[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA]

The Biosynthesis of the Triterpene, Eburicoic Acid[1,2]

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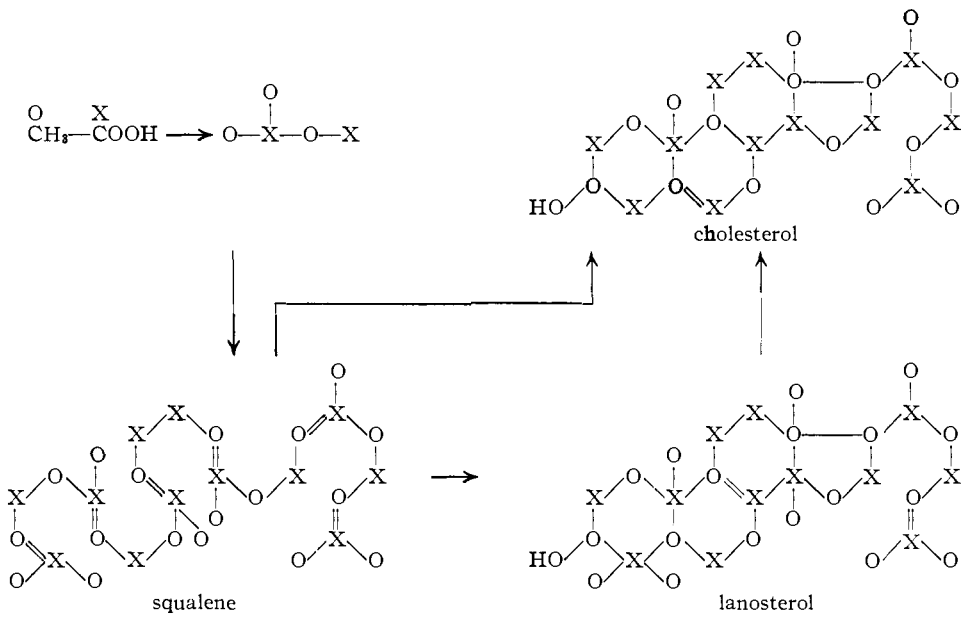
The C_{31} triterpene, eburicoic acid, has been biosynthesized by allowing *P. sulfureus* to grow on a medium containing carboxyl-labeled acetate. Labeled eburicoic acid has been obtained and by stepwise degradation of the molecule the following facts have been established: (a) the acetate is utilized as a two carbon unit, (b) C_4 of ring A and C_{11} and C_{12} of ring C are labeled, and (c) the *gem*-dimethyl group, C_{30} and C_{31}, of ring A, the carboxyl group, C_{21}, and the extra methylene carbon atom, C_{28}, in the side-chain are not labeled. The specific activities of the labeled carbon atoms are those expected for an eburicoic acid molecule containing 12 carbon atoms derived from the carboxyl of acetate. The location of the labeled atoms and their specific activities are those predicted on the basis of the squalene hypothesis as the mechanism of biosynthesis for steroids and triterpenes. Of particular interest is the demonstration that both C_{11} and C_{12} are derived from the carboxyl carbon of acetate since such is a necessary requirement if squalene is to be utilized as an intact molecule.

A number of recent results pertaining to the absolute configuration of triterpenes and steroids[4] have suggested that these two classes of compounds arise *via* the same or very similar biosynthetic pathway. In this regard, the acyclic triterpene squalene has been shown to be an efficient precursor for cholesterol[5] and its conversion has been envisioned[6] as shown below. Such a concept is in complete accord with all the experimental evidence.[7]



Although there has been considerable speculation with regard to a "universal" biosynthetic mechanism[8] leading both to the triterpenes and to the steroids, the only experiments which have been reported have shown that either acetate or squalene will give rise to lanosterol, a triterpene of animal origin, and that this compound, in turn, will give rise to cholesterol.[9] In these latter experiments only a gross conversion has been demonstrated and a constancy of isotope distribution was not established.

A detailed knowledge of the manner of biosynthetic incorporation of acetate into the cholesterol molecule has played a very major role in the elucidation of the mechanism of biosynthesis of this important product. If it could be shown that acetate is incorporated into a triterpene in an exactly analogous fashion, additional evidence to that of the previously demonstrated gross conversion[9]

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(2) This work was supported, in part, by grant No. AT(11-1)-34, Project No. 16, U. S. Atomic Energy Commission.

(3) National Science Foundation Predoctoral Fellow, 1954–1955.

(4) W. Klyne, *J. Chem. Soc.*, 2916 (1952); W. G. Dauben, D. F. Dickel, O. Jeger and V. Prelog, *Helv. Chim. Acta*, **36**, 325 (1953); E. Kyburg, B. Riniker, H. R. Schenk, H. Heusser and O. Jeger, *ibid.*, **36**, 1891 (1953); R. Riniker, D. Arigoni and O. Jeger, *ibid.*, **37**, 546 (1954); A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *ibid.*, **38**, 1890 (1955); K. Schaeffner, R. Viterbo, D. Arigoni and O. Jeger, *ibid.*, **39**, 175 (1956).

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(6) R. B. Woodward and K. Bloch, THIS JOURNAL, **75**, 2023 (1953); W. G. Dauben, S. Abraham, S. Hotta, I. L. Chaikoff, H. L. Bradlow and A. H. Soloway, *ibid.*, **75**, 3038 (1953).

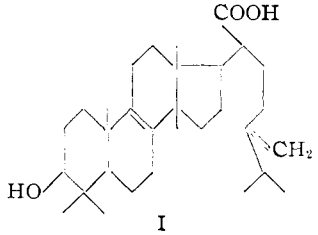
(7) H. N. Little and K. Bloch, *J. Biol. Chem.*, **183**, 33 (1950); J. Wuersch, R. L. Huang and K. Bloch, *ibid.*, **195**, 439 (1952); J. W. Cornforth, G. D. Hunter and G. Popjak, *Biochem. J.*, **54**, 597 (1953); K. Bloch, *Helv. Chim. Acta*, **36**, 1611 (1953); D. J. Hanahan and S. J. Wakil, THIS JOURNAL, **75**, 273 (1953); W. G. Dauben and K. H. Takemura, *ibid.*, **75**, 6302 (1953); K. Bloch, L. C. Clarke and I. Harary, *ibid.*, **76**, 3859 (1954); J. W. Cornforth, *Rev. Pure Applied Chem.*, **4**, 286 (1954); G. Popjak, *Roy. Inst. Chem., Lecture*, No. 2 (1955); F. Diturit, F. Cobey, J. V. B. Warms and S. Gurin, *Federation Proc.*, **14**, 203 (1955).

(8) L. Ruzicka, *Experientia*, **9**, 357 (1953); G. Stork and A. W. Burgstahler, THIS JOURNAL, **77**, 5068 (1955); A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

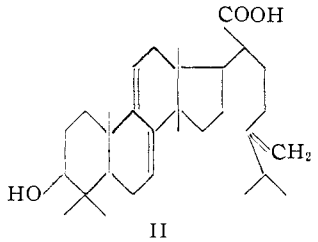
(9) R. B. Clayton and K. Bloch, *J. Biol. Chem.*, **218**, 305, 319, (1956); T. T. Tchen and K. Bloch, THIS JOURNAL, **77**, 6085 (1955).

would thereby be provided that the steroids and the triterpenes are, in fact, elaborated in virtually the same manner. The purpose of the present investigation was to determine whether acetate can serve as a precursor of the triterpenes of plant origin and further to establish whether its manner of incorporation into the triterpene skeleton does, or does not, parallel in detail the biosynthetic incorporation of acetate into cholesterol.

The problem was attacked by following the incorporation of labeled acetate into the tetracyclic triterpene eburicoic acid, a compound which has been shown to have structure I[10-15] and is produced



I



II

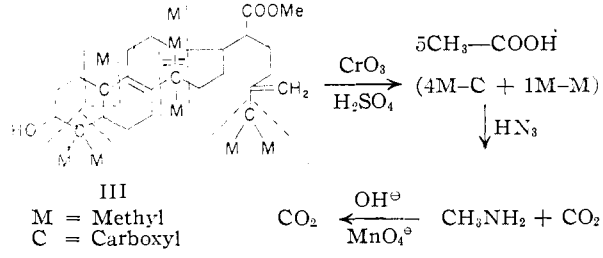
by certain varieties of fungi[12] grown in culture. Eburicoic acid has been related to the C-30 triterpene, lanosterol, which, in turn, has been related to cholesterol.[16] Eburicoic acid, therefore, can be viewed as a typical representative of this class of tetracyclic triterpenes and, further, as a close analog of ergosterol.

Eburicoic acid was isolated from the mycelium of *Polyporus sulfureus* which had been grown on a medium of potato broth–3% glucose to which had been added sodium acetate labeled in the carboxyl group with C^{14}. After a growth period of 3–4 months at 32° (stationary cultures), the mycelium was filtered and dried. The dried mycelium was then extracted for 2 hours with petroleum ether (60–90°). This extract yielded a fat fraction which comprised 3.8% of the dry weight of the mycelium. A determination of the specific activity of this fraction showed that approximately 2% of the labeled acetate had been incorporated into petroleum ether extractable substances (mainly fats).

Subsequent extraction of the mycelium with ether for 24 hours yielded a white crystalline solid (54% of the dry weight of the mycelium). This crude material was methylated with diazomethane and then acetylated with acetic anhydride in pyridine. A determination of the specific activity of this substance indicated that approximately 2% of the added labeled acetate had been incorporated into ether extractable material (principally eburicoic acid). Since the labeled acetate was added at the beginning of a long growth period and due to the high probability of changing metabolic kinetics at various periods in the mycelial development, it is not possible to make any deductions with regard to the relative rates of the various acetate biosynthetic pathways on the basis of the above evidence.

The crude methyl acetyleburicoate, from the ether fraction, was purified by chromatography over alumina. It was not possible to remove a small amount of an impurity which absorbs in the ultraviolet (ϵ^{242}_{max} 192, ϵ^{253}_{max} 125) and which is almost certainly[13] the 7,9(11)-diene (II) present to an extent of less than 1%. The 7,9(11)-diene has not previously been reported as a contaminant of eburicoic acid isolated from the mycelium of *P. sulfureus*, although it has been observed in the mycelium of other strains of this and related fungi.[13] The specific activity of the pure methyl acetyleburicoate isolated from the chromatography was 90% of the specific activity of the crude material. That this observed radioactivity was due to methyl acetyleburicoate and not to some unknown contaminant is amply demonstrated by the constant specific activities of various transformation products discussed below.

It was first necessary to establish that the acetate was utilized as a two carbon unit in its incorporation into eburicoic acid. In this regard, methyl eburicoate (methyl 3-hydroxyeburic-8,24(28)-diene-21-oate, III), biosynthesized from carboxyl-labeled acetate, was oxidized with chromic acid in sulfuric acid (Kuhn-Roth procedure).[1] The acetic acid from the five C-methyl groups which was obtained in an 84% yield was degraded first by reaction with hydrazoic acid to yield carbon dioxide and methylamine. The methylamine then was oxidized to carbon dioxide by alkaline permanganate. The results are listed in Table I and they indicate



III

M = Methyl
C = Carboxyl

that acetate is utilized as a two-carbon unit (at least in those positions removed by Kuhn-Roth oxidation) and that the amount of activity in the carboxyl group of acetic acid so obtained is in complete agreement with that expected on the basis of the "squalene hypothesis" (*i.e.*, in methyl eburicoate there are 12 carbon atoms derived from the carboxyl of acetate and, of the 5 molecules of acetic acid formed in the oxidation, the carboxyl group of

(10) R. M. Gascoigne, J. S. E. Holker, B. J. Ralph and A. Robertson, *Nature*, **166**, 652 (1950).

(11) F. N. Lahey and P. H. A. Strasser, *J. Chem. Soc.*, 873 (1951).

(12) R. M. Gascoigne, J. S. E. Holker, B. J. Ralph and A. Robertson, *ibid.*, 2346 (1951).

(13) R. M. Gascoigne, A. Robertson and J. H. J. Simes, *ibid.*, 1830 (1953).

(14) J. S. E. Holker, A. G. D. Powell, A. Robertson, J. J. H. Simes and R. S. Wight, *ibid.*, 2414 (1953).

(15) J. S. E. Holker, A. G. D. Powell, A. Robertson, J. J. H. Simes, R. S. Wight and R. M. Gascoigne, *ibid.*, 2422 (1953).

(16) R. B. Woodward, A. A. Patchett, D. H. R. Barton, D. A. J. Ives and R. B. Kelly, THIS JOURNAL, **76**, 2852 (1954); D. H. R. Barton, D. A. J. Ives and V. R. Thomas, *J. Chem. Soc.*, 903 (1954).

only 4 of them is derived from the carboxyl of acetate).

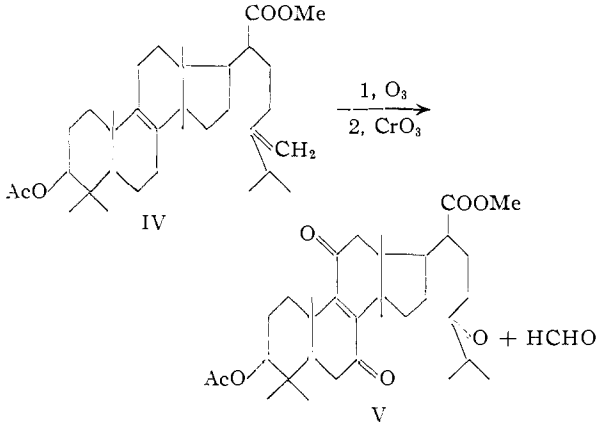
TABLE I

DISTRIBUTION OF C^{14} IN ACETIC ACID FROM THE KUHN-ROTH OXIDATION OF C^{14}-METHYL EBURICOATE BIOSYNTHESIZED FROM CARBOXYL-LABELED ACETATE

Compound	s.a., cts./min./mg. $BaCO_3$ Found	Calcd.[a]
Methyl eburicoate	52	..
Methyl carbon of acetic acid	0	0
Carboxyl carbon of acetic acid	109	110

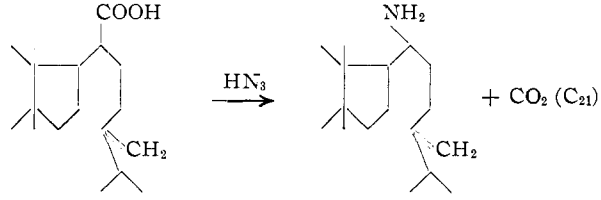
[a] Calculated on the basis of the squalene hypothesis.

Subsequent to the demonstration of the utilization of acetate as a two carbon unit, the exact distribution of the individual carbon atoms of acetate in the eburicoic acid molecule was investigated. In steroids, there is often a one or two carbon fragment attached to the isoöctyl side-chain at C-24. Such is the case in eburicoic acid, a methylene group being appended at this position. As the carbon atom of this methylene group is not part of an isoprene unit, its origin is not predicted on the basis of the above biosynthetic mechanism. A similar carbon attachment is found in ergosterol and Hanahan and Wakil[7] have shown that this carbon (C-28) of ergosterol is not derived from the carboxyl of acetate. The same result has been obtained with eburicoic acid. Methyl acetyleburicoate (methyl 3-acetoxyeburic-8,24(28)-diene-21-oate, IV), prepared from eburicoic acid biosynthesized from carboxyl labeled acetate, was ozonized in acetic acid. Water was then added and formaldehyde (as the Dimedon derivative) was obtained in 65–70% yield. The material was found to be non-radioactive. The triterpene residue after ozonolysis was found to be a mixture of compounds arising from ancillary oxidation at the positions allylic to the 8(9)-double bond. The mixture, therefore, was oxidized with chromic acid in acetic acid and the product chromatographed to yield methyl 3-acetoxy-28-nor-eburic-7,11,24-trione-8-ene-21-oate (V). The specific activity of this material was found to be the same as the starting acetyl ester IV, after allowing for the loss of one unlabeled carbon atom.



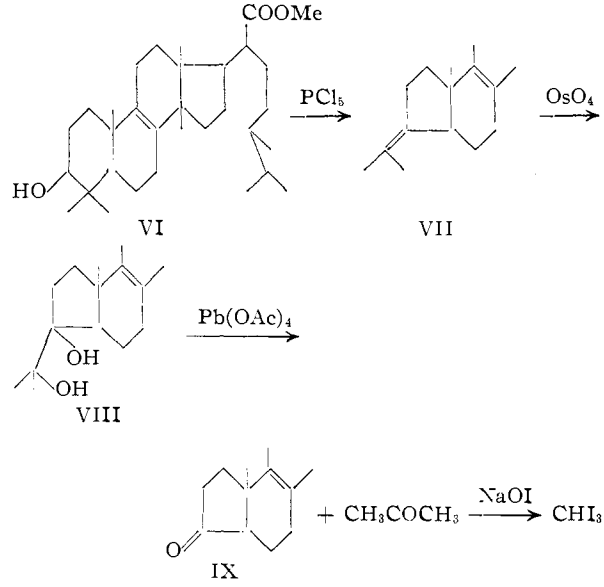
The origin of C_{21} of eburicoic acid is of particular interest. The squalene hypothesis predicts its origin to be in the methyl group of acetate, *i.e.*, a carboxyl group in the triterpenes is derived from a methyl group of acetate, implying that some specific methyl to carboxyl oxidation has occurred at the C_{21} position. It is of further interest to note that in some of the pentacyclic triterpenes (*e.g.*, oleanolic acid) the carbon atom which is biosynthetically equivalent to C_{21} of eburicoic acid occurs also as a carboxyl group.[8] Though in cholesterol the origin of C_{21} is known to be in the methyl group of acetate,[7] there is no experimental evidence yet at hand to indicate that a carboxyl group in a triterpene can have as its precursor the methyl group of acetate.

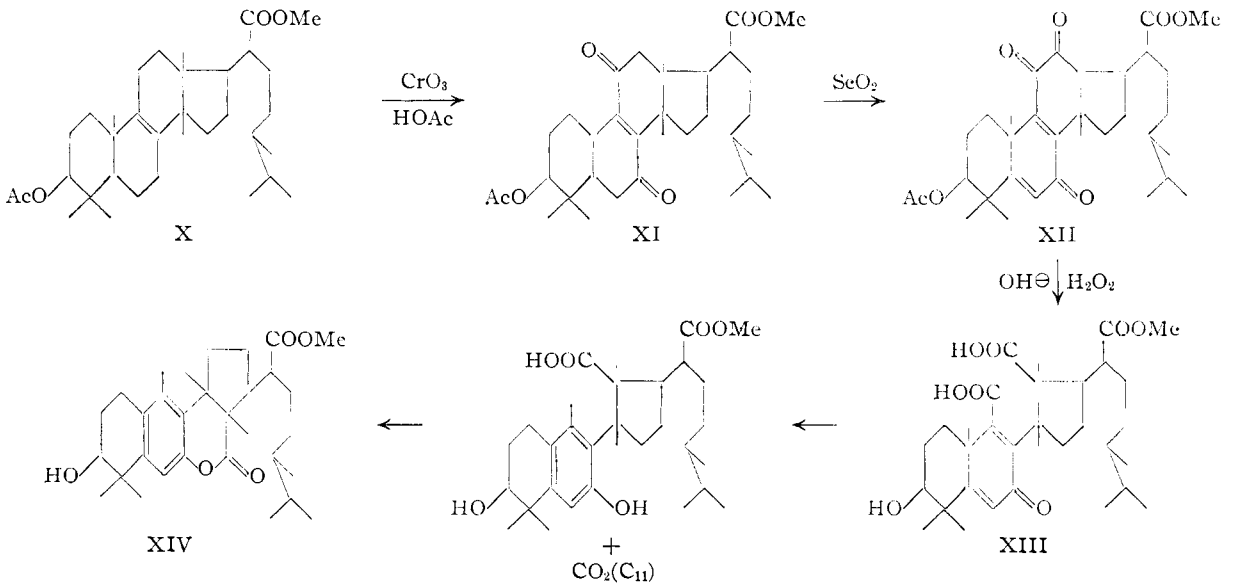
Eburicoic acid, biosynthesized from carboxyl-labeled acetate, was decarboxylated *via* the Schmidt reaction and the evolved CO_2 was found to be devoid of radioactivity. Thus, C_{21} of eburicoic acid does not arise from the carboxyl of acetate and it, therefore, most likely has its origin in the methyl carbon of acetate and further experiments are now in progress to establish definitely this point.



A unique structural feature found in most triterpenes and absent in cholesterol is the presence of the *gem*-dimethyl group at C_4. It has been shown that C_4 of cholesterol is derived from the carboxyl of acetate,[7] but since the two methyl groups are lost in the lanosterol to cholesterol conversion,[9] no evidence has yet been presented as to the origin of these two carbon atoms (C_{30} and C_{31}). According to the squalene hypothesis they arise from the end *gem*-dimethyl groups of squalene and thus should both be derived from the methyl of acetate.[7]

To obtain direct evidence with regard to this question, ring A of methyl 3-hydroxyeburic-8-ene-21-oate VI was rearranged by means of a retropinacol reaction which transforms C_4, C_{30} and C_{31} to an isopropylidene residue attached to a five-mem-





bered ring A. The isopropylidene group then was separated from the nucleus by a two-step oxidation, first with osmium tetroxide to give the diol VIII, followed by treatment with lead tetraacetate in acetic acid to cleave the diol to acetone and a ring A-nor ketone IX. On addition of Deniges reagent,[17,18] acetone was precipitated from the aqueous acetic acid solution as the mercuric sulfate complex. Iodoform was obtained from the methyl groups of the acetone by decomposition of the mercuric sulfate complex in hydrochloric acid followed by steam distillation of the liberated acetone into an alkaline solution of alkaline hypoiodite. It was not possible to obtain the ring A-nor ketone IX in crystalline form and it was, therefore, converted into a crystalline 2,4-dinitrophenylhydrazone derivative. The results of this degradation are summarized in Table II and clearly indicate that C_4 of the triterpene does, and that C_{30} and C_{31} do not, arise from the carboxyl of acetate. These results are in complete accord with the predictions of the squalene hypothesis.

TABLE II

DISTRIBUTION OF C^{14} IN RING A OF EBURICOIC ACID BIOSYNTHESIZED FROM CARBOXYL-LABELED ACETATE

Compound	s.a., cts./min./mg. $BaCO_3$ Found	Calcd.[a]
Methyl 3-hydroxyeburic-8-ene-21-oate (VI)	86	..
Diol VIII	84	86
Nor-ketone IX, DNPH	75	72
Acetone	77	76
Iodoform	2	0

[a] Calculated on the basis of the squalene hypothesis.

The results so far reported fit well into the squalene hypothesis of biosynthesis of triterpenes. However, none of them offer an answer with regard to the concept of an intact utilization of squalene. In order to establish this latter point, it is necessary to consider the unique requirement of the biosynthesis that the intact utilization of the acyclic triterpene presents. Squalene is a symmetrical molecule which can be viewed as being composed of two sesquiterpenic units linked in a head-to-head fashion. Only at this point of the head-to-head fusion should two carbon atoms in juxtaposition both originate from the carboxyl of acetate.[19] On the basis of the cyclization of squalene as shown above, these two carbon atoms should reside at C_{11} and C_{12} of the triterpene or steroid molecule. In order to locate this point of "biosynthetic symmetry" in the triterpene molecule, ring C of eburicoic acid was degraded. This was accomplished by a stepwise oxidation about the B/C ring juncture. Treatment of methyl 3-acetoxyeburic-8-ene-21-oate (X) with chromic acid in acetic acid gave rise to the enedione XI. This compound was further oxidized with selenium dioxide in refluxing nitrobenzene to yield the dienetrione XII. Ring C was readily opened by cleavage of the α-diketone linkage with alkaline hydrogen peroxide and the ring C diacid XIII was formed. Similar cleavages have been performed in the lanosterol series.[20] In addition, it has been shown that such a diacid from lanosterol on heating smoothly undergoes a dienone–phenol type of rearrangement with concomitant decarboxylation, the carbon dioxide evolved originating from the C_{11} atom of the triterpene molecule.[21] When the ring C diacid XIII, in the eburicoic acid series, was heated to its melting point (262°), carbon dioxide was evolved (64% yield) and after chromatography of the residue, the phenolic lactone XIV was isolated in 63% yield.

The ester lactone XIV was subjected to a Schmidt reaction and a 42% yield (based on 2 moles of CO_2 per mole of ester lactone) of carbon dioxide was obtained. The specific activities of the various samples are listed in Table III.

The above results in Table III indicate that C_{11} of eburicoic acid is derived from the carboxyl group of the acetate precursor and that its specific activ-

(17) M. G. Deniges, *Compt. rend.*, **126**, 1868 (1898); **127**, 963 (1898).

(18) D. D. Van Slyke, *J. Biol. Chem.*, **32**, 455 (1927).

(19) J. W. Cornforth and G. Popjak, *Biochem. J.*, **58**, 403 (1954).

(20) J. F. Cavalla and J. F. McGhie, *J. Chem. Soc.*, 744 (1951); D. H. R. Barton, J. S. Fawcett and B. R. Thomas, *ibid.*, 3147 (1951).

(21) C. S. Barnes, D. H. R. Barton, J. S. Fawcett and B. R. Thomas, *ibid.*, 2339 (1952).

TABLE III

DISTRIBUTION OF C^{14} IN RING C OF EBURICOIC ACID BIOSYNTHESIZED FROM CARBOXYL-LABELED ACETATE

Compound	s.a., cts./min./mg. $BaCO_3$ Found	Calcd.[a]
7,11,12-Trione-5,8-diene, XII	69	..
Ring C diacid, XIII	75	73
Carbon dioxide from C_{11}	185	194
Phenolic lactone, XIV	73	69
Carbon dioxide from Schmidt reaction of phenolic lactone, XIV	23	..

[a] Calculated on the basis of the squalene hypothesis.

ity is, within experimental error, in complete agreement with that predicted on the basis of the squalene hypothesis. The result from the Schmidt reaction, however, yields only qualitative data with regard to the source of C_{12}. In this reaction, carbon dioxide can originate both from the carboxyl group of the phenolic lactone and from the carbomethoxy substituent on the side-chain of the molecule. It has been shown previously in this work that the carboxyl group of eburicoic acid is not derived from the carboxyl of acetate and, thus, any radioactivity obtained in the Schmidt reaction on the phenolic lactone XIV must arise from C_{12}. The finding of activity in the carbon dioxide indicates that C_{12}, to some extent at least, is derived from the carboxyl of acetate, a result expected on the basis of the concept of an intact utilization of squalene in the biosynthesis of a triterpene.

The foregoing evidence demonstrates that acetate, as a two-carbon unit, is utilized as a carbon source for the biosynthesis of a tetracyclic triterpene. Further, the manner of the incorporation of this acetate does appear, on the basis of a number of specific degradations of the triterpene molecule, to be identical in detail with that observed in cholesterol biosynthesis and that expected on the basis of an intact utilization of squalene in the biosynthetic reaction. These results lend considerable support to the contention that both triterpenes and steroids do, indeed, arise in nature by the same or very similar biosynthetic pathway, the sterol pathway very likely differing from the route to triterpenes in only the terminal stages.

Experimental[22]

Growth.—A potato broth was prepared by autoclaving 250 g. of peeled sliced potatoes in one liter of water at 160° and 15 lb. p.s.i. for 30 minutes. The resulting soup was filtered through cheese cloth and 30 g. of glucose was added to give a 3% glucose concentration. To this solution was added 180.6 mg. of carboxyl-labeled sodium acetate with an activity of 12.2 μc./mg. (total activity added, 2.203 mc.). An aliquot (100 ml.) of this medium was placed in each of ten 500-ml. erlenmeyer flasks. The flasks were stoppered with cotton plugs and sterilized for 15 minutes in an autoclave at 160° and 15 lb. p.s.i. They were allowed to cool and were then inoculated with a few spores of *Polyporus sulfureus*. The flasks were maintained at 32° (stationary cultures) for a period of four months. The mycelium was filtered, washed well with water and then dried.

Isolation.—The dry mycelium (5.19 g.) was ground and then extracted in a Soxhlet apparatus with petroleum ether (60–90°) for two hours yielding 198 mg. (3.8%) of an oil. A sample of this material was oxidized and found to have s.a. of 3,380 cts./min./mg. $BaCO_3$.

Extraction of the mycelium with ether for 28 hours yielded 2.8 g. of a crude white solid. This was acetylated with acetic anhydride–pyridine and methylated with diazomethane in methanol. Two recrystallizations from methanol afforded 2.3 g. of material, m.p. 155–157°. A sample was oxidized and found to have s.a. of 300 cts./min./mg. $BaCO_3$. The ultraviolet spectrum showed the following bands: λ_{max} 243 mμ (ε 384), λ_{max} 252 mμ (ε 282). This material was dissolved in 150 ml. of hexane and poured onto a column of 70 g. of alumina prepared in hexane. Hexane–benzene (1:1) and benzene removed the main fraction which was twice recrystallized from methanol to afford 725 mg. of material, m.p. 156.4–157.2°. The infrared spectrum of this material was identical with that of authentic methyl acetyleburicoate and the melting point was undepressed on admixture with an authentic sample.

Acetic Acid from Methyl 3-Hydroxyeburic-8,24(28)-diene-21-oate (III).—Saponification of methyl acetyleburicoate with 4% aqueous alcoholic potassium hydroxide yielded methyl eburicoate (methyl 3-hydroxyeburic-8,24(28)-diene-21-oate). Three samples of this material (s.a. 52 cts./min./mg. $BaCO_3$; 52.7 mg., 0.109 mmole total) were oxidized by the Kuhn-Roth procedure. The aqueous solutions of sodium acetate were combined and evaporated to dryness, yielding 36.4 mg. of sodium acetate (82% based on 5 moles of acetic acid per mole of triterpene).

Degradation of Acetic Acid. (a) **Schmidt Reaction of Acetic Acid.**—The sodium acetate from above (34.0 mg., 0.415 mmole) was divided into two portions, each of which was placed in a small vial designed to fit into the center well of a 25-ml. erlenmeyer flask. Recrystallized sodium azide (30 mg., 0.46 mmole) was added to each vial and the flasks fitted with serum-flask rubber stoppers. The flasks were evacuated through a hypodermic needle and 0.2 ml. of 100% sulfuric acid was then added to the vial in the center well *via* a hypodermic needle and syringe. The flasks were placed in an oven at 80° and kept there for one hour. They were allowed to cool to room temperature and 2 ml. of 1 *N* carbon dioxide free sodium hydroxide was injected into the main compartments and the flasks allowed to stand for one hour. The vacuum was released by inserting a hypodermic needle and the cap and vial removed. The flasks were recapped and re-evacuated as described above. To regenerate the carbon dioxide, 5 ml. of 1 *N* sulfuric acid containing 2% hydrogen peroxide was injected into the main compartment. To reabsorb this carbon dioxide, 2 ml. of 1 *N* carbon dioxide-free sodium hydroxide was injected into the center well. The flask was allowed to stand for one additional hour, the vacuum was released and the base from the center well transferred to a 25-ml. erlenmeyer flask. Barium carbonate was precipitated by addition of 2 ml. of 1 *M* ammonium chloride followed by 2 ml. of 1 *M* barium chloride. The yield of barium carbonate from the two runs on 17 mg. of sodium acetate each was 27.0 mg. (66%) and 25.2 mg. (62%). This barium carbonate was found to have s.a. 109 cts./min./mg. $BaCO_3$.

(b) **Oxidation of Methylamine.**—The vial from above which contained the methylamine was transferred to a small distilling flask containing 5 ml. of water and the solution was made distinctly alkaline with 1 *N* sodium hydroxide. The contents of the flask were then distilled and the methylamine distillate (about 5 ml.) was trapped in 0.5 ml. of 1 *N* sulfuric acid contained in an oxidation flask (a round bottomed flask with a very long neck and a joint). To this flask there was added 3 ml. of a 10% potassium permanganate solution and 2 ml. of 1 *N* carbon dioxide-free sodium hydroxide. The flask was connected to an adapter (fitted with a dropping funnel) and a side arm leading to a carbon dioxide absorption train and the entire system evacuated. The mixture was then heated at 80° for 30 minutes and allowed to cool to room temperature. Sulfuric acid (5 ml., 1 *N*) was cautiously added from the dropping funnel and the gas liberated was passed through 30 ml. of 0.3 *N* carbon dioxide free sodium hydroxide solution contained in the conventional bubbler trap. The barium carbonate was precipitated in the usual fashion by the addition of 10 ml.

(22) All analyses were performed by the Microanalytical Laboratory, Department of Chemistry and Chemical Engineering, University of California, Berkeley. The radioactivity was determined as described previously [W. G. Dauben, J. C. Reid and P. E. Yankwich, *Anal. Chem.*, **19**, 828 (1947)] using either a thin-windowed Geiger–Mueller tube or a windowless tube flushed with "Q" gas. The counting error is ±5%. A few determinations were performed using a vibrating-reed electrometer.

of 1 *M* ammonium chloride followed by 10 ml. of 1 *M* barium chloride. This barium carbonate (23.3 mg., 58%) was plated and found to possess no observable activity.

Ozonolysis of Methyl 3-Acetoxyeburic-8,24(28)-diene-21-oate (IV). (a) Dimedon Derivative of Formaldehyde.—C^{14}-Methyl 3-acetoxyeburic-8,24(28)-diene-21-oate (1.0 g., 1.9 mmoles, s.a. 5.8 dis./min./mg. $BaCO_3$) was dissolved in 60 ml. of glacial acetic acid. Ozone (2.4 mmoles) was passed through the solution at 20–25°. The solution was then poured into water (800 ml.). Water (600 ml.) was distilled and to this distillate there was added 20 ml. of a 10% ethanolic solution of Dimedon. After standing overnight, a white precipitate formed which on filtration afforded 362 mg. (65%) of material, m.p. 189.2–190.0°. After recrystallization from aqueous ethanol, there resulted 260 mg. (47%), m.p. 190.6–191.2°. This material was oxidized and counted in an ionization chamber. It was found to have no observable activity.

(b) **Methyl 3-Acetoxy-28-nor-eburic-7,11,24-trione-8-ene-21-oate V.**—The residue remaining after steam distillation of the above was extracted into ether. The ether was distilled (960 mg. residue). This material was dissolved in 70 ml. of glacial acetic acid. The solution was heated to 70° and chromic oxide (1.10 g., 11 mmoles) in 20 ml. of glacial acetic acid was added over a period of 15 minutes. The reaction mixture was maintained at 70° for one hour and then allowed to cool to room temperature and stand overnight. It was poured into water and the aqueous mixture extracted well with ether. The ethereal solution was washed with a saturated sodium bicarbonate solution and water. It was then dried over anhydrous magnesium sulfate, filtered and the ether distilled.

The residue (807 mg.) was dissolved in 80 ml. of hexane–benzene (1:1) and poured onto a column of 25 g. of alumina prepared in hexane. Elution with benzene–ether (1:1) removed the main fraction (497 mg.) which after two recrystallizations from methanol afforded 316 mg. (30%), m.p. 194.6–195.8° (lit.[15] 194–195°). The ultraviolet spectrum showed the following band: λ_{max} 271 mμ (ϵ 8600). A sample of this material was oxidized and counted in an ionization chamber. It had s.a. 5.2 dis./min./mg. $BaCO_3$.

Carbon Dioxide from Carbon-21 of Eburicoic Acid (I).—Eburicoic acid (542.9 mg., 1.16 mmoles, s.a. 37 cts./min./mg. $BaCO_3$) was suspended in chloroform (30 mg.). To this suspension was added 157.3 mg. (2.4 mmoles) of recrystallized sodium azide. The mixture was cooled in an ice-bath and 8.0 mg. of concentrated sulfuric acid was added. The reaction mixture was stirred magnetically and the flask swept with a stream of carbon dioxide-free nitrogen. The effluent gases were passed through a carbon dioxide absorption train. The reaction was maintained at 0° for one hour after which it was allowed to warm to room temperature. Stirring was continued overnight. Barium carbonate was precipitated in the usual manner and found to possess no observable radioactivity.

Ring A Degradation

Methyl 3-Hydroxyeburic-8-ene-21-oate (VI).—A mixture which consisted of C^{14}-methyl 3-acetoxyeburic-8-ene-21-oate (150 mg., 0.284 mmole, s.a. 1,520 cts./min./mg. $BaCO_3$) and non-radioactive material (2.650 g., 5.03 mmoles) was saponified by heating on the steam-bath for 1.5 hours with a 4% aqueous alcoholic potassium hydroxide solution. The reaction mixture was worked up in the usual fashion and after recrystallization from methanol, there resulted 2.4 g. (93%) of VI, m.p. 130–132° (lit.[11] 135–136°). After recrystallization from ethyl acetate, a sample has m.p. 144–145°. A sample was combusted and the barium carbonate found to have s.a. 86 cts./min./mg. $BaCO_3$.

Methyl 3-Isopropylideneburic-A-nor-8-ene-21-oate (VII).—To a solution of C^{14}-methyl 3-hydroxyeburic-8-ene-21-oate (2.3 g., 4.73 mmoles, s.a. 86 cts./min./mg. $BaCO_3$) in petroleum ether (30–60°) was added 1.8 g. (8.7 mmoles) of phosphorus pentachloride. The reaction mixture was vigorously agitated for one hour, after which time all of the phosphorus pentachloride had dissolved giving a pale yellow solution. The reaction mixture was then allowed to stand at room temperature for 24 hours. The petroleum ether solution was washed three times with water and four times with 1 *N* sodium hydroxide until the washings, after acidification with nitric acid, gave no precipitate with a 0.1 *M* silver nitrate solution. After further washing with water, the petroleum ether solution was dried over anhydrous magnesium sulfate and filtered. The petroleum ether was distilled leaving a residue of a yellow oil (2.0 g.).

This residue was dissolved in 100 ml. of hexane and poured onto a column of 30 g. of alumina prepared in hexane. Elution of this column with hexane and hexane–benzene (1:1) removed the main fraction (900 mg., 40%) of material which was not further purified.

Methyl 3-(2-Hydroxy-2-propyl)-3-hydroxy-eburic-A-nor-8-ene-21-oate (VIII).—The crude product from the reaction above (900 mg., 1.85 mmoles) was dissolved in 20 ml. of dry ether and 490 mg. (1.93 mmoles) of osmium tetroxide was added. The reaction mixture was allowed to stand at room temperature for 24 hours at the end of which time it was black. The ether was removed by a stream of dry nitrogen and the resulting black residue then refluxed for 1.25 hours in a solution of 75 ml. of methanol, 75 ml. of water and 15 g. of sodium sulfite. The resulting mixture was filtered, and the solid washed well with methanol. The methanol was distilled from the filtrate and the resulting aqueous mixture extracted three times with ether. The ethereal solution was washed with water, dried over anhydrous magnesium sulfate and filtered. The ether was distilled leaving a residue of 880 mg. of a viscous oil. This residue was dissolved in 50 ml. of hexane and poured onto a column of 30 g. of alumina prepared in hexane. After proceeding through the usual solvent sequence, the main fraction (460 mg.) was eluted with ether. This was recrystallized from methanol, yield 300 mg. (33%) of material with m.p. 159–160° (lit.[12] 158–159°). A sample was oxidized and the barium carbonate found to have s.a. 84 cts./min./mg. $BaCO_3$.

Methyl Eburic-A-nor-3-one-8-ene-21-oate (IX) and Acetone.—To a solution of C^{14}-methyl 3-(2-hydroxy-2-propyl)-3-hydroxyeburic-A-nor-8-ene-21-oate (290 mg., 0.580 mmole, s.a. 84 cts./min./mg. $BaCO_3$) in 5 ml. of glacial acetic acid was added 300 mg. (0.677 mmole) of lead tetraacetate. The reaction mixture was allowed to stand at room temperature for 14 hours and was then poured into water and filtered through Super-cel.

The gummy residue on the filter (283 mg.) was dissolved in 10 ml. of methanol and 127 mg. (0.64 mmole) of 2,4-dinitrophenylhydrazine and 0.1 ml. of concentrated hydrochloric acid were added. The solution was heated on the steam-bath for 5 minutes. It first became clear and then a solid separated. The methanol was evaporated and the residue dissolved in 25 ml. of benzene. This solution was poured onto 6 g. of alumina prepared in benzene. Elution of the column with benzene removed the main fraction which was recrystallized from a large volume of methanol, yield 250 mg. (69%) of material, m.p. 225–226° dec. (lit.[12] 225° dec.). A sample was oxidized and the barium carbonate found to have s.a. 75 cts./min./mg. $BaCO_3$.

To the filtrate from above, 3 ml. of 1 *N* sulfuric acid was added to precipitate the lead and the solution filtered. To this filtrate was added 90 ml. of an aqueous solution which contained 8% mercuric sulfate and 8% sulfuric acid.[16] Water was then added to bring the final volume to 300 ml. The mixture was refluxed for one hour and filtered, yield 440 mg. (66% assuming 20 mg. of solid mercury–acetone complex is equivalent to 1 mg. of acetone). A sample of this was oxidized and the barium carbonate found to have s.a. 77 cts./min./mg. $BaCO_3$.

Iodoform.—The mercury–acetone complex (200 mg.) from above was dissolved in 10 ml. of 3 *N* hydrochloric acid and distilled into 50 ml. of 1 *N* sodium hydroxide. To this solution was added 5 ml. of 0.5 *N* potassium triiodide solution and the mixture warmed on the steam-bath for 10 minutes. The iodoform was collected by filtration and recrystallized from methanol, yield 20 mg. (30%), m.p. 118.5–119.2°. The entire sample was oxidized and the barium carbonate found to have s.a. 2 cts./min./mg. $BaCO_3$.

Ring C Degradation

Methyl 3-Acetoxyeburic-8-ene-21-oate (X).—C^{14}-Methyl 3-acetoxyeburic-8,24(28)-diene-21-oate (725 mg., 1.38 mmoles, s.a. 1,425 cts./min./mg. $BaCO_3$) was dissolved in 100 ml. of ethyl acetate. Platinum oxide (150 mg.) was added and the mixture hydrogenated at 44 lb. p.s.i. The reaction stopped after the uptake of one mole of hydrogen. After filtration, the ethyl acetate was distilled and the residue recrystallized from methanol, yield 670 mg. (92.5%), m.p. 158.2–158.6° (lit.[11] 157–158°).

Methyl 3-Acetoxyeburic-7,11-dione-8-ene-21-oate (XI).—C^{14}-Methyl 3-acetoxyeburic-8-ene-21-oate (420 mg., 0.82

mmole, s.a. 1,430 cts./min./mg. $BaCO_3$) and non-radioactive methyl 3-acetoxyeburic-8-ene-21-oate (6.204 g., 11.8 mmoles) were dissolved in 250 ml. of glacial acetic acid. The solution was warmed to 70° and chromic oxide (7.75 g., 77.5 mmoles) in 150 ml. of glacial acetic acid was added slowly over the period of one hour. The reaction mixture was maintained at 70° for an additional hour and allowed to stand overnight at room temperature. Methanol was added to decompose the excess chromic oxide and the mixture poured into water. It was extracted four times with ether. The ethereal solution was washed twice with water, with saturated sodium bicarbonate solution until the acetic acid had been neutralized and then three times with water. The solution was dried over magnesium sulfate, filtered and the ether distilled. The residue (6.925 g.) was dissolved in 300 ml. of hexane and 50 ml. of benzene and poured onto a column of 210 g. of alumina prepared in hexane. Elution of the column with benzene and benzene–ether (3:1) removed the main fraction which was recrystallized from methanol, yield 3.978 g. (57%), m.p. 173.4–174.3° (lit.[11] 171–172°). The ultraviolet spectrum showed the following band: λ_{max} 271 mμ (ϵ 8500). A sample was oxidized and the barium carbonate found to have s.a. 93 cts./min./mg. $BaCO_3$.

Methyl 3-Acetoxyeburic-7,11,12-trione-5,8-diene-21-oate (XII).—C^{14}-Methyl 3-acetoxyeburic-7,11-dione-8-ene-21-oate (3.948 g., 7.1 mmoles, s.a. 93 cts./min./mg. $BaCO_3$) and non-radioactive material (1.114 g., 2 mmoles) were dissolved in 10 ml. of redistilled nitrobenzene. To this solution there was added 12.12 g. (10.9 mmoles) of selenium dioxide. The mixture was heated in an oil-bath at 210° for two hours. After being allowed to cool to room temperature, the reaction mixture was diluted with ether and filtered. The ethereal solution was washed three times with 1 *N* sodium hydroxide, three times with 2 *N* hydrochloric acid and then well with water. The solution was dried over anhydrous magnesium sulfate, filtered and the ether distilled. The residue (2.4 g.) was dissolved in 300 ml. of hexane–benzene (1:1) and poured onto a column of 80 g. of alumina prepared in hexane. Benzene and benzene–ether (3:1) removed the main fraction which was recrystallized from methanol, yield 1.524 g. (29.6%) of yellow orange plates, m.p. softens 166°, resolidifies and melts 175.4–175.8° (lit.[15] 164.5–165.4°). The ultraviolet spectrum showed the following bands: λ_{max} 295 mμ (ϵ 6450), λ_{max} 414 mμ (ϵ 316). The infrared spectrum was determined in a carbon disulfide solution and showed the following carbonyl bands: 5.70 μ (1755 cm.$^{-1}$), 5.92 μ (1690 cm.$^{-1}$) and 6.05 μ (1655 cm.$^{-1}$). A sample was oxidized and the barium carbonate found to have s.a. 69 cts./min./mg. $BaCO_3$.

3-Hydroxyeburic-8-one-5,8-diene-11,12-seco-11,12,21-trioic Acid 21-Methyl Ester (XIII).—C^{14}-Methyl 3-acetoxyeburic-7,11,12-trione-5,8-diene-21-oate (1.376 g., 2.42 mmoles, s.a. 69 cts./min./mg. $BaCO_3$) was dissolved in 60 ml. of dioxane and 60 ml. of methanol. Potassium hydroxide (870 mg., 15.5 mmoles) was added and after it had dissolved completely, the solution was cooled in an ice-bath and Superoxol (35% hydrogen peroxide, 14.5 ml.) was added. The original orange color was discharged in one hour to give a colorless solution with a small amount of white flocculent precipitate. This mixture was poured into water and extracted with ether. The ethereal solution was extracted three times with 10% sodium hydroxide solution and washed with water. The ethereal solution was then dried over anhydrous magnesium sulfate, filtered and the ether distilled to leave a negligible amount of neutral residue.

The basic filtrate and washings were combined and 15 ml. of concentrated hydrochloric acid added. A white precipitate separated and was extracted into ether. The ethereal solution was washed with water, dried over anhydrous magnesium sulfate and filtered. The ether was distilled.

The residue was saponified by refluxing with 120 ml. of 2% methanolic potassium hydroxide (50% methanol, 50% water) for 40 minutes. The mixture was allowed to cool to room temperature, poured into water and 10 ml. of concentrated hydrochloric acid added. The resulting white precipitate was extracted into ether. The ethereal solution was washed well with water, dried over anhydrous magnesium sulfate, filtered and the ether distilled. The solid residue was recrystallized from ethyl acetate, yield 781 mg. (60%), m.p. 260–262° dec. The ultraviolet spectrum showed the following band: λ_{max} 250 mμ (ϵ 10,900). The infrared spectrum was determined in a Nujol mull and showed the following bands: hydroxyl band at 2.60 μ (3580 cm.$^{-1}$) and three carbonyl bands: 5.88 μ (1725 cm.$^{-1}$), 5.95 μ (1680 cm.$^{-1}$) and 6.05 μ (1650 cm.$^{-1}$).

Anal. Calcd. for $C_{32}H_{48}O_8$: C, 68.62; H, 8.58. Found: C, 68.50; H, 8.47.

A sample was oxidized and the barium carbonate found to have s.a. 75 cts./min./mg. $BaCO_3$.

Pyrolysis of the Ring C Diacid.—The ring C diacid (from above, 700 mg., 1.25 mmoles, s.a. 75 cts./min./mg. $BaCO_3$) was placed in a 25-ml. erlenmeyer flask which was directly connected to a carbon dioxide absorption train. The entire system was evacuated and then the flask was heated in a salt-bath at 270° for 10 minutes and the evolved carbon dioxide collected and precipitated as barium carbonate in the usual fashion. This barium carbonate was found to have s.a. 185 cts./min./mg. $BaCO_3$.

The residue from the pyrolysis was dissolved in 70 ml. of benzene and poured onto 21 g. of alumina prepared in benzene. Elution of the column with ether removed the main fraction (402 mg., 63%). This material could not be induced to crystallize, but remained as a colorless viscous oil. The ultraviolet spectrum showed the following bands: λ_{max} 207 mμ (ϵ 29,900) and λ_{max} 276 mμ (ϵ 940). The infrared spectrum was determined in a carbon disulfide solution and showed the following bands: 5.60 μ (1785 cm.$^{-1}$), 5.80 μ (1725 cm.$^{-1}$), 6.10 μ (1647 cm.$^{-1}$) and 6.20 μ (1613 cm.$^{-1}$).

Anal. Calcd. for $C_{31}H_{46}O_5$: C, 71.59; H, 9.00. Found: C, 71.26; H, 8.96.

A sample was oxidized and the barium carbonate found to have s.a. 73 cts./min./mg. $BaCO_3$.

Schmidt Reaction on the Phenolic Lactone.—To the lactone (370 mg., 0.72 mmole) was added 400 mg. (6.2 mmoles) of recrystallized sodium azide and 8 ml. of concentrated sulfuric acid. The reaction flask was connected to a train for the absorption of carbon dioxide. The mixture was stirred magnetically and the reaction flask swept slowly with a stream of dry carbon dioxide-free nitrogen. Barium carbonate, yield 120 mg. (42% based on two moles of carbon dioxide per mole of lactone), was precipitated in the usual manner. This barium carbonate was found to have s.a. 23 cts./min./mg. $BaCO_3$.

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