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IDENTIFICATION OF A DEPHOSPHORYLATED OXIDATION PRODUCT OF THE MOLYBDENUM COFACTOR AS 2-(1,2-DIHYDROXYETHYL)THIENO[3,2-g]PTERIN

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SUMMARY: A new method was developed for the synthesis of 2-(1,2-dihydroxyethyl)thieno[3,2-g]pterin and related 2-substituted thienopterins. A dephosphorylated fluorescent oxidation product of the molybdenum cofactor isolated from xanthine oxidase (EC 1.2.3.2) was identified as 2-(1,2-dihydroxyethyl)thieno[3,2-g]pterin by comparison of electronic and fluorescence spectra and TLC behaviors with those of the synthetic compound.

The involvement of a molybdenum cofactor common to various molybdenum-containing enzymes other than nitrogenase was first suggested by genetic studies of Aspergillus nidulans in 1964 by Pateman et al. (1) and established by biochemical studies on assimilatory nitrate reductase of Neurospora crassa by Nason and co-workers (2-4). Although isolation of the active cofactor was reported (5), structural elucidation of the cofactor was delayed by its extreme sensitivity to oxygen (4,5). In 1980, Johnson et al. reported the presence of a novel 6-substituted pterin in an oxidized inactive form of the molybdenum cofactor from several molybdoenzymes (6). A fluorescent oxidation product showing an absorption peak at 395 nm in alkaline solution and containing a phosphate group was isolated from denatured molybdoenzymes, independently by Johnson and Rajagopalan (Form B) (7) and by us (C_{395}) (8). This compound can be dephosphorylated by alkaline phosphatase (7,8), but not by phosphodiesterase (8). Johnson

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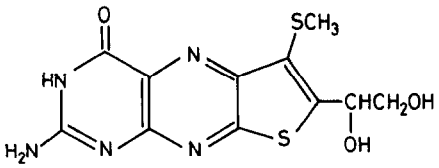

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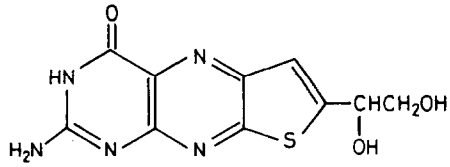

(2)

and Rajagopalan suggested structural and metabolic relationship between the molybdenum cofactor and urothione on the bases of the degradation experiments with alkaline $KMnO_4$ (7) and IO_4^- (7,8) as well as the absence of urothione in the urine samples from patients deficient in the molybdenum cofactor (7). Urothione (1), first isolated from human urine by Koschara (9), is a sulfur-containing pterin, whose structure was established by Goto et al. (10). We demonstrated previously that a metastable oxidation product (C_{395}) of the molybdenum cofactor and its dephosphorylated form (dp-C_{395}) are thieno[3,2-g]pterin derivatives with an unidentified side chain in the 2 position (8). Recently, Johnson et al. have reached the conclusion that Form B (dephospho) is the urothione analog lacking the 3-methylthio function (11). To get a decisive proof for the suggested structure, we developed a new method for the synthesis of several 2-substituted thienopterins and report evidence that dp-C_{395} is 2-(1,2-dihydroxyethyl)thieno[3,2-g]pterin (2). Although one synthesis of this compound has been reported (12), the overall yield was too low to obtain the compound in a sufficient amount (13).

MATERIALS AND METHODS

C_{395} was obtained by denaturation of cow milk xanthine oxidase (Sigma Chemical Co., St. Louis, U.S.A.) in 6 M guanidine·HCl as described before (8). dp-C_{395} was prepared from C_{395} by treatment with Escherichia coli alkaline phosphatase (Worthington Biochemical Corp., Freehold, U.S.A.) and purified by florisil and Sephadex LH-20 column chromatography.

Pterin-6-carboxylic acid, thieno[3,2-g]pterin-2-carbaldehyde and thieno[3,2-g]pterin-2-carboxylic acid were prepared as described before (8). 2-Ethylthienopterin was synthesized from 6-(2-oxobutyl)-7-oxopterin by the method described by Goto et al. (12) and purified by column chromatography on triethylaminoethylcellulose (OH^- form), florisil, and Sephadex LH-20. A homogeneous preparation was obtained by HPLC through a reverse phase column (Du Pont Zorbax ODS 4.6 x 250 mm) using

60 % aqueous methanol as eluting agent. Retention time was 8 min at a flow rate of 1 ml/min. 2-Ethylthienopterin was oxidized to 2-acetylthienopterin by cerium(IV) ammonium nitrate in 50 % acetic acid. 2-Acetylthienopterin isolated by florisil column chromatography was purified to homogeneity by HPLC through the Zorbax ODS column using 40 % methanol. Retention time was 9 min at a flow rate of 1 ml/min. 2-Acetylthienopterin was converted to 2-(2-bromoacetyl)thienopterin by reaction with bromine in glacial acetic acid at 50-55°C for 2 days. After evaporation in vacuo, crude 2-(2-bromoacetyl)thienopterin was completely hydrolyzed to 2-(2-hydroxyacetyl)thienopterin in 12.5 mM potassium phosphate buffer (pH 7.2). Resulting crude 2-(2-hydroxyacetyl)thienopterin in the buffer was reduced to 2-(1,2-dihydroxyethyl)thienopterin by $NaBH_4$ at pH 8 to 8.5. The desired product was isolated by column chromatography on florisil and Sephadex LH-20, and finally purified to homogeneity by HPLC through the Zorbax ODS column using 20 % aqueous methanol. The retention time was 8 min at a flow rate of 1 ml/min. The overall yield of this compound was fairly good (about 50-60 % theoretical, based on acetylthienopterin).

RESULTS AND DISCUSSION

TLC behaviors of 2-(1,2-dihydroxyethyl)thienopterin and related compounds are summarized in Table I (Run No. 1). The electronic and fluorescence spectra of the 2-substituted thienopterins synthesized are shown in Figs. 1 and 2 (A-C).

Table I. Thin layer chromatography of dp-C_{395} and 2-substituted thieno[3,2-g]pterins[a]

Run No.	Sample[b]	R_F Cellulose A	B	C	D	Silica gel E
1	TP-2-CH_2CH_3	0.38	0.68	0.05	0.05	0.76
	TP-2-$COCH_3$	0.15	0.30	0.02	0.03	0.69
	TP-2-$COCH_2Br$	0.21	0.42	0.02	0.02	0.77
	TP-2-$COCH_2OH$	0.15	0.26	0.03	0.03	0.72
	TP-2-$CH(OH)CH_2OH$	0.15	0.18	0.12	0.12	0.62
	Pterin-6-COOH	0.09	0.13	0.32	0.88	0.38
2	dp-C_{395}	0.13	0.19	0.12	0.13	0.67
	IO_4^--treated dp-C_{395}	0.15	0.25	0.03	0.04	0.74
	TP-2-$CH(OH)CH_2OH$	0.13	0.19	0.12	0.13	0.67
	IO_4^--treated TP-2-$CH(OH)CH_2OH$	0.15	0.25	0.03	0.04	0.74
	TP-2-CHO	0.15	0.25	0.03	0.04	0.74
	TP-2-COOH	0.04	0.13	0.04	0.62	0.52
	Pterin-6-COOH	0.10	0.12	0.35	0.87	0.40

[a]Solvents: (A) 2-propanol-1 % NH_4OH (2:1); (B) 1-butanol-acetic acid-water (4:1:1); (C) 3 % NH_4Cl; (D) water; (E) 1-butanol-2-propanol-water (10:7:10).

[b]TP=thieno[3,2-g]pterin

[c]The samples were treated at room temperature for 10 min with 10 mM KIO_4 in 10 mM acetic acid.

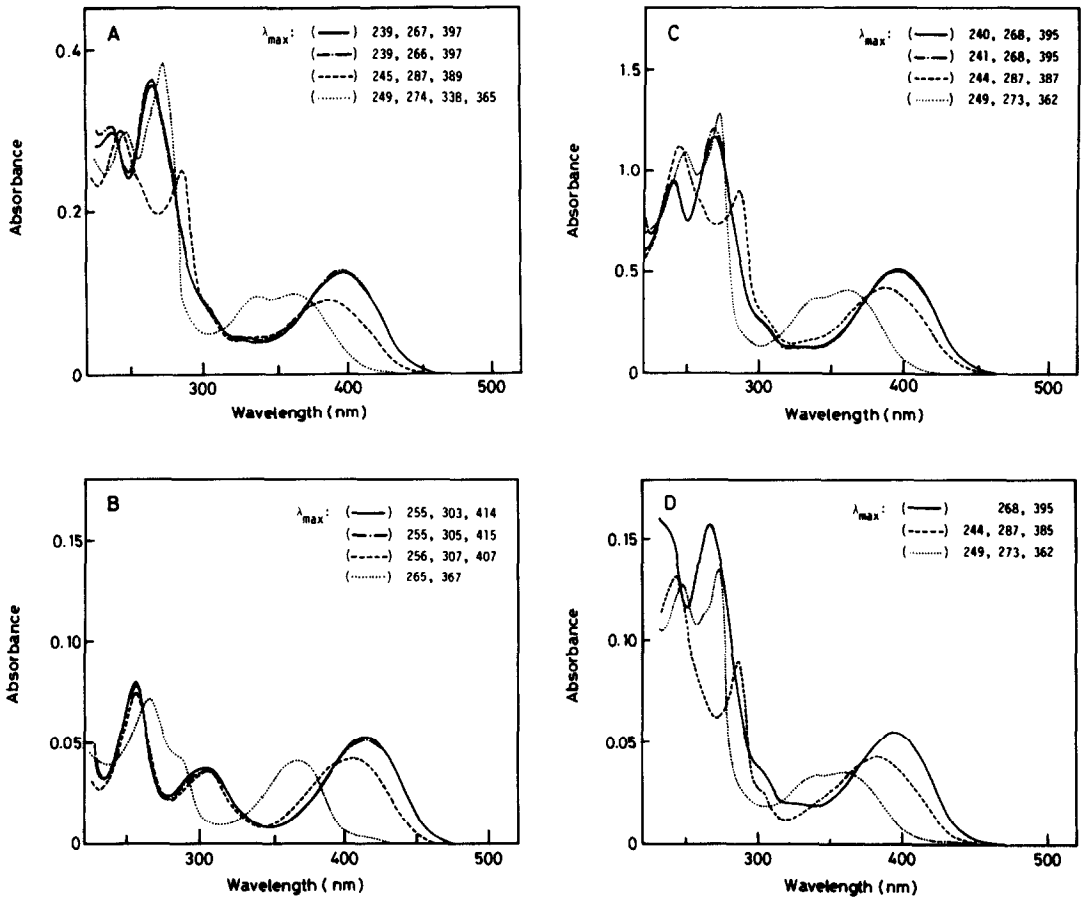

Fig. 1. Electronic spectra of dp-C_{395} and synthetic 2-substituted thieno[3,2-g]pterins. A, 2-ethylthieno[3,2-g]pterin; B, 2-acetylthieno-[3,2-g]pterin; C, 2-(1,2-dihydroxyethyl)thieno[3,2-g]pterin; D, dp-C_{395}. (——), in 1 N NH_4OH; (—·—), in 0.1 N NaOH; (-----), in 0.05 M potassium phosphate buffer (pH 7.0); (·····), in 0.1 N HCl.

Chromatographic behaviors of dp-C_{395} and synthetic 2-(1,2-dihydroxy-ethyl)thieno[3,2-g]pterin were compared by TLC on cellulose and sillica gel. As shown in Table I (Run No. 2), R_F values of dp-C_{395} coincided with those of the synthetic compound in all the solvent systems tested. Electronic spectra of dp-C_{395} were compared with those of the synthetic compound. Fig. 1 (C and D) indicates that both spectra were essentially indistinguishable in either alkaline, neutral or acidic solution. As shown in Fig. 2 (C and D), both fluorescence excitation and emission spectra of dp-C_{395} are also identical with those of the synthetic 2-(1,2-dihydroxy-ethyl)thienopterin. Therefore, the identity of these two compounds was

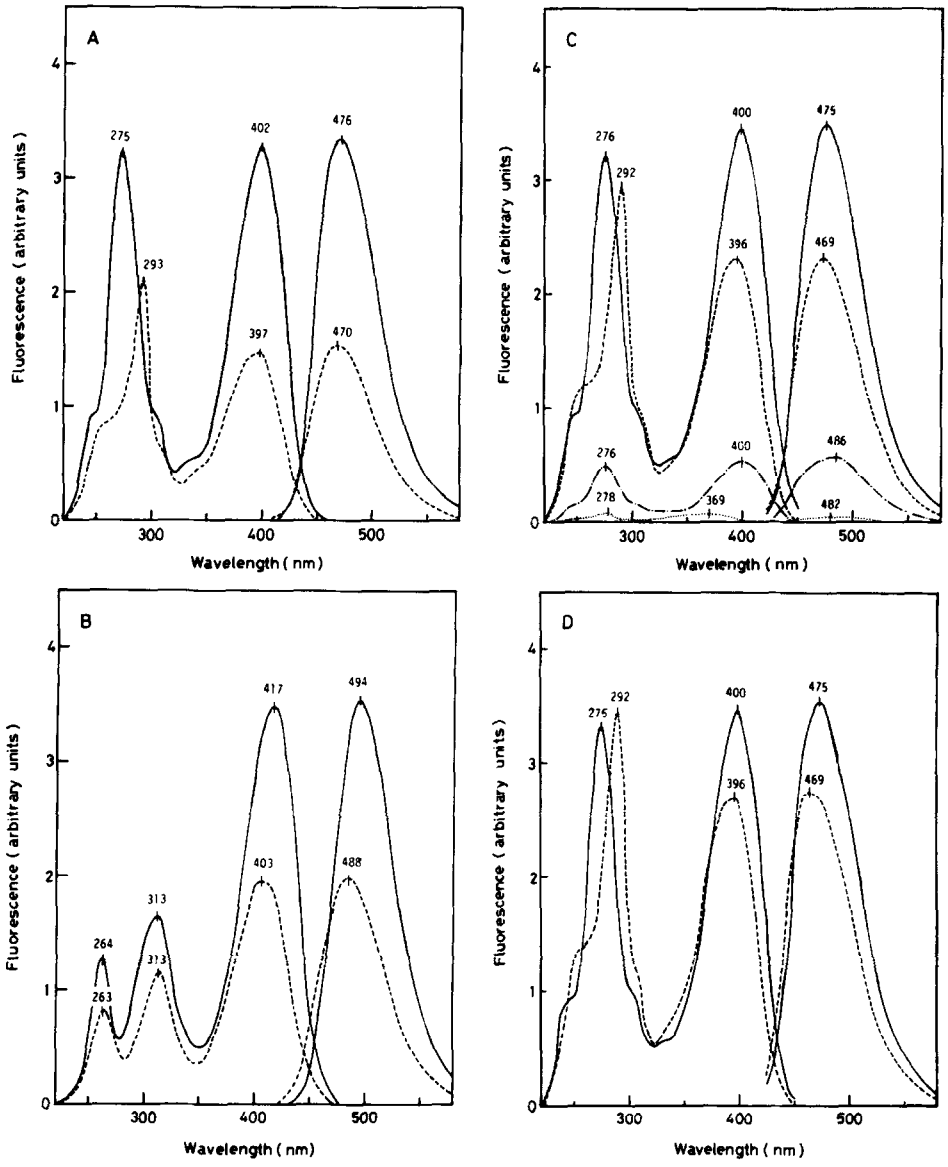

Fig. 2. Fluorescence excitation and emission spectra of dp-C_{395} and synthetic 2-substituted thieno[3,2-g]pterins. A, 2-ethylthieno[3,2-g]-pterin; B, 2-acetylthieno[3,2-g]pterin; C, 2-(1,2-dihydroxyethyl)thieno-[3,2-g]pterin; D, dp-C_{395}. The excitation spectra were measured by emission at wavelengths of emission maximum. (———), in 1 N NH_4OH; (—·—), in 0.1N NaOH; (-----), in 0.05 M potassium phosphate buffer (pH 7.0); (·····), in 0.1 N HCl.

established chromatographically, spectrophotometrically and fluorometrically.

Upon IO_4^- oxidation, both dp-C_{395} and the synthetic compound were rapidly and quantitatively converted to the same product (Table I).

TLC behaviors of this product were identical with those of synthetic thieno[3,2-g]pterin-2-carbaldehyde (Table I). Accompanying this conversion, light-blue fluorescence of dp-C_{395} and the synthetic 2-(1,2-dihydroxyethyl)thienopterin turned to bluish-green one of the resulting thienopterin-2-carbaldehyde. This change in fluorescence offers additional evidence for the conversion of dp-C_{395} and the synthetic compound to thienopterin-2-carbaldehyde upon the IO_4^- treatment.

From all the criteria tested, it was concluded that dp-C_{395} and the synthetic 2-(1,2-dihydroxyethyl)thieno[3,2-g]pterin are the same compound. Since C_{395} contains a phosphate group and is not susceptible to IO_4^- (7,8), it is likely that one of the two hydroxyl groups of the 2-substituent in dp-C_{395} is phosphorylated in the C_{395} molecule.

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