Bunolol Metabolism by Dogs: Isolation and Identification of Two Acidic Metabolites

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Abstract □ The urine collected from three dogs for 24 hr. after oral administration of bunolol-^{14}C contained 61.2% of the administered radioactivity. An acidic metabolite, representing 16% of the urinary ^{14}C, was purified by ether extraction and preparative TLC. The methyl ester of the metabolite was prepared and submitted for analysis by mass spectrometry. The spectrum indicated the compound to be the methyl ester of 3-[(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)-oxy]lactic acid, and this compound was synthesized from 5-hydroxytetralone. The identity of the esterified urinary metabolite and the synthetic compound was established by mass spectrometry. A second unconjugated acid constituted 7% of the urinary ^{14}C. Its identity was established as [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)-oxy]acetic acid by synthesis and comparative TLC. The 2,4-dinitrophenylhydrazones and the methyl esters of the metabolite and the synthetic material were prepared; comparison of their R_f values confirmed the identity of the metabolite.

Keyphrases □ Bunolol, metabolites—isolation, identification, dog urine □ β-(5-Oxytetralonyl)lactic acid—isolation, identification, bunolol metabolite, dogs □ (5-Oxytetralonyl)acetic acid—isolation, identification, bunolol metabolite, dogs □ TLC—isolation, identification □ Mass spectroscopy—identification

A new, highly potent, β-adrenergic blocking agent, bunolol {*dl*-5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride}, was metabolized extensively following oral administration to dogs (1). Urine collected for 24 hr. following bunolol-^{14}C administration contained only 0.7% of the urinary radioactivity as the unaltered drug. An experimental survey of the different classes of bunolol metabolites indicated the presence of as many as 18 urinary metabolites. Among these metabolites were three unconjugated and five conjugated bases and four unconjugated and six conjugated acids. The present report describes the isolation and identification of two acidic metabolites of bunolol from dog urine; β-(5-oxytetralonyl)lactic acid and (5-oxytetralonyl)acetic acid.

MATERIALS AND METHODS

^{14}C-Labeled Bunolol—Bunolol was synthesized with ^{14}C in position 1 (carbonyl-C) of the saturated ring (2). The preparation was 99.0% pure, both chemically and radiochemically, as judged by TLC; it had a specific activity of 4.90 mc./g.

Radioactivity Counting—Quantitative assays for ^{14}C were performed using a liquid scintillation spectrometer[1]. The external standardization method was used for quench corrections.

TLC—Chromatograms for analytical purposes were run on 5 × 20-cm. glass plates coated with 250 μ of silica gel G bound with calcium sulfate. For preparative efforts, 20 × 20-cm. plates were used. One-dimensional chromatograms were developed using the following solvents: 1, *n*-butanol–29.6% ammonia–water (4:1:3, upper phase); 2, *n*-butanol–glacial acetic acid–ether–water (9:6:3:1); 3, *n*-butanol–glacial acetic acid–ether–water (6:3:9:1); 4, methanol–29.6% ammonia–water (12:1:7); 5, methanol–ethyl acetate (1:4); 6, heptane–chloroform–ethanol (1:3:1); 7, chloroform–glacial acetic acid–methanol (15:1:4); and 8, chloroform–glacial acetic acid–methanol (54:1:5). TLC plates were scanned for radioactivity with a radiochromatogram scanner[2]. The area under each peak was determined with a compensating polar planimeter[3]. In this manner, R_f values and relative amounts of each metabolite were determined.

Mass Spectrometry Studies—A mass spectrometer[4] with a direct insertion probe was used. The source temperature was 140–160°, the ionizing energy was 70 ev., and the trap current was 100 μamp. Exact masses were determined by peak matching.

Synthesis of Metabolites—The synthesis of Metabolite V was carried out in an unambiguous manner from 5-hydroxytetralone, sodium hydroxide, and β-chlorolactic acid, utilizing a general method reported previously (3). The synthesis of Metabolite VI was performed similarly, using α-bromoacetic acid in place of β-chlorolactic acid. Mass spectra were taken of these compounds.

Preparation of Methyl Esters of Urinary and Synthetic Metabolites—The procedure of Metcalfe and Schmitz (4) was applied to portions of the urinary and synthetic metabolites. Samples of the metabolites, purified by preparative TLC (40 mcg. to 10 mg.), were dissolved in 0.5 ml. of methanol and combined with 1 ml. of a 14% solution of boron trifluoride in methanol[5]. After boiling for 5 min., the methanol was allowed to evaporate to approximately 0.2 ml., and 5 ml. of distilled water was added to the reaction mixture. The methylated compounds were extracted three times with ether. The ether was evaporated and, for identification by TLC, the residues were dissolved in 0.2–1 ml. of methanol.

Preparation of 2,4-Dinitrophenylhydrazone of Metabolite VI—Aliquots of urinary and synthetic Metabolite VI were diluted to 0.5 ml. with ethanol. A slight excess of a solution of 2,4-dinitrophenylhydrazine in ethanol and 20 μl. of 12 *N* HCl were added. Each mixture was boiled for 30 min. in a water bath with occasional ethanol addition to compensate for evaporative losses. Then aliquots of the reaction mixture were chromatographed in parallel with the 2,4-dinitrophenylhydrazones of the synthetic compounds.

Protocol—Twenty-milligram portions of bunolol-^{14}C were weighed into three gelatin capsules, and enough carrier bunolol was added to provide 10-mg./kg. doses. The capsules were administered to female beagles weighing: Dog 1, 11.5 kg.; Dog 2, 11.5 kg.; and Dog 3, 13.0 kg. The dogs had free access to food and water. Urine collected from 0 to 24 hr. from each dog was kept frozen until needed for investigation. The 0–24-hr. urine samples from the three dogs were pooled before investigation and were identical with those used to determine the extent of bunolol excretion (1).

Purification and Esterification of Metabolite V—To obtain Metabolite V, a 390-ml. aliquot of urine, containing 94-mg. equivalents of bunolol, was adjusted to pH 1.8 and extracted seven times with one-half volumes of ether. The concentrated ether extract was chromatographed using Solvent 7. A band, 26 mm. wide at R_f 0.66, contained most of the ^{14}C; it was scraped from the plate and eluted with methanol. The concentrate was rechromatographed using Solvent 1. The 17-mm. radioactive band at R_f 0.30 was scraped and eluted similarly. The concentrated eluate was again chromatographed in Solvent 7. The radioactive band (16 mm. wide, R_f 0.57) was scraped from the plates. An aliquot of the eluted metabolite was methylated by the boron trifluoride method (4). The methyl ester was chromatographed using Solvent 1. A radioactive band (16 mm. wide, R_f 0.73) was scraped off the plates.

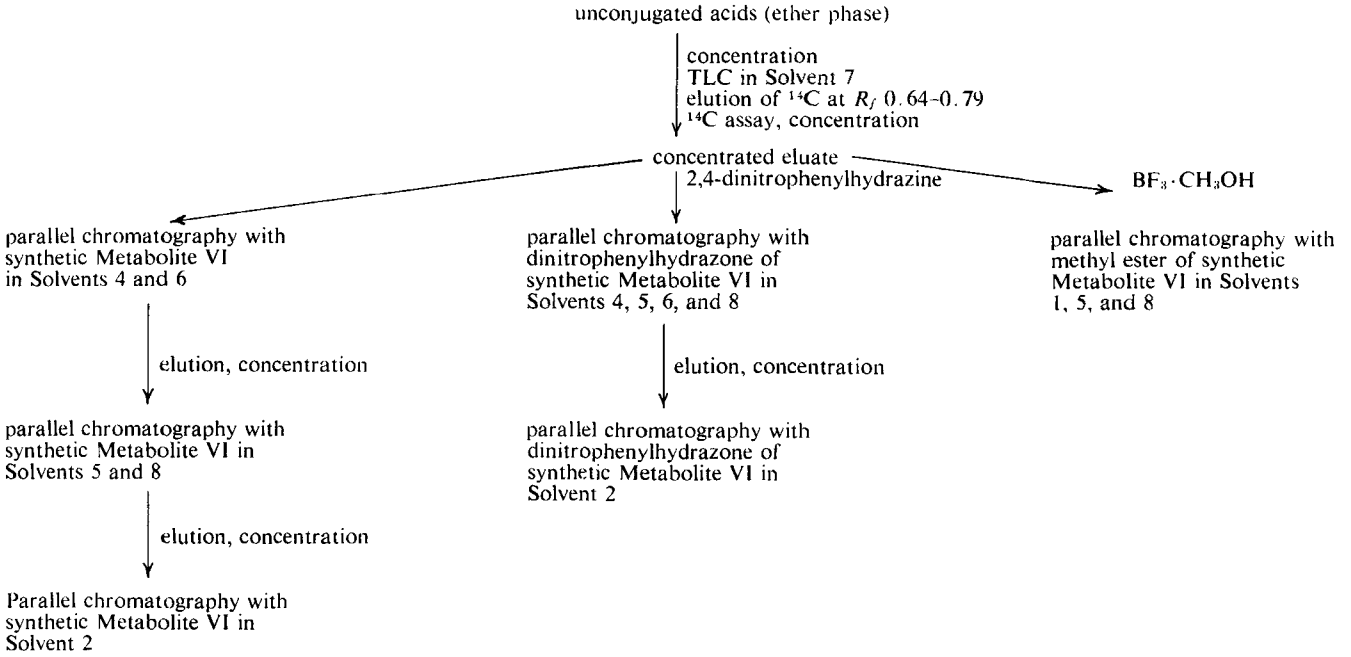
[1] Packard Tri-Carb model 3320.

[2] Packard model 7201.

[3] Keuffel and Esser.

[4] AEI model 902.

[5] Applied Science Laboratories, State College, Pa.



Scheme I—Chromatographic steps used to identify Metabolite VI in dog urine. Parallel chromatography refers to running two preparations side by side on the same plate. Parallel chromatography (as distinguished from cochromatography in which spotting is superimposed) permitted elution of only the radioactive material at the R_f of interest.

The radioactivity was eluted (total 3.77×10^6 d.p.m.), and the eluate was reduced to dryness. After drying *in vacuo* for 3 days, the sample weighed 3.0 mg. UV and mass spectra were taken on this preparation.

Quantity of Metabolite V in Dog Urine—A 56-ml. aliquot of the pooled urine was adjusted to pH 1.8 and extracted with ether. The ether phase contained 41.4% of the urinary radioactivity. An aliquot of the concentrated extract, containing 21,000 d.p.m., was chromatographed in Solvent 7. Radioactivity migrating in parallel to the synthetic compound was eluted and found to represent 41.8% of the ^{14}C in the ether extract. The eluate was concentrated and rechromatographed in Solvent 1. Most of the radioactivity (90.9%) moved parallel to the synthetic compound. The radioactivity was again eluted and rechromatographed in Solvent 2.

Search for Conjugated Metabolite V—For this purpose, the acidified urine after ether extraction was adjusted to 10.5. After extraction with ether to remove unconjugated bases, the aqueous phase was aerated to remove traces of ether. After adjusting the pH to 6.6, 0.46 ml. of a preparation containing 44,000 Fishman units of β-glucuronidase and 23,000 Fishman units of aryl sulfatase[6] was added. Following 16 hr. of incubation, an additional 0.46 ml. of the enzyme preparation was added, and the incubation was continued for 7 hr. Then the pH was adjusted to 1.8, and the solution was extracted three times with two volumes of ether. The ether was evaporated and the residue was taken up in methanol. This fraction represented 4.4% of the urinary ^{14}C. Aliquots of this sample were streaked onto 20 × 20-cm. TLC plates, which were developed previously in methanol and heated in an oven at 140° for 1–2 hr. After development in Solvent 7 and drying, a 5-cm. strip was cut from each plate and the ^{14}C was located by radioscanning. A radioactive band, R_f 0.54, representing 55% of the ^{14}C due to the conjugated acids, was scraped from the plate and eluted with methanol. Preparative TLC was applied to the eluate using Solvent 1. A radioactive band, R_f 0.24, represented 35% of the ^{14}C present on the plate. Chromatography of the eluate from this purification step with synthetic Metabolite V in Solvents 7 and 3 indicated that 74 and 73% of the ^{14}C, respectively, migrated as one peak parallel to the synthetic compound. This metabolite corresponded to 0.6% of the urinary radioactivity. The metabolite thus isolated was chromatographed in four solvents side by side with synthetic Metabolite V.

[6] Calbiochem.

Isolation of Metabolite VI from Urine—The chromatographic procedure used to isolate Metabolite VI is outlined in Scheme I. A 1-ml. aliquot from the urine pool used for the purification of Metabolite V was adjusted to pH 10.5 and extracted three times with 4 ml. of ether. The aqueous layer was adjusted to pH 1.8 and again extracted three times with ether. The ether extract was concentrated, and aliquots were chromatographed in Solvent 7. One radioactive peak, R_f 0.7, was noted to be similar to that of a reference compound, [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)oxy]acetic acid. The chromatography of the ether extract was, therefore, carried out side by side with this synthetic compound, designated tentatively as synthetic Metabolite VI. The radioactive peak, corresponding to that of synthetic Metabolite VI (R_f 0.64–0.79), was scraped off the plate and eluted. The concentrate of this eluate was rechromatographed in parallel with the synthetic Metabolite VI in Solvents 4 and 6. Both chromatograms yielded single radioactive peaks which chromatographed side by side with synthetic Metabolite VI. The radioactive peaks were eluted from both chromatograms. The pooled eluate was concentrated and rechromatographed in parallel with synthetic Metabolite VI in Solvents 5 and 8. Most of the radioactivity migrated the same distance as the synthetic Metabolite VI. The elution of the tagged metabolite was repeated, and the pooled concentrate was rechromatographed in Solvent 2. Only one radioactive peak was found.

Quantity of Metabolite VI in Dog Urine—To estimate the quantity of urinary Metabolite VI, an aliquot of the pooled urine was acidified and extracted with ether. The ether phase was the same sample as that employed for estimating the quantities of urinary Metab-

Table I—Purification Scheme for Metabolite V

Purification Step	Recovery of Radioactivity, d.p.m. × 10^6
Unfractionated urine	171.28
Ether extract (pH 1.8)	50.91
Eluate from TLC, Solvent 7	28.74
Eluate from TLC, Solvent 1	19.18
Eluate from TLC, Solvent 7	8.47
Esterified sample: eluate from TLC, Solvent 1	5.89[a]

[a] Corrected for aliquot taken at previous step.

Table II—Molar Absorptivities of the Methyl Ester of Metabolite V and Bunolol (Free Base)

Compound	Molar Absorptivity[a] (ϵ) at 221 nm.	253 nm.	310 nm.
Metabolite V methyl ester	35,700	10,900	2,600
Bunolol	25,800	9,400	2,500

[a] Solutions employed were 0.06 m*M* in methanol.

Table III—R_f Values of Metabolite V and the Synthetic Compound

Solvent	Metabolite V[a]	Synthetic Compound[b]	Methyl Esters: Metabolite V[a]	Methyl Esters: Synthetic Compound[b]
1	0.32	0.28	0.76	0.76
5	0.02	0.11	0.73	0.75
7	0.50	0.51	0.89	0.87

[a] Detection by radioscanning. [b] Detection by fluorescence under UV ight.

olite V. The volume was reduced to near dryness, and a few drops of methanol were added. The relative amount of Metabolite VI was determined by repeatedly chromatographing this sample side by side with the synthetic sample, with subsequent determination of the relative peak area and elution.

RESULTS

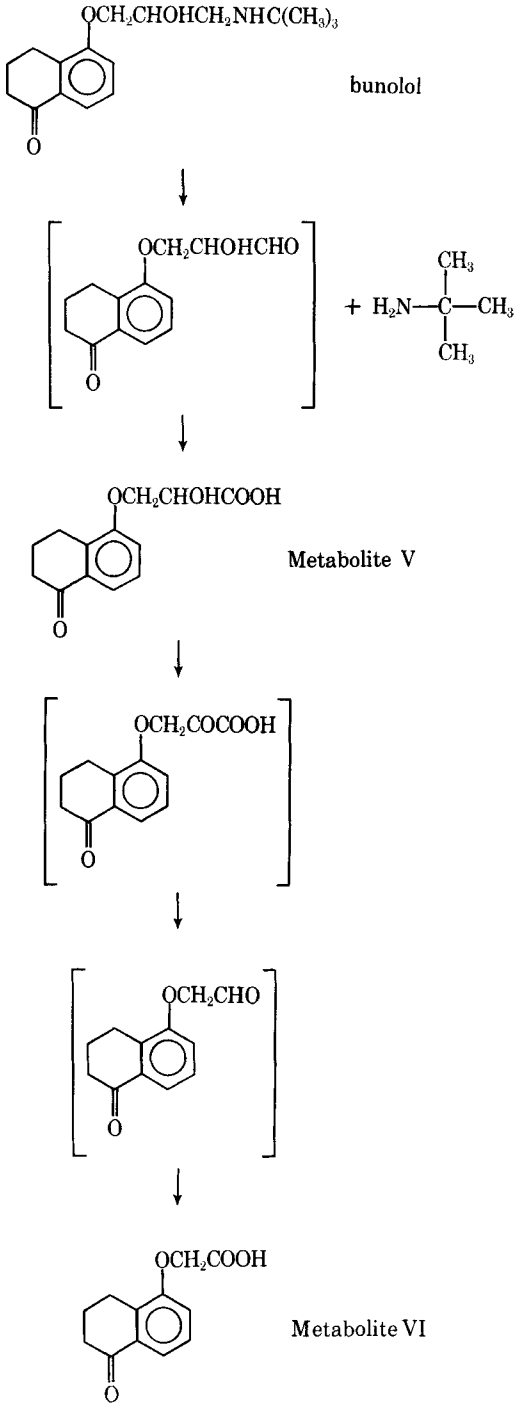
Isolation and Identification of Metabolite V—Table I shows the recoveries at various stages of the isolation of esterified Metabolite V from the urine of dogs treated with bunolol. From the specific radioactivity of bunolol (528,000 d.p.m./μmole), the 3.0 mg. of isolated methyl ester was judged to be approximately 64% pure. When a sample of the free acid was rechromatographed to the point of radiochemical purity, it was found to represent 15.7% of the urinary radioactivity.

The method used to purify Metabolite V was based upon its acidic character, a point confirmed by synthesizing the methyl ester of the metabolite. As indicated in Table II, the UV absorption of the methyl ester was not greatly different from the spectrum of bunolol; this observation suggested that the tetralone ring system was intact.

The mass spectrum of the methyl ester of Metabolite V showed the presence of peaks at *m/e* 264 and 161. The *m/e* 161 peak represented the 5-oxytetralone fragment. The precise mass of the derivatized metabolite was *m/e* 264.101. The presence of the 5-oxytetralone moiety and the methyl ester group suggested that the urinary isolate was β-(5-oxytetralonyl)lactic acid methyl ester ($C_{14}H_{16}O_5$), which has a calculated precise mass of 264.100. After this lactate was synthesized, its mass spectrum was taken and found to exhibit peaks at *m/e* 161 and 264, which were in the same intensity ratio as for esterified urinary Metabolite V. The synthetic compound showed fewer peaks than the urinary isolate, indicating contamination of the latter. The precise mass of the molecular ion of the synthetic ester was *m/e* 264.098. The agreement of the values for the molecular ions of the urinary and the synthetic samples indicates their identity. This identification was confirmed by parallel chromatography of Metabolite V and the synthetic lactic acid and of the methyl esters of both (Table III).

Table IV—R_f Values of an Acid Metabolite Isolated from the Fraction Containing the Conjugated Acid Metabolites; R_f Values of the Metabolite and Its Methyl Ester are Compared with Those of Synthetic Metabolite V and Its Methyl Ester

Solvent	Urinary Metabolite	Synthetic Metabolite V	Methyl Ester: Urinary Metabolite	Methyl Ester: Synthetic Metabolite V
1	0.26	0.30	0.81	0.77
3	0.74	0.71	—	—
5	0	0.08	0.85	0.83
7	0.40	0.43	0.89	0.85



Scheme II

The effort to establish the presence of a glucuronide of Metabolite V in dog urine ended equivocally. Although the urinary aglycone and its methyl ester migrated as their synthetic counterparts in several TLC systems (Table IV), the recovery was so low (0.6% of the urinary ^{14}C) that it might represent unconjugated Metabolite V attributable to incomplete extraction with ether.

Isolation and Identification of Metabolite VI—Metabolite VI was found in the acidic fraction, and its carboxylic character was confirmed by esterification. The presence of an active carbonyl group in the molecule was evident from 2,4-dinitrophenylhydrazone formation. The metabolite was identified as [(5,6,7,8-tetrahydro-5-oxo-1-naphthyl)oxy]acetic acid on the basis of comparative TLC with the synthetic compound and by comparative TLC of its methyl

Table V—R_f Values of Urinary and Synthetic Metabolite VI and the Corresponding 2,4-Dinitrophenylhydrazones and Methyl Esters

Solvent	Urinary Metabolite VI[a]	Synthetic Metabolite VI[b]	2,4-Dinitrophenylhydrazones: Urinary Metabolite VI[a]	2,4-Dinitrophenylhydrazones: Synthetic Metabolite VI[c]	Methyl Esters: Urinary Metabolite VI[a]	Methyl Esters: Synthetic Metabolite VI[b]
1	—	—	—	—	0.75	0.72
2	0.90	0.90	0.97	0.96	—	—
4	0.91	0.92	0.05	0.0	—	—
5	0.25	0.22	0.86	0.83	0.75	0.75
6	0.04	0.04	0.78	0.84	—	—
8	0.47	0.51	0.95	0.94	0.85	0.82

[a] Detection by radioscanning. [b] Detection by UV fluorescence or iodine vapors. [c] Detection by its yellow color.

ester and 2,4-dinitrophenylhydrazone with the corresponding derivatives of the synthetic acid (Table V).

Radioactivity, migrating in parallel with synthetic Metabolite VI in Solvent 7, was eluted and found to represent 17.1% of the radioactivity in the ether extract. The eluate was concentrated and rechromatographed in Solvent 1. Only one radioactive peak was noted. After elution, rechromatography in Solvent 2 indicated that the metabolite was radiochemically pure. Urinary Metabolite VI thus represents 7.1% of the urinary radioactivity.

Search for Conjugated Metabolite VI—A thorough examination disclosed no Metabolite VI in the ether fraction containing the aglycones of conjugated acidic metabolites.

DISCUSSION

The two unconjugated acid metabolites found in dog urine bear a close structural relationship. It appears likely that Metabolites V and VI are linked by a common pathway, as shown in Scheme II.

The initial attack upon bunolol is considered to be oxidative dealkylation of the side chain with the formation of tertiary butylamine and a lactic aldehyde. Oxidation of the aldehyde intermediate would yield Metabolite V. We are postulating that Metabolite VI may be formed by three subsequent reactions: (*a*) oxidation of the secondary alcohol to carbonyl, (*b*) decarboxylation of the α-keto acid to an aldehyde, and (*c*) oxidation of the aldehyde to a carboxyl group.

Metabolism studies with propranolol and pronethalol also revealed the formation of carboxylic end-products resulting from oxidative attacks upon the side chains. Hydroxy acids were the principal urinary metabolites from both of these β-adrenergic blocking agents. Propranolol was converted into a naphthoxylactic acid analogous to bunolol Metabolite V in man (5, 6) and in four rodent species (6). Similarly, pronethalol yielded 2-naphthylglycollic acid in man and three rodent species (7). Subsequent oxidative decarboxylation shortened the side chain of pronethalol (7) in the manner observed to produce Metabolite VI from bunolol, and this observation leads us to consider that the preceding sequence of bunolol degradation observed in the dog may occur in other animal species and in man.

REFERENCES

(1) F.-J. Leinweber, L. J. Haynes, M. C. Crew, and F. J. Di Carlo, *J. Pharm. Sci.*, **60,** 1512(1971).

(2) E. J. Merrill, *ibid.*, **60,** 1589(1971).

(3) C. F. Schwender, S. Farber, C. Blaum, and J. Shavel, Jr., *J. Med. Chem.*, **13,** 684(1970).

(4) L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, **33,** 363 (1961).

(5) P. A. Bond, *Nature*, **213,** 721(1967).

(6) J. W. Paterson, M. E. Conolly, C. T. Dollery, A. Hayes, and R. G. Cooper, *Pharmacol. Clin.*, **2,** 127(1970).

(7) P. A. Bond and R. Howe, *Biochem. Pharmacol.*, **16,** 1261 (1967).

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