MOLYBDENUM COFACTOR: ITS BIOLOGICAL SIGNIFICANCE, STRUCTURAL, AND SYNTHETIC ASPECTS

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Dedicated with profound regards to Professor Edward C. Taylor on the occasion of his seventieth birthday.

Abstract - A brief review of the biological importance, structural and synthetic aspects of the extremely labile molybdenum cofactor (Moco) and its different stable oxidative degradation forms has been presented.

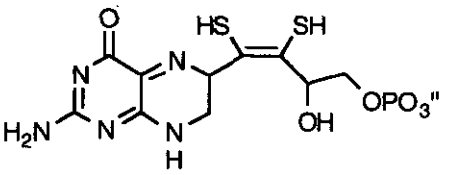
Molybdenum, as an important trace element, participates in a number of biochemical redox reactions which include the oxidation of xanthine and purines and the reduction of nitrate and molecular nitrogen. Mo-Cofactor containing enzymes are present in various biological species including fungi, algae, bacteria, plants, animals and man. Molybdenum is essential in trace amount for human health. The major routes of nitrogen incorporation in plants, and therefore, animals involve nitrate reduction and nitrogen fixation where molybdenum functions in bound form to an enzyme and associated with a coenzyme, flavin adeninedinucleotide (FAD). The biological work on the various enzymes has appeared in a number of articles,[1-6] and symposia. Individual enzyme catalyses steps in the biological nitrogen and sulfur cycles significant of agricultural, environmental and biogeological importance.

The presence of an organic prosthetic group associated with the molybdenum atom was first suggested by Pateman *et al.* [3] and the subsequent proof of the hypothesis of such a common molybdenum cofactor was confirmed by Nason.[7-9] These discoveries suggest that all molybdenum-containing enzymes except nitrogenase are associated with a similar cofactor acting as a universal link to bind the enzyme subunits to form an active enzyme and function as an electron carrier.

Molybdenum enzymes are present playing varied and important role in organisms from primitive bacteria to higher plants and animals including man. Molybdenum thus functions as a key part of the prosthetic group of over fifteen matalloenzymes that catalyse redox reactions[10] where the common organic cofactor for the activity of the enzymes has been termed molybdopterin (**1**). These molybdenum cofactor systems

include oxidases, hydroxylases, reductases and dehydrogenases. However larger variants of molybdopterin are present in enzymes[11-13] which show more complicated dinucleotide structure. Molybdenum cofactor (Moco, 2) is referred to as the complex of molybdenum with molybdopterin. Molybdenum cofactor is now recognised as a universal cofactor for all known molybdenum-containing enzymes[14,15] with the single exception of nitrogenase which has a unique iron-molybdenum[16] cofactor that is not interchangeable with that in other molybdoenzymes.[13]

The structure of molybdopterin seems to be complicated and varies depending on its source. Thus in bacteria, more complex dinucleotide structures[12] represent different molybtopterin structure, although the pterin as well as molybdenum dithiolene moieties are common to all Moco's. The alkylation of the molybdenum cofactor in the bacterial enzyme dimethyl sulfoxide reductase has been identified[13] as molybdopterin guanine dinucleotide by analogy to flavin adenine dinucleotide and nicotinamide adenine dinucleotide suggesting that the pterin-containing cofactor is distributed in at least two different structural forms.



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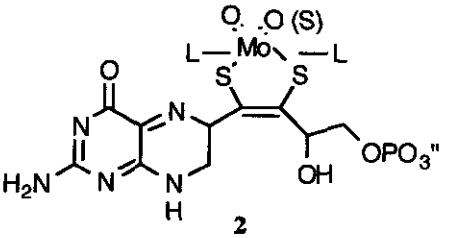

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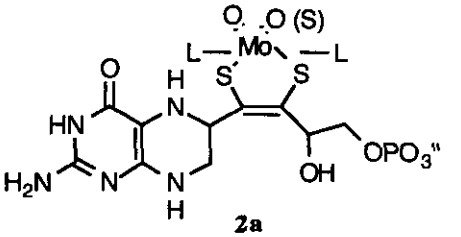
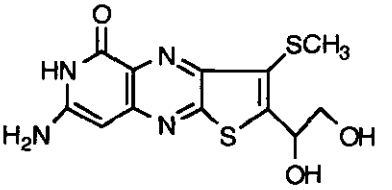


2a



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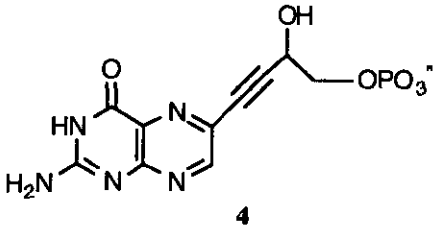

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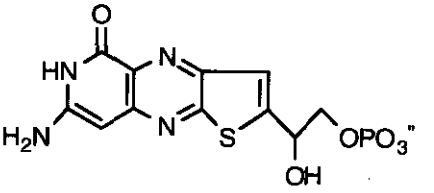

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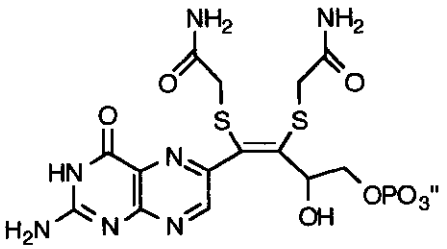

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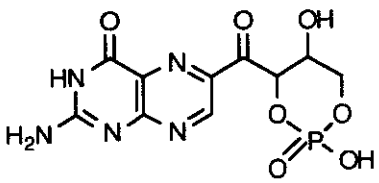

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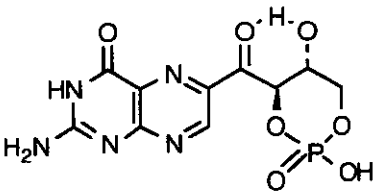
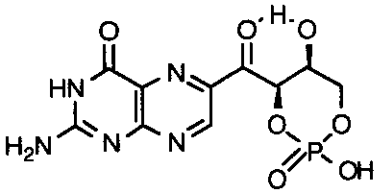
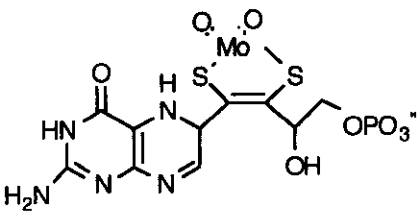


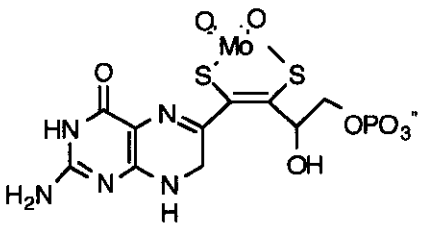
7a : Trans



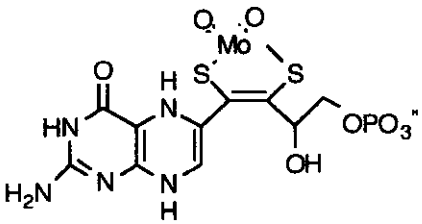
7b : Cis



5,6-Dihydro

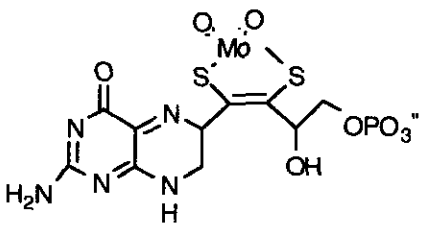


7,8-Dihydro

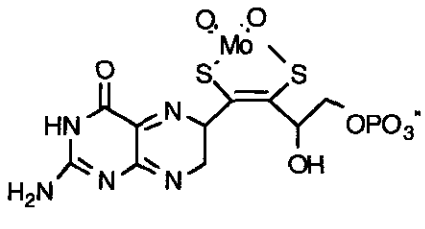


5,8-Dihydro

Figure 1



p-Quinoid-dihydro



o-Quinoid-dihydro

Figure 2

Molybdenum cofactor plays a critical role in the functioning of the Moco-enzymes in fungi, bacteria, plants and mamals. These Moco systems include oxidases, hydroxylases, reductases and dehydrogenases. The Moco enzymes play critical roles in the global carbon, nitrogen and sulfur cycles, in the metabolism of purines and other *N*-heterocycles and in the generation of biological antioxidants in higher organisms. Thus they have a biogeochemical significance. In methanogenic bacteria, the fixation of carbon dioxide and the production of methane is facilitated by Moco-enzymes which are also involved in the generation of carbon dioxide from carbon monoxide or from dehydrogenation of formate. Moco-enzymes are important in the assimilation of nitrogen by plants, algae, bacteria and fungi, by assisting both the reduction of nitrate and the reduction of amine oxides. Thus these enzymes are responsible, as a part of the sulfur cycle, for oxidation of sulfite to sulfate and also for reduction of DMSO to dimethyl sulfide which in turn photo-oxidised in the stratosphere to methane sulfonic acid, which is key to cloud formation, and is eventually metabolised to sulfite by soil bacteria.[17]

Mo enzymes also play significant roles in human health showing their biomedical significance. Moco deficiency is a well recognised entity since the first published case in 1978[18] and also the clinical presentation is well documented. Genetic deficiency in human molybdenum enzymes causes severe clinical abnormalities.[19] Individual human deficient in xanthine oxidase suffers mild myopathy due to deposition of hypoxanthine and xanthine crystals in muscle and renal xanthine calculi, a genetic deficiency of both xanthine oxidase and sulfite oxidase leads to serious physiological and neurological problems like mental and growth retardation, dislocated ocular lenses, tonic-clonic seizures and even death in case of completely lacking Moco. A combined deficiency of xanthine dehydrogenase, sulfite oxidase and aldehyde oxidase is due to an individual' s inability to synthesise active molybdenum cofactor.[20-25] A number of reviews has appeared in books[26-28] and articles[29] about aspects of molybdenum enzymes.

The crucial problem in elucidating the structure and in the total synthesis of the natural cofactor itself lies in its inherent chemical instability in the free state which may be directly ascribable to the unprecedented free enedithiol moiety in the pendant hydroxylated four carbon side chain attached with dihydro or tetrahydro (another difficult prediction) pterin nucleus generated by degradation of the

cofactor during isolation. It has not been possible to isolate the native cofactor from protein for direct structural studies due to the extreme instability of Moco.[30] The lability of the active Mo-cofactor has hindered isolation, handling and direct structural characterisation of the compound. However, a major breakthrough on the basic structure of the organic moiety of the Mo-cofactor was abetted by the observation[31] of blue fluorescent compounds (pterins) which are the rapid degradation products of Moco in aerobic systems. Studies of the biological derivative, urothione[32-34] (3) and the two oxidised degradation products, Form A (4) and Form B (5)[35,36] as well as a carboxamidomethyl derivative (oxidised) (6)[36] have revealed the structural aspects of the molybdenum cofactor.[3,7-9] The characterisation of Form A led to the conclusion that the native cofactor posseses a reduced pterin nucleus with a 6-alkyl substituent and a terminal phosphate ester. Studies on Form B and its chemical and metabolic relationship to urothione provided evidence for pterin ring system in the active cofactor. Presence of sulfur in the side chain is clearly established through the characterisation of Form B permanganate product as pterin-6-carboxylic-7-sulfonic acid. The presence of glycol function is evident from the periodate sensitivity of alkaline phosphatase-treated Form B. The metabolic link[33] between Moco and urothione, suggested by absence of urothione in urine from Moco deficient patients, implies the presence two sulfurs and at least four carbons in the side chain of the active cofactor.

The difference of molybdopterin from other organic enzyme cofactors such as FAD, biotin, pyridoxal phosphate, etc., is that it is extraordinarily unstable in the free form when released from the enzyme and it is rapidly degraded to inactive products. As a consequence, its structure has been probed indirectly by EXAFS examination of the molybdenum active sites, by chemical degradation and by spectroscopic studies of its stable but inactive oxidation products.

The other important aspect to know the possible role of catalytic activity of molybdoenzymes (MPT) is the state of reduction[37] of MPT and hence molybdenum cofactor which was presumed to exist as a tetrahydro form primarily on the basis of the extreme air lability of Moco upon its release from protein[32-34] but like any other pterin the reduction forms may be 5,6-dihydro, 6,7-dihydro, 5,8-dihydro, and quinonoid dihydro (Figure 1 and Figure 2). Xanthine oxidase is an enzyme which exists, as purified, in active and inactive forms. Knowledge of state of reduction of MPT may lead to the understanding of the possible role of MPT in catalytic activity. Although quinonoid dihydropterins are generally highly unstable transient species (such as quinonoid-H_2B, Figure 2), some are relatively stable in HCl solution and it is suggested that MPT in xanthine oxidase is the readily reducible quinonoid dihydro form like the existence of quinonoid dihydrobiopterin in the aromatic amino acid hydroxylase reactions.[37,38] It is possible that molybdopterin, with several possible available levels of reduction takes part in controlling the redox behaviour of the molybdenum centres. Alternatively, the pterin ring of molybdopterin is an electron acceptor-donor, in which the molecule cycles between two or more oxidation states during electron transfer through the internal electron transport chains of the enzymes.

Johnson *et al.*[39,40] reported another compound called compound Z [proposed structure (7)] from molybdenum cofactor as a molybdopterin precursor.[41] In the crucial observation by Johnson, patients were identified who, although unable to biosynthesise compound Z, were capable of converting this intermediate to produce active sulfite oxidase; conversely, another group of patients accumulated compound Z (as found in their urine) but unable to transform it further to active sulfite oxidase (Figure 3). The defects in group A patients and *E. coli* ChlA 1 are a step preceding converting enzyme in the synthesis of molybdopterin but have not been further localised and may be different in each case.[39] Unlike molybdopterin or Moco, compound Z is stable and might be utilised as a prodrug with the former group of patients unable to biosynthesise compound Z, but capable of its subsequent conversion to active enzyme.

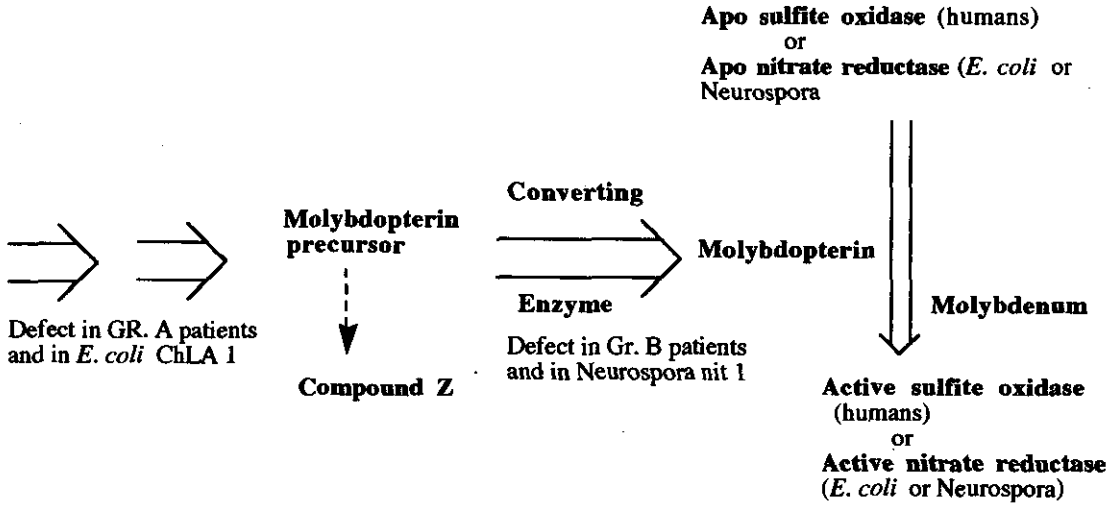


Figure 3 Pathway of Moco biosynthesis[39,40] in humans and microorganisms

Structural and Synthetic Aspects:

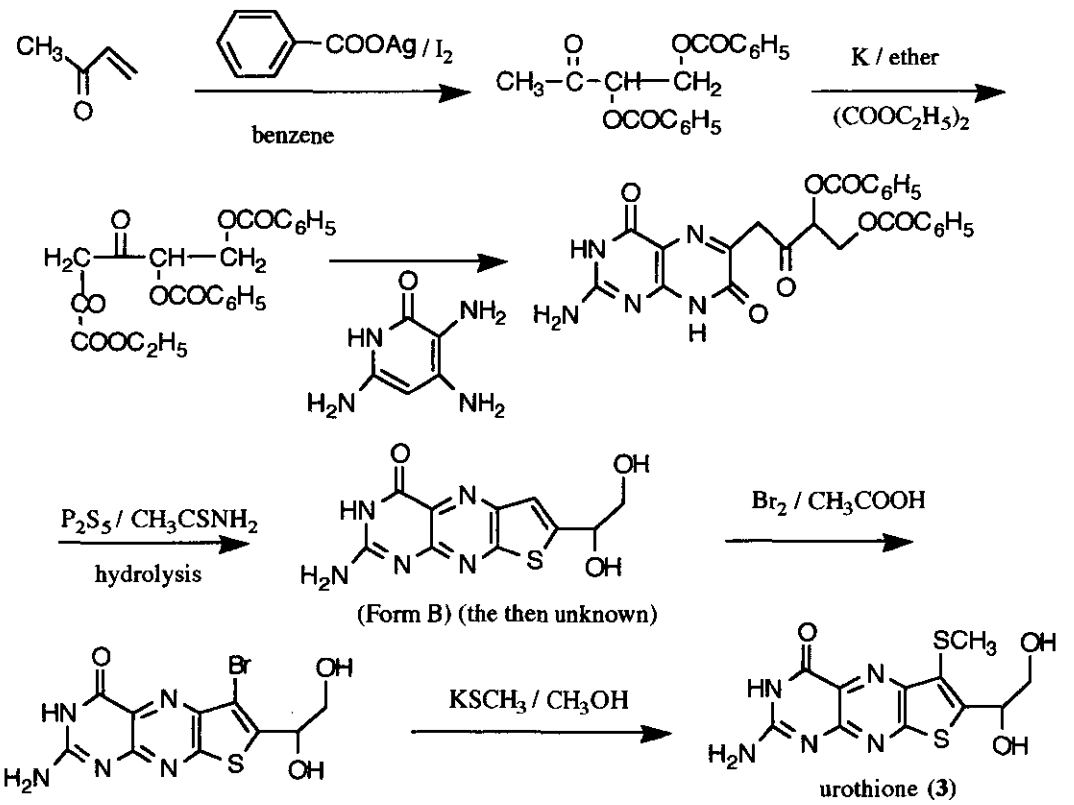
The extractable Moco is labile and has not been isolated in pure state and so far eluded direct structural characterisation. On the basis of persuasive chemical evidence, the tetrahydro structure (2a) was proposed for Moco in hepatic sulfite oxidase and milk xanthine oxidase.

Taylor *et al.* have reported unequivocal and efficient total syntheses of almost all the stable oxidative degradation forms isolated from the unstable Moco or molybdopterin (urothione,[42] Form A[43,44]- both optically active forms by asymmetric synthesis (and hence determination of absolute configuration of the natural Form A) as well as racemic form,[45] and Form B[46] of molybdenum cofactor) by unequivocal efficient routes and thus confirmed the structures. Taylor *et al.* also reported the synthesis of deoxyurothione.[47] Goto *et al.* has first reported the synthesis[48] of urothione[32, 49-52] where Form B was

intermediate but at that time Form B was not known. The extremely unstable Moco makes it too difficult to have synthetic access to the natural cofactor. However Taylor[53,54] and Goswami in collaboration with Pilato and Stiefel have been able to incorporate cyclopentadienylmolybdenum-enedithiol ring[53] in the alkynyl side chain at 6-position of pterin (15) and also synthesised[54] the stable protected Moco having oxidised pterin and cyclopentadienyl molybdenum, as the closest stable target (16) to the natural extremely unstable cofactor. The proposed biological precursor compound Z of molybdopterin and hence Moco has also been synthesised in its protected stable dephospho Forms (9) and (10) (both pure diastereomers) by Goswami and Taylor.[55] The synthetic routes of these forms are outlined as follows:

Goto' s synthesis of urothione[50] (3):

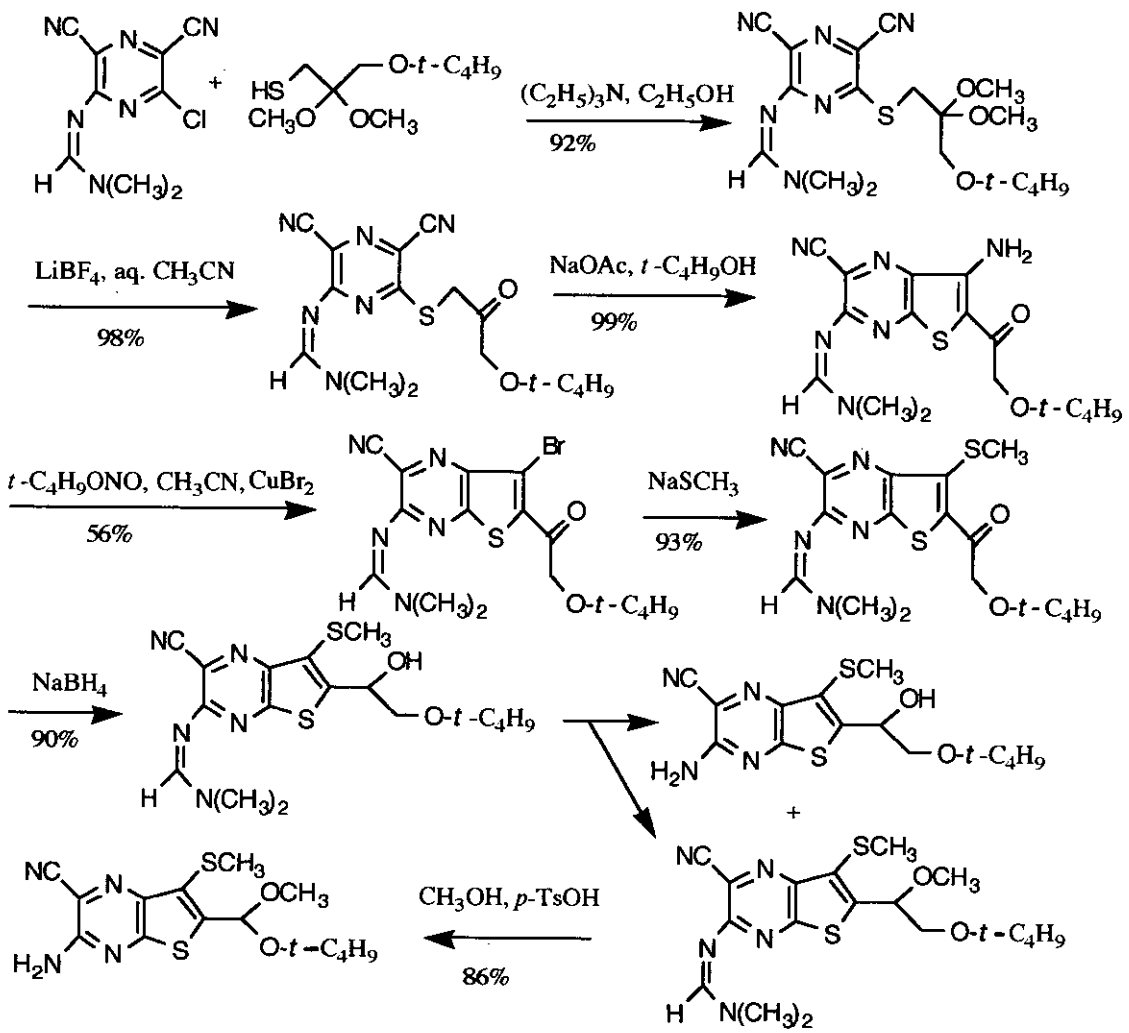
Scheme 1

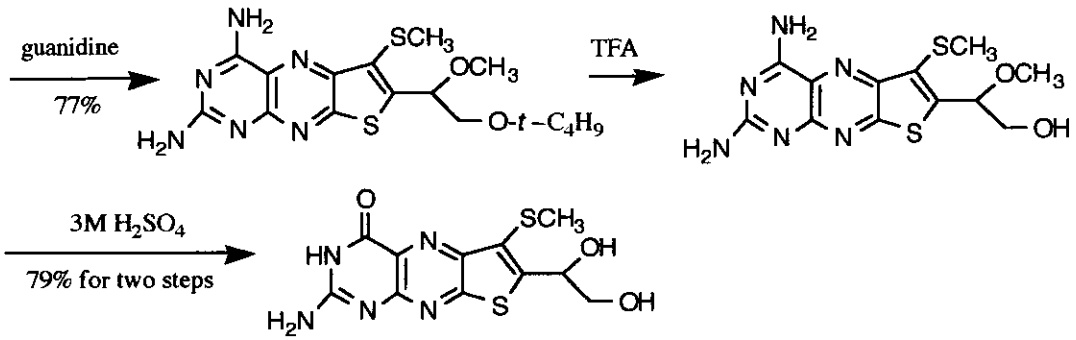


The first isolation of urothione from human urine was reported in 1940 by Koschara[49] and its structure and synthesis were reported in 1968 by Goto *et al.*[48, 50] The possible metabolic link between Moco and urothione has been established by Johnson and Rajagopalan[33,34] by showing that urothione is, in fact, the urinary metabolite[36] of Moco.

Taylor and Reiter: Unequivocal and efficient synthesis of (±)urothione[42] (3): Synthesis of urothione by Taylor and Reiter starts from protected pyrazine which undergoes thione annulation in the first phase and pyrimidine annulation in the last phase as outlined in Scheme 2.

Scheme 2



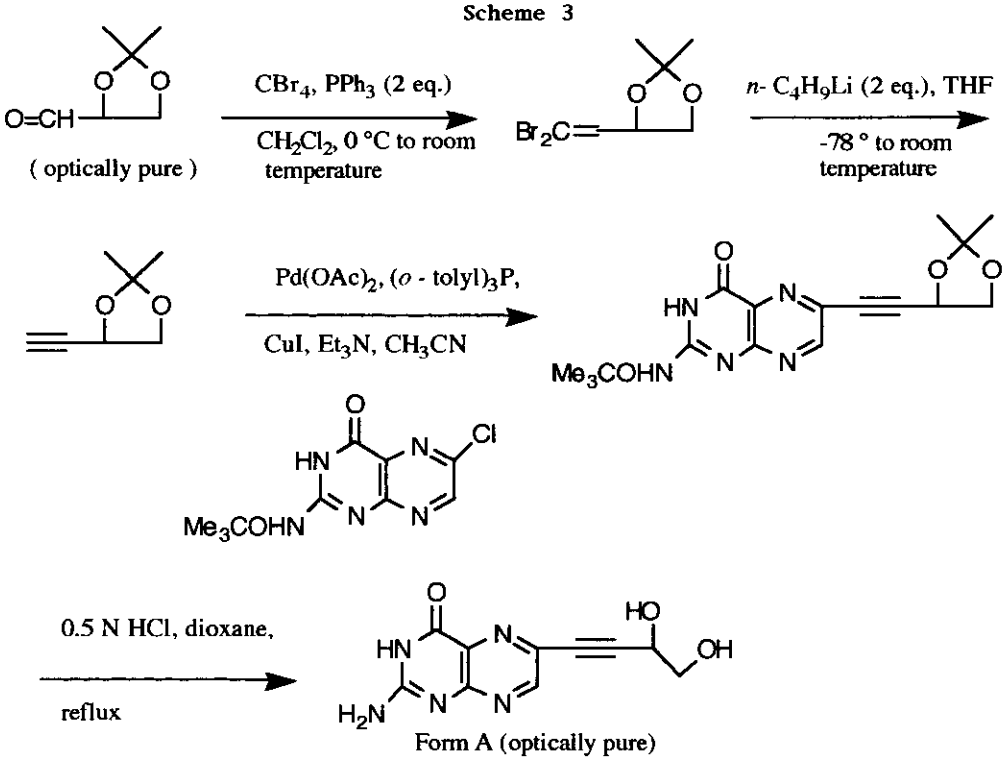


urothione (3)

Taylor and *et al.*: Synthesis of optically pure dephospho Form A (both enantiomers) and determination of absolute configuration:[43,44]

(R-Glyceraldehyde acetonide afforded the S- Form A and S - glyceraldehyde acetonide gave R - Form A).

Scheme 3



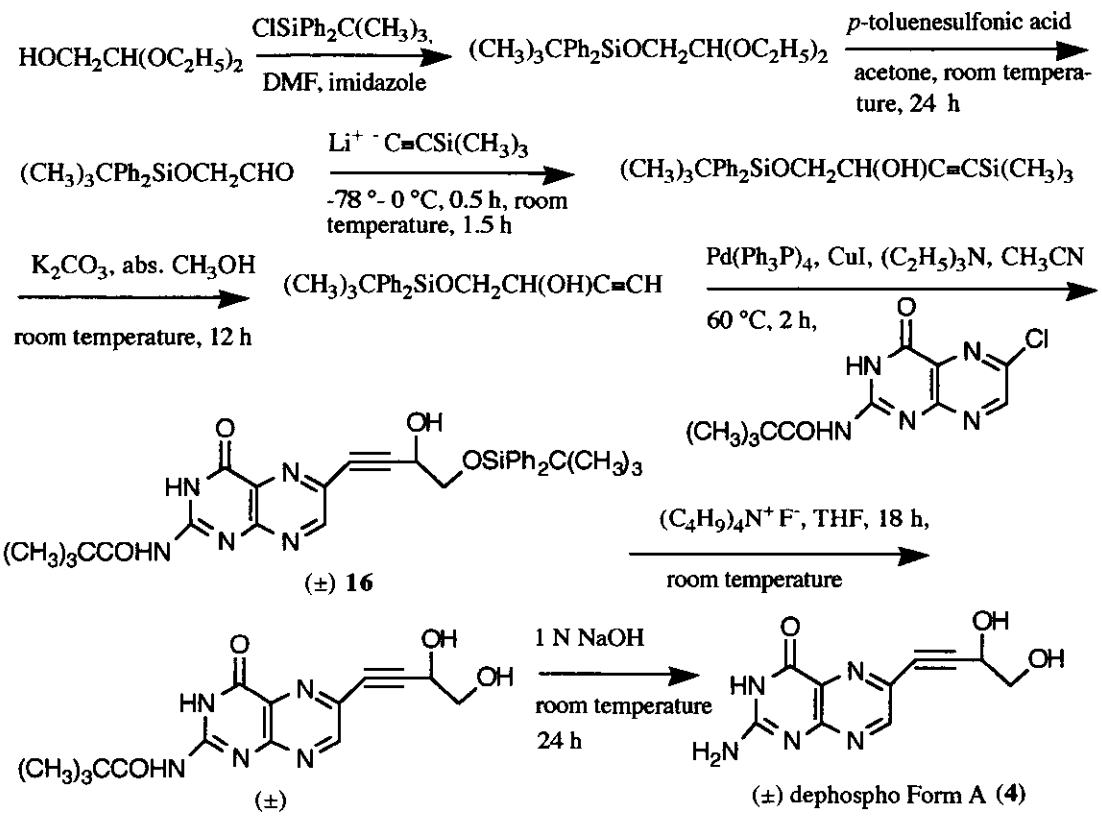
Form A (optically pure)

The determination of the absolute stereochemistry at the side chain asymmetric carbon centre bearing the secondary hydroxyl group in Form A (which is the same in other forms of Moco as well as in the

natural cofactor) has been established by Taylor *et al.*[36] by comparison of CD spectra of synthetic optically pure sample with the Form A of natural cofactor which is thus found to be S.

Taylor and Goswami: Synthesis of (±)dephospho Form A (**4**):[37]

Scheme 4

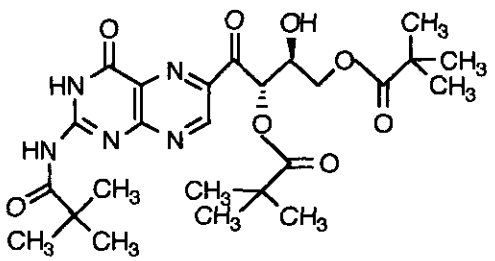


This efficient synthesis of racemic Form A made it possible to make the *t*-butyldiphenylsilyl protected intermediate (**16**) in appreciable amounts for the ultimate success to prepare the silyl protected cyclopentadienyl-Moco (**17**) in racemic form.[54] Since the incorporation of the cyclopentadienyl-molybdenum-enedithiolene moiety required the activation of the triple bond by oxidation of the propargylic alcohol group to the corresponding ketone, we developed this efficient synthesis of racemic intermediate (**16**), the silyl group of which survives in the subsequent oxidation step.[54]

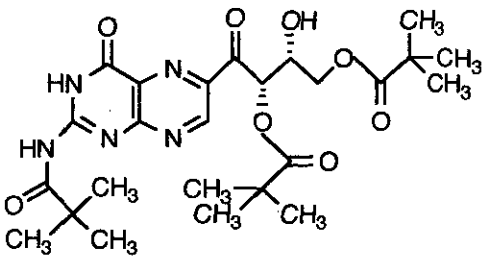
The present author working with Taylor on the total synthesis of compound Z[55] of the natural cofactor as well as Moco[53,54,45] itself has developed a new aldol reaction in the unequivocal synthesis of four

carbon sugars at the 6- position of pterin ring without complete protection of pterin- NH groupings (complete protection of all NH groups is in fact a general problem of pteridine chemistry). This aldol reaction is the key step in the proposed synthesis of compound Z and we have been able to synthesise efficiently both the diastereomers of the protected forms of compound Z and also deprotected it to give the dephospho compound Z which was very difficult to purify. However, the paucity of material and time precluded the further development by the present author of the cyclic six membered phosphate, the natural compound Z. The synthesis of both the diastereomers (**9**) and (**10**) in pure forms, enroute to the cyclic six membered phosphate, gave a clue to the relative stereochemistry of the adjacent two asymmetric centres in the natural compound Z which is otherwise unreported so far.

The separation of the diastereomers was successfully achieved by column chromatography and crystallisation to afford pure *threo* and *erythro* diastereomers of protected stable form of dephospho compound Z. The characterisation of *syn* and *anti* isomers was done by careful analysis of the coupling constants of H-2' and H-3' in the ^{1}H nmr Spectrum of the individual pure diastereomer (considering the predominant conformation)[55] and comparison with that of the natural compound Z (reported[41] coupling constant of H-2' and H- 3' (J<1) suggests that they are *syn* in the six membered cyclic phosphate which is not defined in the original structure[41]) settles that compound Z is *cis* cyclic phosphate originating from the *syn*-10.



9 (or enantiomer)



10 (or enantiomer)

Dithiolene co-ordination in Moco:

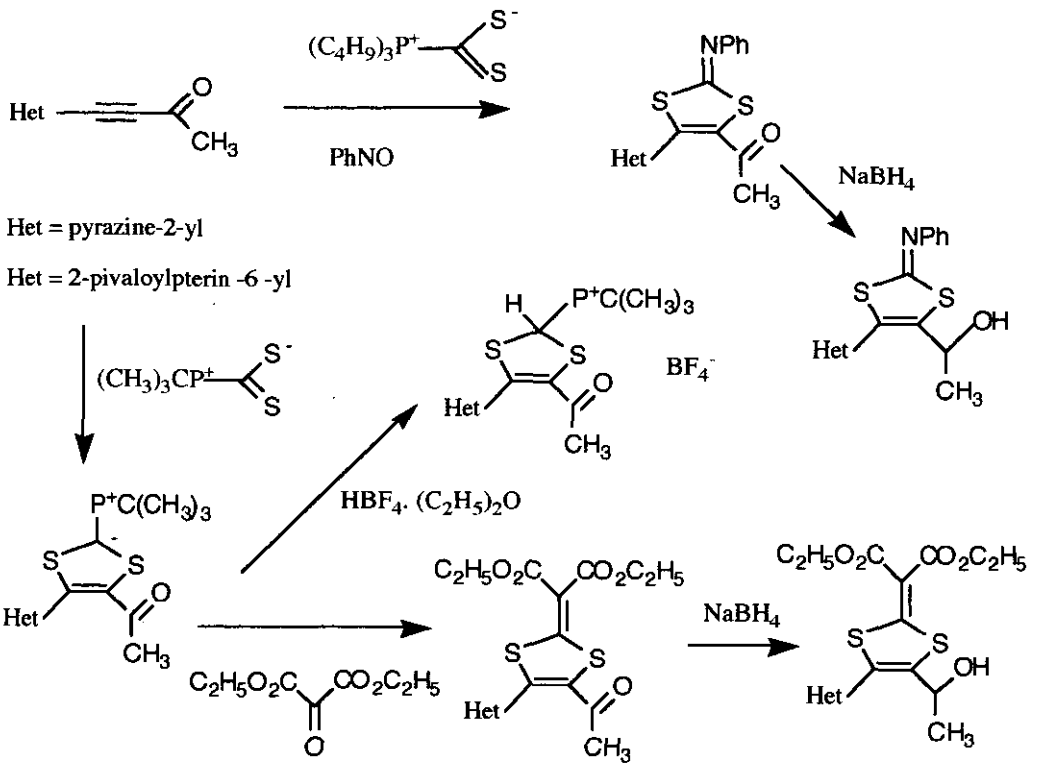
The molybdenum dithiolene ring as well as the underlying enedithiol moiety in Moco are unique among the biomolecules hithertofore known. These functional groups pose a challenging unsolved problem in natural product synthesis. A large number of complexes of Mo in various oxidation states is known[56,57] including complexes with thiols[58,59]and thio amino acids e,g, cysteine.[60] Reports have been made on *molybdenum dithiolate*[61-63] *and Mo-pterin complexes.*[64] The presence of this unusual dithiolene coordintion to molybdenum is also supported from the results of recent Raman studies by Spiro *et al.* on molybdenum dithiolene model complexs and on Moco from flavin-free DMSO reductase.[62,63, 60] Mo-phosphate interactions have also been demonstrated in model compounds of oxo-Mo (v) complexes.[65]

Joule and *et al.* have reported some model studies on quinoxazoline-dithiolene and dithiolane compounds at the 2-position of quinoxazoline.[66-68]

Synthetic models of Mo-enedithiolate moiety in Moco:

Taylor and Dotzer[69] reported novel pterin-substituted 2-alkylidene or 2-phenylimino-1,3-dithioles as shown in scheme 5.

Scheme 5

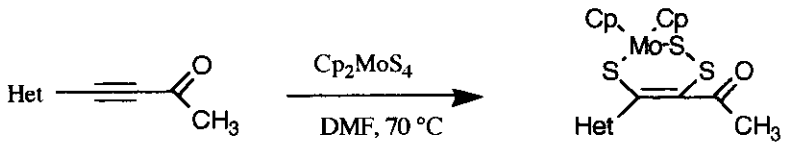


The activated alkynes are known to form 1,3-enedithiol by reaction with the betaine tributylphosphine-carbon disulfide complex followed by Wittig reaction with benzaldehyde.[70] However pterin or pyrazine substituted acetylenes are not reactive enough to give appreciable yields of these cycloaddition products presumably because pterin system is not sufficiently electron deficient to activate the triple bond. Thus the pyrazine or pterin substituted propargyl alcohol was carefully oxidised with Jones reagent at room temperature to the corresponding ketones which reacted smoothly to give the expected 1,3-dithioles in good yields which can generate the alcohol by $NaBH_4$ reduction. However the dithiolene ring failed to undergo cleavage to give the enedithiol intermediate to be isolated as alkyl derivative.

Incorporation of cyclopentadienyl Mo-enedithiol functionality:

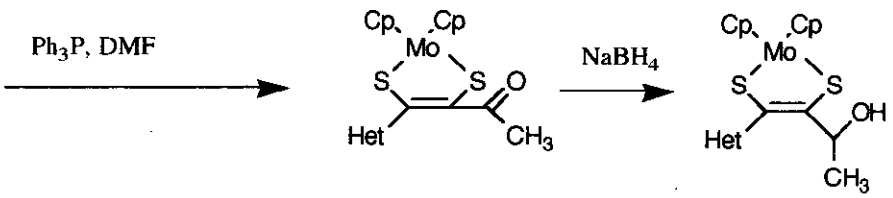
Taylor and Goswami in collaboration with Pilato and Stiefel at Exxon also reported[53] the synthesis of a analogue of the natural cofactor having cyclopentadienyl Mo-enedithiole functionality and characterised for the first time the formation of enetrithiolene (X ray structure, Figure 4) followed by dithiolene (X-ray structure, Figure 5) in course of reaction of pterin-proparzylic ketone (or quinoxazoline analogue) with $C_{p_2}MoS_4$.

Scheme 6



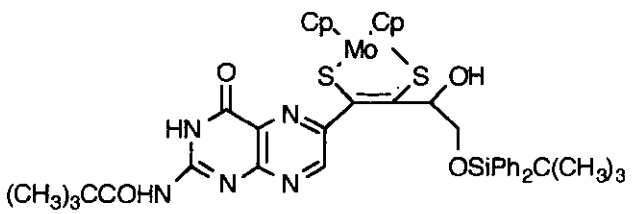
Het = quioxazoline-2-yl

Het = 2-pivaloylpterin-6-yl



15

The closest stable molecule to the natural cofactor (**protected oxidised Moco, 17**) having cyclopentadienyl-molybdenum in the enedithiolene ring with the required terminal hydroxymethyl group in the side chain (protected as *t*-butyldiphenylsilyl group) of the oxidised pterin ring has also been synthesised by Taylor,[54] Goswami, Pilato and Stiefel.



Protected cyclopentadienyl Moco (oxidised)

17

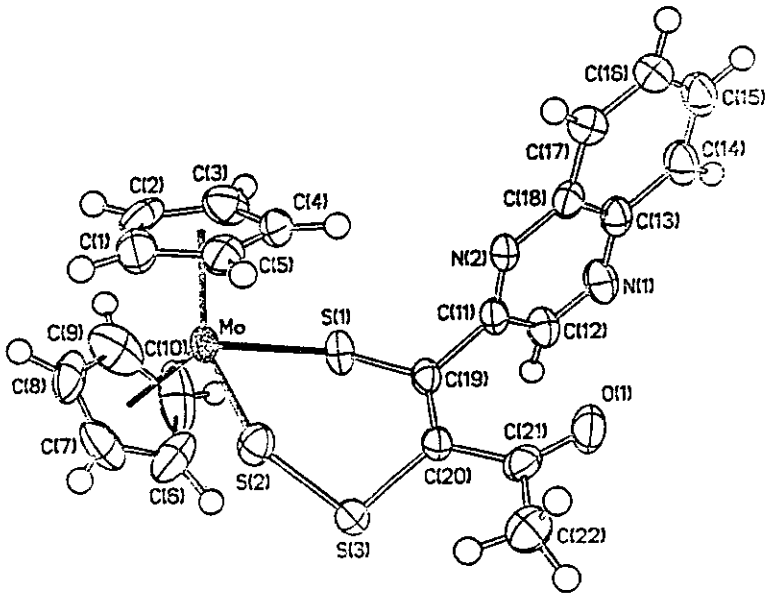


Figure 4

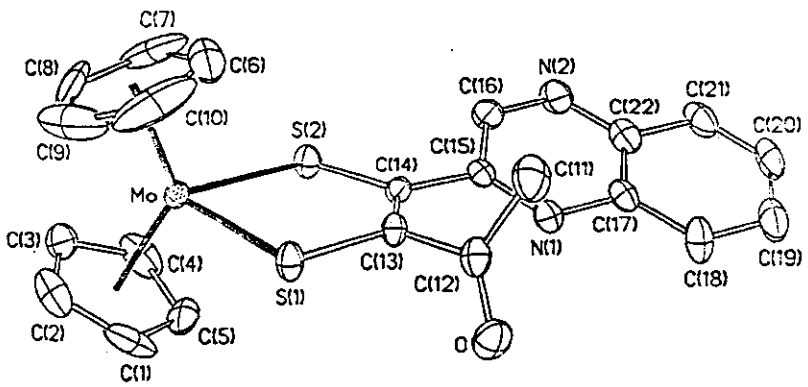


Figure 5

Biosynthesis:

The first biochemical studies of Moco were performed by Ketchum et.al.[71] The biogenesis of Mo-cofactor has been reviewed by Hinton and Dean.[72] The metabolic relationship has been established by Johnson and Rajagopalan.[33] The isolation of compound Z (7) led to the recognition of compound Z as the substrate molybdopterin converting factor.[39] However Mo acquisition by enzymes[73-76] is not a subject of detailed discussion here. Several groups have reported on incorporation of Mo as a trace element for life[77-79] and transport of Mo,[80,81] the essential component of active Mo-cofactor and molybdate.[81]

Though much work has been done on Mo- enzymes and the biological importance of Mo-cofactor is emphasised so much, the final synthesis of the active Mo-cofactor has not been reported. Unfortunately the extreme instability of the natural cofactor makes its synthesis too difficult and the final synthesis still remains an unsolved challenging problem in natural product synthesis.

ACKNOWLEDGEMENT

The author gratefully acknowledges Professor Edward C. Taylor for his guidance to work on this difficult aspect of natural product chemistry and the collaborators, specially Drs. E. I. Stiefel and R. S. Pillato. Princeton University and Exxon Research and Engineering Co., USA are acknowledged for providing excellent facilities. Dr. John Stuart (USA), Dr. Mrs. B. Goswami, Professor P. K. Sen (Presidency College, Calcutta), Dr. M. K. Jani, Dr. V. Sudarsanam, and the Director of B. V. Patel PERD Centre, India are thanked for encouragement.

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Received, 22nd February, 1993