[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Erythromycin. II. Des-N-methylerythromycin and N-Methyl-C^{14}-erythromycin

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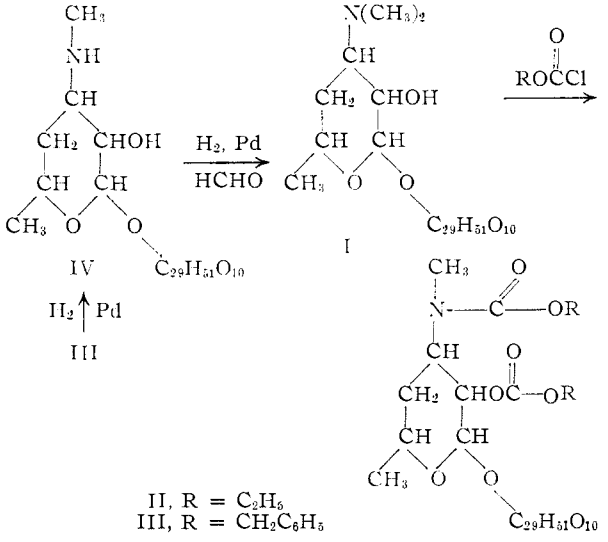
Erythromycin has been converted to O,N-dicarbobenzoxydes-N-methylerythromycin; this was hydrogenolyzed to give des-N-methylerythromycin. Des-N-methylerythromycin was converted to N-methyl-C^{14}-erythromycin.

The reaction between erythromycin[1] and ethyl chloroformate has been reported previously[2] to yield the unexpected product O,N-dicarbethoxydes-N-methylerythromycin (II) in which a methyl group has been displaced from the dimethylamino function originally present in erythromycin. This in itself is a remarkable reaction. Most important, however, is the possibility of using this reaction as a route to des-N-methylerythromycin (IV), which has advantages as a subject for structural studies, for the preparation of C^{14}-labeled erythromycin, and for study of the effect of structure on antibacterial activity. This paper reports the synthesis of O,N-dicarbobenzoxydes-N-methylerythromycin, its conversion to des-N-methylerythromycin and reconversion of the latter to erythromycin (I) and to N-methyl-C^{14}-erythromycin.



If the dicarbobenzoxy derivative III of erythromycin (I) could be prepared, it seemed likely that these groups could be removed by hydrogenolysis since it is known that erythromycin is not reduced by platinum and hydrogen when alcohol is the solvent.[2] The desired O,N-dicarbobenzoxydes-N-methylerythromycin was obtained by using carbobenzoxy chloride as both reaction medium and reagent and sodium bicarbonate as a means of neutralizing the acid produced. Numerous variations in reaction conditions were tried and are described in general terms in the Experimental section. The N-methyl group lost in the reaction was eliminated as methyl chloride, identified by passing nitrogen through the reaction mixture and collecting the evolved gas in an alcohol solution of trimethylamine. Tetramethylammonium chloride was isolated in 23% yield based on the amount of dicarbobenzoxy derivative obtained in this reaction.

O,N-Dicarbobenzoxydes-N-methylerythromycin is a neutral compound which is readily soluble in most organic solvents. Its infrared absorption spectrum exhibited a band at 5.74 μ, and another at 5.90 μ in the carbonyl region, attributed to the carbonate and carbamate groupings, respectively. These bands are in addition to those normally found in the erythromycin absorption spectrum.

Removal of the carbobenzoxy groups by hydrogenolysis was effected readily with palladium black as catalyst. A sodium acetate–acetic acid buffered reaction mixture eliminated variable results which were obtained when an unbuffered system was used. The hydrogenolysis product was extremely susceptible to acid-catalyzed hydrolysis or alcoholysis; the buffer served as a neutralizing agent for trace contaminants of carbobenzoxy chloride which would yield hydrogen chloride by hydrogenolysis. Under the conditions described, yields of des-N-methylerythromycin were 85% or better.

Des-N-methylerythromycin was only 5% as active as erythromycin by bioassay.[3] The spectrum of activity was similar in its qualitative aspects. The compound formed solvated crystals, as does erythromycin. It was less soluble in acetone and crystallized readily from this solvent. The melting point was indefinite but was about 5° higher than erythromycin. To demonstrate that the reactions involved had effected no change in the molecule other than those described, des-N-methylerythromycin was converted to erythromycin by reductive methylation with formaldehyde and hydrogen. The properties of the product obtained in this manner were identical in all respects with the corresponding properties of an authentic sample of erythromycin.

The reductive methylation reaction proved to be of particular value for the preparation of N-$C^{14}H_3$ labeled erythromycin. The labeled antibiotic so obtained is being used in tissue distribution and metabolic studies now underway.

Welles and co-workers[4] have reported the occurrence of des-N-methylerythromycin in the bile after administration of erythromycin to dogs. It seems, therefore, that N-demethylation of erythromycin also can be accomplished *in vivo*.

(1) The Eli Lilly and Company trade mark for the antibiotic erythromycin is "Ilotycin."

(2) E. H. Flynn, M. V. Sigal, Jr., P. F. Wiley and K. Gerzon, THIS JOURNAL, **76**, 3121 (1954).

(3) C. Higgins, R. C. Pittenger and J. M. McGuire, *Antibiotics and Chemotherapy*, **3**, 50 (1953).

(4) J. Welles, R. C. Anderson and K. K. Chen, *Antibiotics Annual*, 1954–1955, Medical Encyclopedia, Inc., New York, New York, 1954, in press. Presented at the Antibiotics Symposium, Washington, D. C., October 25–29, 1954.

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Experimental

O,N-Dicarbobenzoxydes-N-methylerythromycin.—Carbobenzoxy chloride (100 ml., *ca.* 120 g., 0.7 mole; Mann Research Laboratories, Inc., New York) was placed in a 500-ml. 3-neck flask. Dry sodium bicarbonate (74 g., 0.88 mole) was added; while stirring vigorously, 50 g. (0.068 mole) of erythromycin was added in 5-g. portions. About one hour was required to complete the addition. The temperature of the reaction mixture was kept at 35–50° by cooling intermittently with an ice-bath. When three-fourths of the erythromycin had been added, an additional 50 ml. of carbobenzoxy chloride was poured into the thick reaction mixture. After another hour of stirring at room temperature, 250 ml. of benzene was added. The mixture was filtered and the solid was washed with 200 ml. of benzene. The filtrate and washings were combined, cooled in an ice-bath, and shaken with four 120-ml. portions of cold 0.25 *N* hydrochloric acid. A gummy precipitate formed with each of the first three portions of acid, slowing separation of the liquid phases. However, the precipitate remained in the water phase so the benzene layer could be obtained free of this material. The gummy substance is thought to be O-carbobenzoxyerythromycin hydrochloride and is relatively insoluble in both phases. The extraction with acid was followed by two extractions with 120-ml. portions of cold water. The benzene phase was dried with anhydrous magnesium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in 50 ml. of chloroform, and 120 ml. of ether was added. Petroleum ether (Skellysolve B, 1200 ml.) was added gradually. Crystals of O,N-dicarbobenzoxydes-N-methylerythromycin appeared quickly. After several hours the solid was removed by filtration and air-dried. The product (38.1 g., 60% theory, m.p. 186–191°) was recrystallized from the same solvent mixture. The pure substance melted at 193–194°.

Anal. Calcd. for $C_{52}H_{77}NO_{17}$: C, 63.20; H, 7.86. Found: C, 63.38; H, 7.94.

Other preparative runs, made in an analogous manner, gave yields of 40–50% and appeared to be favorably influenced by efficient stirring. Variations of the above procedure included use of inert solvents as the reaction medium, variation of the reaction temperature and the molar ratio of reactants, and change in reaction time. Yields were lower in all cases though some product generally was obtained. When the erythromycin was added as a solution in an inert solvent, lower yields resulted.

An alternative procedure for separating the reaction products was evolved. After reaction with carbobenzoxy chloride had been completed and sodium bicarbonate removed by filtration as described above, sufficient petroleum ether was added to completely precipitate the reaction products. The precipitate then was dissolved in 50% methanol containing 0.1% sodium bicarbonate (a quantity sufficient to maintain a basic medium) and the solution was heated in a water-bath at 40° for four hours. This treatment hydrolyzed O-carbobenzoxyerythromycin, liberating erythromycin. The solvents were removed under reduced pressure and the residue was dissolved in chloroform. The chloroform solution was extracted with five equal volumes of phosphate buffer, *p*H 6.0, and then washed with water. The chloroform solution was dried and concentrated under reduced pressure. The solid was crystallized from acetone by adding cyclohexane or as described in the first procedure.

Des-N-methylerythromycin.—O,N-Dicarbobenzoxydes-N-methylerythromycin (116 g., 0.117 mole) was dissolved in 2.3 l. of 95% ethanol. To this was added 500 ml. of water containing 16.5 g. of sodium acetate, 100 ml. of 50% ethanol containing 10.4 ml. of glacial acetic acid and 3.0 g. of palladium black. Hydrogen was passed through the stirred mixture for 24 hours, by means of a sintered glass sparger in order to obtain good contact. The solution was filtered and concentrated under reduced pressure to a final volume of about 300 ml. Precipitation began at this point. Water (2.5 l.) was added, the mixture warmed to hasten solution, then cooled to 2°. The *p*H of the solution was 5.5. Sodium hydroxide (2.5 *N*) was added dropwise to the cold, stirred solution until a *p*H of 10.3 was reached. Ice was added occasionally to keep the temperature below 5°. The solution was allowed to warm to room temperature during six hours, with occasional stirring. Crystals of des-N-methylerythromycin appeared during this time. After one hour at 35° the suspension was filtered and the product washed with 50 ml. of warm water. The dried product weighed 61.0 g. (72%). An additional 10.0 g. of material was crystallized from the filtrate by cooling and again adjusting the *p*H to 10.3, then placing the solution at 48° overnight; total yield was 72.0 g., 85%.

For recrystallization, 59 g. of the above product was suspended in 200 ml. of acetone and the mixture was heated to the boiling point. The solid dissolved, followed immediately by precipitation of a new crystal form from the boiling solution. After standing overnight at 5°, 49.0 g. was obtained. Pure des-N-methylerythromycin melted indefinitely at 140–145°. It was pig-dried at 56° for 2 hours for analysis.

Anal. Calcd. for $C_{36}H_{65}NO_{13}$: C, 60.06; H, 9.10; N-CH_3, 2.09. Found: C, 60.14; H, 9.20; N-CH_3, 2.06.

When assayed by the procedure used for erythromycin, this preparation assayed 54 mcg./mg. of erythromycin activity. The microbiological spectrum of activity was similar to that of erythromycin in its qualitative aspects. Solubility of des-N-methylerythromycin in water increased as the temperature was lowered. This behavior parallels that of erythromycin.[2]

N-Acetyl-des-N-methylerythromycin.—Des-N-methylerythromycin (1.00 g., 0.00139 mole) was dissolved in 50 ml. of methanol and the solution cooled to 2°. Acetic anhydride (0.35 ml., 0.0027 mole) was added dropwise. After three hours at 2°, the methanol was removed under reduced pressure. The amorphous solid crystallized readily when it was dissolved in 4 ml. of chloroform and petroleum ether (14 ml.) was added. The pure compound melted at 207–209° and was microbiologically inactive. The compound was pig-dried at 100° for two hours *in vacuo* before analysis.

Anal. Calcd. for $C_{38}H_{67}NO_{14}$: C, 59.90; H, 8.86. Found: C, 59.91; H, 9.05.

When dried at 78° *in vacuo*, the substance analyzed as a monohydrate.

Anal. Calcd. for $C_{38}H_{67}NO_{14}\cdot H_2O$: C, 58.51; H, 8.92. Found: C, 58.07; H, 9.00.

N-Methyl-C^{14}-erythromycin from Des-N-methylerythromycin.—Into a 50-ml. flask was placed 740 mg. (0.00103 mole) of des-N-methylerythromycin, 35 ml. of absolute ethanol, 225 mg. of palladium black, and 3 ml. of a 1% aqueous solution of formaldehyde-C^{14} (0.001 mole containing 1 millicurie of C^{14}). The reaction mixture was stirred with hydrogen (one atmosphere, 28°) for 48 hours. One milligram of ordinary formaldehyde and 50 mg. of palladium black were then added and hydrogenation was continued for 24 hours. The catalyst was removed from the reaction mixture by centrifugation and the supernatant was evaporated to dryness under reduced pressure. Water (15 ml.) was added to the residue and the flask kept at −20° for 24 hours. When the solution was warmed to 60°, a crystalline product precipitated, which melted indefinitely at 135–139° and had an X-ray pattern identical with erythromycin dihydrate.[5] Further drying yielded 300 mg. of anhydrous N-methyl-C^{14}-erythromycin, specific activity 1.1 μc./mg. and bioactivity 980 μg./mg. Examination of the product by paper chromatography showed about 99% of the bioactivity to be associated with erythromycin and the remainder with erythromycin B.[6] No radioactive spots could be detected other than that coincident with the erythromycin spot. An additional 315 mg. of N-methyl-C^{14}-erythromycin (specific activity 1.1 μc./mg., bioactivity 620 μg./mg.) was obtained by evaporating to dryness mother

(5) H. A. Rose, *Anal. Chem.*, **26**, 938 (1954).

(6) C. W. Pettinga, W. M. Stark and F. R. Van Abeele, THIS JOURNAL, **76**, 569 (1954).

liquor from the first crop. This material showed the same behavior as the first crop of crystals when examined by paper chromatography. In cold runs, the identity of the methylation product was substantiated further by preparation of the acetone solvated crystal form and comparison of its X-ray pattern with that of an authentic sample of erythromycin. An infrared spectrum obtained from the methylation product was indistinguishable from that obtained from erythromycin of known identity.

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Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids. III. Configuration of the β-Asymmetric Center of Isoleucine

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By virtue of the greater susceptibility of L-acyl-L-amino acids over D-acyl-L-amino acids to the action of renal acylase I, a method of determining the optical configuration of the β-asymmetric center of isoleucine has been developed. For this purpose, *dl*-α-methylbutyryl-L-norleucine was subjected to the action of the enzyme, yielding as a hydrolytic product a mixture of free (+) and (−)α-methylbutyric acids in which the former predominated. Since (+)α-methylbutyric acid is derivable from L-isoleucine (and D-alloisoleucine), the β-asymmetric center of these stereomers has been assigned the L-configuration. On the basis of the known configuration of the β-asymmetric centers of the isomeric isoleucines, threonines and phenylserines, a correlation between the configuration of this center and the extent of susceptibility of the α-asymmetric center to various L- and D-directed enzyme systems has been noted.

Previous papers of this series[1,2] were concerned, in part, with the utilization of the partial molar rotation data of the α- and ω-asymmetric centers of several diasymmetric amino acids for the assignment of configuration to the α-asymmetric center. Such amino acids with two dissimilar centers of optical asymmetry which have thus far been demonstrated in proteins include threonine, hydroxyproline, isoleucine and hydroxylysine. Although these amino acids may exist as four optical stereoisomers, the employment of physical, chemical and biological criteria has, in every instance, led to the assignment of the L-configuration to the α-asymmetric center of the naturally occurring form.[3,4] Demonstration of a D-configuration for the ω-asymmetric center of L-threonine[5] and L-hydroxyproline[6] has been made, whereas the configuration of the δ-asymmetric center of L-hydroxylysine remains uncertain at this time. The present communication is concerned with the configuration of the β-asymmetric carbon atom of isoleucine.

In 1907, Ehrlich[7] demonstrated the presence of (−)α-methylbutanol-1 as a yeast fermentation product of L-isoleucine. Oxidation of this material to the corresponding aldehyde, (+)α-methylbutyraldehyde,[7] and acid, (+)α-methylbutyric acid,[8] was readily effected. The synthesis of a mixture of L-isoleucine and D-alloisoleucine from (+)α-methylbutyraldehyde by Ehrlich,[7] and from (−)α-methylbutanol-1 by White and Smith,[9] *via* methods that did not lead to Walden inversion of the asymmetric center provide unequivocal evidence that the degradation of L-isoleucine by yeast proceeded with retention of configuration of the β-center of asymmetry.[10] The configurational relationship between the β-asymmetric center of L-isoleucine (also D-alloisoleucine) and (+)α-methylbutyric acid, as well as its reduction products, was thereby established.[11]

On the basis of the foregoing discussion, the assignment of a configuration to (+)α-methylbutyric acid relative to some reference compound, as L-serine in the amino acid series or D-glyceraldehyde in the carbohydrate series, will in turn assign the L- or D-configuration to the β-asymmetric carbon atom of each of the four stereoisomeric isoleucines.[12] However, since the asymmetric carbon atom of α-methylbutyric acid is linked to two alkyl substituents, the assignment of a D- or an L-designation to this compound could become quite arbitrary when based solely on absolute configuration. Examination of Fig. 1 will readily reveal how such ambiguity could arise.

In Fig. 1, the configuration of a typical L-amino acid, written in the conventional Fischer diagram, is compared with the levo- and dextrotatory forms of α-methylbutyric acid. These forms were so represented to conform to the findings of recent reports, derived from X-ray[13] and chemical evi-

(1) J. P. Greenstein, N. Izumiya, M. Winitz and S. M. Birnbaum, THIS JOURNAL, **77**, 707 (1955).

(2) M. Winitz, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **77**, 716 (1955).

(3) A. Neuberger, *Advances in Protein Chem.*, **4**, 297 (1948).

(4) J. P. Greenstein, *ibid.*, **9**, 121 (1954).

(5) C. E. Meyer and W. C. Rose, *J. Biol. Chem.*, **115**, 721 (1936); D. P. Shoemaker, J. Donohue, V. Schomaker and R. B. Corey, THIS JOURNAL, **72**, 2328 (1950).

(6) A. Neuberger, *J. Chem. Soc.*, 429 (1945); J. Zussman, *Acta Cryst., Camb.*, **4**, 72 (1951).

(7) F. Ehrlich, *Ber.*, **40**, 2538 (1907).

(8) W. Marckwald, *ibid.*, **37**, 1038 (1904).

(9) H. C. White and H. T. Smith, Abstracts, 126th American Chemical Society Meeting, 3C (1954).

(10) Further evidence in support of this view was provided by the report of W. S. Fones (THIS JOURNAL, **76**, 1377 (1954)) that the degradation of L-isoleucine and D-alloisoleucine with ninhydrin led to the formation of (+)α-methylbutyraldehyde, whereas comparable treatment of their respective optical antipodes resulted in the production of the levorotatory aldehyde.

(11) It has long been the practice to label commercially available synthetic isoleucine as DL-isoleucine, regardless of the proportion of alloisoleucine present. To our knowledge no preparation of pure DL-isoleucine is now available on the American market, but only mixtures consisting (a) of nearly equal amounts of L-isoleucine and D-alloisoleucine, and (b) of DL-isoleucine and DL-alloisoleucine in unknown relative proportions. Identification of pure DL-isoleucine and of the mixtures cited may be made by determination of the melting points of their respective acetyl derivative; *cf.* J. P. Greenstein, L. Levintow, C. G. Baker and J. White, *J. Biol. Chem.*, **188**, 647 (1951); W. A. H. Huffman and A. W. Ingersoll, THIS JOURNAL, **73**, 3366 (1951).

(12) J. P. Greenstein, L. Levintow, C. G. Baker and J. White, *J. Biol. Chem.*, **188**, 647 (1951).

(13) J. Trommel, *Proc. Koninkl. Ned. Akad. Wetenschap.*, Series B, **56**, 272 (1953); *ibid.*, Series B, **57**, 364 (1954).