (with the exception of III, in which case the 11-methyl group was β-oriented). The configuration of the γ-lactone ring did not appear to play an important role in the ability of the pseudoguaianolides to lower serum cholesterol levels, because the *cis*-fused lactones I and IV were approximately as potent as the *trans*-fused V, VII, VIII, and XI.

Germacranolides that contain the α-methylene-γ-lactone moiety had significant antihypercholesterolemic activity, *e.g.*, deoxyelephantopin (XII) and eupahyssopin (XIII). In addition to the essential α-methylene-γ-lactone moiety, XIII contains an ester side chain, a primary hydroxyl group, and an epoxy ring, which may contribute to the pharmacological activity. Compound XII, in addition to the α-methylene-γ-lactone, contains an α,β-unsaturated-γ-lactone ring and an ester side chain. The simple 2-cyclopentenone (XV), 2,3-epoxycyclopentan-1-one (XVI), maleic anhydride (XVII), and α-methylene-γ-lactone (XVIII) were not as active in lowering serum cholesterol, indicating that some other steric, configurational, or conformational arrangement was required for potent activity. The sesquiterpene lactones also were observed to lower serum triglycerides.

The proposed mechanism of action of the sesquiterpene lactones is the formation of adducts with the thiol groups of enzymes by a rapid Michael-type addition of the α-methylene-γ-lactone moiety or cyclopentenone ring system to the thiol nucleophile, thereby inactivating the enzyme (25, 26). Helenalin and tenulin block cellular metabolism by inhibiting key regulatory thiol-bearing enzymes, *e.g.*, DNA polymerase (6, 27) and phosphofructokinase (25). The β-hydroxy-β-methylglutaryl-CoA reductase, acetyl-CoA carboxylase, and fatty acid synthetase reportedly contain exposed thiol groups (28, 29). Further elevated cyclic adenosine monophosphate levels, which helenalin elevated by 194% (30), were related to the reduction of β-hydroxy-β-methylglutaryl-CoA reductase activity (31) and fatty acid synthesis by modulating acetyl-CoA carboxylase, fatty acid synthetase, and cytosol pyruvate levels for acetyl-CoA generation (32). For these reasons, several key regulatory enzymes in the genesis of fatty acid and cholesterol synthesis were measured to evaluate the effects of sesquiterpene lactones *in vivo*.

Studies on the generation of acetyl-CoA for fatty acid and cholesterol synthesis from cytosol acetate and citrate showed that the pseudoguaianolides (helenalin and tenulin) and the germacranolides (deoxyelephantopin and eupahyssopin) equally suppressed the activities of these two enzymes. Acetyl-CoA carboxylase activity, the regulatory enzyme of fatty acid synthesis, was reduced drastically in the presence of the sesquiterpene lactones, with helenalin being the most potent agent. Fatty acid synthetase activity also was reduced in the presence of the sesquiterpene lactones by ~50%. β-Hydroxy-β-methylglutaryl-CoA reductase activity was inhibited equally by the sesquiterpene lactones. The commercially available clofibrate, present at 41 μmoles, resulted in 23% inhibition, indicating that the sesquiterpene lactones were far more potent in inhibiting this enzyme that regulates cholesterol synthesis. Clofibrate required a dose of 300 mg/kg/day in rodents to reduce serum cholesterol levels and had no effect at 20 mg/kg/day. Thus, it can be concluded that the sesquiterpene lactones possess sufficient ability to block lipogenesis to warrant further investigation as antihyperlipidemic agents.

## REFERENCES


(1) I. H. Hall, K. H. Lee, C. O. Starnes, S. A. ElGebaly, T. Ibuka, Y. S. Wu, T. Kimura, and M. Haruna, *J. Pharm. Sci.*, **67**, 1235 (1978).

(2) I. H. Hall, K. H. Lee, C. O. Starnes, Y. Sumida, R. Y. Wu, T. G. Waddell, J. W. Cochran, and K. G. Gerhart, *ibid.*, **68**, 537 (1979).

(3) K. H. Lee, I. H. Hall, E. C. Mar, C. O. Starnes, S. A. ElGebaly, T. G. Waddell, R. I. Hadgraft, C. G. Ruffner, and I. Weidner, *Science*, **196**, 533 (1977).

(4) K. H. Lee and T. A. Geissman, *Phytochemistry*, **9**, 403 (1970).

(5) K. H. Lee, D. C. Anuforo, E. S. Huang, and C. Piantadosi, *J. Pharm. Sci.*, **61**, 626 (1972).

(6) I. H. Hall, K. H. Lee, E. C. Mar, C. O. Starnes, and T. G. Waddell, *J. Med. Chem.*, **20**, 333 (1977).

(7) R. A. Lucas, S. Rovinski, R. J. Kiesel, L. Dorfman, and H. B. MacPhillamy, *J. Org. Chem.*, **29**, 1549 (1964).

(8) K. H. Lee, C. M. Cowherd, and M. T. Wolo, *J. Pharm. Sci.*, **64**, 1572 (1975).

(9) K. H. Lee, T. Kimura, M. Okamoto, C. M. Cowherd, A. T. McPhail, and K. D. Onan, *Tetrahedron Lett.*, **14**, 1051 (1976).

(10) K. H. Lee, T. Kimura, D. Sims, A. T. McPhail, and K. D. Onan, *Phytochemistry*, **16**, 393 (1977).

(11) T. G. Waddell, A. M. Austin, J. W. Cochran, K. G. Gerhart, I. H. Hall, and K. H. Lee, *J. Pharm. Sci.*, **68**, 715 (1979).

(12) K. H. Lee, Y. S. Wu, and I. H. Hall, *J. Med. Chem.*, **20**, 911 (1977).

(13) G. M. Ksander, J. E. McMurry, and M. Johnson, *J. Org. Chem.*, **42**, 1180 (1977).

(14) R. G. Salomon and M. F. Salomon, *J. Am. Chem. Soc.*, **99**, 3501 (1977).

(15) I. H. Hall and G. L. Carlson, *J. Med. Chem.*, **19**, 1257 (1976).

(16) A. T. Ness, J. V. Pastewka, and A. C. Peacock, *Clin. Chim. Acta*, **10**, 229 (1964).

(17) J. M. Chapman, G. H. Cocolas, and I. H. Hall, *J. Med. Chem.*, **22**, 1399 (1979).

(18) G. Hoffmann, L. Weiss, and O. H. Wieland, *Anal. Biochem.*, **84**, 441 (1978).

(19) A. G. Goodridge, *J. Biol. Chem.*, **248**, 4318 (1973).

(20) M. D. Greenspan and J. M. Lowenstein, *ibid.*, **243**, 6273 (1968).

(21) T. P. Cao and S. Rous, *Life Sci.*, **22**, 2067 (1978).

(22) R. O. Brady, R. M. Bradley, and E. G. Trams, *J. Biol. Chem.*, **235**, 3093 (1960).

(23) F. Wada, K. Hirata, and Y. Sakamoto, *J. Biochem.*, **65**, 171 (1969).

(24) G. T. Haven, J. R. Krzemien, and T. T. Nguyen, *Res. Commun. Chem. Pathol. Pharmacol.*, **6**, 253 (1973).

(25) R. L. Hanson, H. A. Lardy, and S. M. Kupchan, *Science*, **168**, 378 (1970).

(26) C. H. Smith, J. Larner, A. M. Thomas, and S. M. Kupchan, *Biochim. Biophys. Acta*, **276**, 94 (1972).

(27) I. H. Hall, K. H. Lee, and S. A. ElGebaly, *J. Pharm. Sci.*, **67**, 1232 (1978).

(28) P. H. E. Groot, H. R. Scholte, and W. C. Hulsmann, *Adv. Lipid Res.*, **14**, 75 (1976).
(29) A. White, P. Handler, E. L. Smith, R. L. Hill, and I. R. Lehman, "Principles of Biochemistry," 6th ed., McGraw-Hill, New York, N.Y., 1978, pp. 584–589, 620.
(30) I. H. Hall, C. O. Starnes, Jr., K. H. Lee, and T. G. Waddell, *J. Pharm. Sci.*, **69**, 537 (1980).
(31) V. W. Rodwell, J. L. Nordstrom, and J. J. Mitschelen, *Adv. Lipid Res.*, **14**, 1 (1976).
(32) R. A. Harris, J. P. Mapes, R. S. Ochs, D. W. Crabb, and L. Stropes, *Adv. Exp. Med. Biol.*, **111**, 17 (1979).

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# Evaluation of Nystatin Stability Using Tristimulus Colorimetry

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**Abstract** □ A tristimulus reflectance spectrophotometer was used to examine the color changes of nystatin during accelerated stability studies, and a relationship was observed between the loss of microbiological potency and the change in color during thermal degradation. By substitution of the measured tristimulus values in the Kubelka–Munk equation, the remission function was calculated and resulted in a linear response with time. Application of the technique to bulk raw materials and formulated products is demonstrated, and uses of the technique are discussed.

**Keyphrases** □ Colorimetry—nystatin, thermal degradation, relationship between loss of microbiological potency and color change □ Degradation, thermal—nystatin, colorimetry, relationship between loss of microbiological potency, and color change □ Nystatin—thermal degradation, colorimetry, relationship between loss of microbiological potency and color change □ Antifungal agents—nystatin, thermal degradation colorimetry, relationship between loss of microbiological potency and color change

The most frequent use of surface color measurements in the pharmaceutical industry has been the monitoring of color stability of coated tablets (1). Alternative uses of the procedure have been described, particularly in preformulation and stability studies. These applications generally involve the determination of the discoloration exhibited by a particular drug–excipient mixture or formulation. By this means, the incompatibility of various mixtures was reported (2–6), and the stability of active ingredients in formulated products was determined (7). Prediction of the shelflife of tablets also may be assessed by this technique (8, 9). A comprehensive review of the pharmaceutical applications of tristimulus colorimetry and diffuse reflectance spectroscopy was published recently (10). The stability of the polyene antibiotic nystatin was evaluated in these laboratories by monitoring the color change at elevated temperatures. The color change at a solid surface is measured quantitatively by diffuse reflectance spectrometry, and monochromatic light generally is used to illuminate the sample. Alternatively, illumination may be by a white light source, and the values of each of the three primary color components (the tristimulus values) of the reflected light are determined. This technique usually is referred to as tristimulus colorimetry.

For nystatin, a visual relationship between the degree of browning observed and the loss of microbiological potency was demonstrated previously[1]. Therefore, tristimulus colorimetry was used to quantitate the observed color changes since the measurement of the tristimulus values closely parallels the color discrimination obtained with the human eye.

## THEORY

To quantitate the observed degradation of nystatin, it was necessary to derive a suitable equation so that a relationship between the color and intensity of the reflected light, as described by the tristimulus values, and the concentration of colored material in the reflecting surface was defined. Kubelka and Munk (11, 12) derived an equation to describe the phenomenon of diffuse reflectance. For the special case of an infinitely thick opaque layer, it is:

$$f(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{k}{s} \qquad \text{(Eq. 1)}$$

where $R_\infty$ is the diffuse reflectance of the layer relative to a nonabsorbing standard, $k$ is the molar absorption coefficient, $s$ is the scattering coefficient, and $f(R_\infty)$ is the remission function. In practice, this requirement will be satisfied by a layer of material several millimeters deep.

The color of light reflected from the sample surface is described by the measured tristimulus values $X$, $Y$, and $Z$. The $Y$ value corresponds to the Munsell value, which measures the degree of sample darkening, without regard to hue or chroma. The tristimulus values are used to calculate the chromaticity coordinates $x$, $y$, and $z$ according to $x = X/(X + Y + Z)$, $y = Y/(X + Y + Z)$, and $z = Z/(X + Y + Z)$.

Tristimulus values are measured relative to a nonabsorbing standard, usually a white ceramic tile. For this study, the color of a sample before and after degradation was compared. This comparison may be achieved if the color of the degraded material is determined using the material prior to degradation as the standard. However, this procedure introduces practical difficulties and necessitates a large supply of original material. To overcome these difficulties, all materials were measured against a white tile as the standard, and the relative color change was calculated from the expression $x_0/x_t$, where $x_0$ is the initial chromaticity coordinate of the sample and $x_t$ is the chromaticity coordinate at time $t$.

Substitution of these values into the Kubelka–Munk equation gives:

$$f(R_\infty) = \frac{(1 - x_0/x_t)^2}{2x_0/x_t} \qquad \text{(Eq. 2)}$$

[1] U. Nager, unpublished data.

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