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RESEARCH ARTICLES

Synthesis and Biological Activity of Deuteriobenzyl-d_7-penicillin

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Abstract ☐ To investigate the deuterium isotope effect on the biological activity of penicillin G, this compound was chemically synthesized by first obtaining phenylacetyl-d_7-chloride from phenylacetic-d_7-acid and thionyl chloride, followed by condensation of the acid chloride with 6-aminopenicillanic acid. The penicillin was isolated as the 1-ethylpiperidine salt. Yield of the deuteriopenicillin was approximately 60% when a 2:1 (6-aminopenicillanic acid–acid chloride) molar ratio was used. The identity of the deuteriated penicillin was confirmed using several physical constants. From NMR data, the presence of deuterium in the benzyl moiety was found to be greater than 95 atom %. The biological activity of the deuteriopenicillin was compared to that of the protioanalog by a turbidimetric assay procedure using *Staphylococcus aureus*. The results of the biological assay indicate that a significant deuterium isotope effect operates in the antistaphylococcal action of benzylpenicillin. With the test organism chosen, the ratio of the antibiotic potencies was 125% H/D.

Keyphrases ☐ Deuteriopenicillin—synthesis ☐ Biological activity—deuteriopenicillin ☐ Turbidimetric assay—deuteriopenicillin antimicrobial activity ☐ NMR spectroscopy—identification ☐ IR spectrophotometry—identification

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Deuterium (^{2}H), a rare stable isotope of hydrogen, was discovered by Urey *et al.* (1) in 1932. Extensive work by several investigators has felled the long-standing opinion that deuterium oxide (D_2O) is incompatible with life. The successful mass culture of algae, yeasts, and certain bacteria under conditions of full deuteriation has provided a useful source of fully deuteriated sugars, amino acids, proteins, and certain drugs (2–5). Substitution of deuterium for ordinary hydrogen and deuteriated substrates for protio metabo-

lites has been shown to produce profound changes in biosystems. Isotope effects have been categorized as being either primary or secondary. Measurement of the magnitude of the effect is usually useful in distinguishing between the larger primary mass isotope effects and the smaller secondary effects.

The substitution of deuterium in several drug molecules has been the impetus for many recent research contributions. Isotopically altered drugs have shown widely divergent pharmacologic effects. Elison *et al.* (6) demonstrated a reduced analgesic potency of morphine deuteriated in the *N*-methyl group. Nona *et al.* (7) found an increased antifungal activity with fully deuteriated griseofulvin. Foreman *et al.* (8) investigated the *in vitro* Dutch rabbit liver metabolism of selectively deuteriated amphetamines. Their studies show that the ratio of apparent rate constants (k_H/k_D) is 1.9, indicating that the deuterioamphetamine is metabolized more slowly than the protioanalog. One major effect of deuterium substitution in drug-producing organisms has been a general suppression of most nonessential metabolism. Mrtek *et al.* (9) demonstrated an inverse effect between the level of deuteriation in *Claviceps* and the amount of clavine alkaloids produced in saprophytic culture. Multimilligram quantities of highly deuteriated elymoclavine were obtained only through the use of replacement culture techniques (10). Mohammed *et al.* (11) demonstrated a similar effect with several strains of *Penicillium*. Carlstedt (12) obtained small amounts of highly deuteriated penicillin from large numbers of shake

cultures of *Penicillium chrysogenum*. Investigation of the deuterium isotope effect on the antibiotic potency of penicillin may provide important additional information concerning benzylpenicillin, the member of the penicillin group to which all others are compared.

Considerable research has substantiated the hypothesis that one mechanism of antibiotic activity is common to all penicillins. Several workers showed that the presence of a free amino group in the side chain increases Gram-negative activity (13), while Doyle *et al.* (14) noticed that electron-attracting groups in the α-position of the side chain increased acid stability. Similarly, hindering rotation about the single bond in the acyl grouping increases the stability of the molecule toward penicillinase (15). Changes in activity, either in spectrum or relative potency, may be attributed to modifications in the acyl group of the basic penicillin molecule. Repeated attempts to alter the penicillin nucleus have resulted in reduced biological activity when compared to the intact penicillanic derivatives. With respect to benzylpenicillin, it would seem reasonable that deuteration of the benzyl group would alter measurably the antibiotic potency if a significant isotope effect is operating.

The objectives of the present work were to synthesize deuteriobenzyl-d_7-penicillin and to compare its antibiotic potency to that of protiobenzyl penicillin.

EXPERIMENTAL

Deuteriobenzyl-d_7-penicillin—The semisynthetic procedure of Sheehan and Henery-Logan (16) was utilized to prepare the isotopically altered penicillin. Phenylacetyl-d_7-chloride was prepared by reacting 1.36 g. (9.15 mmoles) deuteriophenylacetic-d_7-acid[1] (98 atom % D) with 4.42 g. (37.2 mmoles) thionyl chloride in a 15-ml. flask with an attached calcium chloride drying tube. The reaction was allowed to proceed at room temperature for 24 hr. with occasional agitation. The excess thionyl chloride, as well as other volatile components, was removed by aspiration. No further purification of the acid chloride was attempted.

Deuteriobenzyl-d_7-penicillin was prepared by condensing 0.176 g. (approximately 1.0 mmole) of the partially purified deuteriophenylacetyl-d_7-chloride with 0.430 g. (2.0 mmoles) 6-aminopenicillanic acid (6-APA).[2] The phenylacetyl chloride was added dropwise over a 10-min. period to the stirred chilled solution of 6-APA dissolved in 10 ml. 4% sodium bicarbonate solution and 8 ml. reagent grade acetone. The reaction was conducted at 0–4° with stirring in an open 50-ml. conical flask for 30 min. following completion of addition of the acid chloride. Then the mixture was aspirated to remove excess acetone, and the aqueous mixture was washed once with 5 ml. cold ether A.R. which was discarded. The aqueous portion was layered with 10 ml. cold ether and acidified with 10% cold phosphoric acid to pH 2 as determined by short-range pH paper. The acidified mixture was extracted with two additional 10-ml. portions of cold ether in a small Squibb funnel. The combined ether extracts were washed once with 5 ml. cold water and dried with anhydrous magnesium sulfate. The ether solution was separated from the magnesium sulfate by filtration, and 0.122 g. (1.1 mmoles) 1-ethylpiperidine in 3 ml. dry ether was added to precipitate the deuteriobenzylpenicillin as the 1-ethylpiperidine salt. Twelve milliliters of dry reagent grade acetone was added, and the crystalline precipitate was allowed to stand at approximately −5° overnight. The precipitate was filtered and washed with several portions of cold dry ether. The crystals were dried in a vacuum desiccator at room temperature to constant weight and stored in

the desiccator until used. Yield: 296 mg.; 0.65 mmole; 59% of theoretical calculated on phenylacetic-d_7-acid.

Melting points were obtained simultaneously by placing equal size samples of the 1-ethylpiperidine salts of protiobenzylpenicillin and deuteriobenzylpenicillin and a mixture of equal parts of each in separate capillary melting tubes. These tubes were inserted in a Mel-Temp melting point apparatus previously heated to 100°. The temperature was increased (3–4°/min.) to 140°. The temperature was then increased slowly (approximately 1°/min.) until melting occurred. NMR spectra of the compounds were determined by using a Varian A-60 Mc.p.s. spectrometer. Each sample (approximately 0.05 mmole) was dissolved in approximately 1 ml. deuterium oxide, using an external TMS standard. All spectra were determined at probe temperature. Integration of the spectra were performed electronically. IR spectra of samples of protiobenzylpenicillin and deuteriobenzylpenicillin were obtained using a mineral oil mull in a Perkin-Elmer model 257 grating IR spectrophotometer.

Organisms—A lyophilized pellet of *Staphylococcus aureus* ATCC 6538P was added to 10 ml. Difco Penassay Broth and incubated at 35° for 24 hr. Subcultures were prepared by transferring a loopful of the culture to slants of Difco Penassay Seed Agar and incubating for 18 hr. at 35° prior to storage at 4°. Subcultures were prepared at 7–14 day intervals.

A culture of a clinically isolated penicillinase producing *S. aureus* was obtained.[3] This organism was maintained by subculture on Difco Penassay Seed Agar slants at 14-day intervals. Incubation and storage conditions were the same as for *S. aureus* ATCC 6538P.

Solutions—Accurately weighed quantities of authentic samples of the 1-ethylpiperidine salts of protiobenzylpenicillin and synthetic deuteriobenzyl-d_7-penicillin were used to prepare stock solutions. The stock solutions were prepared by dissolving nearly equal quantities (approximately 0.05 mmole) separately in 0.06 M phosphate buffer, pH 6, and diluting to approximately 1 × 10⁻⁹ mole/ml. concentration. Working solutions of desired concentration were prepared by dilution of these stock solutions. All solutions were stored in the dark at 4° for up to 1 week. Solutions for use with the penicillinase organism were prepared identically, except they were diluted to a concentration of 2 × 10⁻⁷ mole/ml.

Inocula—A loopful of *S. aureus* ATCC 6538P was transferred to 25 ml. Penassay Broth and incubated at 35° for 18 hr. The full quantity for overnight culture was transferred to 950 ml. Penassay Broth and incubated for 1 hr. at 37.5° prior to use. A loopful of the clinically isolated penicillinase producing *S. aureus* was transferred to 20 ml. Penassay Broth to which yeast extract (1.5 g./l.) had been added, and it was incubated at 35° for 18 hr. The full quantity of inoculum was added to 400 ml. Penassay Broth with added yeast extract (1.5 g./l.) and incubated at 37.5° for 1 hr. prior to use.

Procedure—Assay tubes were prepared by addition of 0.5 ml. of the desired working solution and 9.0 ml. of the inoculated (preincubated) broth to 16 × 125-mm. tubes. Two to four different concentrations were used for each determination, and four tubes were prepared at each concentration. A total of 8 and 10 different concentrations (ranging from 70 to 1200 × 10⁻¹² mole/tube) of deuteriobenzylpenicillin and protiobenzylpenicillin, respectively, were used to establish the regression equation. Control tubes were prepared identically, except that 0.5 ml. of 0.06 M phosphate buffer pH 6 (0.0 M benzylpenicillin) was used. Blanks were prepared by incorporating buffer and 0.5 ml. 1:3 formaldehyde USP prior to inoculation with the organism. Both control and blanks were incubated with the test dilutions. The tubes (assay, control, and blank) were arranged randomly in a 48-position rack and incubated in a 37.5 ± 0.1° stirred water bath[4] for 3 hr. At the end of the assay period, growth was stopped by addition of 0.5 ml. 1:3 dilution of formaldehyde USP. The turbidity was determined by measuring percent transmittancy, using matched round cuvettes (19 × 150 mm.) in a Coleman Jr. spectrophotometer at 650 mμ.

Assay tubes for use with the penicillinase producing *S. aureus* were prepared by placing 0.5 ml. of the desired test solution and 9.0 ml. of inoculated preincubated broth in 16 × 125-mm. tubes.

[1] Mallinckrodt-Nuclear, Orlando, Fla., lot L-2248.
[2] Sigma Chemical Co., St. Louis, Mo., lot 44B-1550.

[3] Bacteriological Laboratory, University of Illinois Hospital.
[4] Precision Scientific, Chicago, Ill., model 6580.

Eight concentrations were used in each determination. Control and blank tubes were prepared as described. The tubes were incubated in a 48-tube rack in a stirred 37.5 ± 0.1° water bath for 4 hr. Growth was stopped, and turbidity was measured in the same way as before.

RESULTS AND DISCUSSION

Preliminary experiments with protiophenylacetyl chloride and 6-APA were performed to determine a ratio that would optimize the yield of benzylpenicillin based upon a conservation of the acid chloride. Table I summarizes the results and indicates an optimum ratio of 2:1 (6-APA–phenylacetyl chloride). Yields at the optimum ratio were similar when deuteriophenylacetyl-d_7-chloride was used.

Table II presents a summary of the physical constants for the two isotopic penicillins. The absence of proton signals corresponding to the two α-methylene ($\delta = 3.59$ p.p.m.) and five phenyl protons ($\delta = 7.31$ p.p.m.) in the acyl group indicate the substitution of deuterium in these positions to be greater than 95 atom %. Confirmation of the identity of the synthetic penicillins is assumed on the basis of melting points, elemental analysis, chemical shift of the remaining proton signals in the NMR spectrum, and several IR absorption bands.

Inocula broths were preincubated with overnight cultures of *S. aureus* to obtain organisms in the log phase of growth for the assay, since penicillin exerts its antimicrobial action only on rapidly dividing organisms, *i.e.*, those synthesizing new cell walls. Penicillin has little effect on microbial clones that are metabolizing but not growing or dividing.

Most linear biological assays are commonly based upon a graphical presentation of a transformed measure of some effect that is proportional to the microorganism number, such as turbidity, *versus* log concentration of the test compound (17). Plots of turbidity *versus* dose result in sigmoid curves which may be linearized by suitable transformations. One convenient method is the logarithmic transformation.

In the determination of an isotope effect, the units selected for expression of concentration of penicillin per tube as the independent variable were log (moles $\times$ 10^{-12}) rather than a more common expression of weight (microgram or unit) per tube. When dealing with antibiotics of different molecular weights, the toxic effect of drugs on bacterial growth is proportional to the number of moles present but not to the particular weight of the substance used. When concentrations of two isotopically related drugs are expressed as weight per volume, a factor relating the relative change in molecular weight is confounded with the slope of the dose–response relationship. If the molecular weights are not identical as in the case of isotope hybrids, the slope of the regressions for analogs would be anticipated to be different from each other, at least by a factor proportional to the relative molecular weights. Thus, the slopes of the dose–response lines for the two isotopic benzylpenicillins in which log weight is used as an independent variable would not be truly comparable since they differ in their components. A totally linear regression may be expressed as

$$\log \%T = \theta \log \text{moles} + K \qquad \text{(Eq. 1)}$$

when the independent variable is expressed as moles. In the converse situation, it may be expressed as

$$\log \%T = \theta' \log \text{amt.} + K' \qquad \text{(Eq. 2)}$$

when the independent variable is expressed as micrograms or units, $\theta, \theta' = $ slopes of regressions, and $K, K' = $ intercepts. Since the turbidity term is proportional to the moles of substance present, the units of slope relating the concentration term to a measure of the turbidity term may be shown as

$$\theta = \frac{\log \%T}{\log \text{moles}} \qquad \text{(Eq. 3)}$$

The slope, θ, contains no molecular weight factor. Conversely, if the data are presented as weight of substance, then the components of the slope may be seen as

$$\theta' = \frac{\log \%T}{(\log \text{moles})(\log \text{MW})} \qquad \text{(Eq. 4)}$$

Table I—Effect of Ratio of Reactants on Yield of Benzylpenicillin

Molar Ratio of 6-APA–Phenylacetyl Chloride	Yield of Benzylpenicillin,[a] %
1.0:1.0	60
1.5:1.0	63
2.0:1.0	69
5.0:1.0	65

[a] Calculated on phenylacetyl chloride.

where MW is the mole weight of the substance. Thus, comparing slopes for the isotopic penicillins, from Eq. 3,

$$\frac{\theta_H}{\theta_D} = \frac{\log \%T/\log \text{moles}_H}{\log \%T/\log \text{moles}_D} = \frac{\log \text{moles}_D}{\log \text{moles}_H} \qquad \text{(Eq. 5)}$$

and since the units are the same,

$$\frac{\theta_H}{\theta_D} = 1 \qquad \text{(Eq. 6)}$$

Conversely, from Eq. 4,

$$\frac{\theta'_H}{\theta'_D} = \frac{\log \%T/(\log \text{moles}_H)(\log \text{MW}_H)}{\log \%T/(\log \text{moles}_D)(\log \text{MW}_D)} =$$

$$\frac{(\log \text{moles}_D)(\log \text{MW}_D)}{(\log \text{moles}_H)(\log \text{MW}_H)} = \frac{\theta_H \log \text{MW}_D}{\theta_D \log \text{MW}_H} \qquad \text{(Eq. 7)}$$

and

$$\frac{\theta'_H}{\theta'_D} = \frac{\log \text{MW}_D}{\log \text{MW}_H} > 1 \qquad \text{(Eq. 8)}$$

From these equations, using a drug concentration term expressed in moles rather than amount as the independent variable, confounding of the isotope mass differences is avoided. In testing for parallelism between the regression lines for the two antibiotics, if the null hypothesis is accepted, then only random sampling error remains to account for the observed differences between the two slopes, θ_H and θ_D.

The distribution of log $\%T$ has been established to be normal (18). The model developed for this work is univariate, where Y equals log $\%T$ as the random variable. The variance of Y throughout the range of X equals log moles (concentrations of penicillins) was established as homogeneous. Table III summarizes the calculated parameters used in the statistical analysis.

The observed difference between the regression coefficients for the two isotopic benzylpenicillins was tested for level of significance by a t test. The null hypothesis was that there is no difference between the slopes. (See the *Appendix* for the equations used.) No evidence for a difference other than zero was found, and

Table II—Summary of Physical Constants for Isotopic Benzylpenicillin Salts

	Protio	Deuterio
Molecular formula	$C_{23}H_{33}N_3O_3S_1$	$C_{23}H_{26}D_7N_3O_3S_1$
Elemental analysis, %	—	Calcd. C, 60.76; H, 7.44; N, 9.24 Found C, 60.66; H, 7.37; N, 9.04
Melting point	150–152° (dec.)	150–152° (dec.)
IR spectra	1370,[a] 1390,[a] 1570,[b] 3190,[c] 3240,[c] cm.$^{-1}$	1365,[a] 1390,[a] 1575[b] 3180,[c] 3230,[c] cm.$^{-1}$
NMR spectra[d]	1.51,[e] 1.42[e] 3.59[f] 7.31[h]	1.51,[e] 1.42[e] —[g] —[g]

[a] C—H bend in 2-dimethyl. [b] Carbonyl bend in β-lactam. [c] N—H stretch in amine salt. [d] Chemical shift, p.p.m. (δ) (TMS = 0). [e] 2-Dimethyl. [f] Acyl methylene. [g] Indicates lack of signal corresponding to deuterium substitution. [h] Phenyl.

Table III—Summarized Calculations for Regression Analysis of Isotopic Benzylpenicillin Analogs

	Deuterio	Protio
N	44	60
Σx^2	4.46598	6.05798
Σy^2	0.14444	0.16299
Σxy	0.65940	0.91238
b_{YX} (regression coefficient)	0.14765	0.15061
F ratio (nonzero slope)	$82.5(p < 0.001)$	$259.8(p < 0.001)$
$\bar{X}$ [log (moles $\times$ 10^{-12})]	2.65763	2.64677
$\bar{Y}$ [log ($\%T$)]	1.85164	1.86463
$\hat{Y}_{bYX}$ (regression line)	$1.45471 + 0.14935X$	$1.46933 + 0.14935X$
$SS_{\text{deviations from regression}}$	0.04705	0.02649

parallelism of the regression equations is established on the basis of probability. Furthermore, these data corroborate the suggestion that both deuteriobenzylpenicillin and protiobenzyl-penicillin inhibit bacterial growth by the same mechanism. The regression coefficient is a quantitative expression of the association between variables. Since the regression coefficients for protio-benzylpenicillin and deuteriobenzylpenicillin are not significantly different, it is reasonable to conclude that the presence of deuterium in the acyl group of benzylpenicillin does not alter the mechanism of action of the drug with respect to the test organism.

To obtain a valid estimate of a real difference in the antimicrobial potency of the two compounds, the assumption that the two lines are not identical must be tested. Establishment of parallelism between regression lines does not preclude the possibility of identical intercepts. The null hypothesis for the determination of the identity of the two lines tests the significance of the difference between the intercepts of two parallel lines. If the null hypothesis is accepted on the basis of a t test, a statement can be offered in terms of probability that the two regression lines are estimates of the same locus. (See the *Appendix* for summarized calculations.) Application of the test to the data for the two benzylpenicillins indicates that the two intercepts are significantly different ($p < 0.001$). This information, along with the identity of the slopes, indicates that the regression lines for the two benzylpenicillins, although parallel, describe different loci. Satisfaction of the requirement for parallelism and establishment of significantly different intercepts permit the direct comparison of the two lines in terms of horizontal displacement.

The horizontal displacement as a measure of the relative potency of the two drugs may be calculated and confidence limits generated (*Appendix*). Table IV summarizes the results of these calculations. The introduction of deuterium into the acyl group of penicillin has no effect on the mechanism of antimicrobial action. However, a deuterium isotope effect is present and is reflected in a 25% reduction in potency of the deuterium compound compared with its protio-analog.

Comparative assays were performed on several days and grouped together to increase reliability of the relative potency estimate. The absolute antistaphylococcal activity of the antibiotics varied widely from day to day, as indicated by the overlap of data points throughout the concentration range in Fig. 1. Therefore, day-to-day predictions of absolute activity for each antibiotic could not be made. However, day-to-day effects should not be expected to operate on the relative position of the two regression lines with respect to each other (relative potency estimate). On each day the protioanalog regression line was always displaced above the regression line for the deuterioanalog. The variation in displacement as a function of days was assessed for the presence of a significant trend. The log relative potency was graphed as a function of the day on which the experiment was performed (Fig. 2). No significant regression of the displacement on days was found.

The regression equation calculated for the data in Fig. 2 does not possess a slope that varies significantly from zero ($p \cong 0.70$). As expected, there is no correlation between the log relative potency and the day on which the experiment was completed. If a difference in the rate of decomposition of the two isotopic antibiotics did exist, a significant regression of log relative potency on days could be determined. This information would contribute toward the quantization of the difference in decomposition rate of the two compounds.

Results of preliminary studies obtained with a clinically isolated penicillinase producing *S. aureus* indicate reduced but nearly equal biological activity for the two isotopic penicillins. Direct comparison of the results obtained for *S. aureus* ATCC 6538P and the penicillinase-producing organism may not be appropriate because of the more fastidious nature of the clinically isolated test organism. Further investigation is underway to permit a complete analysis of the deuterium isotope effect on a penicillinase-producing organism (21).

The introduction of deuterium into the benzyl moiety of penicillin G has reduced the antimicrobial activity against a strain of *S. aureus* with respect to the protioanalog. This decrease may result from an altered rate of penetration by penicillin to the site of action in the cell wall or rate of association of penicillin with a crosslinking enzyme. Deuterium introduced into other pharmacologically active compounds has resulted in a decreased rate of binding to the enzyme receptor, with a concomitant increase in the strength of the bond. Since the binding of protiobenzylpenicillin to albumins is mediated by the benzyl moiety and not the β-lactam-thiazolidine nucleus (19), changes in the rate and strength of binding may account for the decreased biological activity of the deuterioanalog. Further work is in progress which will help elucidate the effect of deuterium in the acyl group on the kinetics and extent of binding of benzylpenicillin to macromolecules.
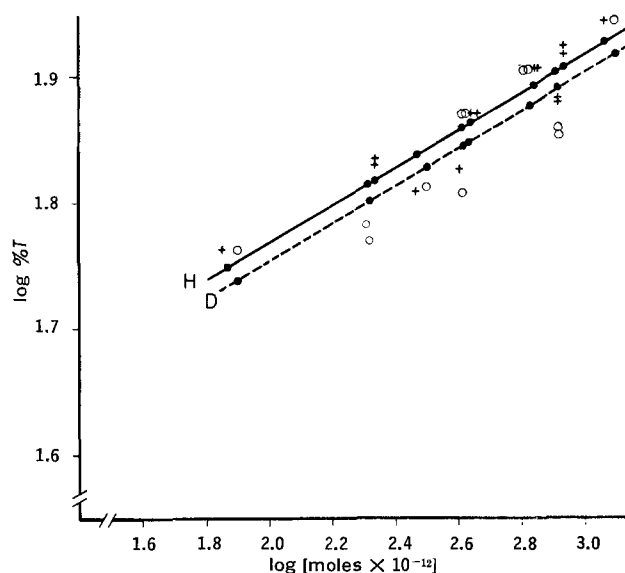
Table IV—Relative Potency and Confidence Limits for Isotopic Benzylpenicillin Salt

	Percent
Relative potency (H/D)	125
95% Confidence interval	114–138
99% Confidence interval	106–148

Figure 1—*Regression lines calculated for isotopic penicillins. Key:* $\hat{Y}_H = 1.46933 + 0.149352X$, *and* $\hat{Y}_D = 1.454718 + 0.149352X$.
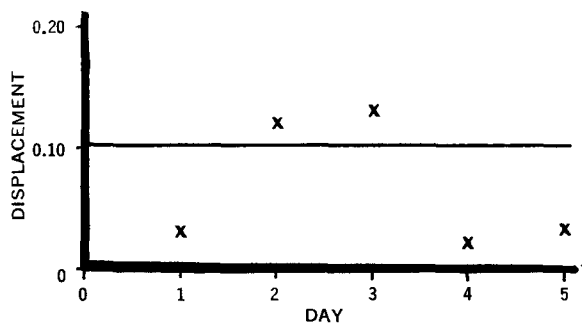
Figure 2—*Effect of days on displacement of regression lines (log relative potency). Key: —, displacement calculated for combined data; and ×, displacement for day. (See text for explanation.)*

SUMMARY

1. Deuteriobenzyl-d_7-penicillin has been chemically synthesized. Phenylacetyl-d_7-chloride was first prepared from phenylacetic-d_7-acid. Subsequently, the acid chloride was condensed with 6-APA, and the penicillin G was isolated as the 1-ethylpiperidine salt.

2. The presence of deuterium in the benzyl moiety was confirmed using several physical constants.

3. The antimicrobial activity of the deuteriopenicillin was compared to its protioanalog by a turbidimetric assay procedure using *S. aureus* as the test organism.

4. Identity of the regression coefficients for the isotopic penicillins was established by statistical analysis. This identity indicates that the regression lines are parallel. Parallelism indicates a similar mechanism of antistaphylococcal action for the two isotopic penicillins.

5. Nonidentity of the regression lines was determined using statistical analysis, permitting calculation of a valid estimate of relative potency.

6. The relative potency (H/D), as a quantitative estimate of the deuterium isotope effect, was calculated to be 125%. Neither the 95% nor the 99% confidence interval generated for the estimate includes a relative potency estimate of 100%.

APPENDIX (20)

Slope Test—

$$t = \frac{b_H - b_D}{s_{Db}} \quad \text{(Eq. 1A)}$$

where

$$s_{Db} = \left[\bar{\sigma}_{YX}^2 \left(\frac{1}{SS_{\text{dev}.H}} + \frac{1}{SS_{\text{dev}.D}} \right) \right]^{1/2} \quad \text{(Eq. 2A)}$$

and

$$\bar{\sigma}_{YX}^2 = \frac{SS_{\text{dev}.H} + SS_{\text{dev}.D}}{n_H + n_D - 4} \quad \text{(Eq. 3A)}$$

$$t = \frac{2.96}{2.08} \times 10^{-2} = 0.014 \ (100\ df) \ \text{(n.s.)} \quad \text{(Eq. 4A)}$$

Identity of Lines—

$$t = \frac{\hat{b} - \bar{b}}{s_{(\hat{b} - \bar{b})}} \quad \text{(Eq. 5A)}$$

where

$$s_{(\hat{b} - \bar{b})} = \left\{ s_{YX}^2 \left[\frac{1}{(X_H - X_D)^2} \left(\frac{1}{n_H} + \frac{1}{n_D} \right) + \frac{1}{\sum x_H^2 + \sum x_D^2} \right] \right\}^{1/2} \quad \text{(Eq. 6A)}$$

and

$$\hat{b} = \frac{\bar{Y}_H - \bar{Y}_D}{\bar{X}_H - \bar{X}_D} \quad \text{(Eq. 7A)}$$

and

$$s_{YX}^2 = \frac{SS_{\text{dev}.H} + SS_{\text{dev}.D} + \left[\frac{(b_H - b_D)^2}{1/(SS_{\text{dev}.H}) + 1/(SS_{\text{dev}.D})} \right]}{n_H + n_D - 3} \quad \text{(Eq. 8A)}$$

$$t = 8.86(101\ df)(p < 0.001) \quad \text{(Eq. 9A)}$$

Horizontal Displacement—

$$p_x = \left| \bar{X}_D - \bar{X}_H - \frac{(\bar{Y}_H - \bar{Y}_D)}{b_{YX}} \right| \quad \text{(Eq. 10A)}$$

Confidence Limits of P_x—

$$CL = \left| \bar{X}_D - \bar{X}_H - \frac{(\bar{Y}_D - \bar{Y}_H)}{b_{YX}(1 - K^2)} \right| \pm$$

$$\frac{K}{b_{YX}(1 - K^2)} \left[\left(\frac{1}{n_H} + \frac{1}{n_D} \right) b_{YX}(1 - K^2) \left(\sum x_H^2 + \sum x_D^2 \right) + (\bar{Y}_D - \bar{Y}_H)^2 \right]^{1/2} \quad \text{(Eq. 11A)}$$

where

$$K = \frac{t^2 s_{bYX}^2}{b_{YX}^2} \quad \text{(Eq. 12A)}$$

and

$$s_{bYX}^2 = \frac{s_{YX}^2}{\sum x_H^2 + \sum x_D^2} \quad \text{(Eq. 13A)}$$

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Synthesis of Azabiotin Analogs as Potential Cofactors for Biotin-Dependent Enzymes

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Abstract ☐ As part of a program to synthesize azabiotin analogs and homologs as potential substitutes for the natural coenzyme, several 4- and 5-substituted derivatives of *cis*-hexahydropyrrolo-[3,4-*d*]imidazole-2-one have been prepared. Spectral data and certain side reactions in the synthetic scheme used point to the stereochemistry of the 4-substituted compounds.

Keyphrases ☐ Azabiotin analogs—synthesis ☐ Biotin-dependent enzymes—potential cofactor, azabiotin analogs ☐ NMR spectroscopy—structure ☐ UV spectrophotometry—structure ☐ IR spectrophotometry—structure ☐ Mass spectroscopy—structure

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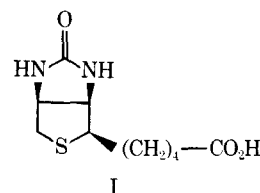
Biotin (I) is a cofactor required for several enzyme-catalyzed carboxylation reactions and, as such, plays a significant role in carbon dioxide fixation reactions. Although a wide variety of compounds closely related to the vitamin have been prepared, very few have been found to possess significant biochemical and growth-promoting activity in microorganisms and animals.

Recently (1–3), controversy has arisen about the significance of the sulfur atom of biotin with regard to interactions between the enzyme protein and the cofactor. NMR data have indicated that both the ureido oxygen and the sulfur atom should be considered as

Table I—NMR Spectra at 60 Mc. in CDCl₃ Using TMS Reference (Pyrrolidino Compounds)[a]

Compound	Data	N-Acetyl	Ester CH₂	Ester CH₃	N—H	5—CH₃	Miscellaneous
III	δ p.p.m.	—	4.17	1.28	6.60	—	C-2 and C-5 4.25
	J, c.p.s.	—	q, 7.0	t, 7.0	s	—	s
XV	δ p.p.m.	—	4.26	1.41	6.02	1.22	C-2 4.61 (1H)
	J, c.p.s.	—	q, 7.0	t, 7.0	s	d, 6.0	C-5 4.25 (2H)
IV	δ p.p.m.	2.20	4.25	{ 1.30 \ t, 7.1	4.90	—	
	J, c.p.s.	s	q, 7.0	{ 1.33 \ t, 7.1	d, 7.4		
XVI	δ p.p.m.	2.19	4.22	1.30	5.54	2.18	C-5 4.25 (2H)
	J, c.p.s.	s	q, 7.0	m	s		
XVII	δ p.p.m.	2.37	4.43	{ 1.36 \ t, 7.1	7.95	2.20	C-2 7.81 (1H)
	J, c.p.s.	s	q, 7.0	{ 1.46 \ t, 7.1	s	s	
V	δ p.p.m.	1.94	4.20	1.25	7.03	—	
	J, c.p.s.	s	q, 7.0	t, 7.0	d, 7.0	—	
XVIII	δ p.p.m.	2.01	4.17	1.25	7.1	1.20	
	J, c.p.s.	s	q, 7.1	t, 7.2	d, 9.5	d, 6.0	
VI	δ p.p.m.	1.99	4.16	1.26	7.36	—	
	J, c.p.s.	s	q, 7.1	t, 7.0	d, 7.0	—	
XXI	δ p.p.m.	2.01	4.17	1.25	7.29	1.10	
	J, c.p.s.	s	q, 7.1	t, 7.1	d, 7.0	d, 6.0	

[a] s = singlet, d = doublet, m = multiplet, t = triplet, and q = quartet.