XII with Potassium Permanganate.—To three liters of dilute potassium permanganate solution (30 g. of $KMnO_4$ in 3000 ml. of water) was added 10.0 g. (0.04 mole) of XII, and the mixture stirred for 16 hours at room temperature. The manganese dioxide, which had formed, was filtered, the filtrate evaporated to about 300 ml., acidified with glacial acetic acid and then evaporated to dryness. The resulting solid was pulverized, extracted several times with boiling absolute ethanol and the ethanol distilled. A viscous oil remained and this material crystallized after standing for several weeks to give 3.5 g. of VII, m.p. 162–163° (from aqueous ethanol) alone and when mixed with a sample of the material described in IV (a).

(b) **The Reaction of 2-Vinylpyridine with 2,5,6-Trimethylhepten-4-one-3 (XI).**—A mixture of 8.5 g. (0.05 mole) of XI, 5.2 g. (0.05 mole) of 2-vinylpyridine and 0.2 g. (0.01 mole) of sodium was refluxed for six hours and worked up in the regular way to give 4.0 g. (29.1%) of XII, b.p. 170° (4 mm.) and 153–155° (0.5 mm.). The picrate of this material melted at 226–228° alone and when mixed with the same compound obtained as a by-product in the pyridylethylation of methyl isopropyl ketone.

VI. The Structure of Monopyridylethylated Methyl *n*-Amyl Ketone (XVI).—A mixture of 110.0 g. (0.60 mole) of ethyl α-*n*-butylacetoacetate, 63.0 g. (0.60 mole) of 2-vinylpyridine and 1 g. (0.04 mole) of sodium was refluxed for 12 hours and worked up to give some pyridylethylated material and 106 g. of unreacted β-ketoester. This material was dried and treated with more 2-vinylpyridine and sodium. The bases isolated from both runs were combined and distilled to give 11.5 g. (3.4%) of ethyl α-*n*-butyl-α-(β-(2-pyridyl)-ethyl)-acetoacetate, b.p. 145–185° (4 mm.). This material was dissolved in a mixture of 30 ml. of concentrated hydrochloric acid and 30 ml. of water and refluxed for 10 hours to give, after being worked up in the customary manner, 5.0 g. (55.5%) of 3-*n*-butyl-5-(2-pyridyl)-2-pentanone, b.p. 150–156° (4 mm.); semicarbazone, m.p. 155–156° alone and when mixed with a sample obtained from the direct monopyridylethylation of methyl *n*-amyl ketone.

VII. The Structure of Monopyridylethylated Methyl Benzyl Ketone (XVII).—On oxidizing 4.0 g. (0.02 mole) of XVII with potassium hypochlorite in the customary fashion 1.1 g. (27.3%) of 2-phenyl-4-(2-pyridyl)-butanoic acid, m.p. 159–160°, was obtained. *Anal.* Calcd. for $C_{15}H_{15}NO_2$: N, 5.81. Found: N, 5.81. A mixed melting point of this acid with that obtained from the saponification of monopyridylethylated ethyl phenylacetate[9] showed no depression.

(9) H. Reich and R. Levine, unpublished observations from this Laboratory.

PITTSBURGH, PENNSYLVANIA

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

Mycomycin. III. The Structure of Mycomycin, an Antibiotic Containing Allene, Diacetylene and *cis*, *trans*-Diene Groupings[1]

BY WALTER D. CELMER AND I. A. SOLOMONS

RECEIVED SEPTEMBER 18, 1952

The antibiotic mycomycin, $C_{13}H_{10}O_2$, is an optically active, eightfold unsaturated, carboxylic acid which yields *n*-tridecanoic acid upon catalytic hydrogenation. Chemical and spectral data disclose allene, diacetylene and conjugated diene groupings in mycomycin and lead to its formulation as (−)-3,5,7,8-*n*-tridecatetraene-10,12-diynoic acid (Ia). The 3,5-diene in mycomycin is further characterized as possessing a *trans,cis* stereoconfiguration. Mycomycin undergoes a unique rearrangement in aqueous alkali, involving an allene to acetylene conversion, a dual acetylenic migration and a *trans,cis* to *trans,trans* isomerization, yielding optically inactive isomycomycin, 3(*trans*),5(*trans*)-*n*-tridecadiene-7,9,11-triynoic acid (IIa). Mycomycin represents the first reported example of an optically active allene of natural origin.

Mycomycin,[2a] $C_{13}H_{10}O_2$, is an optically active, $[\alpha]^{25}D$ −130°, highly unsaturated carboxylic acid, shown to be (−)-3,5,7,8-*n*-tridecatetraene-10,12-diynoic acid (Ia).[2b] This antibiotic is inherently

$$HC{\equiv}C{-}C{\equiv}CCH{=}C{=}CHCH{=}CHCH{=}CHCH_2CO_2R$$
Ia, R = H; Ib, R = CH_3

unstable. The crystalline compound rapidly darkens at room temperature (losing one-half of its antibiotic activity in three hours) and explodes at its melting point, 75°.

In the presence of dilute aqueous alkali, mycomycin is rapidly converted to an isomeric acid, isomycomycin, which has the structure 3,5-*n*-tridecadiene-7,9,11-triynoic acid (IIa).[3]

$$CH_3C{\equiv}CC{\equiv}C{-}C{\equiv}CCH{=}CHCH{=}CHCH_2CO_2R$$
IIa, R = H; IIb, R = CH_3

It is the purpose of this paper to discuss in detail the chemical and spectral properties of mycomycin which led to a previous abbreviated announcement[2b] of its structure. Herein, mycomycin and isomycomycin are further characterized in regard to the stereoconfiguration of their 3,5-diene structural units. It is shown that mycomycin undergoes in addition to an allene to acetylene conversion and a dual acetylenic migration,[3] a *trans,cis* to *trans, trans* isomerization during its alkaline-induced rearrangement to isomycomycin.

Unbranched Chain.—Early characterization work on mycomycin revealed that complete catalytic hydrogenation required eight moles of hydrogen and gave a quantitative yield of *n*-tridecanoic acid.[2] The linear nature of this reduction product eliminates the possibility of branching and/or ring structure and establishes the chain length in the original mycomycin molecule.

—C≡CH.—The presence of a monosubstituted acetylene is indicated by the reactivity of both mycomycin and its methyl ester with acetylenic hydrogen reagents such as alcoholic silver nitrate.[4] This grouping is further substantiated by intense, well-defined, infrared absorption exhibited by the ester near 3280 cm.$^{-1}$ (Fig. 1) which is the characteristic hydrogen stretching frequency associated

(1) Presented before the Division of Medicinal Chemistry at the Atlantic City Meeting of the American Chemical Society, September 17, 1952. Abstracts of Papers, p. 17L.

(2) (a) W. D. Celmer and I. A. Solomons, THIS JOURNAL, **74**, 2245 (1952); (b) W. D. Celmer and I. A. Solomons, *ibid.*, **74**, 1870 (1952).

(3) W. D. Celmer and I. A. Solomons, Abstracts 121st American Chemical Society Meeting, Milwaukee, Wis., April, 1952, p. 93K; THIS JOURNAL, **74**, 3838 (1952).

(4) A. Behal, *Ann. chim.*, **15**, 408 (1888).

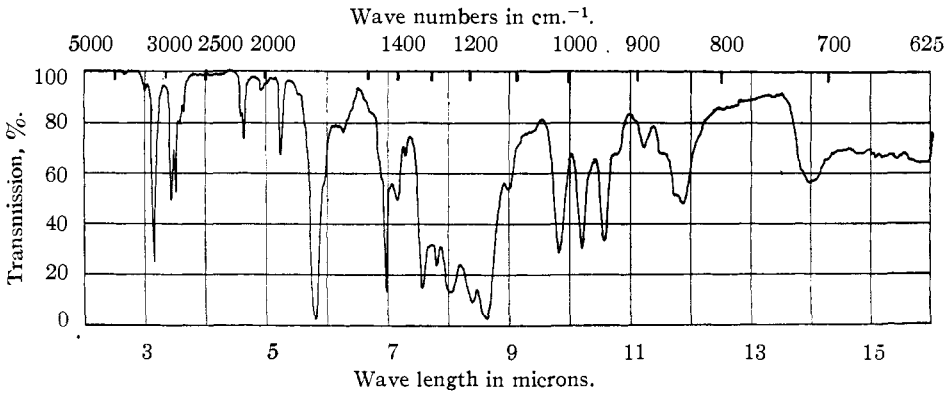
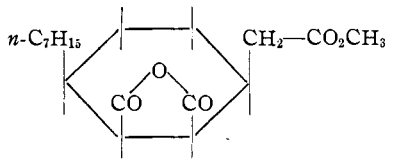


Fig. 1.—Infrared spectrum of mycomycin methyl ester recorded on a Baird infrared spectrophotometer using a 20% solution in carbon tetrachloride in a cell of 0.1 mm. thickness.

with a R—C≡C—H function.[5] A low intensity absorption band near 2040 cm.$^{-1}$, an expected monosubstituted C≡C stretching frequency, is also present.[6] A negligible Kuhn–Roth C-methyl value (0.48%) is in accord with the assignment of a terminal triple bond.

—C≡C—C≡CH.—The infrared spectra of mycomycin[2a] and its methyl ester (Fig. 1) exhibit a prominent absorption band near 2200 cm.$^{-1}$, interpreted as a disubstituted C≡C stretching frequency.[5] This band is readily distinguishable from monosubstituted acetylenic absorption and is intensified in the infrared spectrum[3] of isomycomycin (II) which contains three disubstituted triple bonds. Hence, mycomycin contains two distinct types of acetylenic bonds. The ultraviolet absorption spectrum of mycomycin exhibits two well-defined peaks ($\lambda_{max}^{m\mu}$ 267 and 281) whose fine structure spacing ($\Delta\nu'$ 1900 cm.$^{-1}$) gives evidence that the two recognized acetylenic bonds are in conjugation[3,7]; therefore, since one is terminal they are located in the 10- and 12-positions of the mycomycin carbon chain.

—CH=CH—CH=CH—CH_2—CO_2H.—The presence of a 3,5-diene grouping in isomycomycin has been previously deduced.[3] The ease with which isomycomycin and its methyl ester undergo a Diels–Alder reaction requires a *trans,trans* configuration.[8] Substantiation of these conclusions was obtained by the identity of the dihydro derivative of the maleic anhydride adduct of methyl 3(*trans*),5(*trans*)-*n*-tridecadienoate, 6-(*cis*)-*n*-heptyl-1(*cis*),2(*cis*)-cyclohexanedicarboxylic anhydride 3-acetic acid methyl ester[9,10] and the tetradecahydro derivative of the maleic anhydride adduct of isomycomycin methyl ester[3] (III).



III

After a 3(*trans*),5(*trans*)-diene unit was established in the structure of isomycomycin, the occurrence of a similar grouping in mycomycin was postulated.[2b] Attempts to demonstrate the presence of a conjugated diene by means of the Diels–Alder reaction were unrewarding. Infrared spectra–structure correlations, however, have afforded direct evidence in support of the assigned 3,5-diene in mycomycin. Interestingly, the mycomycin conjugated diene possesses a different stereoconfiguration than that found in isomycomycin.

The 900 to 1000 cm.$^{-1}$ range of the out-of-plane hydrogen bending region has been especially useful in differentiating olefinic configurations. In general, a monoethylenic compound possessing a *trans* configuration discloses strong infrared absorption at 970 cm.$^{-1}$, whereas, the corresponding *cis* isomer or saturated compound exhibits little or no absorption in this region.[11]

Stereoisomeric, conjugated dienes can be differentiated similarly.[9] Conjugated (*trans*),(*trans*)-methyl linoleates are characterized by a single strong band at 988 cm.$^{-1}$ and the (*cis*),(*trans*) isomers by two bands of medium intensity at 948 and 982 cm.$^{-1}$.[12]

(5) (a) H. W. Thompson, *J. Chem. Soc.*, 328 (1948); (b) J. H. Wotiz and F. A. Miller, THIS JOURNAL, **71**, 3441 (1949); (c) N. B. Colthup, *J. Opt. Soc. Am.*, **40**, 397 (1950).

The ≡C—H stretching frequency in the spectrum of mycomycin (dioxane solution, ref. 2a) is somewhat masked by strong carboxyl OH absorption, whereas, it is cleanly resolved in the spectrum of the methyl ester. In general, the ester is preferable to the free acid for extensive infrared studies because of greater solubility in solvents such as carbon tetrachloride and carbon disulfide, which exhibit excellent transparency in critical infrared regions. Direct spectra determinations on the crystalline ester or acid (Nujol mull) have not been satisfactory.

(6) It was necessary to use highly concentrated solutions of mycomycin methyl ester (in carbon tetrachloride) in order to detect the 2040 cm.$^{-1}$ band in its infrared spectrum. The corresponding band in model mono-*n*-alkyl substituted acetylenes and diacetylenes was also found to be inherently weak.

(7) (a) K. W. Hausser, R. Kuhn and G. Seitz, *Z. physik. Chem.*, **B29**, 391 (1935); (b) T. Bruun, C. M. Haug and N. A. Sorensen, *Acta Chem. Scand.*, **4**, 850 (1950); (c) E. R. H. Jones, M. C. Whiting, J. B. Armitage, C. L. Cook and N. Entwistle, *Nature*, **168**, 900 (1951); (d) M. Anchel, THIS JOURNAL, **74**, 1588 (1952).

(8) (a) D. Craig, *ibid.*, **72**, 1678 (1950); (b) K. Alder and M. Schumacher, *Ann.*, **571**, 87 (1950); (c) J. D. Von Mikusch, *Angew. chem.*, **62**, 475 (1950).

(9) Mycomycin. IV. To be published.

(10) The stereospecificity of the Diels–Alder reaction requires the all *cis* relationship of cyclohexane substituents, as indicated. Consult ref. 8a and references cited therein.

(11) (a) R. S. Rasmussen, R. R. Brattain and P. S. Zucco, *J. Chem. Phys.*, **15**, 135 (1947); (b) O. D. Shreve, M. R. Heether, H. B. Knight and D. Swern, *Anal. Chem.*, **22**, 1261, 1498 (1950).

(12) J. E. Jackson, R. F. Paschke, W. Tolberg, H. M. Boyd and D. H. Wheeler, *J. Am. Oil Chem. Soc.*, **29**, 229 (1952).

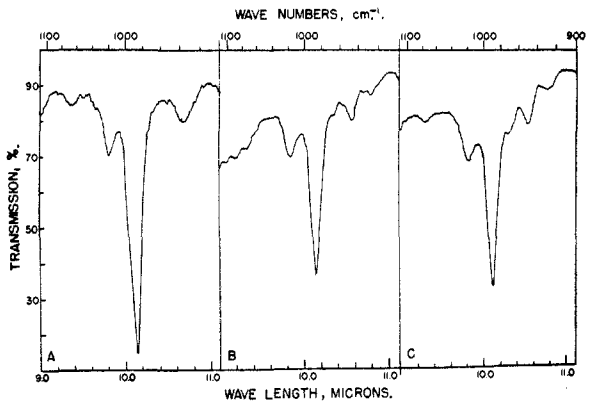


Fig. 2.—Infrared spectra in carbon tetrachloride solution: A, isomycomycin methyl ester (5%); B, methyl 3(*trans*),5(*trans*)-*n*-nonadienoate (2.5%); C, methyl 3(*trans*),5(*trans*)-*n*-tridecadienoate (10%). All spectra recorded on a Baird infrared spectrophotometer in cells of 0.1 mm. thickness.

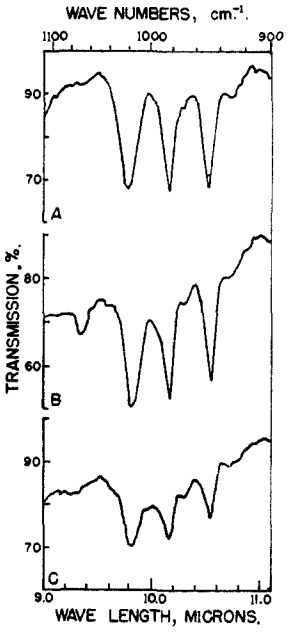


Fig. 3.—Infrared spectra in carbon tetrachloride solution: A, mycomycin methyl ester (6%); B, methyl 3-(*trans*),5(*cis*)-*n*-nonadienoate (12%); C, methyl 3-(*trans*),5(*cis*)-*n*-tridecadienoate (10%). All spectra recorded on a Baird infrared spectrophotometer in cells of 0.1 mm. thickness.

The pronounced spectral differences in this region between isomycomycin methyl ester (IIb) and mycomycin methyl ester (Ib) (compare Figs. 2A and 3A) prompted the synthesis of model stereoisomeric 3,5-diene fatty acid esters.[1,9] Spectral comparisons with these models (Fig. 2) reveal striking consistencies among IIb, methyl 3(*trans*),5(*trans*)-*n*-nonadienoate and methyl 3(*trans*),5(*trans*)-*n*-tridecadienoate near 990 cm.$^{-1}$. Significantly, Fig. 3 reveals that Ib, methyl 3(*trans*),5(*cis*)-*n*-nonadienoate and methyl 3(*trans*),5(*cis*)-*n*-tridecadienoate, are all characterized by three well-defined bands at 950, 985 and 1020 cm.$^{-1}$.

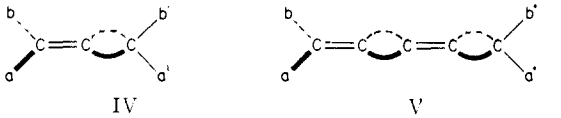
These spectral comparisons give evidence that mycomycin contains a conjugated diene which most likely exists in a 3(*trans*),5(*cis*) stereoconfiguration.[13] Although a *cis,cis* configuration may be safely excluded, an alternate 3(*cis*),5(*trans*) structure is not entirely disregarded in the present absence of appropriate model compounds with this configuration.

Neither mycomycin, its methyl ester, nor any of the four model 3,5-diene esters mentioned above exhibits characteristic absorption in the C=C stretching region near 1600 cm.$^{-1}$. In the case of isomycomycin and its methyl ester, the presence of well-defined bands of moderate intensity at 1580 and 1620 cm.$^{-1}$ appears to be due to further conjugation of the 3,5-diene with a triacetylene grouping. However, all of the esters in question exhibit characteristic unconjugated ester carbonyl absorption at 1733 ± 2 cm.$^{-1}$ (in carbon tetrachloride solution).[3]

—CH=C=CH—.—Infrared studies on allenic compounds in this Laboratory and elsewhere show that the C=C=C stretching frequency observed in the 1900–2000 cm.$^{-1}$ region is usually strong, and is generally the most reliable band for characterizing this grouping.[14] Accordingly, the strong band near 1930 cm.$^{-1}$ exhibited by mycomycin and its methyl ester is interpreted as evidence for an allenic bond.

The assigned 3,5-diene and 10,12-diyne in mycomycin limits the location of the allenic double bonds to positions 7 and 8, as illustrated in I. This position of the allenic grouping in the structure of mycomycin is further substantiated by its serving to explain two important properties of the compound, namely, (1) the high order of observed optical activity, $[\alpha]^{25}D$ −130°, and (2) the observed ultraviolet absorption centering around two maxima at 281 and 267 mμ and an inflection at 256 mμ.

(1) **Optical Activity of Mycomycin.**—According to van't Hoff's classical concepts of the tetrahedral nature of the carbon bonds, appropriately substituted compounds containing an even number of cumulated bonds of the type IV and V, etc., can exist in enantiomorphic forms due to substituents a b and a′b′ residing in planes perpendicular to one another.



a = a′ or a ≠ a′
b = b′ or b ≠ b′

This view has been proved experimentally by the successful preparation of optically active allenic compounds[15] and extensive dipole measurements of variously substituted allenes.[16]

The fact that optically active mycomycin yields *n*-tridecanoic acid upon complete catalytic saturation with hydrogen can be reconciled only with an allenic bond located within the mycomycin chain.

(13) The Diels–Alder reaction invariably fails or proceeds very poorly when the intended conjugated diene contains a *cis* bond (refs. 8 and 9). Thus, the detection of this reaction in the case of mycomycin would not be expected on the basis of its stereoconfiguration. The extreme instability of mycomycin, however, precluded earlier interpretations of the inability to isolate a Diels–Alder adduct.

(14) J. H. Wotiz and W. D. Celmer, THIS JOURNAL, **74**, 1860 (1952). Symmetrically substituted allenes, *i.e.*, tetraphenylallene, do not exhibit infrared absorption in this region, presumably because of their symmetry.

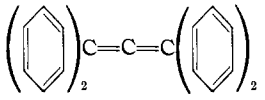
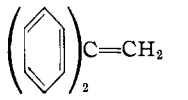
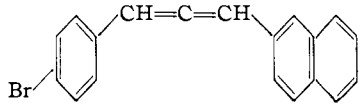
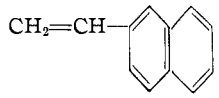
(15) P. Maitland and W. H. Mills, *Nature*, **135**, 994 (1935); E. P. Kohler, J. T. Walker and M. Tishler, THIS JOURNAL, **57**, 1743 (1935); J. H. Wotiz and R. J. Palchak, *ibid.*, **73**, 1971 (1951).

(16) E. Bergmann and G. C. Hampson, *J. Chem. Soc.*, 989 (1935).

TABLE I

LIGHT ABSORPTION OF ALLENIC COMPOUNDS

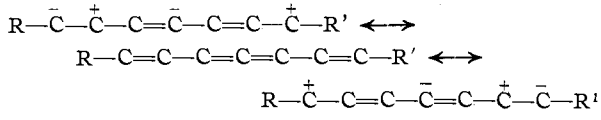
$R_1 = C_2H_5$, $R_2 = C_4H_9$, $R_3 = CH_3$

No.	Allenic compound	λmμ/max.	Ref.	Analogous ethylenic compound	λmμ/max.	Ref.
1	$R_1—CH=C=CH_2$	170	[a]	$R_1—CH=CH—R_2$	185	[a]
2	$R_2—C(CO_2R_3)=C=CH_2$	210–215	[b,g]	$R_3—C(CO_2R_1)=CH_2$	212–214	[c]
3		267	[d,g]		250	[a]
4		298[h]	[e]		295[h]	[f]

[a] E. A. Braude, *Chem. Soc. Annual Reports*, 105 (1945). [b] We are indebted to Dr. J. H. Wotiz for a sample of this compound. [c] H. E. Ungnade and I. Ortega, THIS JOURNAL, **73**, 1564 (1951). [d] We are indebted to Dr. W. Bergmann for a sample of this compound. [e] T. L. Jacobs and S. Singer, *J. Org. Chem.*, **17**, 475 (1952). [f] Spectrum No. 229, R. A. Friedel and M. Orchin, "Ultraviolet Spectra of Aromatic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1951. [g] Spectrum determined in this Laboratory. [h] Longest wave length maxima of approximately log ε 4.0 intensity.

Thus, among many of its unique features, mycomycin represents the first reported example of an optically active, allenic compound of natural origin.

(2) **Ultraviolet Absorption.**—It is well known that resonance interaction between chromophoric systems decreases as deviation from a planar configuration increases. Accordingly, in compounds of the type IV and V, where substituents a and a′ are chromophores, *i.e.*, conjugated multiple bonds, little resonance interaction is to be expected between a and a′. However, the chromophoric contribution of the allene double bonds themselves, must be taken into consideration in predicting an over-all ultraviolet absorption spectrum. Either double bond of the allene grouping may be considered comparable to an alicyclic ethylenic bond in its availability for resonance with chromophoric groups attached to it. The inability of resonance interaction completely across the allene bond can be appreciated by formulating the following resonance



hybrids. In effect, two isolated chromophores exist in such a molecule.[17]

Table I illustrates the observed chromophoric behavior of allenic compounds. When a single chromophoric substituent is attached directly to an allene bond, as in No. 2, the contribution of the allene to the light absorption is essentially that of an ethylenic bond. When the allene is substituted in both the 1- and 3-positions by chromophoric groups, the resulting light absorption confirms the view that little resonance interaction occurs across the allenic bond although either double bond of the allene may enter into conjugation with its vicinally substituted chromophore (Nos. 3 and 4).

The ultraviolet absorption spectrum of mycomycin can be related to the chromophoric role of its allene grouping. The Δ^7-allenic double bond in conjugation with the 3,5-diene and the Δ^8-allenic double bond in conjugation with the 10,12-diyne would be expected to constitute two isolated chromophores each containing three multiple bonds in conjugation, *i.e.*, a triene and enediyne. In Table II, the longest wave length maxima exhibited by mycomycin and known triene and enediyne compounds are in good agreement.

TABLE II

LIGHT ABSORPTION CORRELATIONS

Compound	Longest wave length maxima, mμ			Ref.
Mycomycin	256[g]	267	281	[a,f]
$C_2H_7—C\equiv C—C\equiv C—CH=CH—CH_2OH$	252	266.5	282.5	[b]
$CH_3—CH=CH—C\equiv C—C\equiv CH$	251	264	280	[c]
$CH_3—(CH_2)_2—(CH=CH)_3—(CH_2)_7—CO_2H$	257	267	278	[d,f]
$CH_2=CH—CH=CH—CH=CH_2$	248	258	268	[e]

[a] Ref. 2a. [b] Ref. 7b. [c] Ref. 7c. [d] We are indebted to Dr. B. F. Daubert for a sample of β-eleostearic acid. [e] G. F. Woods and L. H. Schwartzman, THIS JOURNAL, **70**, 3394 (1948). [f] Spectrum determined in this Laboratory. [g] Inflection.

Alkali-Isomerization of Mycomycin.—The facile alkali-induced isomerization of mycomycin to isomycomycin has no reported precedent of such complexity. However, observations in this Laboratory[9] and elsewhere[18] have stressed that even in relatively simple synthetic compounds, allene–acetylene interconversions may occur under mild alkaline conditions, such as normal sodium hydroxide or sodium bicarbonate solutions at room temperature. Although the 3,5-diene is not positionally disturbed during the alkali isomerization of mycomycin, it is not unexpected that a *trans,cis*

(17) Compounds containing an odd number of cumulated double bonds would not be expected to behave similarly because (1) chromophoric substituents such as a and a¹ would lie in the same plane and (2) hybrids can be formulated which allow complete resonance through the cumulene grouping. The ease of electronic vibrations in such systems is reflected by observed ultraviolet absorption at even longer wave lengths than similar compounds in which the cumulated double bonds are replaced by the same number of conjugated multiple bonds. R. Kuhn and K. Wallenfels, *Ber.*, **71**, 783 (1938); N. A. Sorensen and K. Stavholt, *Acta Chem. Scand.*, **4**, 1080 (1950).

(18) E. R. H. Jones, paper presented before the New York City Meeting of the American Chemical Society, September 6, 1951.

to *trans,trans* configurational change takes place in a reaction which obviously involves a multitude of prototropic shifts.

Acknowledgments.—The authors wish to express deep appreciation to Dr. Wilbur A. Lazier for his active interest in the investigation. We are grateful to Dr. R. C. Lord for helpful discussion. We are indebted to Dr. John Means and Mr. Glenn B. Hess for the spectral data.

BROOKLYN 6, N. Y.

[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Steric Configuration of β-Benzoylpropionate Ion in Aqueous Solution as Determined by Immunochemical Means[1,2]

BY DAVID PRESSMAN AND MALCOLM SIEGEL

RECEIVED NOVEMBER 1, 1952

Immunochemical evidence is presented for the existence of a coiled or *cis* configuration of the β-benzoylpropionate ion in aqueous solution. A study of other structural features important in the combination of haptens with antibodies prepared against the β-benzoylpropionate ion shows that the antibody fits closely around the homologous ion.

It has been postulated that β-benzoylpropionate ion either exists in or readily assumes a coiled (*cis*) configuration in aqueous solution.[3] This postulate was based on the observation that β-benzoylpropionate ion combines strongly with antibodies formed against the *p*-azosuccinanilate ion grouping. These antibodies are known to be complementary to the *cis* configuration of the succinanilate ion since they combine strongly with the maleanilate ion (which must exist in the *cis* configuration), and do not combine with the fumaranilate ion (which must exist in the *trans* configuration). In order to determine whether antibodies prepared against the β-benzoylpropionate ion grouping also reflect this *cis* configuration a study was made of antisera prepared against the β-(*p*-azobenzoyl)-propionate ion grouping. The results are reported here. The study also included the determination of other structural features which are important in the combination of hapten with these antibodies.

Experimental Methods

Materials.—With two exceptions the simple substances used have been described previously,[3,4] or were commercial preparations crystallized to the correct melting point and neutral equivalent. β-Benzoylacrylic acid was prepared according to the method of Papa, Schwenk, Villani and Klingsberg.[5] On recrystallization from water it melted at 63–65°.

β-(*p*-Aminobenzoyl)-propionic acid was prepared as follows: **β-(*p*-acetaminobenzoyl)-acrylic acid** was prepared according to the method of Papa, Schwenk, *et al.* 6.2 g. of β-(*p*-acetaminobenzoyl)-acrylic acid was dissolved in 95% ethanol by the addition of 4 *M* sodium hydroxide. The mixture was catalytically reduced (1 g. of 5% Pd on $BaSO_4$) for 1.75 hours at an initial hydrogen pressure of 42.3 lb./in.^{2} On acidification with HCl 2.7 g. (43% yield) of **β-(*p*-acetaminobenzoyl)-propionic acid** was obtained, m.p. 197–198°. **β-(*p*-Aminobenzoyl)-propionic acid** was prepared by refluxing the acetyl derivative for 2 hours in 10% HCl. A 61% yield of crystals melting at 184–186° was obtained.

Whole beef serum was stored in the lyophilized form. Ovalbumin was the crystallized Armour preparation.

Protein Antigens.—The antigen used for injection was prepared by coupling the diazotization product from 0.54 g. (0.0028 mole) of β-(*p*-aminobenzoyl)-propionic acid with 4 g. of regenerated lyophilized beef serum at *p*H 10. After standing overnight at 3–5° the azoprotein was dialyzed *vs.* cold saline until a colorless dialysate was obtained.

The test antigen was prepared by similarly coupling 350 mg. of ovalbumin at *p*H 10 with the diazonium salt from 54 mg. of β-(*p*-aminobenzoyl)-propionic acid. The azoprotein was purified by dialyzing against cold saline, precipitating three times at *p*H 3.85, washing with 4 portions of cold 60% acetone, and redissolving in 50 ml. of saline at *p*H 7.4. The last traces of acetone were removed by dialysis against saline.

Preparation of Antisers.—Antisera were prepared by methods described previously.[4] High titer serum from rabbits injected with each antigen were pooled.

Reaction of Antiserum with Antigen and Hapten.—Equal volumes of antigen, antisera and hapten were mixed and incubated for about 1 hour at 37° (or room temperature) then allowed to stand for 2–4 days at 3–5°. A concentration of antigen diluted with borate buffer[6] was used which yielded a maximum amount of precipitate. The precipitates were washed 3 times with 8 ml. of saline, dissolved in 1 *M* NaOH, and analyzed by a modified Folin procedure.[7]

Hapten stock solutions were prepared by dissolving a weighed quantity of hapten in the calculated quantity of NaOH and adjusting *p*H to 7–9. The ionic strength of these stock solutions was adjusted to 0.16. Dilutions were made with 0.16 *M* NaCl.

Binding Measurement.—10.0-ml. portions of a threefold borate buffer dilution of normal rabbit serum (19.2 mg. protein/ml. by Nessler analysis) were placed in dialysis bags and immersed in vials containing 10.0-ml. portions of a borate solution of hapten. The vials were rocked for 4 days to reach equilibrium. The concentration of the hapten both inside and outside the dialysis bag was determined by measurements of optical density. The per cent. of the hapten inside the dialysis bag bound to the protein was calculated either from the difference in optical density of the hapten inside and outside the bag or from the change in optical density of the outer phase.

Results

The Extent of Combination of Haptens with Antibody.—The extent of combination of hapten with antibody was determined by measuring the ability of hapten to inhibit the precipitation of anti-BzP antibody (antibody against the β-benzoylpropionate ion grouping) with BzP–oval (β-benzoylpropionate ion coupled to ovalbumin).

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(7) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **51**, 357 (1943).