TABLE IV

Compound	Color with $CuCl_2$[14] After 1 min.	After 12 hr.	Color with $CoCl_2$ After 1 min.
N,N'-Tetramethyl-2-deoxystreptamine (III)	Bright emerald green	Green ppt. formed	Deep blue violet
Tri-O-acetyl-N,N'-tetramethyl-2-deoxystreptamine (IV)	Pale yellow green (negative)	No change	Pink (negative)
Symmetric di-O-acetyl-N,N'-tetramethyl-2-deoxytreptamine (V)	Pale green	Green, ppt. formed	Pale violet
Asymmetric di-O-acetyl-N,N'-tetramethyl-2-deoxystreptamine (VI)	Pale yellow green	Pale green, ppt. formed	Pink violet
Diacetate of unknown structure (R_f 0.95)	Pale yellow green (negative)	No change	Pink (negative)

epimerization, although thermodynamically not favored in a pentasubstituted all-*trans* and equatorial cyclohexane system, is a definite possibility, since a third diacetate of N,N'-tetramethylstreptamine of unknown configuration has been obtained and is described in the Experimental part.

Table IV describes the color phenomena observed in the complex formation of several deoxystreptamine derivatives with methanolic cupric chloride, a tool which has been used for configurational assignments of streoisomeric amino alcohols.[14]

The study of the lability of acetate esters derived from simpler model systems, such as 3α (and β)-N-dimethyl-$1\alpha,2\beta$(and α)-cyclohexanediols[15] will be of interest.

Another element of relatively minor significance in the control of ester group lability in this series is seen in the comparison of the hydrolytic steps of tertiary esters with their corresponding mono- or diquaternary derivatives. Quaternization leads to a definite decrement in hydrolysis velocity at each stage of reaction. This result affords a curious contrast with the previously observed[3] behavior in the fully acetylated streptamine series; an acetoxy function in the 2-position causes the quaternary derivative I to surpass the tertiary parent compound in initial hydrolysis rate by a factor of about 5, at *p*H 7.7. Apparently two effects are operative: a *field effect* in the quaternary compounds accelerates nucleophilic attack without being able to transfer directly a proton to the ester site, whereas a *charge transfer effect* is operative in the protonated tertiary amines which are capable of transferring their charge to a neighboring ester[15a] aided by over-all conformational factors. In the monoquaternary streptamine derivative I both effects may be additive. If this picture is correct future studies might show that rates of hydrolysis in the diquaternary series are less dependent on *p*H than in the ditertiary series. That neighboring ammonium ions may have a protective influence on labile groups is known from 6-deoxy-6-aminomethyl-α-glucoside which is stable to acid hydrolysis.[16]

The new concept of labilization through over-all conformational effects should add to the interest in cyclic aminopolyols, their partial symmetric and asymmetric esters and acetals such as are present in kanamycin, a 4,6-disubstituted deoxystreptamine,[17] and paromomycin, a 5,6(4,5)-disubstituted deoxystreptamine.[18]

Acknowledgment.—We are indebted to Dr. Louis Cohen of this Institute for his assistance to us in the interpretation of n.m.r. spectra, to Mr. Robert Badley for the measurement of the n.m.r. spectra and to Dr. Edward Garrett for his valuable comments.

(14) *Cf.* G. Drefahl, *Ber.*, **93**, 509, 514 (1960).

(15) *Cf.* R. A. Barnard and L. R. Hawkins, *Can. J. Chem.*, **36**, 1241 (1958).

(15a) *Cf.* E. R. Garrett, THIS JOURNAL, **79**, 5206 (1957), **80**, 4049 (1958).

(16) F. Cramer, H. Otterbach and H. Springmann, *Ber.*, **92**, 384 (1959).

(17) M. J. Cron, D. L. Evans, F. M. Palermiti, D. F. Whitehead, I. R. Hooper, Paul Chu and R. U. Lemieux, THIS JOURNAL, **80**, 4741 (1958).

(18) Th. H. Haskell, J. C. French and Q. R. Bartz, *ibid.*, **81**, 3482 (1959).

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC., GROTON, CONN.]

2-Acetyl-2-decarboxamidoöxytetracycline

BY F. A. HOCHSTEIN, M. SCHACH VON WITTENAU, FRED W. TANNER, JR., AND K. MURAI

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A new antibiotic, $C_{23}H_{25}NO_9$, has been isolated from the fermentation beers of a strain of *Streptomyces rimosus*. Consideration of its physical properties, and acid hydrolysis to water, acetic acid, dimethylamine and decarboxamidoterrinolide (V) shows it to be 2-acetyl-2-decarboxamidoöxytetracycline (VIII). The new antibiotic resembles oxytetracycline in its antibacterial spectrum, though it is less active.

2 - Acetyl - 2 - decarboxamidoöxytetracycline (ADOT)[1] was first detected as a minor compound in the broth of an oxytetracycline-producing strain of *Streptomyces rimosus*. Further mutation of this actinomycete yielded a strain which produces predominantly ADOT and only low levels of oxytetracycline. The new antibiotic was isolated as a yellow crystalline hydrochloride, m.p. 200–203° dec., with the composition $C_{23}H_{25}NO_9 \cdot HCl$; ADOT resem-

(1) We have chosen 2-acetyl-2-decarboxamidoöxytetracycline as a simple generic name for this substance. The abbreviated form, ADOT, is used henceforth in this article.

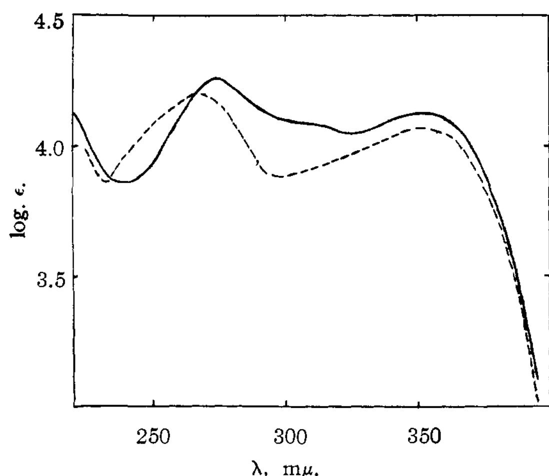


Fig. 1.—Ultraviolet spectra: ———, ADOT in 0.01 N HCl; - - - -, oxytetracycline in 0.01 N HCl.

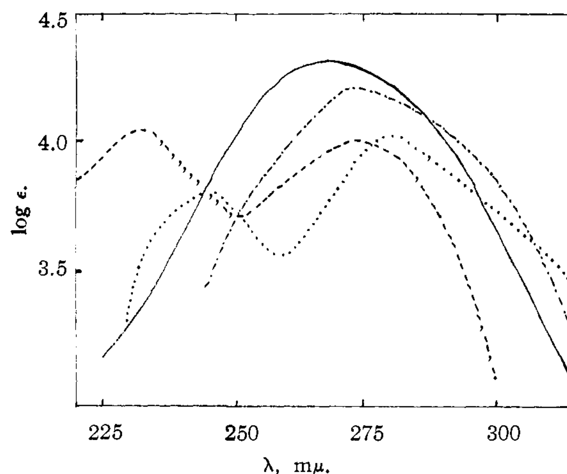
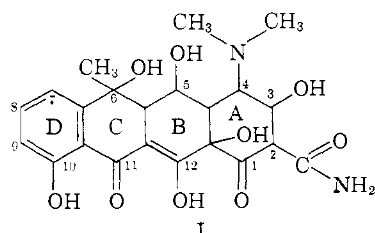


Fig. 2.—Ultraviolet spectra: -.-.-., ADOT chromophore in methanol–0.01 N NaOH; ———, 2-acetyldimedone in methanol–0.01 N NaOH; . . . ., ADOT chromophore in methanol–0.01 N HCl; --------, 2-acetyldimedone in methanol–0.01 N HCl.

bles oxytetracycline I[2] in many respects. The antibacterial spectra of the two compounds are similar, although ADOT has only about one-tenth the activity of oxytetracycline. The ultraviolet absorption spectra in several solvents show a close resemblance to those of oxytetracycline (I) in the

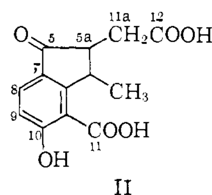


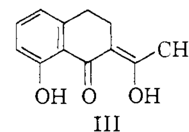
regions above 330 mμ but differ at lower wave lengths (Fig. 1). Titration of the hydıochloride in aqueous solutions shows pK_a values of 3.3, 7.1, 9.2, similar to those of oxytetracycline, 3.27, 7.32, 9.11.[3] Addition of calcium chloride effects pK_a shifts similar in magnitude and direction for both antibiotics. The new compound is more stable in aqueous acid than in base but, in general, shows slightly less stability than oxytetracycline.

ADOT analyzes for one nitrogen and for two C–methyl groups in contrast to the previously known tetracycline antibiotics all of which show two nitrogens and not more than one C–methyl group. It differs too in that the infrared absorption spectrum shows a carbonyl band at 5.92 μ, an unusually low wave length for a tetracycline.

It has been shown for oxytetracycline that the chromophore in the B, C and D rings is alone responsible for the ultraviolet absorption spectrum above 330 mμ.[2] The absorption below 330 mμ is largely due to the A ring chromophore. Since ADOT shows absorption above 330 mμ virtually identical to that of oxytetracycline, it seemed probable that the main difference between these compounds lay in the structure of the A ring. Alkaline degradation of ADOT supported this hypothesis. A small amount of terracinnoic acid (II)[2] was isolated and identified by its ultraviolet absorption spectrum and by paper chromatographic comparison with an authentic sample.[4] The nitrogen was all liberated as dimethylamine.

In order to learn more about the chromophore responsible for the ultraviolet absorption below 330 mμ, difference curves were drawn for ADOT and 2-acetyl-8-hydroxytetralone (III). The latter substance has been used previously as a model for the B, C and D ring system.[2] The resultant curves (Fig. 2) which must approximate those of the A





ring chromophore of ADOT show a single maximum at 273 mμ in base and two maxima at 245 and 279 mμ in acid. The corresponding difference curves for oxytetracycline and 2-acetyl-8-hydroxytetralone show only a single maximum for either solvent. Thus the short wave length chromophore of ADOT resembles that of molecules which have a tricarbonylmethane group in their structures, rather than the cross-conjugated carboxamido-β-diketone of oxytetracycline. 2-Acetyldimedone (IV), for example, has a single maximum at 268 mμ in basic medium and two maxima at 232 and 276 mμ in acid.[5] Consideration of these facts strongly suggested that ADOT was a 2-acetyl-2-decarboxamido-oxytetracycline, and the subsequent experiments were designed to test this hypothesis.

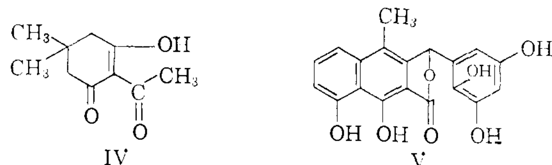
It is known[2] that acid hydrolysis of oxytetracycline proceeds with loss of water (at C.5a and C.6), carbon dioxide and ammonia (from the carboxamide group), and dimethylamine (from C.4), to

(2) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, THIS JOURNAL, 75, 5455 (1953).

(3) C. R. Stephens, K. Murai, K. J. Brunings and R. B. Woodward, *ibid.*, 78, 4155 (1956).

(4) For a discussion of the formation of terracinnoic acid from the B C and D rings of oxytetracycline see ref. 2.

(5) W. R. Chan and C. H. Hassall, *J. Chem. Soc.*, 3495 (1956).

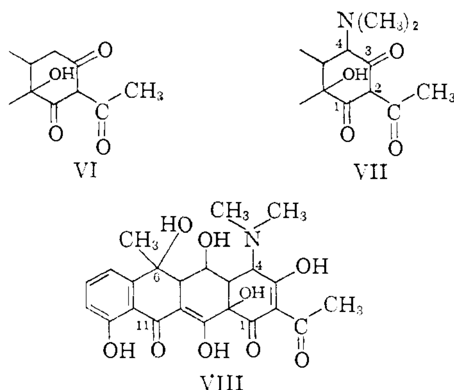


yield the rearrangement product decarboxamidoterrinolide (V). Since acetyldimedone (IV) loses acetic acid readily in aqueous acid, it could be predicted that 2-acetyl-2-decarboxamidoöxytetracycline should lose water, dimethylamine and acetic acid to yield the same product, decarboxamidoterrinolide.

Hydrolysis of ADOT in hot dilute acid proceeded as expected. Dimethylamine and acetic acid were formed and isolated in excellent yield and decarboxamidoterrinolide was isolated and identified as its pentaacetate. The formation of this degradation product V establishes the fact that ADOT has a linear tetracyclic carbon skeleton with a methyl group in the 6-position. The positions of seven oxygen atoms at C.1, 3, 5, 10, 11, 12 and 12a are also fixed. This acid-catalyzed decomposition must resemble that of other tetracyclines in that the first step involves a loss of water at C.5a–C.6, and production of a naphthalenediol system.[2,6,7] An eighth oxygen atom can therefore be placed at C.6. The formation of terracinnoic acid (II) on alkaline degradation is further evidence for this assignment.

Since alkaline hydrolysis of this new antibiotic yields only 0.4 mole of acetate, while acid hydrolysis yields one mole, ADOT cannot be an O-acetate, but must be a labile methyl ketone. It is evident, therefore, that the A ring of ADOT must have a structural unit of the dimedone type, and it could thus be represented as VI. The only other position possible for such a labile acetyl group would be at C.11a, but this is excluded by the nature of the long wave ultraviolet spectrum.

The position of the dimethylamine group has not been considered in detail heretofore. The ready loss of this group on acid hydrolysis, and particularly the very marked similarity of the pK_a values of ADOT hydrochloride to those of oxytetracycline hydrochloride, leave no choice but to place it at C.4 as in VII.



(6) C. R. Stephens, L. H. Conover, R. Pasternack, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, This Journal, **76**, 3658 (1954).

(7) J. S. Webb, R. W. Broschard, D. B. Cosulick, W. J. Stein and C. F. Wolf, *ibid.*, **79**, 4563 (1957).

In view of these chemical characteristics, the similarity of the pK_a values to those of oxytetracycline and the spectral evidence, ADOT must have structure VIII. It differs from oxytetracycline only in that the carboxamide group is replaced by an acetyl group.

Experimental

Bioassays were determined by a plate assay method using *Bacillus cereus* var. mycoides[8] against an oxytetracycline standard. Melting points were determined in capillaries, and are corrected.

Fermentation of 2-Acetyl-2-decarboxamidoöxytetracycline (2-Acetyl-4-dimethylamino-4a,5a,6,12a-tetrahydro-3,-5,6,10,12,12a-hexahydroxy-6-methyl-1,11-(4H,5H)-naphthacenedione).—Although several mutants of *S. rimosus* were found to produce ADOT, strain 7478 was preferred because the antibiotic produced was essentially devoid of oxytetracycline.

S. rimosus 7478 is readily cultured in media suitable for the original oxytetracycline-producing strain (NRRL 2234).[9] One such medium contains 40 g. of soybean meal and 5 g. of sodium nitrate per liter. The medium is adjusted to *p*H 6.8–7 before autoclaving and is used for both the inoculum and fermentation stages. When employed in tank fermentations the medium is supplemented with 20 ml. of soybean oil per gallon before autoclaving, and sterile soybean oil is added periodically as required to control foaming.

Spores of *S. rimosus* 7478 on Emerson slants were inoculated into 1000 ml. of the above medium in Fernbach flasks, and incubated for 40–48 hours on a rotary shaker at 28°. Four such flask cultures were conveniently employed to inoculate 100 gallons of the same medium which had been batch sterilized at 20 p.s.i. for 1 hour in a stainless steel fermentor equipped with the usual mechanical agitator. Sterile air was supplied at the rate of 1 volume per minute. During the fermentation, only small *p*H changes were observed. Broths were usually harvested at about 90 hours, and contained 15–25 micrograms/ml. of oxytetracycline activity. (+)-Diaminosuccinic acid and rimocidin are also present in the fermented broth.

Isolation of 2-Acetyl-2-decarboxamidoöxytetracycline.—The broth, 970 l., was adjusted to *p*H 2 with hydrochloric acid, and filtered from mycelium. 1-Butanol, 350 l., was added, and the mixture saturated with sodium chloride. The butanol layer was separated, and the extraction repeated with 350 l. of butanol. The combined organic phases were concentrated *in vacuo* at 40° to about 20 l., and filtered from a small precipitate of sodium chloride. This concentrate contained 85% of the biopotency originally present in the filtered broth. The butanol concentrate (18 l.) was extracted three times with an equal volume of 0.05 *N* hydrochloric acid, and the combined aqueous extracts, which contained virtually all the ADOT, were washed twice with hexane to remove butanol. One liter of a solution containing 360 g. barium chloride dihydrate and 48 g. of magnesium chloride hexahydrate was added and the solution adjusted to *p*H 8.5 with 10 *N* sodium hydroxide. The insoluble barium–magnesium–ADOT complex which formed was aged for 4 hours and filtered with the aid of 630 g. of Supercel. Assay of the cake showed 50% yield from filtered broth.

Two kg. of the wet cake were slurried in 700 ml. of water, and 18 *N* sulfuric acid was added to *p*H 1.5. The mixture was filtered to remove Supercel and barium sulfate, the filter cake was reslurried in 700 ml. of dilute acid (*p*H 1.5) and refiltered. The combined filtrates, 2300 ml., containing 5.5 million units of activity, were adjusted to *p*H 7 with 10 *N* sodium hydroxide, refrigerated for several hours, and the amorphous precipitate of crude ADOT separated by filtration, and washed with cold water. The dried precipitate, 110 g., assayed 43 u./mg. (4.75 million units).

This crude ADOT (30 g.) was suspended in 90 ml. of 0.01 *N* HCl saturated with butanol, and concentrated HCl added to the stirred mixture to maintain *p*H 2. The mixture was filtered, and the small insoluble residue washed with sufficient 0.01 *N* HCl to give a total of 120 ml. of filtrate. This was used for a nine-plate countercurrent distribution in the

(8) D. C. Grove and W. A. Randall, "Assay Methods of Antibiotics," Medical Encyclopedia, Inc., New York, 1955, p. 52.

(9) B. A. Sobin, A. C. Finlay, J. H. Kane, U. S. Patent 2,516,080 (1950).

system butanol–0.01 N HCl, using 300 ml. of butanol as the stationary phase and 120 ml. of 0.01 N HCl in each tube as the moving phase. Following distribution, addition of 600 ml. of hexane to each fraction forced the antibiotic into the aqueous phases, which were washed with hexane and then freeze-dried. Fractions 2, 3 and 4 contained about 70% of the antibiotic in up to 90% purity and could be readily crystallized; the other fractions could be used as starting material for a new countercurrent distribution.

Seven and one-half grams of the purer fractions obtained by countercurrent distribution were dissolved in 68 ml. of methanol and 4.5 ml. of a saturated solution of calcium chloride in methanol. The solution was filtered and 40 ml. isopropyl alcohol was added. After concentration *in vacuo* to 25 ml. and addition of 2 ml. of methanol, 14.3 ml. of 5.5 N hydrochloric acid in isopropyl alcohol was added slowly with stirring. Crystallization of ADOT hydrochloride occurred spontaneously. Ethyl acetate (42 ml.) was added dropwise to complete the crystallization and the mixture was cooled for a few hours. The crystalline precipitate was filtered, washed with 1:1 ethyl acetate–isopropyl alcohol and dried over calcium chloride *in vacuo;* weight 5.7 g., bioassay 64 u./mg., yield 15% (from broth).

Recrystallization from isopropyl alcohol–ethyl acetate yielded pure ADOT hydrochloride as yellow crystals, m.p. 200–203° dec., $[\alpha]^{25}_{D}$ −47°, (c 0.9 in 0.1 N hydrochloric acid); bioassay, 65 oxytetracycline u./mg.

Anal. Calcd. for $C_{23}H_{25}NO_9 \cdot HCl$: C, 55.70; H, 5.29; N, 2.82; Cl, 7.15; $C-CH_3$ (2), 6.06; acetyl (1), 8.68. Found: C, 55.74; H, 5.46; N, 2.57; Cl, 6.90; $C-CH_3$, 5.99; acetyl (acid hydrolysis), 9.33; acetyl (base hydrolysis), 3.53.

The ultraviolet absorption spectrum of ADOT in methanol–0.01 N HCl has λ_{max} 240sh (ϵ 10,580), 277 (15,000), 316sh (10,620) 357 (13,870). In methanol–0.01 N NaOH, it shows λ_{max} 237 (ϵ 10,300). 269 (18,300), 376 (15,600). The infrared spectrum (KBr) shows peaks in the carbonyl region at 5.92 μ, and at 6.1–6.3 μ.

Countercurrent distribution studies showed ADOT to have K = 1.1 in 1-butanol–2% acetic acid, K = 0.9 in 1 butanol–0.01 N hydrochloric acid, K = 2.6 in ethanol-water–isopropyl alcohol–chloroform, 1:1:2:2. Oxytetracycline has K = 0.4, 0.5 and 1.6 in these three systems.

Titration of ADOT hydrochloride was performed in 0.1 M potassium chloride solution, and in this solvent with five molar equivalents of added calcium chloride. Calculations of similar titration data for oxytetracycline have been described.[3,10] ADOT hydrochloride shows pK_a values of 3.3, 7.1 and 9.2. In the presence of calcium chloride, the pH at 50% neutralization shifts from 3.8, 7.0 and 8.6 to 3.8, 6.4 and 7.6. Under identical conditions, oxytetracycline shows shifts from 4.0, 7.2 and 8.7 to 4.0, 6.7 and 7.7. It may be concluded that the stability of the proton-liberating complex of ADOT with calcium is of the same order of stability as that of oxytetracycline.

Unlike other tetracycline antibiotics,[11,12] ADOT has not yielded a C.4 epimer when held in acetic acid solution, or under other conditions which effect epimerization of the previously known tetracycline antibiotics.

Alkaline Degradation of ADOT—ADOT hydrochloride (50 mg., 0.1 mmole) was dissolved in 5 ml. of 20% NaOH and heated to reflux under a stream of nitrogen for 24 hours. The effluent nitrogen was passed through two absorbers containing 4 ml. of 0.1 N HCl. Titration of the absorbers showed that 0.082 mmole of volatile base had been collected. The reacidified absorber contents were concentrated to dryness and sublimed at 110°, 0.03 mm. to yield 7 mg. of dimethylamine hydrochloride, m.p. 168–168.5°, completely soluble in 1 ml. of chloroform. The identification was confirmed by examination of the infrared spectrum and by conversion to N,N-dimethyl p-toluenesulfonamide, m.p. 80.5–81.5°.

The alkaline solution from the hydrolysis was acidified to pH 2 and extracted successively with ether, ethyl acetate and butanol. The ethyl acetate extract (10 mg.) was examined for terracinoic acid by paper chromatography on two systems (chloroform–ethanol–ammonia and formic acid–ethanol) and showed the major component to have an R_f equal to that of terracinoic acid. A portion was sublimed to yield 2 mg. of a yellow oil, whose ultraviolet spectrum showed peaks at 223, 238 and 270 mμ in 0.01 N hydrochloric acid, at 239, 282 and 320 mμ in 0.01 N sodium hydroxide as does terracinoic acid.[13] However, pure terracinoic acid was not isolated.

Acid Degradation of ADOT—ADOT hydrochloride (355 mg., 0.71 mmole) was heated to reflux in 2 N sulfuric acid (54 ml.) under a nitrogen atmosphere for 16 hours. Octanol was used as an anti-foaming agent. The cooled mixture was filtered from the insoluble residue of crude decarboxamidoterrinolide (176 mg.) and the filtrate held for further investigation. The residue was dissolved in 6 ml. of acetone and the acetone solution, after filtration from a trace of insoluble material, was run through a 1 × 10 cm. column of acid-treated Fluorisil. Evaporation of the eluent yielded impure decarboxamidoterrinolide which was dissolved in 2 ml. of acetic anhydride and heated with 100 mg. of anhydrous sodium acetate on a steam-bath for 100 minutes. After standing for 17 hours at room temperature, 10 ml. of water and 10 ml. of chloroform were added. The mixture was stirred for 10 minutes and the phases separated. The aqueous phase was extracted three times more with 10-ml. portions of chloroform, the combined chloroform extracts were washed with 5 ml. of water, dried over anhydrous sodium sulfate and filtered. Removal of the solvent afforded crude pentaacetyldecarboxamidoterrinolide (220 mg., 0.39 mmole) which was crystallized from boiling acetone. The crystals (116 mg., 27.51%) which melted at 246–249° dec. were recrystallized twice from dioxane. The purified product, m.p. 245–247° dec., showed a mixture melting point of 248–250° dec. with an authentic sample of pentaacetyldecarboxamidoterrinolide, m.p. 248–251° dec. The infrared spectra (KBr) of the two samples were identical. *Anal.* Calcd. for $C_{29}H_{24}O_{12}$: C, 61.70; H, 4.29; acetyl, 38.12. Found: C, 62.00; H, 4.55; acetyl, 37.01. The sample showed no optical activity.

The sulfuric acid hydrolysis solution was distilled after addition of 3 ml. of concentrated sulfuric acid until 45 ml. of a distillate had been collected. The distillate was neutralized with 0.094 N sodium hydroxide solution (0.75 mmole) and freeze dried. The solid residue was identified as sodium acetate by its infrared spectrum.

The residual sulfuric acid solution was made alkaline by the addition of sodium hydroxide. A stream of nitrogen was bubbled first through this solution and then through a trap containing 16 ml. of 0.1 N hydrochloric acid for 6.5 hours. The contents of the acid trap was neutralized by 10.5 ml. of 0.094 N sodium hydroxide solution, indicating 0.61 mmole of volatile base. The acidified trap solution was evaporated and the residue sublimed. The sublimate, m.p. 170°, was identified as dimethylammine hydrochloride by its infrared spectrum.

Acknowledgments.—We are indebted to Dr. R. L. Wagner and his associates for the analytical data and physical measurements. We should like to acknowledge too the very competent technical assistance of Mr. A. Adriansen.

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