above-described denitration procedure and the product was isolated as an acetate in the form of an amorphous powder. The yield of water-insoluble product employing the hydrogen chloride promoter was practically quantitative whereas that from the pyridine procedure was low (*ca.* 60%). Both products were highly degraded, as the viscosity data of Table II indicate. Nevertheless the procedure employing hydrogen chloride may prove useful in investigations wherein subsequent hydrolysis of the denitrated product is envisioned.

Experimental

Simultaneous Denitration and Acetylation of Nitrate Polyesters Using Zinc, Acetic Anhydride and Hydrogen Chloride.—A solution of 2 g. of the crystalline nitrate ester (*cf.* Table I) in 25 ml. of acetic anhydride was cooled by immersion in a bath containing 8 liters of water at 10–15°. This solution was treated under mechanical stirring with zinc dust (added in small portions to the total extent of 6–7 g.) and a stream of anhydrous hydrogen chloride was led into the mixture at such a rate as to maintain the temperature of the reaction mixture at 30–35°. The stream of hydrogen chloride was discontinued when a negative test for the nitrate group was obtained with the diphenylamine reagent (no blue coloration with a 0.2% solution of diphenylamine in 90% sulfuric acid). This required approximately one hour. Stirring was then maintained for fifteen minutes whereupon the mixture was poured slowly with stirring onto *ca.* 500 g. of ice and water. After one hour the mixture was extracted with chloroform and the extract washed with a saturated aqueous solution of sodium bicarbonate until neutral to moist litmus paper, dried and concentrated to a crystalline residue under reduced pressure. Recrystallization was effected from 95% ethanol. The yields and constants found are shown in Table I.

Dry cellulose nitrate (2 g., 13% N) was treated in the manner described above. Denitration was slower and a total of 8 g. of zinc dust was required over a five hour period. The precipitate obtained on pouring the reaction mixture onto the ice and water was dissolved in 25 ml. of acetone and reprecipitated by pouring into 600 ml. of water. The light brown, amorphous powder was removed by filtration; yield practically quantitative. The viscosity of the product is shown in Table II.

Simultaneous Denitration and Acetylation of Nitrate Polyesters using Zinc, Acetic Anhydride and Pyridine.—A solution of 5.0 g. of D-mannitol hexanitrate in 50 ml. of dry pyridine and acetic anhydride (1:7 by volume) was treated under stirring with zinc dust added in small portions while maintaining the temperature of the reaction mixture at 40–45°. A negative diphenylamine nitrate test (*cf.* above) was obtained after the addition of 10 g. of zinc over a period of ninety minutes. The reaction mixture was then poured slowly and with stirring onto 800 g. of ice and water. After one hour, the solution was extracted with chloroform and the extract washed with a saturated aqueous solution of sodium bicarbonate until neutral to moist litmus paper, dried and concentrated to a black sirup. The sirup was extracted with acetone, the extract concentrated to dryness and crystallized (decolorizing charcoal) from 95% ethanol. The yield and constants found are shown in Table I.

Dry cellulose nitrate (5 g., 13% N) was dissolved in 200 ml. of the pyridine and acetic anhydride mixture and treated in the manner described above. Denitration required the addition of 18 g. of zinc over a period of two hours. The product was isolated and purified as described above for the denitration of cellulose nitrate with the hydrogen chloride promoter. The product was a light yellow, amorphous powder; yield 58%. The viscosity of the product is shown in Table II.

Acknowledgment.—The work herein reported was carried out under a contract (W-33-019-ord-3978; supervising agency, Ballistic Research Laboratory, Aberdeen Proving Ground, Maryland) between the Ordnance Department and The Ohio State University Research Foundation (Project 212). Acknowledgment is made for the laboratory assistance of Mr. Percy McWain and for the counsel of Mr. E. E. Dickey of this Laboratory.

Summary

1. Nitrate esters (of polyhydric alcohols and of sugar derivatives) are simultaneously denitrated and acetylated by acetic anhydride and zinc in the presence of either hydrogen chloride or pyridine.

2. Cellulose nitrate is also denitrated and acetylated but is very considerably degraded in the process.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF PARKE, DAVIS AND COMPANY]

Hydrogenation of Vitamin Bc (Pteroylglutamic Acid)[1] and Related Pterines

BY B. L. O'DELL, J. M. VANDENBELT, E. S. BLOOM AND J. J. PFIFFNER

Soon after crystalline vitamin Bc was isolated by Pfiffner, *et al.*,[2] the presence of a pyrimidopyrazine ring in its molecular structure was suspected[3] and studies were undertaken to compare the response of vitamin Bc and pterines of known structure to catalytic reduction. The substituted pteridines shown in the formulas were investigated.

Kuhn and Ströbele[4] have shown that riboflavin can be reduced in dilute alkali through a series of colored intermediates to the dihydro compound, leucoflavin, which in turn can be readily dehydrogenated by shaking with oxygen. By vigorous catalytic hydrogenation of flavines, Karrer, *et al.*[5]

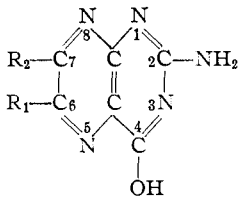
(1) By synthesis Angier, *et al.* [*Science*, **103**, 667 (1946)] have proved the structure of the liver *L. casei* factor to be N-[4-[[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino]-benzoyl]-glutamic acid and have named the compound pteroylglutamic acid. In our laboratories a comparison of vitamin Bc with the synthetic compound generously supplied by the Lederle Laboratories has shown them to be identical.

(2) Pfiffner, Binkley, Bloom, Bird, Emmett, Hogan and O'Dell, *Science*, **97**, 404 (1943).

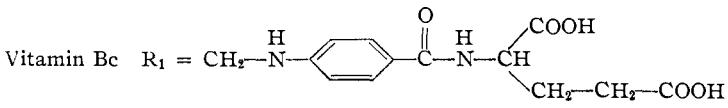
(3) Bloom, Vandenbelt, Binkley, O'Dell and Pfiffner, *Science*, **100**, 295 (1944).

(4) Kuhn and Ströbele, *Ber.*, **70**, 753 (1937).

(5) Karrer and Ostwald, *Rec. trav. chim.*, **57**, 500 (1938).



Xanthopterine	R_1 = OH	R_2 = H
Isoxanthopterine	R_1 = H	R_2 = OH
Leucopterine	R_1 = OH	R_2 = OH
Acid #1[6,7]	R_1 = COOH	R_2 = H



Vitamin Bc R_1 = CH_2—N(H)—⟨benzene⟩—C(=O)—N(H)—CH(COOH)—CH_2—CH_2—COOH R_2 = H

obtained octahydroflavines which are oxidized by air to the hexahydroflavines. Koschara[8] studied the reduction of xanthopterine and found, on the basis of the subsequently proved formula, that it added one mole of hydrogen to give dihydroxanthopterine which in alkaline solution was readily oxidized by air to the original compound.

Since vitamin Bc was found to react analogously to xanthopterine and riboflavin, the latter of which functions in oxidation–reduction enzyme systems, it appeared desirable to record our observations. The results are consistent with the view that vitamin Bc also may function in the animal body as one component in an oxidation–reduction system.

Experimental

Apparatus and Methods.—The microhydrogenation apparatus used was essentially the same as the one described by Smith.[9] The catalyst and 15 cc. of solvent were introduced into the reaction vessel and the sample was suspended in a glass container. After the catalyst was saturated with hydrogen, the sample was introduced and at intervals the decrease in volume of gas was noted. When the uptake of hydrogen ceased, the shaking was discontinued and a sample (0.1 cc.) was removed for specific ultraviolet absorption determinations. In case it was desired to dehydrogenate the reaction mixture, the apparatus was swept out with nitrogen which was then replaced by oxygen while the catalyst lay quietly on the bottom. Then while shaking the apparatus, the oxygen uptake was measured.

The ultraviolet absorption studies were made with a Beckmann spectrophotometer using a hydrogen discharge tube as a source of light. Most of the reduced compounds were so sensitive to air that it was very difficult to isolate pure products In experiments in which the reduced products were not isolated, ultraviolet absorption studies were made on the reduced compounds by cautiously removing aliquots from the reaction solution and making the observations as rapidly as possible. The molecular extinction of such compounds is based on the weight of the starting material and hence is subject to greater error than is usually the case.

Dihydrovitamin Bc.—Vitamin Bc (36 mg.) was dissolved in 15 cc. of 0.1 *N* sodium hydroxide and shaken over 25 mg. of platinum oxide. The amount of hydrogen consumed was 2.0 ml. (STP); theoretical for 1 mole is 1.78 ml. During the reaction the yellow color of the original solution gradually faded to give finally a clear colorless solution. A small sample of this solution which was allowed to stand in the air gradually regained its yellow color and its ultraviolet absorption curve indicated that the parent compound was reformed. Oxidation did not occur as readily in acid solution. Because of the great ease of oxidation it was difficult to isolate an analytical specimen of the dihydro-vitamin; attempts at recrystallization always gave the original vitamin. In this preparation the catalyst was removed and the solution immediately adjusted to *p*H 3.0 which precipitated the dihydro compound. The precipitate was washed four times with 0.01 *N* hydrochloric acid and once with water. After drying over calcium chloride in a vacuum desiccator there was 15 mg. of product. For analysis it was dried at 145° in high vacuum giving a volatile loss of 8.15%.

Anal. Calcd. for $C_{19}H_{21}O_6N_7$: C, 51.47; H, 4.77. Found: C, 51.39, 51.16; H, 4.77, 4.57 (corrected for ash, 2.38, 2.09%).

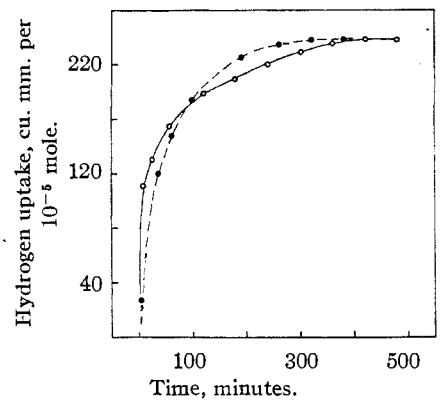


Fig. 1.—Rate of hydrogenation: —, vitamin Bc; – –, xanthopterine.

The specific ultraviolet absorption properties of this compound are shown in Fig. 2.

Oxidation of Dihydrovitamin Bc.—Vitamin Bc (26.7 mg.), when hydrogenated in 0.1 *N* sodium hydroxide over palladium-on-charcoal, consumed 1.43 ml. of hydrogen (calcd. for 1 mole is 1.35 ml.) and the reaction mixture gave the typical ultraviolet absorption curve for dihydrovitamin Bc. This solution was then shaken with oxygen to regenerate the parent vitamin. The catalyst was removed and the solution adjusted to *p*H 3.0. The resulting precipitate was washed three times with water and dried; yield 15 mg. The compound was dissolved in 1 *N* hydrochloric acid and the insoluble material removed. After precipitation by adjusting to *p*H 3.0 it was recrystallized from 50 ml. of hot water. To remove ash the product was extracted with three 5-ml. portions of 0.01 *N* hydrochloric acid and again recrystallized from 30 ml. of hot water. There resulted 8 mg. of a yellow crystalline compound which was identical with the starting material. For analysis it was dried at 145° in high vacuum giving a volatile loss of 8.4%.

Anal. Calcd. for $C_{19}H_{19}O_6N_7$: C, 51.71; H, 4.34. Found: C, 52.16; H, 4.10 (corrected for 0.2% ash).

The ultraviolet absorption curves of vitamin Bc, dihydrovitamin Bc and the oxidized dihydro compound are shown in Fig. 2.

Hydrogenation of Vitamin Bc and Related Pterines.—The hydrogenation studies were performed under various conditions using either 0.1 *N* sodium hydroxide or glacial acetic acid as solvents and a palladium or platinum catalyst with or without a carrier. Table I shows the molar

(6) Acid #1 refers to an oxidative degradation product of vitamin Bc obtained by Wittle, *et al.*[5] It was shown by Angier, *et al.*, [*Science*, **103**, 667 (1946)] to be 2-amino-4-hydroxy-6-carboxypteridine.

(7) Wittle, O'Dell, Vandenbelt and Pfiffner, in press.

(8) Koschara, *Z. physiol. Chem.*, **250**, 161 (1937).

(9) Smith, *J. Biol. Chem.*, **96**, 35 (1932).

TABLE I

Compound	Solvent	Catalyst	Cat. wt., mg.	Sample Wt., mg.	Sample Moles × 10^{-5}	H_2 uptake, moles × 10^{-5}	O_2 uptake,[a] moles × 10^{-5}
Vitamin Bc	0.1 *N* NaOH	Pd.-$BaSO_4$	200	29.0	6.57	6.43	
Vitamin Bc	0.1 *N* NaOH	Pd.-char.	50	26.7	6.05	6.03	
Vitamin Bc	Gl. HAc	PtO_2	25	30.0	6.80	15.52	5.26
Vitamin Bc	Gl. HAc	PtO_2	25	22.1	5.02	11.78	6.47
Xanthopterine	0.1 *N* NaOH	Pd.-$BaSO_4$	200	20.2	11.3	11.00	6.39
Xanthopterine	0.1 *N* NaOH	Pd.-char.	100	14.9	8.35	8.93	
Xanthopterine	Gl. HAc	PtO_2	25	25.2	14.0	13.75	14.82[b]
Xanthopterine	Gl. HAc	Pd.-$BaSO_4$	200	19.3	10.8	10.71	9.28[b]
Isoxanthopterine	0.1 *N* NaOH	Pd.-$BaSO_4$	200	23.7	13.2	0	
Leucopterine	0.1 *N* NaOH	PtO_2	25	20.2	10.3	0	
Acid #1[6]	0.1 *N* NaOH	Pd.-char.	100	17.8	8.60	21.62	8.35
Acid #1	0.1 *N* NaOH	Pd.-char.	100	14.2	6.85	14.20	8.93
2-Amino-4-hydroxy-6-carboxy-7-methylpteridine	0.1 *N* NaOH	Pd.-char.	100	22.6	10.2	20.10	9.60
	Gl. HAc	PtO_2	25	20.3	9.2	17.60	8.66

[a] Oxygen consumed in dehydrogenating the reduction mixtures. [b] Leucopterine was formed.

equivalents of hydrogen uptake as well as the oxygen used to dehydrogenate the reaction mixture.

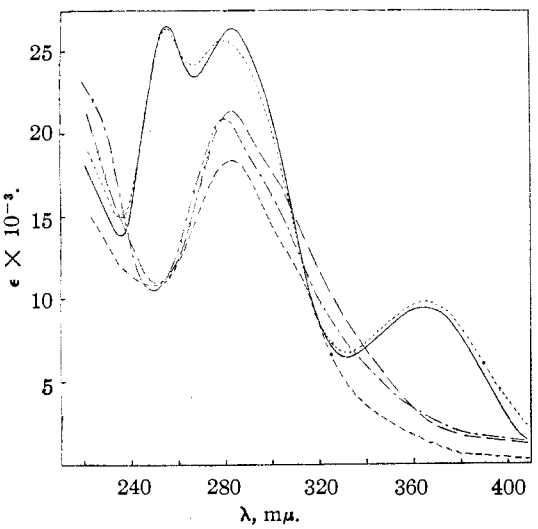


Fig. 2.—Ultraviolet absorption spectra: —, vitamin Bc at *p*H 11.00; — — —, dihydrovitamin Bc at *p*H 11.0; — - —, dihydrovitamin Bc at *p*H 3.1; - - -, tetrahydrovitamin Bc at *p*H 11.0; - - -, oxidized dihydrovitamin Bc at *p*H 11.0.

(a) **Vitamin Bc.**—In dilute alkali over palladium vitamin Bc adds one mole of hydrogen to give dihydrovitamin Bc which in the presence of oxygen is readily oxidized to the parent compound. This ease of reduction and subsequent oxidation is analogous to the behavior of xanthopterine reported by Koschara.[8] Wieland and Purrmann[10] observed that xanthopterine in dilute acetic acid over platinum is oxidized to leucopterine. Under our experimental conditions neither vitamin Bc nor xanthopterine in dilute alkali is oxidized catalytically as evidenced by the measurement of the oxygen uptake of the compounds themselves and their dihydro derivatives. The similar response of vitamin Bc and xanthopterine to catalytic hydrogenation in dilute alkali is further shown by their comparative rates of hydrogen uptake illustrated in Fig. 1.

In glacial acetic acid over platinum vitamin Bc takes up two moles of hydrogen in contrast to xanthopterine which consumes only one mole. When the reduction mixture containing tetrahydrovitamin Bc is shaken with oxygen one mole is consumed, an amount sufficient to account for its dehydrogenation. Vitamin Bc does not take up oxygen over platinum in glacial acetic acid under these conditions.

As shown in Fig. 2 the ultraviolet absorption curve of vitamin Bc in alkaline solution has maxima at 255, 282 and 365 mμ but the curve of dihydrovitamin Bc has only one maximum, located at 284 mμ; molecular extinction 21,300 at *p*H 11.0. The curve of the reduced compound at *p*H 3.1 is very similar to the one at *p*H 11.0, the peak shifting only slightly to the shorter wave lengths. Oxygenation of dihydrovitamin Bc produces a compound which exhibits essentially the same ultraviolet absorption properties as that of the parent vitamin. When 2 moles of hydrogen are introduced into the molecule to give tetrahydrovitamin Bc, the ultraviolet absorption properties are not materially different from those of the dihydro compound as is shown by the curve in Fig. 2, determined on the reduction mixture.

(b) **Xanthopterine.**—The clear colorless solution of dihydroxanthopterine, prepared by hydrogenation in either dilute alkali or glacial acetic acid, is readily oxidized in air. An aliquot of the alkaline reduction mixture shows ultraviolet absorption properties similar to those of dihydrovitamin Bc. Catalytic oxygenation of the dihydroxanthopterine in glacial acetic acid consumes one mole of oxygen, giving leucopterine as the final product. This is in agreement with the findings of Wieland and Purrmann.[10]

Figure 3 shows the ultraviolet absorption curves of xanthopterine, dihydroxanthopterine and oxidized dihydroxanthopterine. In alkaline solution xanthopterine has maxima at 255 and 385 mμ while dihydroxanthopterine possesses only one maximum located at 276 mμ. In acid solution dihydroxanthopterine shows maxima at 263 and 310 mμ. Treatment of a solution of dihydroxanthopterine with oxygen regenerates the typical xanthopterine curve.

(c) **6-Carboxypterines.**—Acid #1[7] (2-amino-4-hydroxy-6-carboxypteridine), a degradation product of vitamin Bc, and the synthetic homolog, 2-amino-4-hydroxy-6-carboxy-7-methylpteridine, absorb two moles of hydrogen in dilute alkali. Allowing the reduced solutions to stand in air rapidly regenerates the parent compound and catalytic oxygenation consumes the calculated amount (1 mole) of oxygen to account for their dehydrogenation.

In glacial acetic acid 2-amino-4-hydroxy-6-carboxy-7-methylpteridine also absorbs two moles of hydrogen, but acid #1 consumes no hydrogen under these conditions probably due to its extreme insolubility in glacial acetic acid.

The effect on the ultraviolet absorption properties of introducing 2 moles of hydrogen in the 6-carboxypterines

(10) Wieland and Purrmann, *Ann.*, **544**, 163 (1940).

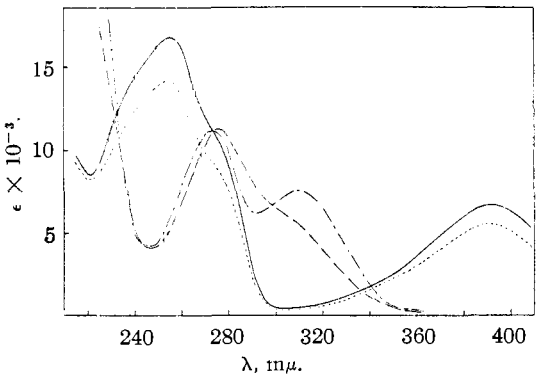


Fig. 3.—Ultraviolet absorption spectra: —, xanthopterine at *p*H 11.0; — — —, dihydroxanthopterine at *p*H 11.0; — - —, dihydroxanthopterine at *p*H 3.0; - - -, oxidized dihydroxanthopterine at *p*H 11.0.

is shown in Fig. 4. In dilute alkali (*p*H 11.0) the ultraviolet absorption curve of acid #1 has maxima at 262 and 365 mμ whereas the tetrahydro derivative possesses maxima at 253 and 380 mμ. In contrast to the pronounced shift of the acid #1 curve with change in *p*H,[7] the curve of the reduction product at *p*H 3.0 is not greatly different from its *p*H 11.0 curve, the maxima shifting to only slightly longer wave lengths. As in the case of the parent compounds,[7] reduced acid #1 and its reduced 7-methyl homolog show similar specific ultraviolet absorption.

Discussion

The ease of reduction of the pyrimido-pyrazine ring system as evidenced by the catalytic hydrogenation of riboflavin,[4] xanthopterine[8] and vitamin Bc coupled with the fact that their dihydro-derivatives are readily oxidized to the parent compounds when allowed to stand in air strongly suggests that these compounds may perform their respective metabolic functions in a common manner, namely, as hydrogen acceptors in oxidation–reduction enzyme systems. Wieland and Liebig[11] have shown that xanthopterine is oxidized to leucopterine by an enzyme present in liver, but that leucopterine in contrast to xanthopterine is not present in urine. In addition to these workers' suggestion of a physiological function of xanthopterine, Simmons and Norris[12] have demonstrated its hemopoietic effect in young salmon, and Totter, *et al.*,[13] have reported xanthopterine to be active in delaying nutritional cytopenia in the monkey.

Since leucopterine and isoxanthopterine are not catalytically hydrogenated in dilute alkali, showing that the enolic double bonds and the 5,6 double bonds in the pteridine ring are quite stable to reduction, whereas xanthopterine readily adds one mole of hydrogen, it would appear that

(11) Wieland and Liebig, *Ann.*, **555**, 146 (1944).

(12) Simmons and Norris, *J. Biol. Chem.*, **158**, 449 (1945).

(13) Totter, Shukers, Kolson, Mims and Day, *ibid.*, **152**, 147 (1944).

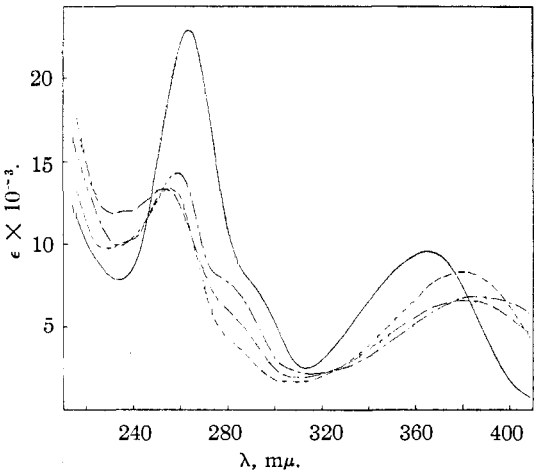


Fig. 4.—Ultraviolet absorption spectra: —, acid #1 at *p*H 11.0; — — —, tetrahydro-acid #1, at *p*H 11.0; — - —, tetrahydro-acid #1 at *p*H 3.0; - - - -, 2-amino-4-hydroxy-6-carboxy-7-methyltetrahydropteridine at *p*H 11.

it is the 7,8 double bond which is readily reduced.

In view of these facts one would expect that it is the 7,8 double bond of the pteridine ring in vitamin Bc which is readily reduced. The benzene ring in vitamin Bc is not reduced under these experimental conditions as evidenced by the fact that the parent compound is easily regenerated by oxygen. In contrast to the vitamin, two double bonds of the degradation product, 2-amino-4-hydroxy-6-carboxypteridine, are saturated in dilute alkali. The presence of the carboxyl group in the 6-position may contribute to its greater ease of hydrogenation and account for the fact that the ultraviolet absorption curve of its tetrahydro derivative has a band at 380 mμ in contrast to the absence of such a band in the curve of tetrahydrovitamin Bc.

Acknowledgment.—We wish to thank Dr. E. L. Wittle for his advice and suggestions in this work and Mr. A. W. Spang for the microanalyses.

Summary

Dihydrovitamin Bc has been prepared by catalytic hydrogenation of vitamin Bc in dilute alkali. It has been found that, like dihydroxanthopterine, dihydrovitamin Bc is readily oxidized to the parent compound. In glacial acetic acid over platinum two moles of hydrogen are consumed by the vitamin.

The degradation product, 2-amino-4-hydroxy-6-carboxypteridine, absorbs two moles of hydrogen in dilute alkali to give the tetrahydro-derivative which is also readily oxidized to the parent compound. The specific ultraviolet absorption curves of the reduced compounds are presented.

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