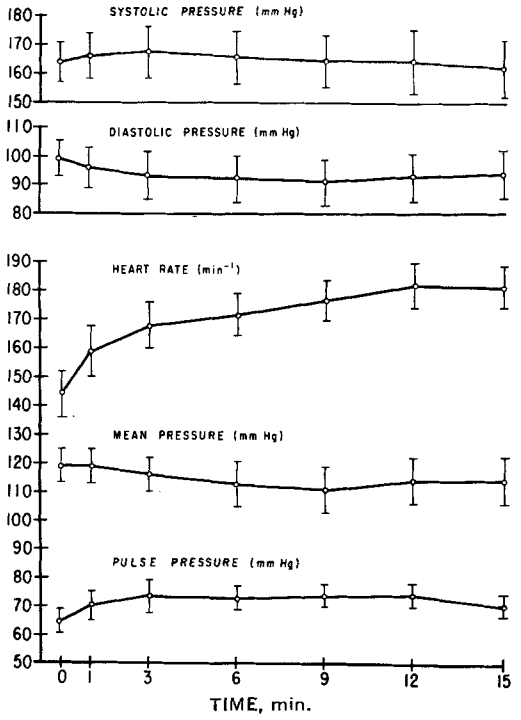

Fig. 3—Cardiovascular effects following 10 inhalations of metaproterenol sulfate (2%). The standard error is represented by vertical lines.

proterenol was observed on the heart rate which showed significant elevation at every observation time. Whereas the maximum increase in heart rate after isoproterenol inhalation was 7% and occurred 1 min. after the drug had been administered, the maximum increase following metaproterenol inhalation was 26% and occurred 12 min. after the drug had been administered.

REFERENCES

(1) Engelhardt, A., Hoefke, W., and Wick, H., *Drugs Made Germany*, **4**, 123(1961).
(2) Konzett, H., and Rossler, R., *Arch. Exptl. Pathol. Pharmakol.*, **195**, 71(1940).
(3) Widdicombe, J. G., *Physiol Rev.*, **43**, 1(1963).
(4) Familiar, R. G., Wardell, J. R., Jr., and Greene, L. C., *J. Pharm. Sci.*, **56**, 768(1967).
(5) Amdur, M. O., and Mead, J., *Am. J. Physiol.*, **192**, 364(1958).
(6) Diamond, L., *Arch. Intern. Pharmacodyn.*, **168**, 239 (1967).
(7) Aviado, D. M., and Patel, V. A., *ibid.*, **150**, 336(1964).
(8) Lovejoy, F. W., Constantine, H., and Dautrebande, L., *Proc. Soc. Exptl. Biol. Med.*, **103**, 836(1960).
(9) Robillard, E., Lepine, C., and Dautrebande, L., *Can. Med. J.*, **86**, 362(1962).
(10) Karczewski, W., and Dautrebande, L., *Arch. Intern. Pharmacodyn.*, **148**, 287(1964).

Keyphrases

Metaproterenol—β-adrenergic stimulant
Bronchodilatory effects—metaproterenol
Pulmonary resistance, complicance—determined
Cardiovascular effects—metaproterenol
Histamine antagonism—metaproterenol

Effect of Deuterium Oxide on the Culturing of *Penicillium janczewskii* II

Isolation of Fully Deuterated Griseofulvin

By D. A. NONA, M. I. BLAKE, H. L. CRESPI, and J. J. KATZ

Fully deuterated griseofulvin was isolated in pure form from *P. janczewskii* grown in a completely deuterated medium. Direct fermentation and a replacement culture technique were used. The isolated antibiotic was purified by thin-layer chromatography and characterized by ultraviolet, infrared, and nuclear magnetic resonance spectra. The extent of incorporation of deuterium during biosynthesis at the various proton sites in the molecule was determined.

In a previous paper (1) the effect of heavy water both on the growth of the mold *Penicillium janczewskii* and on its production of antibiotic was reported. Nutritional requirements for optimal growth in D_2O and the general culture techniques were described in detail. Griseofulvin production was severely impaired when the organism was grown in pure D_2O media by the usual surface culture method, but the griseofulvin titer was improved when the D_2O culture medium was supplemented with vitamin B complex. In the present study a replacement culture method was employed to produce deuterated griseoful-

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vin. Deuterated antibiotic biosynthesized by the organism in replacement culture was isolated, purified, and characterized by chemical means.

EXPERIMENTAL

Replacement Culture Method—The griseofulvin-producing strain *Penicillium janczewskii* (NRRL 2301) was used throughout this study. Mycelial contents of shake cultures grown in an H_2O medium were used as starting tissue for preparative replacement cultures. After 7 days' growth the vegetative tissue from 60 ml. of an H_2O medium culture contained in a 250-ml. conical flask was separated from the medium by filtration. The tissue retained by the filter was resuspended in 75 ml. of sterile D_2O and the suspension was again filtered. This process was repeated twice. Then the mycelium was dispersed in 60 ml. of deuterated medium. The composition and preparation of the nutrient medium have been described previously (1). Fermentation was allowed to take place in 250-ml. conical flasks stoppered with cotton and sealed with aluminum foil. Cultures were returned to the shaker and incubation resumed for a period of 4–6 weeks. All manipulations involving exposed cultures were performed in a bacteriologic hood.

Surface cultures were prepared by inoculating 500 ml. of H_2O medium contained in 3-L. conical flasks with a spore suspension of the organism. Replacement was effected after a 7-day growth period. The water medium was removed from beneath the floating mat by introducing the tip of a sterile 2-ml. pipet below the mat. A section of Tygon tubing was attached to the other end of the pipet, and the medium was carefully siphoned away. The underside of the mat was washed with 100 ml. of sterile D_2O added by slow infusion through a reservoir joined to the pipet by Tygon tubing. After swirling several times, the D_2O wash was siphoned away and fresh sterile heavy water was added. This procedure was repeated twice. After the third washing, 500 ml. of deuterated replacement medium was slowly introduced under the mycelial mat with the aid of a pipet. The fluid flow rate was carefully controlled by adjustment of the reservoir height. For successful preparation of a replacement culture it was essential that the mycelial mat remain floating and that there be no liquid on the upper surface of the mat. In surface culture antibiotic production does not occur when the mat sinks into the medium.

Analysis for deuterium content was performed periodically throughout the fermentation period by the technique of Crespi and Katz (2). At no time was the final deuterium oxide concentration observed to be more than 2% below the initial concentration, indicating that little isotopic dilution had occurred.

Extraction Procedure—At harvest, the mycelium was separated from the fermentor broth by filtration, and both the mycelium and filtrate were extracted for griseofulvin. The filtrate was extracted with chloroform in a 1-L. liquid–liquid extractor for 76 hr. Preliminary studies indicated that this length of time was required for complete extraction of the antibiotic. The chloroform extract was reduced in volume to about 10 ml. on a flash evaporator. Antibiotic was recovered from the concentrate in pure form by preparative thin-layer chromatography.

The mycelium was treated according to the procedure of Oxford *et al.* (3). After drying in an oven at 100° for 24 hr., the mycelium was reduced to a fine powder in a mortar and transferred to an extraction thimble. The thimble was placed in the extractor of a Soxhlet continuous extraction apparatus. The powdered mycelium was extracted with ether for 76 hr. The ether extract was evaporated to dryness in a flash evaporator, the residue redissolved in chloroform, and products separated by preparative thin-layer chromatography.

Preparative Thin-Layer Chromatography—Silica Gel G preparative thin-layer plates, 500 μ in thickness, were prepared with a Desaga variable gauge applicator. Concentrated chloroform extracts were streaked across the plate at the origin (a line parallel to one side of the plate and 3.5 cm. from the edge) with an automatic applicator and streaker (Rodder Instruments, Los Altos, Calif.). All chromatograms were spotted with a chloroform solution of reference standard griseofulvin to serve as a control. Plates were placed in a standard developing tank which was lined with filter paper to ensure saturation of the chamber. The developing solvent consisted of ether–acetone, 3:2. The development time was about 25 min., which was the time required for the solvent front to reach the end of the plate. Since griseofulvin fluoresces strongly, its location on the plate was readily detected by scanning with an ultraviolet lamp. The fluorescent band having an R_f value of 0.62 was scraped from the chromatographic plate with a razor blade. Griseofulvin adsorbed to the Silica Gel G was isolated by shaking at 200 r.p.m. for 30 min., with a mixture of ether–acetone, 1:3; the extract was recovered by centrifugation. The extract after filtration was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in chloroform and rechromatogramed on a preparative thin-layer plate using as the developing solvent, ether–acetone, 4:1. Griseofulvin was removed from the plate as described previously. A cream-colored powder of purified griseofulvin was obtained.

Silica Gel Chromatopile Technique—As an alternate procedure for obtaining purified griseofulvin from the fermentor broth and mycelium, a preparative chromatopile technique was also employed. A cylindrical form was shaped by rolling a section of chromatography paper to a dimension of 14 cm. in length and 3 cm. in diameter at the base. The paper form was covered with aluminum foil and placed on end. A thick suspension of Silica Gel G (75 Gm. of gel in 35 ml. of water) was poured into the cylindrical mold. After 3 hr. the aluminum foil was removed and the chromatopile was allowed to air dry overnight. Finally, the paper mold was peeled away exposing a cylindrical bar of Silica Gel G.

A concentrated solution of griseofulvin in chloroform was placed in a 50-ml. evaporating dish. The base of the chromatopile was dipped into the solution. When the solvent front reached a height of about 1.5 cm., the chromatopile was removed, air dried, and returned to the evaporating dish. This process was continued until the entire chloroform concentrate had been adsorbed on the lower 1.5 cm. of the chromatopile. The band of sample at

the origin was developed by dipping into a solvent mixture of ether–acetone (3:2) contained in a 50-ml. evaporating dish. Development was continued until the griseofulvin band reached a height of about 4 cm. as determined by fluorescence under ultraviolet light. The chromatopile was then placed into the TLC developing tank containing developing solvent, and development was continued for approximately 25 min., after which it was removed and air dried. The band containing the griseofulvin was sliced from the column with a razor blade, reduced to a powder, and extracted as described previously.

Infrared Spectrum—A 0.5% solution of griseofulvin in chloroform was examined over the 4,000 to 650 cm.$^{-1}$ spectral region in a Beckman spectrophotometer, model IR 7, equipped with a 0.1-mm. ultramicro cavity cell.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of a solution of griseofulvin in ethyl acetate was obtained with a Beckman DG-B grating spectrophotometer provided with a Sargent recorder (model SR).

Nuclear Magnetic Resonance Spectroscopy—NMR spectra of the griseofulvin samples prepared as 5% solutions in Silanar-C (deuteriochloroform with 1% TMS as internal standard; Merck Sharp and Dohme, Canada, Ltd.) were obtained on a Varian A-60 NMR spectrometer and a Varian HA-100 NMR spectrometer, equipped with a Varian C-1024 computer of average transients.

RESULTS AND DISCUSSION

Good growth does not necessarily imply good antibiotic production. It was noted, however, that good antibiotic production was always associated with good growth. The decreasing griseofulvin titers observed with increasing D_2O solvent concentrations were, for the most part, accompanied by inferior mycelial felts. A replacement culture method allows for the separate analysis of the effects of deuterium isotope replacement on vegetative growth and on griseofulvin production, and it provides a high degree of control over each phase. When the replacement medium consisted of pure D_2O and deuterio sugars, a fully deuterated antibiotic was obtained, and when pure D_2O and protio sugars were employed, the isolated griseofulvin was partially deuterated. Milligram amounts of fully deuterated griseofulvins were obtained from 500-ml. preparative replacement cultures. A comparison of antibiotic yields from varying culture conditions is listed in Table I. After the nutrient was replaced, the cultures were allowed to ferment for an additional 28 days. Both mycelium and fermentor broth were then extracted for antibiotic. Protio griseofulvin was not found to accumulate in mycelium grown in a protioculture before the fifteenth day of incubation. Therefore, if the protio medium is replaced with a deuterated medium before such a time, the mycelium originating from H_2O cultures may be extracted for deuterio griseofulvin at the conclusion of the fermentation period free of contamination with protio griseofulvin. The 50–60 days for maximum antibiotic production by direct fermentation can thereby be reduced to about 30 days by the replacement technique.

Since laboratory scale antibiotic production under the described conditions involves the isolation of small amounts of griseofulvin, the usual preparative procedures cited in the scientific and patent literature were not applicable in this study. A thin-layer chromatographic procedure was developed which served effectively to identify and separate the griseofulvin from its fermentation congeners. The method not only enabled the isolation of small quantities of pure griseofulvin, but was less tedious than solvent extraction and recrystallization techniques, and the small quantitites of griseofulvin produced were obtained in a chromatographically pure state. Purification of the extracts obtained from larger batches of mycelium and fermentor broth required at least five or six TLC plates. The large number of plates is needed because a good separation cannot be effected if the sample is very concentrated and the plates overloaded. The use of a chromatopile thus provided a second method for purification and had the advantage of adsorbing larger amounts of material without impairing separation.

TABLE I—GRISEOFULVIN PRODUCTION IN SURFACE CULTURE BY *P. janczewskii*

Medium	Growth Conditions	Fermentor Broth Titer, mcg./ml.	Recovery, mg./500 ml.
0% D_2O, protio sugars	Direct fermentation	110–120	13.0
99.6% D_2O, protio sugars and vitamins	Direct fermentation	40–50	4–6
99.6% D_2O, protio sugars	Replacement culture	45–50	4–6
99.6% D_2O, deuterio sugars and vitamins	Direct fermentation	45–50	5.2
99.6% D_2O, deuterio sugars	Replacement culture	45–50	4.6

Chloroform solutions of ordinary, partially deuterated, and fully deuterated griseofulvin prepared in this laboratory were characterized by infrared spectrometry. The fully deuterated griseofulvin samples were obtained by both direct fermentation and replacement culture with fully deuterated media. Partially deuterated griseofulvin was obtained from replacement cultures using pure D_2O and protio sugars. The spectra were compared with those obtained with chloroform solutions of authentic griseofulvin and spectral assignments were made according to the interpretation of Page and Staniforth (4).

Infrared spectra of protio griseofulvin isolated in this laboratory from H_2O cultures were identical to that of reference standard griseofulvin. The infrared spectra of chloroform solutions of fully deuterated griseofulvin were found to be identical to that of ordinary griseofulvin with one striking exception, namely, the replacement of the C—H absorption at 2,850 cm.$^{-1}$ by a C—D absorption at 2,120 cm.$^{-1}$. This is the usual isotope effect on the C—H stretch vibration, and the isotope shift confirms the incorporation of deuterium.

The NMR spectra of deuteriochloroform solutions of griseofulvin and selected analogs have been

examined and interpreted independently by Green *et al.* (5) and Arison *et al.* (6). These workers were able to assign each spectral line or group of lines to a particular proton or group of protons, and conversely, to correlate each proton with a particular line or group of lines. The spectra of partially deuterated compounds may be compared directly with the spectrum of the compound which contains only ordinary hydrogen, and in this way information can be obtained about the amount of deuterium present at each molecular site. The effects of the deuterons on the proton resonances may be observed indirectly, but the deuterons themselves will not contribute any signals to the recorded spectrum. Therefore, the situation that is created when the proton magnetic resonance bands are observed for partially deuterated griseofulvin is no different from that which is observed in the fully protiated griseofulvin. Since the NMR signal is proportional to the number of protons contributing to the resonance line in partially deuterated griseofulvin, the proton integral for a particular molecular position will be diminished by a factor which is proportional to the extent of proton replacement by deuterium. If all of the molecules in the collection that forms the sample are fully deuterated at a given position, no signal would be observed and the proton integral for the proton resonances of the position would be zero.

Fully Deuterated Griseofulvin—NMR spectra of griseofulvin samples obtained by direct fermentation and replacement cultures employing fully deuterated media showed a complete absence of resonance peaks at 6.15, 5.53, 4.04, 4.00, 3.63, 2.70, and 0.98 δ.[1] The only absorption peaks in the spectra were the TMS (standard reference signal) peak at 0, and a peak at 7.39 δ due to protons in the deuteriochloroform solvent. When the amplitude was adjusted to the upper limits of the Varian A-60, the noise level was understandably increased, but even so no absorption peaks were observed. These results indicate that the griseofulvin isolated under these fermentation conditions contains essentially no protons, and that there is complete isotopic replacement of hydrogen with deuterium.

High resolution spectra of the fully deuterated griseofulvin samples were then obtained on a Varian HA-100 spectrometer. The spectra obtained exhibited a complete absence of absorption peaks, confirming the spectral results obtained with the Varian A-60. Spectra were also obtained with a computer of average transients (C.A.T.). A readout after multiple scans showed only minor absorption in the C—CH_3 and CH_2—CH absorption region, and no other absorption peaks were observed. The proton resonance observed indicated the presence of some hydrogen in the molecule, but the amount was clearly very small. The origin of these protons is probably due to residual H_2O in the 99.6% D_2O used. From these NMR results it can be concluded that the griseofulvin produced is essentially fully deuterated.

Partially Deuterated Griseofulvin—The extent of deuterium incorporation into griseofulvin isolated from replacement cultures of partially deuterated media, composed of protioglucose (7%) and pure D_2O, was determined by NMR spectroscopy. The spectra obtained were compared directly to that for protio griseofulvin. Examination of the spectrum indicated that no significant chemical shift differences exist between ordinary and partially deuterated griseofulvin. Since the NMR signal integral is proportional to the number of protons contributing to the resonance line, the proton integral for a particular molecular position will be diminished by a factor which is proportional to the total number of nuclei contributing to the signal. By comparing the integrals for these areas to those of a standard signal by a known number of protons, the various regions of the spectrum were calibrated as to the number of protons which were represented.

Sample I was prepared by dissolving an exact amount of partially deuterated griseofulvin, which was obtained from replacement cultures, in exactly 300 μl. of $CDCl_3$ (Silinar-C.). The same quantity (accurately weighed) of ordinary hydrogen griseofulvin, isolated in this laboratory from H_2O cultures, was dissolved in the same volume of Silinar-C and designated as Sample II. The NMR spectra of the two samples were then obtained with a Varian A-60 spectrometer. A spectrum amplitude of 80 was required to obtain a readable spectrum with Sample I, whereas an amplitude of only 40 was needed for Sample II, reflecting the marked difference in proton content of the two samples. The proton content was quantitated by integrating the spectra. Because the volume of deuteriochloroform in each sample was exactly 300 μl., a comparison can be made of the area integrated under the absorption peak of deuteriochloroform arising from the small amount of residual $CHCl_3$ present in the $CDCl_3$. At an amplitude of 80 (Sample I) and 40 (Sample II) the area under the $CHCl_3$ absorption peak was represented by 15 units and 5 units, respectively. Therefore, if the griseofulvin molecule was fully protiated, the expected area under each absorption peak in the spectrum of Sample I (at an amplitude of 80) would be three times that of the spectrum of Sample II (at a spectrum amplitude of 40). Thus the areas under the resonance peaks can be normalized and the relative amounts of hydrogen at the various molecular positions in partially deuterated griseofulvin can be established.

Normalization shows that there is a considerable replacement of hydrogen by deuterium at various

Table II—Isotope Fractionation Factors for Partially Deuterated Griseofulvin Obtained by Biosynthesis in D_2O from Hydrogen Substrates

Resonance	Chemical Shift (δ, p.p.m.)	Partially Deuterated Griseofulvin—Expected Area[a] (Units)	Partially Deuterated Griseofulvin—Observed Area[a] (Units)	Isotope Fractionation Factor K_H/K_D
$CHCl_3$ (present in $CDCl_3$)	7.39	15	15	—
Aromatic *H*	6.15	54	11	0.26
Vinyl *H*	5.53	54	11	0.26
Aromatic OCH_3 (6-methoxyl)	4.04	75	17	0.30
Aromatic OCH_3 (4-methoxyl)	4.00	27	7	0.35
Vinyl OCH_3	3.63	42	10	0.31
CH_2—C*H*	2.70	54	18	0.50
C—CH_3	0.98	48	14	0.41

[a] Per proton, spectral amp. 80.

[1] Chemical shifts are given in parts per million relative to tetramethylsilane (TMS) with downfield shifts positive.

molecular positions in the partially deuterated griseofulvin (Table II), which results from the incorporation of deuterium during biosynthesis at the various proton sites in the molecule. The amount of deuterium incorporated varies with the particular proton site. From Table II it can be seen that replacement of hydrogen by deuterium is maximal at the aromatic and vinyl positions. These molecular sites apparently afford little opportunity for biosynthetic incorporation of proton from the protio substrate, and a maximum of solvent D_2O equilibration occurs at these loci. Decreasing amounts of isotopic substitution occur at the OCH_3, C—CH_3, and CH_2—CH positions, indicating that the protio sugars have a more extensive role in the biosynthetic mechanisms leading to the formation of these moieties. In addition, these sites probably have a decreased capability for equilibration with the solvent. The isotope fractionation factor K_H/K_D calculated as the average of all molecular positions in partially deuterated griseofulvin was found to be 0.36. This indicates a ratio of approximately one hydrogen atom for every three deuterium atoms in the molecule, and an average deuterium isotope replacement of about 75%.

CONCLUSIONS

Griseofulvin extracted from the mycelium and culture filtrates of *P. janczewskii* was purified by preparative thin-layer chromatography and characterized by visible, infrared and nuclear magnetic resonance spectra. Fully deuterated griseofulvin, the first fully deuterated antibiotic so far prepared, was obtained from organisms grown on a fully deuterated medium by direct fermentation. A yield of over 5 mg. was obtained from 500 ml. of culture medium. When a protio medium used for vegetative growth was replaced with a fully deuterated medium, fully deuterated griseofulvin was isolated. Approximately the same amount of antibiotic was obtained from 500 ml. of replacement medium. By this procedure the 50–60 days necessary for maximum deuterio griseofulvin production by direct fermentation could be reduced by about one-half. When the replacement medium was only partially deuterated (protio sugars and pure D_2O), an antibiotic was isolated which was deuterated in part. A yield of 4–6 mg. was obtained from 500 ml. of partially deuterated replacement medium.

Subsequent studies are concerned with the determination of the effect of deuterium on the antibiotic activity. If detoxification of griseofulvin requires rupture of carbon-to-hydrogen bonds it is expected that the deuterio analog should exhibit an enhanced activity since carbon-to-deuterium bonds are generally more stable. A statistical analysis of antifungal activity of deuterio griseofulvin will be the subject of the next report.

REFERENCES

(1) Nona, D. A., Blake, M. I., and Katz, J. J., *J. Pharm. Sci.*, **56**, 1063(1967).
(2) Crespi, H. L., and Katz, J. J., *Anal. Biochem.*, **2**, 274 (1961).
(3) Oxford, A. E., Raistrick, H., and Simonart, R., *Biochem. J.*, **33**, 240(1939).
(4) Page, J. E., and Staniforth, S. E., *J. Chem. Soc.*, **1952**, 1292.
(5) Green, G. F. H., Page, J. E., and Staniforth, S. E., *ibid.*, **1964**, 144.
(6) Arison, B. H., Wendler, N. L., Taub, D., Hoffsommer, R. D., Kuo, C. H., Slates, H. L., and Trenner, N. H., *J. Am. Chem. Soc.*, **85**, 627(1963).

Keyphrases

Penicillium janczewskii II—cultures
Deuterium oxide—*P. janczewskii II* cultures
Griseofulvin, fully deuterated—isolation
TLC—separation
Silica gel chromatohile—separation
NMR spectrometry—identity
IR spectrophotometry—structure
UV spectrophotometry—structure

Synthesis of 2-(*N*,*N*-Dialkylaminomethyl)-7-phenylthioindoles

By ROBERT L. DUNCAN, JR.* and JOHN ANDRAKO†

A series of 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles was prepared. The Japp-Klingemann reaction followed by Fisher cyclization with polyphosphoric acid gave 7-phenylthioindole-2-carboxylic acid. The lithium aluminum hydride reduction of amides derived from this acid provided the corresponding aminomethyl indoles. Infrared and NMR data are reported.

As part of a program directed at the synthesis and pharmacological evaluation of dialkylaminoalkyl-7-substituted indoles, the authors wish to report the synthesis of a series of 2-(*N*,*N*-dialkylaminomethyl)-7-phenylthioindoles (VI). Relatively few 7-substituted indoles and indole derivatives have been reported and there is an almost total lack of pharmacological data for compounds of this type. Hiremath and Siddappa (1) reported the synthesis of 5-methoxy-7-

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