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Blocked dihydropteridines as nitric oxide synthase activators

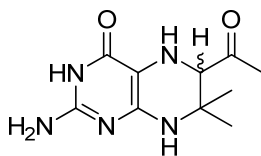
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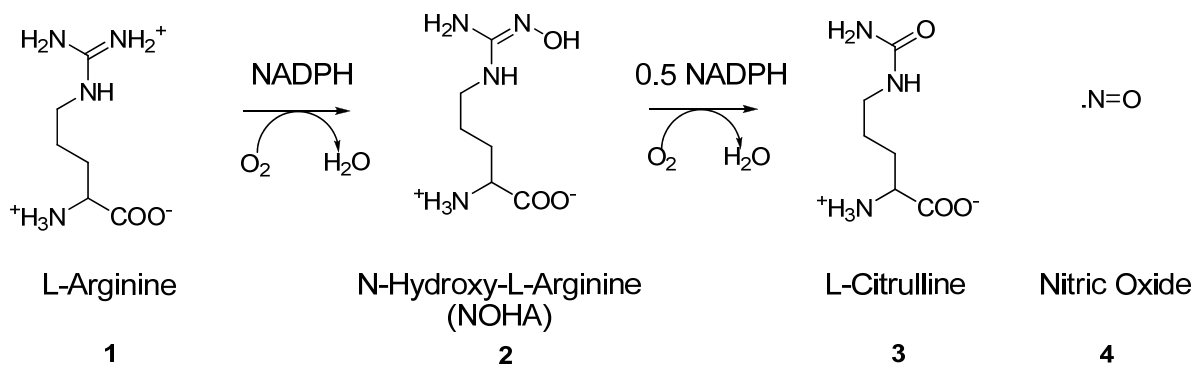
Abstract

It has been shown that 6-acetyl-7,7-dimethyl-5,6,7,8-tetrahydropteridin-4(3*H*)-one can act as a competent cofactor for the production of nitric oxide by neuronal nitric oxide synthase (nNOS). More information was sought on the structural features that could contribute to strong binding within the enzyme whilst maintaining a fast electron transfer rate. This study was concerned with expansion at the C2-position of the pteridine scaffold. The evidence suggests that expansion at the C2-position had a deleterious effect with respect to K_m and as a consequence electron transfer rate. Unexpectedly, several lines of evidence suggested that a methyl substituent on nitrogen at C2 reduced the electron density in the pyrimidine and dihydropterin rings.



Introduction

For many years it has been well established that nitric oxide plays an important role in mammalian biology as an intercellular messenger.¹⁻⁶ Nitric oxide **4** is derived from L-arginine **1** by the action of nitric oxide synthase (NOS) **Scheme 1**, a multi-domain enzyme which has three known isoforms. Nitric oxide has been implicated in three very important biological roles namely immune responses by macrophage cells (inducible NOS), neural signalling cascades in the central nervous system (neuronal NOS), and vasodilation by the relaxation of arterial smooth muscle cells (endothelial NOS). There is a high degree of sequence homology between the three isoforms (50-60%) and the mechanism of action of all three has been well studied. It is now widely accepted that a deficiency of an important cofactor 5,6,7,8-tetrahydrobiopterin (BH4) **5** has a crucial role in disease states such as endothelial dysfunction.⁷⁻⁸ A drop in the bioavailability of BH4 causes the uncoupling of the NOS homodimer which in turn halts the synthesis of nitric oxide. This down-regulation of nitric oxide, caused by a BH4 deficiency has many deleterious effects, from the production of reactive oxygen species (ROS) such as superoxides and peroxynitrites, to the further depletion of nitric oxide by these ROS. From this, it can be seen that in certain disease states, where there is a deficiency of BH4, it is desirable to return this function to the NOS enzyme. It is believed that BH4 plays a vital role in the shuttling of electrons through the enzyme to the substrate, arginine, while also playing a central role in stabilising the NOS dimer.⁷

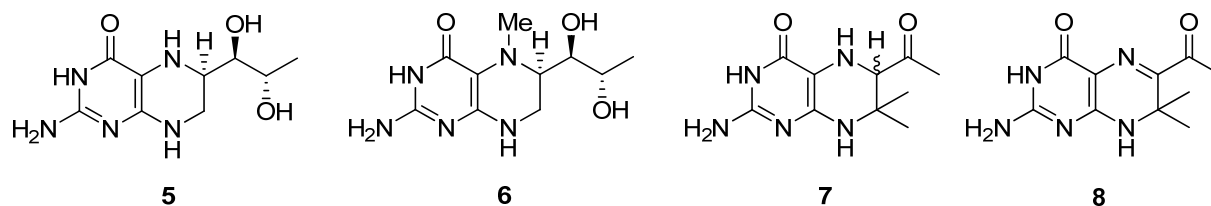


Scheme 1

Until recently, it was thought that BH₄ itself is not suitable as a drug due to its low bioavailability and poor stability *in vivo*. However with the advent of Kuvan, a synthetic form of tetrahydrobiopterin, this is no longer the case. It has been reported that Kuvan has an increased bioavailability compared to BH₄ itself, with an increase in C_{max} of 84% and an increase in AUC of 87%.⁹ Kuvan is currently prescribed for patients suffering from BH₄-responsive phenylketonuria (PKU). This is a disease which affects phenylalanine hydroxylase (PAH) and is a subset of PKUs. PAH is the enzyme responsible for controlling phenylalanine blood levels and its activity can be up-regulated by the administration of BH₄ or Kuvan. However, to the best of our knowledge, no data has been presented to suggest that Kuvan displays any significant activity towards any of the NOS enzymes. This highlights the possibility that other tetrahydropteridines could be useful in the treatment of BH₄ deficiency in NOS production. This possibility has previously been suggested by the BH₄ derivative, 5-methyltetrahydrobiopterin **6**, which also acts as a NOS activator.¹⁰

In the 1980's Wood, Suckling and colleagues synthesised so-called blocked dihydropteridines in which the 7-position contained geminal alkyl groups preventing the compounds from aromatising.¹¹ At the time of the original synthesis, the biochemistry of nitric oxide and NOS

was not known and this class of compound was first evaluated with NOS in 2002, at which point **8** was identified as able to relax precontracted rat aortic rings, and hence a putative activator of eNOS. In 2008 it was observed that the action of blocked dihydropteridines as activators of NOS occurred only after reduction to their corresponding tetrahydro oxidation state **7** from studies with nNOS.¹



Modelling studies suggested that **7** bound to nNOS in the same pocket and in the same orientation as BH₄, conserving the important hydrogen bonding interactions. It was thought that **7** could be stabilising the NOS dimer and participating in the electron transfer cascade. It was of interest, therefore, to find out what properties of **7** made it such a suitable cofactor for NOS and hence to establish a structural activity relationship. With the potential for therapeutic application in mind, we planned to build upon this information to discover a compound with suitable qualities with respect to binding constant and rate of nitric oxide production than **7**. In addition, the physicochemical properties of the corresponding dihydro compound **8** were unsuitable for therapeutic application, primarily due to low solubility.

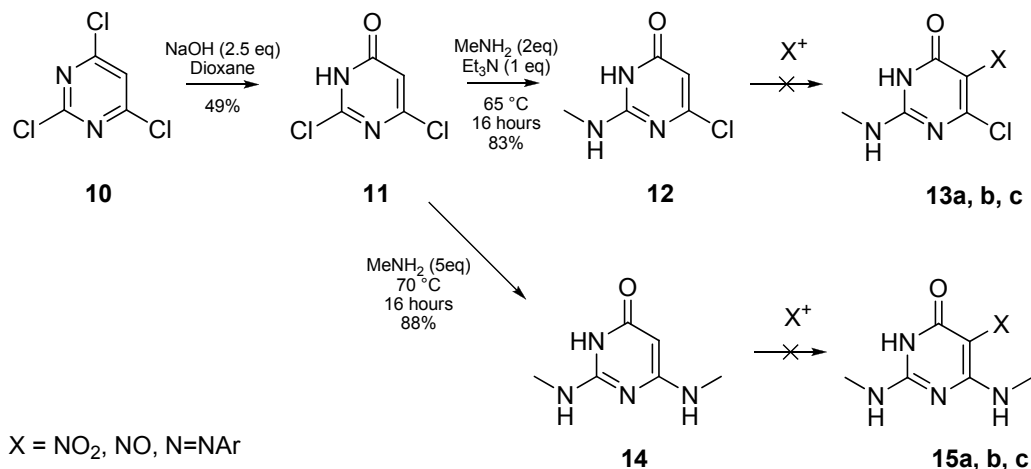
Results and Discussion

X-ray crystallographic studies had shown that the hydrogen bonding interactions between the pyrimidine ring of BH₄ and the haem ring of NOS were of high importance.⁷ To investigate

these interactions we aimed to synthesise a BH4 analogue based on **7** with a 2-methylamino group. Such a substitution might increase the electron density within the pteridine ring due to the inductive effect of the methyl group and hence increase the electron donating ability of the tetrahydropterin bound to NOS, a change that might influence electron transfer at the NOS active site.

C2 Expansion

The first synthetic route undertaken paralleled the typical Boon approach and started from the commercially available 2,4,6-trichloropyrimidine, **10**. Hydrolysis at the 4-position was carried out with sodium hydroxide.¹² The 2-methylamino group was introduced with methylamine in THF keeping the reaction temperature at or below 65 °C to avoid the formation of the bis(methylamino)pyrimidine **14**.

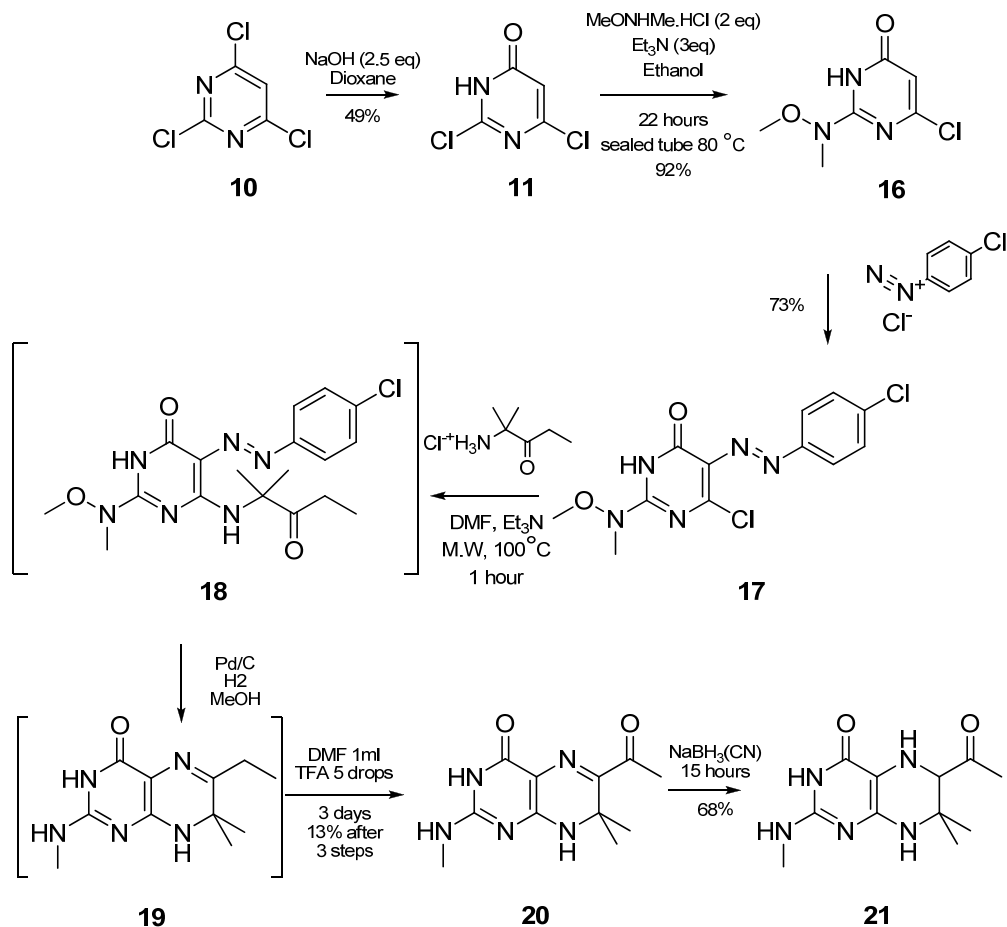


Scheme 2

Surprisingly, it was impossible to insert any nitrogen based electron-withdrawing group at the 5-position with any of the typical electrophilic reagents for this reaction. Nitration by classical nitration mixture, nitronium tetrafluoroborate in various solvents, and acetyl nitrate all failed.

Nitrosation by isoamyl nitrite and diazotisation with the diazonium salt derived from *p*-^tbutylaniline both failed. However all of these reagents react with 2-amino-6-chloropyrimidin-4(3*H*)-one and hence, it appeared that the methyl group was somehow unexpectedly deactivating the pyrimidine ring towards electrophilic aromatic attack. This suggests that the addition of a methyl group to the 2-amino group was paradoxically reducing the electron density in the pyrimidine ring. An alternative synthetic approach to 2-*N*-substituted pteridines involving 2-methoxy-*N*-methylaminopyrimidine **16** had previously been used in pteridine synthesis and it was known that the methoxy group attached to the methylamino group could be cleaved under a variety of conditions.¹³ It appeared that this would be a suitable choice as it was known to be possible to couple a diazonium salt to this type of pyrimidine ring facilitating pteridine synthesis,

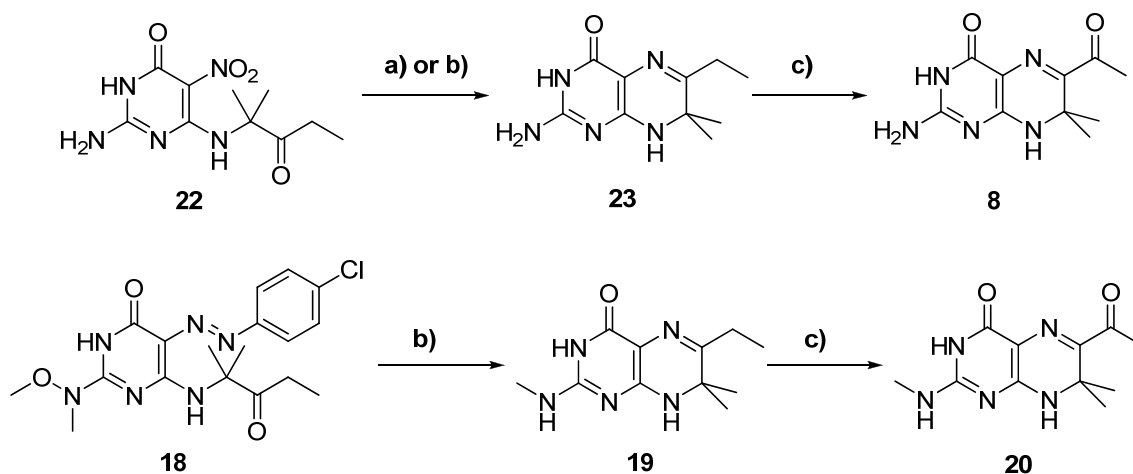
Scheme 4.



Scheme 4

Accordingly 2,4,6-trichloropyrimidine **10** was converted into the diazo pyrimidine **17** in three steps (Scheme 4).¹³ This diazo intermediate is unstable and was immediately coupled with the α -aminoketone which was carried out using microwave heating. The intermediate was immediately reduced, hydrogenolysed and cyclised in situ to give the dihydropteridine, **19**. It was necessary to purify **19** by hplc and during this process it was noted that after 1 day the initially colourless solution had become slightly yellow. After 3 days no further change was observed and the product proved to be the 2-*N*-methyl-6-acetylpteridine **20**. Compound **20** was converted into its tetrahydro derivative **21** by a sodium cyanoborohydride reduction in quantitative yield. **20** was taken on for biological testing. (See biological results)

In blocked dihydropterins with C6 ethyl groups or larger alkyl groups, oxidation typically takes place at the side chain under mild conditions and proceeds at an optimum rate in propan-2-ol at 60 °C; ¹³ oxidation of the side chain at room temperature was therefore unexpected. The rate of oxidation in DMF was not known and a suitable comparison was needed. The *N*-methyl group would normally be expected to donate electron density into the ring; if this were the case, an increased rate of oxidation would be expected. However, previously it had been observed that the *N*-methyl group appeared not to be activating the pyrimidine ring towards electrophilic substitution. It was also possible residual palladium from the reduction of the diazo intermediate influenced side chain oxidation. For the cause of oxidation to be established, the rates of oxidation of three differently prepared compounds would have to be compared **scheme 5**. Compound **22**, an intermediate in the synthesis of **7**, provides a basis for comparison with the corresponding *N*-methyl precursor whilst also providing the starting point for the comparison of C-H activation against solvent effects.



Scheme 5: a) Na₂S₂O₇, NaOH b) Pd/C, H₂, MeOH c) DMF, TFA,

Reduction and ring closure of compound **22** was triggered either by sodium dithionite **a)** or by hydrogenation **b)**. When ring closure was triggered by sodium dithionite, only partial oxidation of **22** to **8** was observed within a 24 hour period (Table 1). It was found that the oxidation of **23** was accelerated by the presence of trace amounts of palladium. Comparing these results with those for the oxidation of the *N*-methyl substituted compound **19**, it was found that even when activated by trace amounts of palladium, oxidation of **19** took at least two days more to proceed to completion. As it would typically be expected that oxidation at a C6 substituent would be accelerated by an electron donating substituent at C2, the relative rates of oxidation could be an indication that pteridines containing the methylamino group as seen in **19** and **20** have a lower electron density than expected compared their amino analogues, such as **23** and **8**. These results parallel those of the pyrimidine series with regard to electrophilic substitution described above.

Compound	Extent of oxidation at 1 day
23a	9 %
23b	25%
19	None observed

Table 1

These properties could have biological implications. If these 2-*N*-methyl pteridines have a lower electron density than the corresponding pterins and bind to NOS, they could be poorer electron donors. This would be undesirable quality for therapeutic applications but could be helpful in mechanistic studies.

Biological results

The *N*-methylpteridine **21** was tested as an activator of nNOS using BH₄-free nNOS in the manner described previously.¹ Neither activation nor inhibition of nNOS was observed when **21** (20 μM) was incubated with nNOS in the presence of arginine as substrate. These results show **21** does not bind to nNOS and suggests that the expansion at the C2 position is an unfavourable strategy for BH₄ mimetics

Conclusion

From the data obtained, expansion into the C2 region is unfavourable with respect to NOS activation, probably due to steric interactions of the methyl group that prevent proper orientation for electron transfer. However the study has revealed unexpected reactivity patterns in 2-*N*-methyl pteridines that can be accounted for by a reduced overlap of the non-bonded electrons of the 2-nitrogen with the conjugated pyrimidine ring. Possible origins of this unexpected behaviour are being investigated by computer modelling.

Experimental procedures can be found in the Supplementary Information.

References

1. Suckling, C. J.; Gibson, C. L.; Huggan, J. K.; Morthala, R. R.; Clarke, B. Kununthur, S.; Wadsworth, R. M.; Daff, S.; Papale, D. *Bioorganic & Medicinal Chemistry Letters*. 2008, 18, 1552
2. Wadsworth, R.; Suckling, C.; Gibson, C. U.S. Pat. Appl. Publ. (2009), 17pp., Cont.-in-part of U.S. Ser. No. 322,914. CODEN: USXXCO US 2009075992 A1 20090319 CAN 150:322673 AN 2009:336538 CAPLUS
3. Pfeiffer, S.; Mayer, B.; Hemmens, B. *Angew. Chem. Int. Ed. Engl.* 1999, 38, 1714.
4. Stuehr, D. J. *Biochim. Biophys. Acta* 1999, 1411, 217.

5. Alderton, W. K.; Cooper, C. E.; Knowles, R. G. *Biochem. J.* 2001, 357, 593.
6. Huang, H.; Younghee, L.; Zhang, H. Q.; Fast, W.; Riley, B.; Silverman, R. B. In *Medicinal Chemistry into the Millennium*; Campbell, M. M., Blagborough, I. S., Eds.; Royal Society of Chemistry: Cambridge, 2001; p 325.
7. Wei, C.-C.; Crane, B. R.; Stuehr, D. J. *Chem. Rev.* 2003, 103, 2365.
8. Sorlie, M.; Gorren, A. C. F.; Marchal, S.; Shimizu, T.; Lange, R.; Andersson, K. K.; Mayer, B. J. *Biol. Chem.* 2002, 272, 45002.
9. Food and Drug Administration, FDA labeling information (online)
<http://www.kuvan.com/HCP/PI/PrescribingInfo.aspx>
10. Riethmuller, C.; Gorren, A. C. F.; Pitters, E.; Hemmens, B.; Habisch, H. J.; Heales, S. J. R.; Schmidt, K.; Werner, E. R.; Mayer, B. J. *Biol. Chem.* 1999, 274, 16047.
11. Al-Hassan, S. S.; Cameron, R. J.; Curran A. W. C.; Lyall W. J. S.; Nicholson, S. H.; Robinson, D. R.; Stuart, A.; Suckling, C. J.; Stirling, I.; Wood, H. C. S. *J. Chem. Soc. Perkin Trans. 1.* 1985, 1645.
12. Huebsch, W.; Pfeleiderer, W. *Helvetica Chimica Acta.* 1989, 72, 4, 738
13. R. R. Morthala, Ph. D. Thesis, University of Strathclyde, 2007, p 103
14. Roman, L. J.; Sheta, E. A.; Martasek, P.; Gross, S. S.; Liu, Q.; Masters, B. S. S. *Proc. Natl. Acad. Sci. U.S.A.* 1995, 92, 8428.
15. Sagami, I.; Daff, S.; Shimizu, T. *J. Biol. Chem.* 2001, 276, 30036.
16. Daff, S.; Sagami, I.; Shimizu, T. *J. Biol. Chem.* 1999, 274, 30589.