tion of oxidant. The oxidants used were not observed to oxidize succinate nonenzymatically at 22°, but they do oxidize albumin and other proteins at low but measurable rates. All results were corrected for these (usually negligible) rates.

Reactions were initiated by the addition of enzyme followed immediately thereafter by oxidant(s). Readings were begun usually 5–10 sec after mixing. A Cary Model 14 recording spectrophotometer was used in most cases.

The Biosynthesis of the Aflatoxins

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Abstract: Degradative studies on radioactive aflatoxin-B_1 prepared by fermentation of added [1-^{14}C]- and [2-^{14}C]-acetates and of methyl-^{14}C-methionine are described. The origin of thirteen of the seventeen carbon atoms present was determined and the results show that the carbon skeleton is derived entirely from acetic acid. The activity of the labeled carbon atoms was equal throughout the molecule suggesting the intermediacy of a single polyacetate chain. A hypothetical pathway for the biosynthesis of aflatoxins and related mold metabolites, consonant with the distribution of labels determined, is proposed.

The aflatoxins constitute a family of secondary metabolites produced by some *Aspergillus* species. Because of their pronounced toxicity and extreme carcinogenicity in many animal species they have become the subject of intense investigation. The chemistry of the various aflatoxins and their interrelationships are well in hand, but work on the mode of action is only in its beginning stages.[2,3] The biosynthesis of the aflatoxins has been a topic of much conflicting speculation.[4-7] An early incorporation study[8] implicated phenylalanine and tyrosine as precursors but more recent work has shown this to be erroneous.[9] To clarify the biogenetic origins of the toxins we have determined the distribution of labels in aflatoxin-B_1 (**1**) derived from methyl-^{14}C-methionine, [1-^{14}C]- and [2-^{14}C]-acetate.[10]

Administration of methyl-^{14}C-methionine yielded radioactive aflatoxin-B_1 (**1**)[8] which on Zeisel degradation gave methyl iodide, isolated as triethylmethyl ammonium iodide, containing 97.8% of the total activity.[11] In agreement with this finding, degradation of aflatoxin-B_1 derived from [1-^{14}C]- and [2-^{14}C]-acetate[9] yielded triethylmethylammonium iodide containing only 0.32 and 0.31% of the radioactivity, respectively. The methoxyl carbon atom thus arises from the C_1 pool and in particular from methionine as expected from analogy with other mold metabolites (*e.g.*, mycophenolic acid[12]).

The most notable feature in the structure of the aflatoxins is the bisdihydrofuran moiety and it became of much interest to compare the actual distribution of labels in this portion of the molecule with that predicted by the previously cited postulates. Treatment of tetrahydrodesoxoaflatoxin-B_1 (**2**), obtained by catalytic reduction of aflatoxin-B_1 (**1**), with aluminum chloride yielded the furocoumarin **3**. Tosylation to give the ester **4**, followed by reduction with lithium aluminum hydride and methylation afforded the dimethoxybenzofuran **5**. Modified Kuhn-Roth oxidation of the latter with 2 *N* chromic acid afforded a mixture of propionic and acetic acids. These were separated by preparative gas-liquid phase chromatography and the pure acids were degraded stepwise to give each carbon atom sequentially as carbon dioxide (Scheme I). Degradation of aflatoxin-B_1 (**1**) derived from [1-^{14}C]-acetate gave the distribution of isotope outlined in Table I.

Table I. [1-^{14}C]-Acetate Incorporation[a]

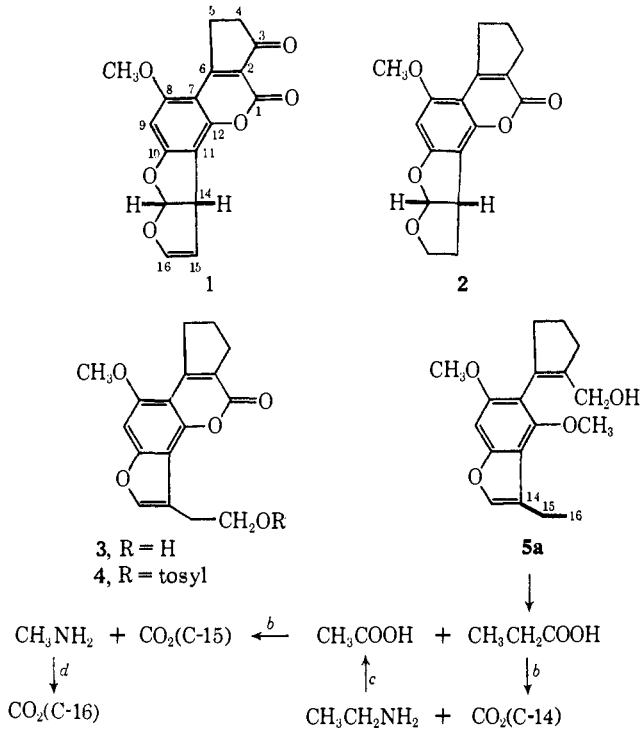
Carbon atoms	Estimated as	% of total radioactivity
C-14–C-16	*p*-Bromophenacyl-propionate	12.01
C-14	CO_2	1.35
C-15, C-16	*p*-Bromophenacyl-acetate	11.21
C-15	CO_2	8.61
C-16	CO_2	1.21

[a] Theoretical activity per carbon atom = 11.1% for 9 labels.

The results indicate that both propionic and acetic acids contain one label with the preponderance of the activity residing in the carboxyl carbon of the acetic acid. This labeling pattern, unfortunately, was not

(1) National Institutes of Health Predoctoral Fellow, 1965–1966.
(2) G. N. Wogan, *Bacteriol. Rev.*, 30, 460 (1966).
(3) R. I. Mateles and G. N. Wogan, *Advan. Microbial. Phys.*, 1, 25 (1967).
(4) D. P. Moody, *Nature*, 202, 188 (1964).
(5) J. G. Heathcote, J. J. Child, and M. F. Dutton, *Biochem. J.*, **95**, 23P (1965).
(6) J. S. E. Holker and J. G. Underwood, *Chem. Ind.* (London), 1865 (1964).
(7) R. Thomas in "Biogenesis of Antibiotic Substances," Z. Vaněk and Z. Hoštálek, Ed., Academic Press, New York, N. Y., 1965, p 155.
(8) J. Adye and R. I. Mateles, *Biochim. Biophys. Acta*, **86**, 418 (1964).
(9) J. A. Donkersloot, D. P. H. Hsieh, and R. I. Mateles, *J. Amer. Chem. Soc.*, **90**, 5021 (1968).
(10) Preliminary communications: M. Biollaz, G. Büchi, and G. Milne, *ibid.*, **90**, 5017 (1968); *ibid.*, **90**, 5019 (1968); Proceedings of the Joint U. S.–Japan Conference on Mycotoxins, Honolulu, Hawaii, 1968.
(11) We are indebted to Dr. S. Brechbühler for this determination.
(12) A. J. Birch, R. J. English, R. A. Massey-Westropp, M. Slaytor, and H. Smith, *J. Chem. Soc.*, 365 (1958).

Scheme I



[a] E. Weissenberger, *Microchim. Acta*, **33**, 51 (1948). [b] Schmidt degradation: J. J. Britt, Dissertation No. 2948, Eidgenössische Technische Hochschule, Zürich, 1959. [c] Oxidation with $KMnO_4$: S. Brechbühler, Dissertation No. 3544, Eidgenössische Technische Hochschule, Zürich, 1964. [d] Oxidation with $KMnO_4$: J. J. Britt, Dissertation No. 2948. We are indebted to Professor D. Arigoni for having called the three procedures cited in footnotes *b*, *c*, and *d* to our attention.

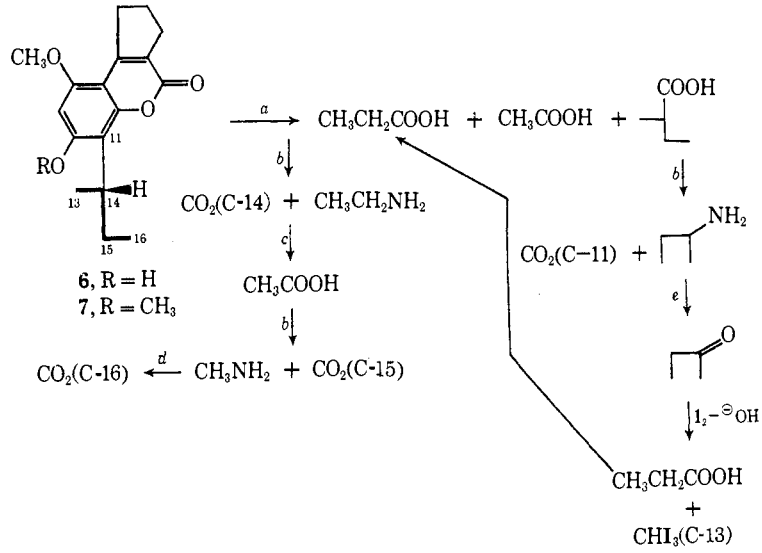
fying work was performed with this particular sample of labeled toxin and the degradative scheme was abandoned in favor of a more efficient one.

In the second method employed to determine the distribution of isotope in the bisdihydrofuran portion, we utilized the previously described tricyclic phenol **6**[13] as starting material. Modified Kuhn-Roth oxidation of the corresponding dimethyl ether **7** afforded 2-methylbutanoic acid (90%), propionic acid (5%), and acetic acid (5%). These acids were separated in the form of their *p*-bromophenacyl esters or N-(α-naphthyl) amides and degraded as indicated in Scheme II. Oxidation of 2-butanone with sodium hypoiodite provided an additional source of propionic acid. The results for [1-^{14}C]- and [2-^{14}C]-acetate derived aflatoxin-B_1 are presented in Tables II and III, respectively.

Examination of Table II reveals the presence of two labels (assuming a total of nine labels) in the four carbon atoms of the side chain, one of which must reside in the propionic acid unit. This implies, by subtraction, that the C-13 atom must be acetate carboxyl derived. This labeling pattern was verified for [2-^{14}C]-acetate derived B_1 by direct measurement of each carbon atom, as indicated in Table III. The ester activities found are in good agreement with those calculated for seven labels.

Since the acetic acid obtained in the Kuhn-Roth oxidation of the coumarin **7** can arise from either of the two C-methyl groups it could not be used to ascertain the distribution of labels. It is of some interest to note, however, that the acetic acid must arise equally from each source because the radioactivity in the acid obtained by degradation of [1-^{14}C]- and [2-^{14}C]-acetate

Scheme II



[a] See Scheme I, footnote *a*. [b] See Scheme I, footnote *b*. [c] See Scheme I, footnote *c*. [d] See Scheme I, footnote *d*. [e] Oxidation with NaOCl: H. Ruschig, W. Fritsch, J. Schmidt-Thomé, and W. Haede, *Chem. Ber.*, **88**, 883 (1955).

delineated as sharply as in any of the degradative studies to be discussed. This may indicate some redistribution of label in the incorporated unit caused by participation of acetic acid in the tricarboxylic acid cycle. An alternative explanation involves partial randomization of label during the degradation. We have no further information regarding this point because no clari-

derived coumarin **7** was distributed evenly between the two carbon atoms.

The next phase of this investigation was concerned with the isotope distribution in the cyclopentenone moiety. Reduction of tetrahydrodesoxoaflatoxin-B_1 (**2**)

(13) S. Brechbühler, G. Büchi, and G. Milne, *J. Org. Chem.*, **32**, 2641 (1967).

Table II. [1-^{14}C]-Acetate Incorporation

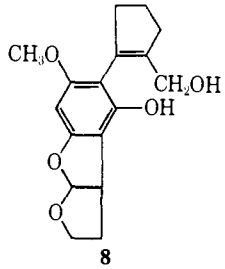
Carbon atoms	Estimated as	% of total radio-activity
C-11, C-13–C-16	N-(α-Naphthyl)-2-methylbutanamide	22.31
C-11	CO_2	0.13
C-14–C-16	N-(α-Naphthyl)-propionamide	11.14

Table III. [2-^{14}C]-Acetate Incorporation[a]

Carbon atoms	Estimated as	% of total radioactivity
C-11, C-13–C-16	*p*-Bromophenacyl-2-methylbutanoate	43.26
C-11	CO_2	12.83
C-13	CHI_3	0.49
C-14–C-16	*p*-Bromophenacyl-propionate	28.61
C-14	CO_2	12.78
C-15	CO_2	0.49
C-16	CO_2	12.72

[a] Theoretical activity per labeled atom = 14.19% for 7 labels.

with lithium aluminum hydride afforded the anticipated allylic alcohol **8** and minor amounts of an isomer **9**. The formation of isomer **9** requires opening of the benzofuran ring followed by reclosure in the alternate direction. On one occasion reduction of the acetal **2** under seemingly routine conditions gave the tricyclic triol **10** in 95% yield. A common intermediate in these transformations would be the oxonium ion **11** in which the phenoxide anion is stabilized by a Lewis acid present in the reaction medium. This oxonium ion **11** could ultimately combine with either of the phenolic hydroxyl functions to give the acetals **8** and **9** or with hydride to furnish the tricyclic tetrahydrofuran **10**. Incidentally, the structural assignments for the two acetals **8** and **9** are not based on experimental evidence and it is only assumed that the major product has the unrearranged structure **8**.



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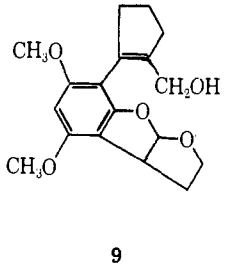

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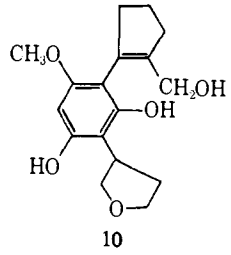

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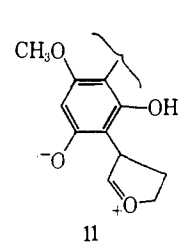
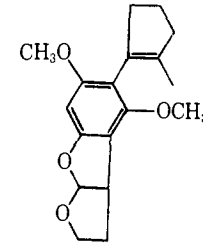
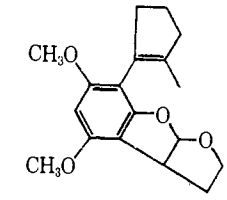


11

Hydrogenolysis of the crude mixture of the two allylic alcohols **8** and **9** over a palladium on carbon catalyst followed by methylation with methyl iodide afforded a mixture of the methylcyclopentenes **12** (84%) and **13** (6%) and the methylcyclopentane **16** (3%) separable by chromatography. Catalytic reduction of the olefin **12** produced the dihydro derivative **16**. Similarly, hydrogenolysis of the tricyclic compound **10** followed by methylation gave the olefin **14** (81%) and its reduction product **17** (10%).



12



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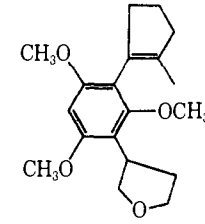

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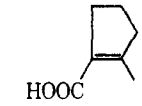
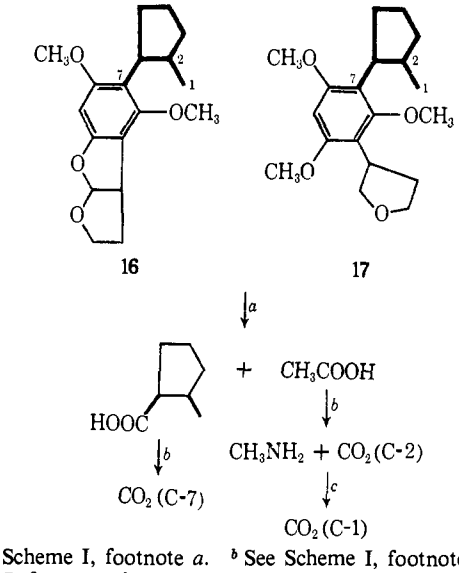


15

The mixture of acids obtained on Kuhn–Roth oxidation of the two methylcyclopentanes **16** and **17** consisted of the previously unreported *cis*-2-methylcyclopentanoic acid (**18**) and acetic acid. An authentic sample of the former was prepared from the known 2-methylcyclopent-1-enoic acid (**15**)[14] by catalytic reduction which yielded a 45:55 mixture of *cis* and *trans* acids. Attempts to change this ratio in favor of the *cis* isomer were negative yet hydrogen addition to both styrenes **12** and **14** must be exclusively *cis* because no *trans*-2-methylcyclopentanoic acid was observed in the Kuhn–Roth oxidation. The further degradations of the acid **18** and the concomitantly formed acetic acid are outlined in Scheme III.

Scheme III

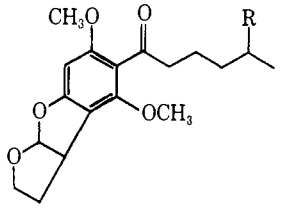


[a] See Scheme I, footnote *a*. [b] See Scheme I, footnote *b*. [c] See Scheme I, footnote *d*.

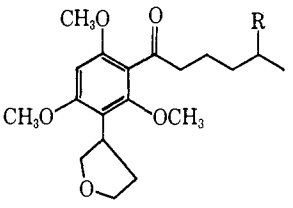
Oxidation of the methylcyclopentenes **12** and **14** with osmic acid and potassium chlorate[15] gave the respective

(14) L. E. King and R. Robinson, *J. Chem. Soc.*, 465 (1941).
(15) M. F. Clark and L. N. Owen, *ibid.*, 319 (1949).

diketones **19** and **21** directly. The intermediate osmate ester resulting from addition of osmium tetroxide to a tetrasubstituted olefin occasionally is oxidized more rapidly (*e.g.*, by hydrogen peroxide[16]) than it is hydrolyzed to the corresponding glycol. The resulting osmium-(VIII) ester then decomposes with carbon-carbon bond cleavage to produce a diketone.



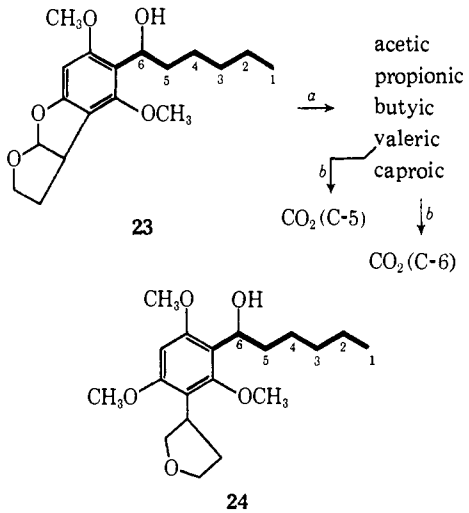
19, R = O =
20, R = OH



21, R = O =
22, R = OH

Selective reduction of the diketones **19** and **21** with lithium aluminum tri-*t*-butoxy hydride afforded the hydroxyketones **20** and **22**. Their tosylates on hydrogenolysis with lithium aluminum hydride were transformed to the secondary alcohols **23** and **24** whose further degradations are outlined in Scheme IV.

Scheme IV



23

24

[a] See Scheme I, footnote *a*. [b] See Scheme I, footnote *b*.

The distribution of radioactivity ascertained by this degradative sequence using [1-^{14}C]-acetate derived aflatoxin-B_1 (**1**) is presented in Table IV. Four of the nine labels are present in the seven carbon atoms of *cis*-2-methylcyclopentanoic acid (**18**) and three of these must reside in atoms C-6, C-5, and C-1 as determined by direct measurement. Since the propionic acid contains two labels and the acetic acid only one, C-3 must contain the fourth label. The values for butyric acid and for carbon dioxide produced by Schmidt degradation of valeric acid are low because the measurements were performed on very small quantities of material.

Supplementary work with [2-^{14}C]-acetate as substrate fully verified these conclusions and the results are summarized in Table V.

Table IV. [1-^{14}C]-Acetate Incorporation

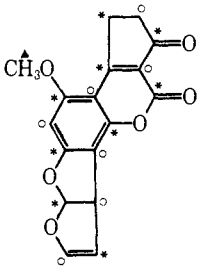
Carbon atoms	Estimated as	% of total radioactivity
C-1–C-7	*p*-Bromophenacyl-*cis*-2-methylcyclopentanoate	43.84
C-7	CO_2	0.14
C-1, C-2	*p*-Bromophenacylacetate	10.37, 11.05
C-2	CO_2	0.21
C-1	CO_2	8.52
C-1–C-6	*p*-Bromophenacylcaproate	44.48
C-6	CO_2	8.89
C-1–C-5	*p*-Bromophenacylvalerate	32.57
C-5	CO_2	6.77
C-1–C-4	*p*-Bromophenacylbutyrate	18.57
C-1–C-3	*p*-Bromophenacylpropionate	21.80

Table V. [2-^{14}C]-Acetate Incorporation

Carbon atoms	Estimated as	% of total radioactivity
C-1–C-7	*p*-Bromophenacyl-*cis*-2-methylcyclopentanoate	43.06
C-7	CO_2	12.86
C-1, C-2	*p*-Bromophenacylacetate	14.10
C-2	CO_2	12.62
C-1	CO_2	0.35
C-1–C-6	*p*-Bromophenacylcaproate	28.56
C-6	CO_2	0.26
C-1–C-5	*p*-Bromophenacylvalerate	28.67
C-5	CO_2	0.35
C-1–C-4	*p*-Bromophenacylbutyrate	28.47
C-1–C-3	*p*-Bromophenacylpropionate	14.28

In summary, our findings establish specific incorporation and a comparison of measured and calculated radioactivities demands the presence of nine labels (theoretical activity 11.1% per labeled carbon atom) in [1-^{14}C]-acetate derived aflatoxin-B_1 and seven labels (theoretical activity 14.3% per labeled carbon atom) in radioactive toxin originating from [2-^{14}C]-acetate.

The distribution of labels portrayed in formula **25** is not in accord with that proposed or implied in the previously proposed biogenetic schemes.[4-7]



$CH_3\overset{\circ}{C}\overset{*}{C}O_2H$

$CH_3SCH_2CH_2CH(NH_2)CO_2H$

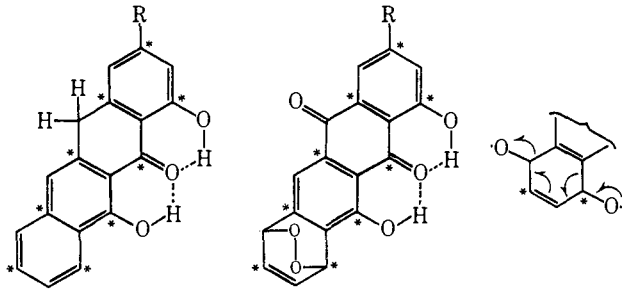
25

We wish to advance a new hypothesis consonant with the experimental evidence now in hand. In designing a biogenesis we were aware of the existence of O-methylsterigmatocystin (**41**)[17] and versicolorin-C (**31**),[18] cometabolites of aflatoxin-B_1 (**1**) in *A. flavus* containing one (C_{17}) and two (C_{18}) additional skeletal carbon atoms, respectively. It is assumed that the C_{18}-polyhydroxynaphthacene **26** (R = H or OH) formed from a nonaacetyl chain by cyclization and removal of oxygen is oxidized to the endoperoxyanthraquinone **27** which in turn rearranges *via* the diradical **28** or the zwitterion to the aldehyde **29**. A further isomerization, similar to

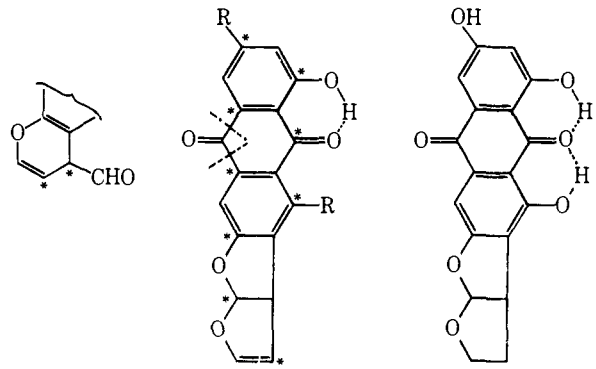
(16) R. Criegee, *Ann.*, **522**, 75 (1936).

(17) H. L. Burkhardt and J. Forgacs, *Tetrahedron*, **24**, 717 (1968).
(18) J. Heathcote and M. Dutton, *ibid.*, **25**, 1497 (1969).

the one encountered in the *in vitro* synthesis of aflatoxin-B_1[19] leads to versicolorin-A (**30**, R = OH)[20] and aversin (**30**, R = OCH_3),[21] metabolites of *A. versicolor*.

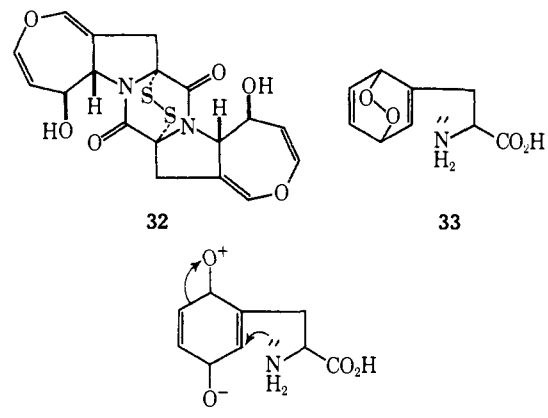


26 **27** **28**



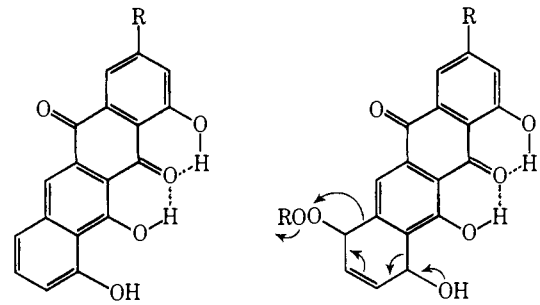
29 **30** **31**

The rearrangement of the endoperoxide **27** to the pyran **29** seems to be without chemical precedent, yet it does provide an exceedingly economical, and mechanistically not unreasonable, pathway to the bisfuran moieties of metabolites elaborated by the genus *Aspergillus*. Aranotin (**32**)[22] a metabolite of *Aspergillus terreus* could arise by a similar rearrangement (arrows in formula **34**) of endoperoxyphenylalanine **33** in which the nitrogen lone pair rather than an oxygen atom serves as the nucleophile.[23]



32 **33** **34**

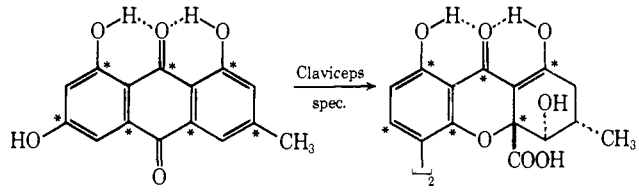
Returning to the biogenesis of the aflatoxins we should mention that 1,4-addition of a hydroperoxide to the naphthacenequinone **35** and subsequent rearrangement of the intermediate peroxide **36** represents an alternate route to the pyran **29**.[24] This seems less likely in view of the growing body of evidence for the intermediacy of singlet oxygen in biological oxidations, *e.g.*, the specific oxidation of *o*-cresol to *cis*-muconic acid by one oxygen molecule[25] and the oxidation of 3-hydroxyflavones.[26]



35 **36**

Two adjacent methyl acetate derived carbon atoms in the bisfuranoid portion of the aflatoxin-B_1 molecule might result also from a condensation of a C_{14} moiety with a C_4 unit. Such a sequence has recently been postulated in a communication on the biosynthesis of sterigmatocystin (**40**).[27] The proposal was based on a slightly lower incorporation of acetate into the furanoid side chain than into the rest of the molecule. However, the difference in radioactivities was scarcely large enough to be conclusive. While an analogous biosynthetic route to aflatoxin cannot be excluded the excellent agreement of labeling levels throughout the molecule makes such a proposal much less likely than one starting from a single polyacetate chain.

It had previously been suggested that the difuroxanthone O-methylsterigmatocystin (**41**) is derived from an anthraquinone by oxidative ring cleavage at the dotted lines in structure **30**.[7] Such an oxidative opening of a quinone ring has recently been established in the biosynthesis of ergochrome BB (**38**) from emodin (**37**).[28]



37 **38**

A similar cleavage of the anthraquinone **30** (R = H) to the carboxylic acid **39** followed by cyclization, decarboxylation, and oxidation (not necessarily in this sequence) would lead to sterigmatocystin (**40**) and thence to O-methylsterigmatocystin (**41**). This pathway results in the loss of one acetate *methyl* derived carbon atom.

(19) G. Büchi, D. M. Foulkes, M. Kurono, G. F. Mitchell, and R. S. Schneider, *J. Amer. Chem. Soc.*, **89**, 6745 (1967).
(20) T. Hamasaki, Y. Hatsuda, N. Terashima, and M. Renbutsu, *Agr. Biol. Chem.* (Tokyo), **31**, 11 (1967).
(21) E. Bullock, D. Kirkaldy, J. C. Roberts, and J. G. Underwood, *J. Chem. Soc.*, 829 (1963).
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(24) Proposed by Professor E. M. Burgess, Georgia Institute of Technology, in the course of a discussion.
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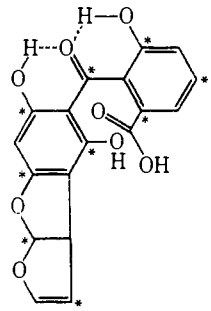

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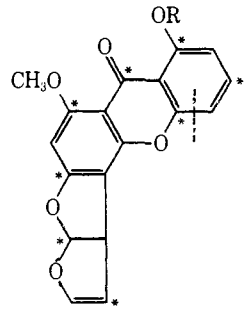
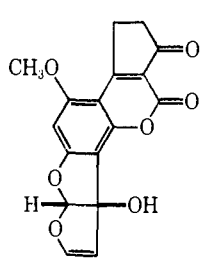


40, R = H
41, R = CH_3

The structural similarity between sterigmatocystin (**40**) and aflatoxin-B_1 (**1**) as well as the previously mentioned coexistence of O-methylsterigmatocystin (**41**) and aflatoxins in *A. flavus*[17] has led to the postulate that a difuroxanthone is an intermediate in the biosynthesis of aflatoxins. Two detailed schemes were presented[6,7] but only one[7] involving oxidative ring cleavage (dotted line in **40**), cyclization to a cyclopentenone, followed by expulsion of an acetate *methyl* derived carbon atom in the form of carbon dioxide leads to the distribution of labels demanded by our experiments. The ring cleavage can be envisaged as occuring *via* a dioxetane as observed by Baldwin in the *in vitro* cleavage of a substituted anthracene.[29] In the preliminary communication[12] we discussed a biogenesis of the aflatoxins involving a polyhydroxybenzanthracene isomeric with the naphthacene **26**. We consider this route unlikely for the following reasons: naphthacenes are much more common in nature than benzanthracenes. Second, fully aromatic naphthacenes are intermediates in the biosynthesis of tetracyclines[30] and finally the cometabolites aflatoxin-B_1 (**1**) and O-methylsterigmatocystin (**41**) can, by our route, be derived from a common hydroxynaphthacene but not from the same benzanthracene precursor.

Aflatoxin-M_1 (**42**)[31] and aspertoxin (**43**)[32] are almost certainly derived from aflatoxin-B_1 (**1**) and sterigmatocystin (**40**), respectively, rather than *vice versa* because the additional hydroxy groups present in these metabolites are attached to an acetate *methyl* carbon. Finally, aflatoxin-G_1 (**44**) as compared to B_1 (**1**) exhibits further branching of the carbon skeleton and consequently should be derived from the latter by further oxidation.



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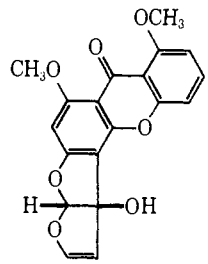

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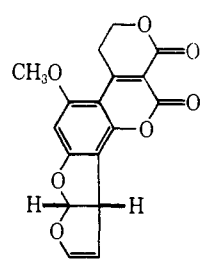

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(29) J. E. Baldwin, H. H. Basson, and H. Krauss, Jr., *Chem. Commun.*, 984 (1968), and references cited.
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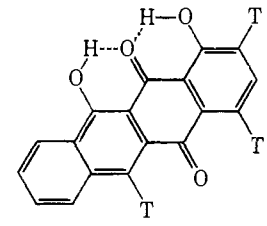
Evidence for, or against, the biogenetic scheme presented will have to come from feeding experiments with labeled intermediates. Fermentation of *A. flavus* in the presence of labeled sterigmatocystin (**40**) produced inactive aflatoxin-B_1 (**1**).[6] Similarly attempts to incorporate the two naphthacenequinones **45** and **46** and the two benzanthrones **47** and **48**,[33] prepared by tritiation[34,35] of the protium precursors, into aflatoxin-B_1 (**1**) by resting cell cultures and freeze dried, mechanically broken cells of *A. flavus* were unsuccessful.[36] The lack of incorporation hopefully is due only to problems of permeability and incorporation studies will be continued as soon as a cell-free system becomes available.



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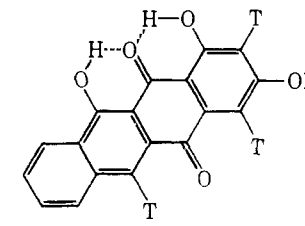

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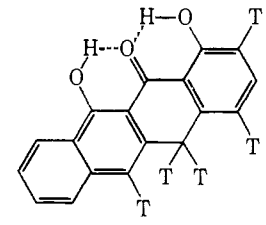

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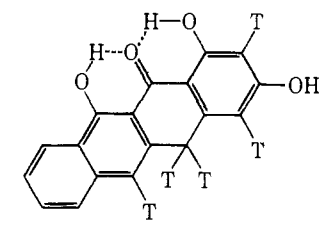

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Experimental Section

General Remarks. Melting points were determined on a hot-stage microscope and are uncorrected. Infrared (ir) spectra were recorded on a Perkin-Elmer Model 237 Infracord. Ultraviolet (uv) spectra were recorded on Cary 14 and Perkin-Elmer 202 recording spectrophotometers. The nuclear magnetic resonance (nmr) spectra were obtained with Varian A-60, T-60, and HA-100 instruments and are given in parts per million downfield from tetramethylsilane as an internal standard. The abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet, and multiplet, respectively. Mass spectra were determined at 70 eV on Hitachi RMU-6D and CEC 21-104 instruments using the direct inlet system; only the strongest peaks are reported. The abbreviation M refers to the molecular ion. High-resolution mass spectra were measured on a CEC 21-110B instrument. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in a 1-dm cell. All radioactivity determinations were made on a Packard Model 3375 liquid scintillation spectrometer. Samples of carbon dioxide liberated from barium carbonate and trapped in Hyamine 10-X solution (Packard) were measured in 20 ml of scintillator solution [3.0 g of 2,5-diphenyloxazole (PPO) in 1.0 l. of toluene]. All other ^{14}C samples were measured in Bruno solution[37] (15 ml/vial). Activity measurements of tritiated compounds were performed using 15 ml of a solution of 300 mg of dimethyl POPOP, 7.0 g of PPO, and 100.0 g of naphthalene in 1 l. of dioxane. Thin layer chromatograms (tlc) were made with Merck Silica Gel PF_{264}. Preparative tlc were made with Silica Gel PF_{254} as were column chromatograms unless otherwise indicated. All labeled compounds were recrystallized to constant activity.

Kuhn-Roth oxidations were performed according to the procedure of Wiesenberger.[38a] Mild Kuhn-Roth oxidations designed

(33) G. Büchi, G. Milne, and S.-S. Yang, unpublished.
(34) A. R. Battersby and J. Martin, *J. Chem. Soc.*, 1785 (1967).
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(36) We wish to thank Professor R. I. Mateles and Messrs. J. A. Donkersloot and D. P. H. Hsieh, Department of Food Science and Nutrition, M.I.T. for these incorporation studies.
(37) G. A. Bruno and J. E. Christian, *Anal. Chem.*, 33, 1216 (1961).
(38) (a) See Scheme I, footnote *a*. (b) See Scheme I, footnote *b*. (c) See Scheme I, footnote *c*. (d) See Scheme I, footnote *d*.

to give the higher steam volatile acids were performed by a modified procedure (*vide infra*).

Treatment of a given compound (50 mg) with 2 ml of a mixture of 4 *N* chromic acid and 1 part of concentrated sulfuric acid and water (4 ml) gave, on heating and immediate steam distillation, a mixture of acids containing primarily the longest chain acid. Distillation and heating were carried out as described by Wiesenberger for the distillation of the acetic acid obtained from the exhaustive oxidation. Schmidt degradations were carried out by the procedure of Britt.[38b] The oxidation of ethylamine to acetic acid was carried out as described in the dissertation of Brechbühler.[38c] The oxidation of methylamine to carbon dioxide was performed as described in the dissertation of Britt.[38d] *p*-Bromophenacyl esters were prepared by the method of Reid and Judefind.[39] N-α-Naphthylamides were prepared according to the procedure of Leete.[40]

Homologous carboxylic acids obtained from Kuhn–Roth oxidations were separated either by preparative glpc [4 ft × 0.25 in., 4% phosphoric acid, 20% diethylene glycol succinate on Chromosorb W (60–80 mesh)] or by chromatography of the corresponding *p*-bromophenacyl esters on silica gel followed by hydrolysis with 20% hydrochloric acid and steam distillation.

Tetrahydrodesoxoaflatoxin (2). Aflatoxin-B_1 (**1**) (3.0 g, 9.6 mmol) was dissolved in glacial acetic acid (600 ml) and hydrogenated in the presence of 10% Pd–C catalyst (4.8 g) for 18 hr. Hydrogen uptake was complete after 660 ml of hydrogen had been consumed. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The catalyst was washed with 10% methanol–chloroform (5 × 800 ml) and these filtrates, on evaporation and combination with acetic acid filtrate, yielded 2.8 g (96%) of residue. Chromatography on 200 g of silica gel PF_{254} (column 4 cm × 44 cm) and elution with chloroform (0.75% EtOH) yielded 2.61 g of pure tetrahydrodesoxoaflatoxin (**2**) (8.66 mmol, 90%). Recrystallization from chloroform–hexane gave tetrahydrodesoxoaflatoxin as colorless plates: mp 249–250°; $[\alpha]^{25}D$ −398° (*c* 0.78, $CHCl_3$); ir, uv, and nmr identical with those of authentic **2**.

Continued elution with 3% methanol–chloroform yielded an additional 240 mg of lower R_f components including aflatoxin-B_1.

Furocoumarin Alcohol 3. A stirred solution of tetrahydrodesoxoaflatoxin-B_1 (**2**) (310 mg, 1.0 mmol) in methylene chloride (110 ml) was treated with aluminum chloride (4.5 g) and the reaction was stirred for 6 hr at ambient temperature. Work-up by addition of ice water (65 g) and extraction with chloroform (3 × 150 ml) followed by chromatography on silica gel (3% MeOH–$CHCl_3$) gave 302 mg (97%) of crystalline furocoumarin alcohol **3** (needles): mp 223–224° (sub); ir (KBr) 3460, 1700, 1630, 1600, 1585, 1480, 1130, and 1100 cm^{-1}; uv max (95% C_2H_5OH) 223, 247 (s), 254, 272, and 314 mμ (ε 18,600, 14,900, 18,900, 17,000 12,000); nmr ($CDCl_3$–CD_3OD) δ 7.4 (s, 1), 6.82 (s, 1), 3.9 (s, 3), 3.6–4.0 (m, 2), 2.5-3.5 (m, 7); 2.16 (broad q, 2, *J* = 8 Hz).

Anal. Calcd for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found: C, 67.78; H, 5.47.

Furocoumarin Tosylate 4. A stirred solution of furocoumarin alcohol (**3**) (289 mg, 0.97 mmol) in pyridine (8 ml, dried over Ba(OH)$_2$) was treated with recrystallized *p*-toluenesulfonyl chloride (312 mg, 1.7 mmol) and stirred for 18 hr at room temperature. The reaction was halted by the addition of ice water (20 g). The furocoumarin tosylate **4** (397 mg, 91%) crystallized out of solution and was collected by filtration: mp 166–167°; ir ($CHCl_3$) 1710, 1630, 1600, 1585, 1480, 1360, and 1180 cm^{-1}; uv max (95% C_2H_5OH) 223, 246 (s), 254, 272, and 314 mμ (ε 15,400, 8100, 10,400, 9050, 6600); nmr ($CDCl_3$) δ 7.55 (d, 2, *J* = 8 Hz), 7.3 (s, 1), 7.01 (d, 2, *J* = 8 Hz), 6.65 (s, 1), 4.4 (d of d, 2, *J* = 6.5 Hz), 3.8 (s, 3), 2.9–3.4 (m, 4), 2.5–3.3 (m, 2), 2.25 (s, 3), 1.8–2.2 (m, 2).

Anal. Calcd for $C_{29}H_{22}O_7S$: C, 63.43; H, 4.88. Found: C, 63.31; H, 4.88.

3-Ethyl-5-[1-(2-methylcarbinolcyclopent-1-ene)]-4,6-dimethoxybenzofuran (5). A stirred solution of **4** (386 mg, 0.85 mmol) in freshly distilled THF (50 ml) was treated with lithium aluminum hydride (1.0 g) added over 1 hr and then allowed to stir overnight. The reaction was halted by the addition of a saturated solution of sodium potassium tartrate. The reaction mixture was filtered, the precipitate was washed with THF (3 × 50 ml) and the filtrates were evaporated to dryness. Treatment of an acetone solution (15 ml) under nitrogen with methyl iodide (250 mg) and potassium carbonate (260 mg) gave, on chromatography on silica gel (10% hexane–chloroform), pure **5**; mp 126–127°; ir ($CHCl_3$) 3500, 1620, 1600, 1580, 1460, 1420, 1320, and 1310 cm^{-1}; uv max (95% C_2H_5OH) 250, 258 (s), and 295 mμ (ε 11,200, 9800, 3100); nmr ($CDCl_3$) δ 7.4 (s, 1), 6.75 (s, 1), 4.3 (m, 2), 3.7 (s, 3), 3.6 (s, 3), 1.5–3.5 (m, 8), 1.3 (t, 3).

Anal. Calcd for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33. Found: C, 71.31; H, 7.64.

Kuhn–Roth Oxidation of 5, Derived from [1-^{14}C]-Acetate Labeled Aflatoxin-B_1 (1). Kuhn–Roth oxidations of **5** (average activity, 7.959 × 10^4 dpm/mmol; **1**, 8.107 × 10^4; **2**, 7.928 × 10^4; **3**, 7.889 × 10^4; and **4**, 7.914 × 10^4) under exhaustive conditions gave exclusively acetic acid and, under mild conditions, a mixture of propionic (64%) and acetic acid (36%). The *p*-bromophenacyl acetate and the *p*-bromophenacyl propionate had activities of 9.55 × 10^3 dpm/mmol and 8.94 × 10^3 dpm/mmol, respectively.

Degradation of the Acids Obtained from the Kuhn–Roth Oxidation of 5 ([1-^{14}C]-Acetate). (a) Propionic Acid. Schmidt degradation of propionic acid gave barium carbonate (1.073 × 10^3 dpm/mmol). Oxidation of the ethylamine to acetic acid and Schmidt degradation of the acetic acid gave barium carbonate (6.85 × 10^3 dpm/mmol). The methylamine was oxidized with basic potassium permanganate to barium carbonate (9.66 × 10^2 dpm/mmol).

(b) Acetic Acid. Schmidt degradation gave methylamine and barium carbonate (6.86 × 10^3 dpm/mmol). Oxidation of the methylamine gave barium carbonate bearing 1.21% of the label (9.61 × 10^2 dpm/mmol).

5,7-Dimethoxy-8-(2-butyl)cyclopenta[*c*]coumarin (7). A stirred solution of 5-methoxy-7-hydroxy-8-(2-butyl)cyclopenta[*c*]coumarin (**6**) (286 mg, 1.0 mmol) in acetone (20 ml) was heated with methyl iodide (480 mg) and potassium carbonate (520 mg) and allowed to stir for 12 hr. The reaction mixture on evaporation to dryness, extraction with chloroform, and chromatography of the chloroform extract on silica gel (chloroform) gave pure **7**: mp 144–145°; $[\alpha]^{25}D$ +4.49° (*c* 0.51, $CHCl_3$); ir ($CHCl_3$) 1700, 1610, 1595, 1450, 1350, and 1130 cm^{-1}; nmr ($CDCl_3$) δ 6.33 (s, 1), 3.85 (s, 6), 2.5–3.6 (m, 5), 1.5–2.2 (m, 4), 1.3 (d, 3, *J* = 7 Hz), 0.8 (t, 3, *J* = 8 Hz).

Anal. Calcd for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33. Found: C, 71.34; H, 7.01.

Kuhn–Roth Oxidation of 7 Obtained from [1-^{14}C]-Acetate Labeled Aflatoxin-B_1. Mild Kuhn–Roth oxidation of **7** (activity of **1**, 5.895 × 10^4 dpm/mmol; **6**, 5.943 × 10^4 dpm/mmol; average activity for **7**, 5.919 × 10^4 dpm/mmol) gave a mixture of 2-methylbutanoic acid (90%), propionic acid (5%), and acetic acid (5%) which were characterized as the corresponding N-α-naphthylamides. N-(α-Naphthyl)-2-methylbutanamide had 1.319 × 10^4 dpm/mmol, N-α-naphthylpropionamide had 6.604 × 10^3 dpm/mmol, and N-α-naphthylacetamide had 6.594 × 10^3 dpm/mmol.

Degradation of 2-Methylbutanoic and Acetic Acid from the Kuhn–Roth Oxidation of 7 ([1-^{14}C]-Acetate). Schmidt degradation of 2-methylbutanoic acid gave barium carbonate with a millimolar activity of 7.582 × 10^1 dpm. Schmidt degradation of the acetic acid gave $BaCO_3$ with 3.27 × 10^3 dpm/mmol and the oxidation of the methylamine obtained in the Schmidt reaction gave barium carbonate which had 3.25 × 10^3 dpm/mmol.

Degradation of the Mixture of Acids obtained on Kuhn–Roth Oxidation of 7 ([2-^{14}C]-Acetate Incorporation). The average millimolar activity for **1**, **2**, **7** (5.017 × 10^5), and **13** was 4.994 × 10^5 dpm. The *p*-bromophenacyl esters of 2-methylbutanoic, propionic and acetic acids had 2.161 × 10^5, 1.429 × 10^5, and 7.128 × 10^5 dpm/mmol, respectively.

(a) 2-Methylbutanoic Acid. The Schmidt degradation gave barium carbonate having 6.406 dpm/mmol and 2-butylamine. The reaction mixture from the Schmidt degradation of 20 mg of 2-methylbutanoic acid was made basic with 20% sodium hydroxide solution (5 ml) and the 2-butylamine was distilled into a sulfuric acid trap (2 ml of 0.5 *N*). The solution was then transferred to a round-bottomed flask equipped with magnetic stirrer and treated with 0.6 ml of 0.8 *N* sodium hypochlorite (0.8 *N* NaOH) and 0.5 *N* sulfuric acid (3 ml). After 4 hr the reaction was made basic with 4 *N* sodium hydroxide (1.2 ml) and stirring was continued for 3 hr. Acidification with 10% hydrochloric acid (2 ml) followed after 1 hr by the addition of 4 *N* sodium hydroxide (2.1 ml) and potassium iodide–iodine solution (6.2 ml). The precipitated iodoform (37 mg, 47%) was collected by filtration and recrystallized from methanol–water. The millimolar activity of the iodoform was 2.453 × 10^3 dpm.

(b) Propionic Acid. Schmidt degradation gave barium carbonate (6.386 × 10^4 dpm/mmol) and ethylamine. Oxidation of the ethylamine to acetic acid and subsequent Schmidt degradation gave barium carbonate (2.433 × 10^3 dpm/mmol) and methylamine

(39) E. Reid and H. Judefind, *J. Amer. Chem. Soc.*, **42**, 1043 (1920).
(40) E. Leete, H. Gregory, and E. G.Gros, *ibid.*, **87**, 3475 (1965).

which gave barium carbonate (6.353×10^4 dpm/mmol) on oxidation with basic potassium permanganate.

(c) **Acetic Acid.** Schmidt degradation of the acetic acid gave barium carbonate (3.110×10^4 dpm/mmol) and methylamine (3.218×10^4 dpm/mmol) measured by conversion to barium carbonate.

Methylcyclopentene Derivatives 12 and 13 and *cis*-Methylcyclopentane Derivative 16. To a suspension of lithium aluminum hydride (5.6 g, 0.147 mol) in anhydrous ether (800 ml) under a nitrogen atmosphere in a 2 l. round-bottomed flask equipped with a magnetic stirrer, was added tetrahydrodesoxoaflatoxin (**2**) (2.6 g, 8.65 mmol) in small portions over 45 min. After stirring at ambient temperature for 7.0 hr, the reaction was halted by the dropwise addition of methanol with cooling (ice bath), followed by water (100 ml) and 2 *N* sulfuric acid to pH ~4. The reaction mixture was extracted continuously with ether for 36 hr. The ethereal extract was washed with water and saturated sodium chloride solution and dried ($MgSO_4$). Evaporation to dryness yielded 2.5 g (96%) of a white solid containing **8** and **9**: ir ($CHCl_3$) 3600, 3480, 1625, 1605, 1595, 1475, 1450, 1125, 1095, and 960 cm^{-1}; uv max (95% C_2H_5OH) 215, 252 (s), and 283 (s) mμ; nmr ($CDCl_3$) δ 6.25 (d, 1, J = 6 Hz), 6.0 (s, 1), 4.05 (broad s, 4), 3.7 (s, 3), 2.4–3.0 (m, 3), 1.5–2.4 (m, 6).

A solution of **8** and **9** in ethanol (300 ml) was stirred in a hydrogen atmosphere in the presence of 10% palladium on carbon catalyst (510 mg) until 1 equiv of hydrogen had been absorbed (195 ml). The reaction mixture was filtered, the catalyst washed with chloroform (3 × 150 ml), and the combined filtrates were evaporated to dryness. To a stirred solution of the residue in acetone (100 ml) was added potassium carbonate (4.0 g) and methyl iodide (4.5 g). After 6 hr an additional 2.1 g of potassium carbonate and 2.6 g of methyl iodide were added and stirring was continued for 18 hr. Evaporation of the reaction mixture to dryness *in vacuo*, extraction with chloroform, and chromatography of the chloroform soluble material on silica gel (chloroform) gave 2.1 g (84%) of **12**, 151 mg (6%) of **13**, and 82 mg (3%) of **16**. An analytical sample of **12** was recrystallized from methanol–chloroform: mp 124–126°; ir ($CHCl_3$) 1615, 1600 (s), 1470, 1450, 1125, 1100, and 950 cm^{-1}; nmr ($CDCl_3$) δ 6.3 (d, 1, J = 6 Hz), 6.2 (s, 1), 3.8–4.2 (m, 2), 3.7 (s, 6), 1.7–2.8 (m, 9), 1.5 (broad s, 3); uv max (95% C_2H_5OH) 246 mμ (ε 1250).

Anal. Calcd for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33. Found: C, 71.52; H, 7.34.

An analytical sample of **13** recrystallized from chloroform–methanol had: mp 105–106°; ir ($CHCl_3$) 1620, 1605, 1500, 1465, 1145, 1100, and 950 cm^{-1}; uv max (95% C_2H_5OH) 245 mμ (ε 1300); $[\alpha]^{26}D$ −75.1° (*c* 0.193, $CHCl_3$); nmr ($CDCl_3$) δ 6.35 (d, 1, J = 6 Hz), 6.13 (s, 1), 3.6–4.2 (m, 2), 3.9 (s, 3), 3.85 (s, 3), 2.3–2.8 (m, 3), 1.8–2.3 (m, 6), 0.72 (broad s, 3).

Anal. Calcd for $C_{18}H_{22}O_4$: C, 71.50; H, 7.33. Found: C, 71.63; H, 7.46.

An analytical sample of **16** was recrystallized from chloroform–methanol: mp 101–104°; ir ($CHCl_3$) 1610, 1590, 1465, 1440, 1230, and 960 cm^{-1}; nmr ($CDCl_3$) δ 6.18 (d, 2, J = 6 Hz), 6.1 (s, 1), 3.3–4.2 (m, 3), 3.76 (s, 3), 3.67 (s, 3), 1.2–2.3 (m, 8), 0.8 (d, 3, J = 6 Hz); mass spectrum *m/e* 304 (M).

Anal. Calcd for $C_{18}H_{24}O_4$: C, 71.02; H, 7.95. Found: C, 71.31; H, 7.85.

Methylcyclopentene Derivative 14 and Methylcyclopentane Derivative 17. Reduction of 1.34 g of tetrahydrodesoxoaflatoxin (**2**) with lithium aluminum hydride (2.8 g) and work-up as previously described gave 1.31 g of **10**: ir ($CHCl_3$) 3450, 3200, 1630, and 1590 cm^{-1}. Hydrogenolysis over 10% Pd-C, followed by treatment with methyl iodide and potassium carbonate gave on chromatography on silica gel (chloroform) 173 mg (14%) of pure **17**, 88 mg (7%) of a mixture of **17** and **14**, and 560 mg (40%) of crystalline **14**: mp 103–104°; ir ($CHCl_3$) 1595, 1585, 1420, and 1200 cm^{-1} **7**; uv max (95% C_2H_5OH) 242 mμ (ε 1100); nmr ($CDCl_3$) δ 6.25 (s, 1), 3.8–4.2 (m, 2), 3.8 (s, 3), 3.75 (s, 3), 3.55 (s, 3), 1.7–2.8 (m, 9), 1.55 (broad s, 3); mass spectrum *m/e*, 318 (M).

Anal. Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.63; H, 8.52.

Kuhn–Roth Oxidation of 16 ([1-^{14}C]-Acetate Incorporation). Exhaustive Kuhn–Roth oxidation of **16** (average millimolar activity 2.514×10^5 dpm; activity of **1** and **2**: 2.465×10^5 and 2.564×10^5 dpm/mmol, respectively) gave acetic acid. The acetic acid was purified and characterized as the corresponding *p*-bromophenacyl-acetate bearing 2.66×10^4 dpm/mmol. Schmidt degradation gave carbon dioxide having 5.290×10^2 dpm/mmol and methylamine which, on oxidation with potassium permanganate, yielded carbon dioxide bearing 2.185×10^4 dpm/mmol.

Kuhn–Roth oxidation of **16** under mild conditions gave a mixture of acetic acid (11%) and *cis*-2-methylcyclopentane carboxylic acid (**18**) (89%) identified by the glpc retention time of the acid and the preparation of *p*-bromophenacyl-*cis*-2-methylcyclopentanoate identical with that prepared from authentic **18**. The *p*-bromophenacyl-*cis*-2-methylcyclopentanoate contained 1.124×10^5 dpm/mmol and on Schmidt degradation gave carbon dioxide, isolated as barium carbonate, having 3.592×10^3 dpm/mmol.

Kuhn–Roth Oxidation of 16 ([2-^{14}C]-Acetate Incorporation). The average millimolar activity of **1, 2, 7,** and **13** for this incorporation was 4.994×10^5 dpm. Kuhn–Roth oxidation of **16** gave a mixture of acetic and *cis*-2-methylcyclopentane carboxylic acid (**18**) isolated as *p*-bromophenacylacetate (2.150×10^5 dpm/mmol and *p*-bromophenacyl-*cis*-2-methylcyclopentanoate (7.045×10^4 dpm/mmol). Schmidt degradation of **18** gave carbon dioxide having 6.421×10^4 dpm/mmol. The Schmidt degradation of acetic acid gave carbon dioxide having 6.304×10^4 dpm/mmol) and methylamine having 1.768×10^3 dpm/mmol as determined by potassium permanganate oxidation to carbon dioxide.

Synthesis of *cis*-2-Methylcyclopentane Carboxylic Acid (18) and *trans*-2-Methylcyclopentane Carboxylic Acid. A stirred solution of 2-methylcyclopent-1-ene carboxylic acid (**15**) (85 mg), prepared from 2-methylcyclopentanone by the method of Robinson and King,[14] in absolute ethanol (10 ml) gave on hydrogenation over platinum oxide 75 mg of a mixture of 45% *cis*-2-methylcyclopentane carboxylic acid (**18**) and 55% of the known *trans*-2-methylcyclopentane carboxylic acid separable by glpc [4 ft, 4% phosphoric acid, 20 DEGS, Chromosorb W (60–80 mesh)] and by chromatography on silica gel (chloroform–hexane) of their respective *p*-bromophenacyl esters. An analytical sample of *p*-bromophenacyl-*trans*-methylcyclopentanoate had: mp 64.5–65°; mmp with the *cis* ester 49–51°; ir ($CHCl_3$) 1735, 1702, and 1585 cm^{-1}; nmr ($CDCl_3$) δ 8.1 (A_2B_2, 4) 5.5 (s, 2), 1.4–2.7 (m, 8), 1.12 (d, 3, J = 6 Hz).

Anal. Calcd for $C_{15}H_{17}O_3Br$: C, 58.45; H, 5.23. Found: C, 58.26; H, 5.14.

An analytical sample of *p*-bromophenacyl-*cis*-methylcyclopentanoate was recrystallized from methanol–water; mp 63.5–64.0°; ir ($CHCl_3$) 1735, 1700, and 1585 cm^{-1}; nmr ($CDCl_3$) δ 8.1 (A_2B_2, 4), 5.5 (s, 2), 2.9–3.2 (m, 1), 1.4–2.7 (m, 7), 0.98 (d, 2, J = 8 Hz).

Anal. Calcd for $C_{15}H_{17}O_3Br$: C, 58.45; H, 5.23. Found: C, 58.32; H, 5.19.

1,5-Diketone 19. To a stirred solution of **12** (220 mg, 0.72 mmol) in tetrahydrofuran (10 ml) was added a solution of potassium chlorate (200 mg) in water (15 ml) and osmic acid (0.2 g). After stirring for 6 hr at room temperature, the reaction mixture was saturated with hydrogen sulfide. After 0.5 hr $Pb(OAc)_2 \cdot 3H_2O$ was added; the reaction mixture was extracted continuously with ether. The extract was washed with water and dried ($MgSO_4$) and the residue from evaporation (197 mg, 83%) was chromatographed on silica gel (chloroform) to give pure **19** (170 mg); ir ($CHCl_3$) 1700 (broad), 1620, 1595, 1475, 1448, 1130, and 960 cm^{-1}; uv max (95% C_2H_5OH) 222 and 275 mμ, (weak); nmr ($CDCl_3$) δ 6.22 (d, 2, J = 6 Hz), 6.1 (s, 1), 3.8–4.2 (m, 2), 3.75 (s, 3), 3.65 (s, 3), 1.0–2.9 (m, 9), 0.82 (t, 3, J = 6 Hz); mass spectrum *m/e* 334 (M), 249 (loss of CH_3COCH_2-CH_2CH_2−). This compound was unstable and decomposed on treatment with acid or base and on chromatography.

Hexyl Alcohol 23. To a stirred suspension of lithium aluminum tri-*t*-butoxy hydride (360 mg, 62% active) in anhydrous ether (5 ml) at −5° was added, dropwise, diketone **19** (235 mg, 0.7 mmol) in 0.9 ml of ether. After stirring for 35 min at −5°, water (0.1 ml) was added, the reaction mixture filtered, and the filtrate evaporated to dryness. Chromatography on silica gel gave keto alcohol **20** (224 mg): ir ($CHCl_3$) 3500, 1685, 1610, 1590, 1200, 1138, 1105, and 960 cm^{-1}. The purified **20** was dissolved in pyridine (3.0 ml), treated with *p*-toluenesulfonyl chloride (240 mg), and stored overnight at 10° and then 5 hr at room temperature. Evaporation to dryness and purification by chromatography on silica gel gave the corresponding keto tosylate (240 mg): ir ($CHCl_3$) 1695, 1610, 1590, 1192, 1185, 1165, 950, 915, 900, and 810 cm^{-1}. The keto tosylate (240 mg) dissolved in anhydrous ether (20 ml) was added dropwise to a suspension of lithium aluminum hydride (880 mg) in ether (12 ml) at −10°. After stirring for 1.5 hr at −10° and 1.5 hr at 0°, the reaction mixture was worked up by the addition of methanol followed by water. Filtration of the reaction mixture through Celite and evaporation gave hexyl alcohol **23** (138 mg, 57% yield from **19**): bp 65° (oil, 0.1 mm); ir ($CHCl_3$) 3550, 1620, 1600, 1480, 1450, 1420, 1200, 1130, and 960 cm^{-1}; nmr ($CDCl_3$) δ 6.1 (d, 1, J = 5 Hz), 6.06 (s, 1), 4.7 (m, 1), 3.4–4.2 (m, 2), 3.84 (s, 3), 3.76

(s, 3), 2.6 (broad m, 1, disappears with D_2O), 1.0–2.1 (m, 11), 0.87 (m, 3); mass spectrum *m/e* 322 (M).

Anal. Calcd for $C_{18}H_{26}O_5$: C, 67.06; H, 8.13. Found: C, 66.94; H, 7.99.

Hexyl Alcohol 24. Oxidation of **14** (510 mg, 1.67 mmol) with osmic acid and potassium chlorate under reaction conditions analogous to those described for the oxidation of **12** yielded 1,5-diketone **21** (460 mg); ir ($CHCl_3$) 1710, 1685 (s), 1595, 1580, 1460, 1167, and 1155 cm^{-1}. Selective reduction of **21** as described for **19** gave keto alcohol **22**: ir ($CHCl_3$) 3600, 3400 (broad), 1690, 1597, 1580, 1460, 1170, and 1155 cm^{-1}. Tosylation of the crude keto alcohol **22** with 350 mg of *p*-toluenesulfonyl chloride in pyridine (4 ml), afforded the corresponding keto tosylate which was reduced with lithium aluminum hydride, as described for the tosylate of **20**, giving 214 mg of crude hexyl alcohol **24**. Chromatography on silica gel with chloroform gave 187 mg (35% from **10**) of hexyl alcohol **24**: ir ($CHCl_3$) 3350, 1620, 1600, 1480, and 1445 cm^{-1}; nmr ($CDCl_3$) δ 6.1 (s, 1), 3.8–4.1 (m, 4), 3.8 (s, 3), 3.75 (s, 3), 2.7 (m, 1, removed by D_2O), 1.0–2.2 (m, 11), 0.82 (m, 3); mass spectrum *m/e* 322 (M).

Kuhn–Roth Oxidation of 24 ([1-^{14}C]-Acetate Incorporation). Kuhn–Roth oxidation of **24** (average millimolar activity = 1.726×10^5 dpm) under mild conditions gave a mixture of caproic (60%), valeric (16%), butyric (3.3%), propionic (8.2%), and acetic acid (13%). Preparation of the *p*-bromophenacyl esters of the mixture of acids and chromatography on silica gel gave *p*-bromophenacylcaproate (7.711×10^4 dpm/mmol), valerate (5.615×10^4 dpm/mmol), butyrate (3.202×10^4 dpm/mmol), propionate (3.758×10^4 dpm/mmol), and acetate (1.914×10^4 dpm/mmol). The caproic and valeric acid were purified by preparative glpc and, on Schmidt degradation, gave carbon dioxide containing 1.463×10^4 dpm/mmol and 1.113×10^4 dpm/mmol, respectively.

Kuhn–Roth Oxidation of 23 ([2-^{14}C]-Acetate Incorporation). Kuhn–Roth oxidation of **23** (average millimolar activity = 4.994×10^5 dpm) gave a mixture of caproic and lower acids as reported for the oxidation of **24**. Preparation of the *p*-bromophenacyl esters of the acid mixture and chromatography on silica gel gave *p*-bromophenacylcaproate (1.426×10^5 dpm/mmol), valerate (1.432×10^5 dpm/mmol), butyrate (1.422×10^5 dpm/mmol), propionate (7.130×10^5 dpm/mmol), and acetate (7.046×10^5 dpm/mmol). The caproic and valeric acids were purified by preparative glpc and on Schmidt degradation afforded carbon dioxide containing 1.748×10^3 dpm/mmol and 1.767×10^3 dpm/mmol, respectively.

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Microanalysis by Successive Isotopic Dilution. A New Assay for Racemic Content[1]

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Abstract: An isotopic dilution assay for racemate with sensitivity in the important 1–0.001% concentration range is outlined, and results are reported for its application to syntheses of the Anderson peptide, ethyl benzyloxycarbonylglycylphenylalanylglycinate, and the Young peptide, ethyl benzoylleucylglycinate, by isoxazolium salt couplings and by the acyl azide procedure. The Anderson test is used to explore the effect of solvent on racemate formation during *p*-nitrophenyl ester couplings, and the triethylamine-catalyzed racemization rate of the Anderson peptide in ethanol is reported. The Young test appears to have roughly ten times the sensitivity of the Anderson test. Under optimal conditions, acylazide and 3-acyloxy-2-hydroxy-N-ethylbenzamide couplings generate similar levels of racemate—0.015% by the Anderson and 0.25% by the Young assay.

Optical purity sets one of the more vexing limitations on synthetic approaches to large peptides. As the following expression indicates, trace levels of epimer at each of the *n* chiral sites of a polypeptide must detract nearly independently from its homogeneity, and as

$$(1 - X)^n = 1 - nx, \; x \text{ small}$$

has been stressed in recent reviews,[2] an average chiral purity of 99.5% per amino acid residue must be regarded as a marginally acceptable lower limit on the integrity required of starting materials and synthetic operations, if a reasonable yield of a large, chirally homogeneous peptide is to be attained.

The development in recent years of a variety[3] of peptide-coupling procedures which appear to be racemization free when judged by assays with limiting sensitivities of 0.5–2.0% racemate points up the need for a convenient, accurate assay for racemic or diastereomeric content in the range of 0.001 to 1.0%. Although much attention has been given the problem of assessing peptide optical purity,[4] only enzymatic assay[5] and tritium incorporation[6] appear to offer sensitivities in this range; unfortunately, neither is ideal. The enzymatic assay is

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