were assayed microbiologically for AN factor. The data obtained are summarized in Table VI. The contents of plates 35–42 were combined and concentrated *in vacuo* to dryness. The residuum which weighed 26 mg. contained 400,000 μg. of AN factor as biotin/g. This material was dissolved in 0.5 ml. of water and the solution clouded with acetone. The colorless crystalline material which formed on standing melted at 236–238° and contained by microbiological assay with *Neurospora crassa* about 750,000 μg. of AN factor as biotin/g.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES, SHARP AND DOHME DIVISION AND CHEMICAL DIVISION, MERCK & CO., INC.]

Biotin *l*-Sulfoxide. III. The Characterization of Biotin *l*-Sulfoxide from a Microbiological Source

BY LEMUEL D. WRIGHT, EMLEN L. CRESSON, JOHN VALIANT, DONALD E. WOLF AND KARL FOLKERS

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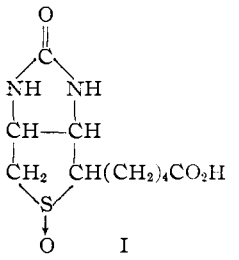
Crystalline AN factor isolated from *Aspergillus niger* culture filtrate, where growth had taken place in the presence of added pimelic acid, has been identified as biotin *l*-sulfoxide. Characterization involved a number of chemical and microbiological comparisons of the isolated material with an authentic sample of the sulfoxide.

The AN factor[1,2] as encountered in culture filtrates or partially purified concentrates was found to have a characteristic spectrum of microbiological activity, to have the same acid strength as biotin, and to have the cyclic urea ring intact as evidenced by avidin combinability.[3] Under appropriate conditions the factor could be reduced to biotin or to desthiobiotin. Acid hydrolysis yielded small amounts of biotin or "biotin sulfoxide" as determined by microbiological assay. Alkaline hydrolysis led to complete inactivation of the factor. These properties were such as to suggest that the AN factor is quite similar to biotin, differing only in the state of oxidation of the sulfur atom. Three such derivatives of biotin have been described in the literature, only one of which has previously been isolated from natural material. Biotin sulfone has been prepared and studied microbiologically.[4] This compound was found in the present study to be an antimetabolite of biotin against *Neurospora crassa* and for this reason could be dismissed from consideration as the AN factor. Two isomeric forms of biotin sulfoxide (I) have been described.[5]



I

The two sulfoxides designated as the *dextro*- and *levo*-forms[6] were prepared by the oxidation of biotin with an equivalent of hydrogen peroxide. The *dextro*-form which is obtained in the larger amount in the oxidation is as active as biotin for *Lactobacillus arabinosus* and *Saccharomyces cerevisiae*. This isomer was also isolated from milk.[5] The *levo*-form of biotin sulfoxide is reported to be only about 5% as active as biotin for *Lactobacillus arabinosus* and less than one-thousandth as active as biotin for *Saccharomyces cerevisiae*.[5] It has not been isolated previously from natural sources.

In the present investigation attention was directed to the sulfoxides of biotin since in some bioautographic studies of biotin, using *Neurospora crassa* as the assay organism, the presence of two sulfoxides of biotin could be observed. These presumably originate by oxidation of biotin during the filter paper chromatography. One of these, occurring in the smaller amount, had an R_f value in 1-butanol–acetic acid–water identical with that of the AN factor. Through the courtesy of Dr. D. B. Melville, samples of the *dextro*- and *levo*-isomers of biotin sulfoxide have been obtained for chemical and microbiological examination. With the availability of the AN factor in crystalline form, it has been possible to make a number of chemical and microbiological comparisons with the two biotin sulfoxides. These comparisons, described in this paper, demonstrate conclusively that the AN factor and biotin *l*-sulfoxide are identical.

The crystalline AN factor and authentic biotin *l*-sulfoxide were found to have the same melting point which was not depressed when the two materials were mixed. The infrared absorption spectrum of the isolated substance corresponds with that of the known compound. The two compounds have the same spectrum of microbiological activity against a number of organisms. R_f values for the AN factor and biotin *l*-sulfoxide in a number of solvents are the same. The two compounds are equally labile to acid and alkaline hydrolysis. Although a comparison of crystalline AN factor with the AN factor-activity as it occurs in culture filtrates was of necessity less direct and dependent entirely upon the results of microbiological evaluations, the factor in culture filtrates has a spectrum of microbiological activity corresponding with that of the isolated substance. R_f values of the factor in culture filtrate agreed by one dimensional paper

(1) L. D. Wright and E. L. Cresson, THIS JOURNAL, **76**, 4156 (1954).

(2) L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf and K. Folkers, *ibid.*, **76**, 4160 (1954).

(3) V. du Vigneaud, K. Dittmer, K. Hofmann and D. B. Melville, *Proc. Soc. Expil. Biol. Med.*, **50**, 374 (1942).

(4) K. Dittmer and V. du Vigneaud, *Science*, **100**, 129 (1944).

(5) D. B. Melville, D. S. Genghof and J. M. Lee, *Federation Proc.*, **9**, 204 (1950); D. B. Melville, *J. Biol. Chem.*, **208**, 495 (1954); D. B. Melville, D. S. Genghof and J. M. Lee, *ibid.*, **208**, 503 (1954).

(6) This nomenclature refers to the optical rotation of the sulfoxides and is not necessarily related to their spatial configuration.

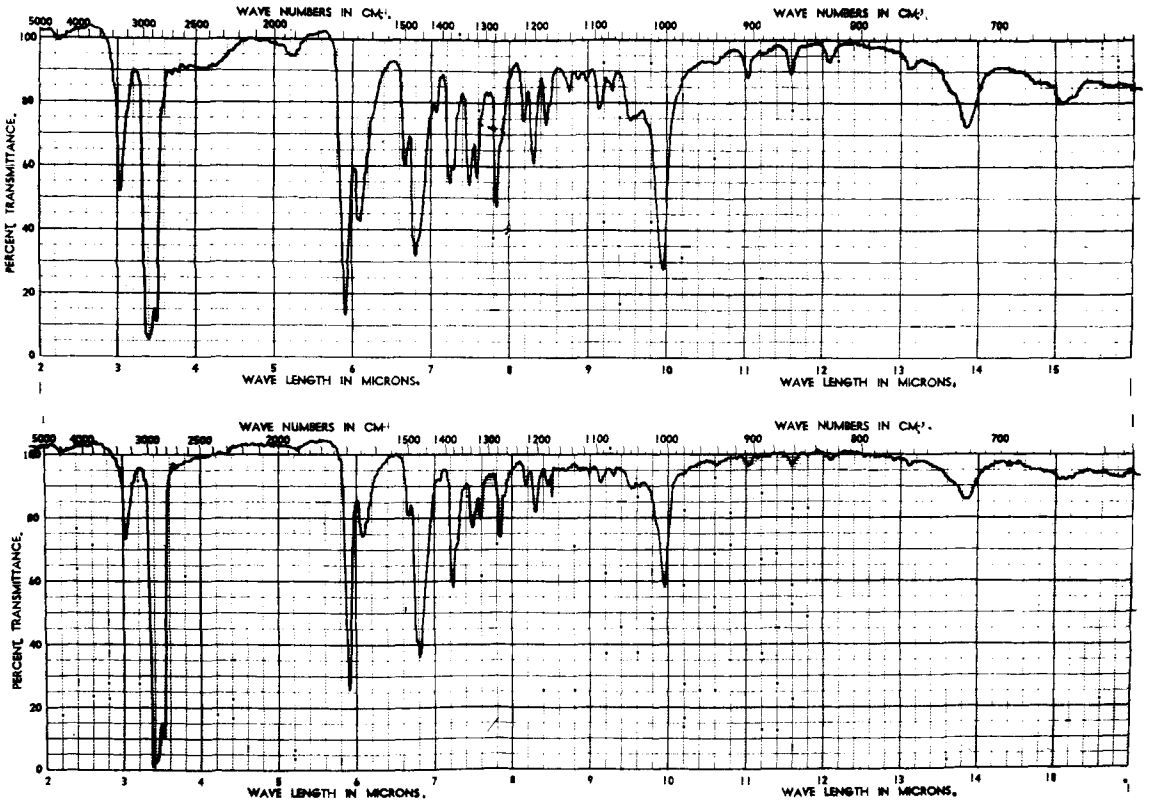


Fig. 1.—Upper curve, infrared spectrum of biotin *l*-sulfoxide. Lower curve, infrared spectrum of biotin *l*-sulfoxide isolated from *Aspergillus niger* culture filtrates.

chromatography with those of the isolated compound in a number of systems. Where agreement was not good, presumably because of interference by extraneous matter of the culture filtrate, it could be shown that a combination of culture filtrate and authentic biotin *l*-sulfoxide migrate as an entity. Agreement was obtained readily by carrying out a preliminary separation of the microbiologically active component from extraneous matter by paper chromatography of the culture filtrate in one direction prior to comparison with the authentic sulfoxide at right angles to that of the preliminary separation.

Consideration has been given to the possibility that biotin *l*-sulfoxide is an artifact of oxidation as a consequence of aeration which favors growth of *Aspergillus niger*. Even though aeration brought about an increase in the AN factor activity, the factor was present in culture filtrates which had not been aerated and which were not supplemented with pimelic acid.[1] As noted earlier, oxidation of biotin with peroxide yields, as expected theoretically, a mixture of the two isomeric sulfoxides. The *dextro*-form is the predominant product of this *in vitro* oxidation. A number of bioautographic studies of *Aspergillus niger* culture filtrates obtained after growth with or without added pimelic acid and AN factor concentrates of varying degrees of potency have failed to show the existence in these filtrates or concentrates of detectable amounts of biotin *d*-sulfoxide. In addition, an experiment was conducted in which biotin, in an amount equivalent to the AN factor usually found, was added to sterile *Aspergillus niger* culture medium at the *p*H which exists after growth has taken place in the presence of pimelic acid. The mixture was shaken under conditions simulating growth with respect to both time and temperature. Differential microbiological assays and bioautography of the solution showed the existence of unchanged biotin. Furthermore, when a similar amount of biotin was added to an *Aspergillus niger* fermentation in lieu of pimelic acid, differential microbiological assays and bioautography of the final culture filtrate indicated that the added biotin was quantitatively changed to biotin *l*-sulfoxide. Finally, if a similar amount of biotin was added to a completed *Aspergillus niger* fermentation, the mixture autoclaved to stop all growth and enzymatic activity, and the flask then shaken under conditions simulating growth with respect to both time and temperature, differential microbiological assays and bioautography of the filtrate showed the existence of unchanged biotin. It would appear, therefore, that biotin *l*-sulfoxide originates in the present studies from an enzymatic reaction and is not an artifact of chemical oxidation by air or a heat stable component of the culture. Originating as it does from an enzymatic reaction it may be presumed to have metabolic significance.

Experimental

Melting Points.—As noted in a previous paper,[2] crystalline AN factor melts at 236–238° (micro-stage). Biotin *l*-sulfoxide was found to melt at 236–238°. This melting point was not depressed when the two samples were mixed.

Infrared Absorption Data.—The infrared absorption spectra[7] of the crystalline AN factor and authentic biotin *l*-sulfoxide were determined as Nujol mulls using a Baird spectrophotometer. Principal absorption bands of the two samples are in good agreement (see Fig. 1).

Microbiological Procedures.—The organisms and procedure employed here are the same as described in previous papers of this series.[1,2] Data demonstrating that the isolated crystalline material has a spectrum of microbiological activity corresponding to biotin *l*-sulfoxide and different from that of biotin *d*-sulfoxide are summarized in Table I.

TABLE I

MICROBIOLOGICAL SPECTRA OF ACTIVITY OF ISOLATED CRYSTALS AND THE SULFOXIDES OF BIOTIN

Organism	Biotin activity[a] Biotin *d*-sulfoxide	Isolated crystals or Biotin *l*-sulfoxide
Neurospora crassa	100	100
Saccharomyces cerevisiae	100	0.001–0.1[b]
Lactobacillus arabinosus	100	0–5[c]
Lactobacillus casei	0	1–5[d]
Leuconostoc dextranicum	*ca.* 75	0

[a] Biotin = 100. [b] Value obtained is dependent upon the level of assay. 0.001% at 0.01–0.04 μg./tube, 0.1% at 0.0001–0.0004 μg./tube. [c] Value obtained is dependent upon the incubation period. Essentially inactive at 16–18 hours. Utilization up to 5% only after 48 hours. [d] Value obtained is dependent upon method of testing. The higher value obtained in the presence of "strepogenin."

Bioautography.—Quantities of 0.01 μg. of isolated crystalline material and 0.01 μg. of biotin *l*-sulfoxide were chromatographed on Whatman No. 1 paper. Details of the paper chromatography are described in a previous paper.[1] Localization of activity was obtained either on seeded plates with *Lactobacillus arabinosus* for which the factor has about 5% of the activity of biotin or by cutting the chromatograms into sections which were assayed for activity with *Neurospora crassa* as described previously.[1] The data of Table II indicate agreement between the corresponding R_f values of the isolated crystalline material and the synthesized product. Although the data are not shown, biotin *l*-sulfoxide and biotin *d*-sulfoxide were found to have corresponding R_f values in all solvents studied with the exception of the 1-butanol–acetic acid–water mixture where the R_f values were 0.46 and 0.58, respectively.

TABLE II

R_f VALUES OF ISOLATED CRYSTALLINE MATERIAL AND BIOTIN *l*-SULFOXIDE

Solvent system	R_f values Isolated crystals	Biotin *l*-sulfoxide
1-Butanol (40%), water (50%), acetic acid (10%)	0.46[a]	0.46[a]
Phenol (sat. with H_2O)	.85[b]	.85[b]
Ethanol (50%), water (50%)	.82[b]	.81[b]
Isopropanol (50%), water (50%)	.77[b]	.76[b]
Pyridine (70%), water (30%)	.63[b]	.63[b]
Isopropanol (70%), water (30%)	.46[b]	.45[b]
Pyridine (anhyd.)	.00[b]	.00[b]

[a] One dimensional paper chromatography, localization of activity with *Neurospora crassa*. [b] One dimensional paper chromatography, localization of activity with *Lactobacillus arabinosus*.

Comparisons of the microbiological activity of *Aspergillus niger* culture filtrate with biotin *l*-sulfoxide were carried out as described above except that, in some instances, a preliminary separation of activity from much extraneous material was carried out by chromatography in 50% ethanol, benzyl alcohol (saturated with water) or phenol (saturated with water) prior to the final comparison against the reference sulfoxide. The data summarized in Table III show that the microbiological activity of *Aspergillus niger* culture filtrates has the same R_f value in a number of solvent systems as does biotin *l*-sulfoxide.

TABLE III

R_f VALUES OF *Aspergillus niger* CULTURE FILTRATE AND BIOTIN *l*-SULFOXIDE

Solvent system	R_f values Biotin *l*-sulfoxide	Culture filtrate
Phenol (satd. with H_2O)	0.83[a]	0.83[a]
Ethanol (50%), water (50%)	.80[a]	.75[a]
Ethanol (50%), water (50%)	.75[b]	.71[b]
Isopropyl alc. (50%), water (50%)	.71[a]	.78[a]
Isopropyl alc. (50%), water (50%)	.70[d]	.70[d]
Isopropyl alc. (70%), water (30%)	.41[d]	.39[d]
Isopropyl alc. (70%), water (30%)	.41[c]	.46[c]
Pyridine (70%), water (30%)	.56[a]	.58[a]
1-Butanol (40%), water (50%), acetic acid (10%)	.49[a]	.45[a]
Isoamyl alcohol (satd. with H_2O)	.03[a]	.08[a]
Isobutyric acid (satd. with H_2O)	.74[a]	.72[a]
Isobutyric acid (satd. with H_2O)	.70[b]	.69[b]
1-Butanol (satd. with H_2O)	.03[c]	.03[c]

[a] One dimensional paper chromatography, localization of activity with *Lactobacillus arabinosus*. [b] Two dimensional paper chromatography, localization of activity with *Lactobacillus arabinosus*. Preliminary chromatography in ethanol–water (1:1). [c] Two dimensional paper chromatography, localization of activity with *Lactobacillus arabinosus*. Preliminary chromatography in benzyl alcohol (satd. with water). [d] Two dimensional paper chromatography, localization of activity with *Lactobacillus arabinosus*. Preliminary chromatography in phenol (satd. with water).

Acid and Alkali Stability.—These determinations were carried out as described previously[1] on 1-μg. quantities of crystalline AN factor or biotin *l*-sulfoxide. The data summarized in Table IV and Table V indicate that the crystalline material and biotin *l*-sulfoxide have the same stabilities to acid and alkali at 120° for one hour. No significance is attached to the fact that both compounds appear to be

TABLE IV

ACID STABILITY OF ISOLATED CRYSTALLINE MATERIAL AND BIOTIN *l*-SULFOXIDE

Acid, *N*	Apparent biotin content assay *Neurospora crassa* Isolated crystals, μg./ml.	*Neurospora crassa* Biotin *l*-sulfoxide, μg./ml.	*Lactobacillus arabinosus* Isolated crystals, μg./ml.	*Lactobacillus arabinosus* Biotin *l*-sulfoxide, μg./ml.
0	0.75[a]	0.88[a]	0.049	0.046
0.6	.74	.68	.053	.049
1.2	.75	.70	.072	.070
3	.47	.45	.111	.105
6	.23	.24	.034	.030
9	.15	.19	.022	.021

[a] Theoretical content = 0.94 μg./ml., assuming that biotin *l*-sulfoxide is 100% as active as biotin for *Neurospora crassa*.

TABLE V

ALKALI STABILITY OF ISOLATED CRYSTALLINE MATERIAL AND BIOTIN *l*-SULFOXIDE

Alkali, *N*	Activity destroyed by assay *Neurospora crassa* Isolated crystals, %	*Neurospora crassa* Biotin *l*-sulfoxide, %	*Lactobacillus arabinosus* Isolated crystals, %	*Lactobacillus arabinosus* Biotin *l*-sulfoxide, %
0.3	52	53	40	34
0.6	67	64	47	42
1.5	86	85	64	62
3.0	96	95	83	82
4.5	99	99	96	95

(7) We are indebted to Mr. Robert W. Walker of the Physical Chemistry Laboratories of the Chemical Division, Merck & Co., Inc., Rahway, New Jersey, for these data.

somewhat more stable to alkaline hydrolysis as determined by *Lactobacillus arabinosus* assay than by *Neurospora crassa* assay.

Biotin in Relation to the Metabolic Activity of *Aspergillus niger*.—A 500-ml. amount of *Aspergillus niger* medium (no added pimelic acid) was brought to *p*H 2.5 which is the *p*H that results from the growth of *Aspergillus niger* under the conditions that have been described. Biotin in an amount of 25 μg. was added (0.050 μg./ml.), the mixture was autoclaved and then shaken at 30° for 5 days. At the end of this time differential assay (*Neurospora crassa* and *Lactobacillus arabinosus*) as well as bioautography (paper chromatography with butanol–acetic acid–water and localization of activity with *Neurospora crassa*) indicated unchanged biotin (*Neurospora crassa* assay 0.045 μg./ml. (90% recovery), *Lactobacillus arabinosus* assay 0.045 μg./ml. (90% recovery), R_f 0.87). In a second experiment 25 μg. of biotin (0.050 μg./ml.) was added to 500 ml. of *Aspergillus niger* culture medium. Growth was carried out in the usual way. At the end of this time differential assay as well as bioautography indicated biotin *l*-sulfoxide equivalent to the biotin added (*Neurospora crassa* assay, 0.046 μg. as biotin/ml. (92% recovery), *Lactobacillus arabinosus* assay, 0.0053 μg. as biotin/ml. (11% recovery, biotin *l*-sulfoxide is only about 5% as active as biotin for *Lactobacillus arabinosus*), R_f 0.47). In a third experiment *Aspergillus niger* was grown with aeration at 30° for 5 days. After growth, 25 μg. of biotin (0.050 μg./ml.) was added to the fermentation and the mixture was immediately autoclaved to stop growth and enzymatic activity. The mixture was then shaken at 30° for 5 additional days. At the end of this time differential assay as well as bioautography indicated unchanged biotin (*Neurospora crassa* assay, 0.051 μg./ml. (102% recovery), *Lactobacillus arabinosus* assay 0.051 μg./ml. (102% recovery), R_f 0.86).

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[CONTRIBUTION FROM ROHM AND HAAS COMPANY]

5-Alkoxymethylenerhodanines and their Reactions with Rhodanines[1]

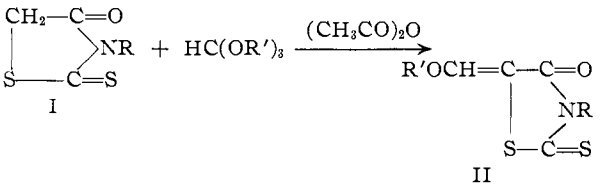
BY CHIEN-PEN LO AND W. J. CROXALL

RECEIVED MARCH 8, 1954

Reaction of 5-unsubstituted rhodanines (I) with alkyl orthoformates in the presence of acetic anhydride yields 5-alkoxymethylenerhodanines (II). Condensation of I and II in the presence of a tertiary amine gives the amine salt of 5,5-methylidynebisrhodanines which upon treatment with hydrochloric acid yield the 5,5′-methylidynebisrhodanines. The acidic property of the latter compounds is believed to be due to the stabilization of the enolate ion by resonance. That the enolate ions are actually hybrids of two extreme resonant forms is supported by experiments. This two-step synthesis furnishes a satisfactory method of preparation of 3,3′-unsymmetrically substituted 5,5′-methylidynebisrhodanines which have not been previously reported in the literature.

Alkyl orthoformates react with active methylene compounds such as malonic, acetoacetic, cyanoacetic esters and others to give either the alkoxymethylene derivatives or the methylidynebis compounds according to the experimental conditions used.[2] The reactivity of the methylene group in rhodanine and 3-substituted rhodanines has long been established. For example, they react with aldehydes and ketones to give 5-alkylidenerhodanines,[3] with isatin to give rhodanine-($\Delta^{5,3'}$-oxindole),[4] with *p*-nitrosodimethylaniline to give 5-(*p*-dimethylaminophenylimino)-rhodanines[5] and with diphenylformamidine to give 5-anilinomethylenerhodanine.[6]

We have found that rhodanine condensed with ethyl orthoformate in the presence of acetic anhydride to yield 5-ethoxymethylenerhodanine (II, R = H; R′ = C_2H_5).



The assignment of the structure of this product was based on analysis and molecular weight determination. The use of methyl orthoformate in the above reaction yielded 5-methoxymethylenerhodanine (II, R = H; R′ = CH_3). This definitely eliminated the possible methylidynebisrhodanine structure. The generality of this reaction was demonstrated by its successful application to a number of 3-substituted rhodanines including 3,3-ethylenebisrhodanine. The 5-alkoxymethylenerhodanines thus prepared are characterized in Table I.

It was reported by Kendall and Fry[7] that the reaction of alkyl orthoformates and rhodanines in the presence of a tertiary amine yielded dyestuffs to which they assigned the structure of 5,5-methylidynebisrhodanines. Since the 5-alkoxymethylene rhodanines obtained above are the logical intermediates for the formation of the compounds of Kendall and Fry, they are expected to react with another molecule of rhodanine with the formation of 5,5′-methylidynebisrhodanines. While the method of Kendall and Fry would yield only symmetrical compounds, ours, if successful, should also be applicable to the synthesis of 3,3′-unsymmetrically substituted 5,5′-methylidynebisrhodanines.

(1) Presented at the Miniature Meeting of the Philadelphia Section of the American Chemical Society, January 29, 1953.

(2) (a) For a review and bibliography on earlier work, see H. W. Post, "The Chemistry of the Aliphatic Orthoesters," Reinhold Publ. Corp., New York, N. Y., 1943, p. 81; (b) R. C. Jones, THIS JOURNAL, **73**, 3684 (1951).

(3) Some of the pertinent references are (a) M. Nencki, *Ber.*, **17**, 2277 (1884); (b) C. Gränacher, M. Gero, A. Ofner, A. Klopfenstein and E. Schlatter, *Helv. Chim. Acta*, **6**, 458 (1923); (c) P. Julian and B. Sturgis, THIS JOURNAL, **57**, 1126 (1935); (d) F. C. Brown, C. K. Bradsher, S. G. McCallum and M. Potter, *J. Org. Chem.*, **15**, 174 (1950); (e) F. C. Brown, C. K. Bradsher, S. M. Bond and M. Potter, THIS JOURNAL, **73**, 2357 (1951).

(4) (a) R. Andreasch, *Monatsh.*, **38**, 138 (1917); (b) C. Gränacher and A. Mahal, *Helv. Chim. Acta*, **6**, 467 (1923); (c) R. V. Jones and H. R. Henze, THIS JOURNAL, **64**, 1669 (1942).

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(6) F. B. Dains and A. E. Stephenson, THIS JOURNAL, **38**, 1843 (1916).

(7) J. D. Kendall and D. J. Fry, British Patent 540,577 (1941).