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6-Acetyl-7,7-dimethyl-5,6,7,8-tetrahydropterin is an activator of nitric oxide synthases

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Abstract—6-Acetyl-7,7-dimethyl-7,8-dihydropterin **3** has been shown to be able to substitute for the natural cofactor of nitric oxide synthases, tetrahydrobiopterin **1**, in cells and tissues that contain active nitric oxide synthases (NOSs). In both macrophages, which produce iNOS, and endothelial cells, which produce eNOS, in which tetrahydrobiopterin biosynthesis has been blocked by inhibition of GTP cyclohydrolase **1**, dihydropterin **3** restored production of nitric oxide by these cells. In tissues, **3** caused relaxation in preconstricted rat aortic rings, again in which tetrahydrobiopterin biosynthesis had been inhibited, an effect that was blocked by the NOS inhibitor, L-NAME. However, dihydropterin **3** was not itself an active cofactor in purified NOS (nNOS) preparations free of tetrahydrobiopterin suggesting that intracellular reduction to 6-acetyl-7,7-dimethyl-5,6,7,8-tetrahydropterin **4** is required for activity. Compound **4** was prepared by reduction of the corresponding 7,8-dihydropterin with sodium cyanoborohydride and has been shown to be a competent cofactor for nitric oxide production by nNOS. Together, the results show that the 7,7-dimethyl-7,8-dihydropterin is a novel structural framework for effective tetrahydrobiopterin analogues.

Nitric oxide is now well established as an intercellular mediator with a wide range of functions in different cell types.[1–4] In the vascular endothelium, nitric oxide release promotes smooth muscle relaxation and corresponding vasodilation. In the CNS, nitric oxide takes part in neuronal signalling and has been implicated in the pathology of many diseases. Both the endothelial NOS (eNOS) and the neuronal NOS (nNOS) are constitutive enzymes, however a third isoform, inducible NOS (iNOS) is produced by macrophages as part of the immune response leading to the formation of reactive species such as peroxynitrite. As with nNOS, excessive production of nitric oxide by iNOS can be harmful. Pathology can result from excess of NOS activity due to increased production or to insufficient nitric oxide due to inadequate enzyme levels or to inadequate supplies of one of the essential cofactors, tetrahydrobiopterin, **1**. Clearly a balance of availability of nitric oxide in all tissues is important for well-being.[3]

Nitric oxide donors such as *S*-nitroso compounds, glycerin nitrate, are widely used in the treatment of cardiovascular disorders in which endothelial relaxation may be compromised. However, such compounds are systemic and their effectiveness as drugs is limited by side effects. Unsurprisingly with this range of actions, the enzymes that synthesise nitric oxide, nitric oxide synthases (NOSs) have attracted much attention as targets for therapeutic intervention by inhibitors. Both the substrate (arginine) binding site[5–7] and the cofactor (tetrahydrobiopterin **1**) binding site have been investigated. In the latter case, many pteridine derivatives have been shown to be inhibitors of NOSs.[8–11]

The mechanism of action of NOSs has been thoroughly investigated[12,13] and the consensus is that tetrahydrobiopterin has both a structural role in maintaining the ac-

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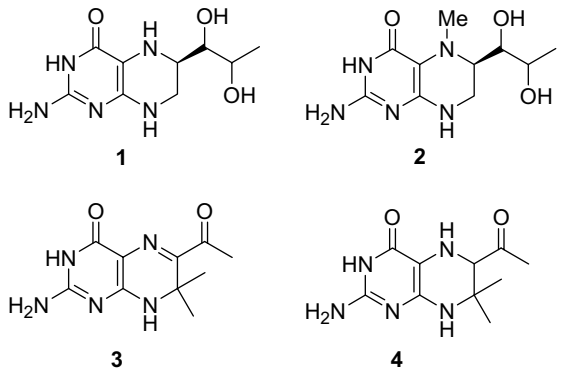
tive conformation of the dimeric enzymes and in electron transfer to the haeme iron at which oxidation takes place. It is therefore reasonable to expect that competent activators of NOSs must have structures closely related to tetrahydrobiopterin itself. Indeed very few such compounds have been discovered the most potent of which is 5-*N*-methyl-tetrahydrobiopterin, **2**.[14] The tetrahydro oxidation state also appears to be obligatory. Pterin biosynthesis, however, largely takes place at the dihydro oxidation state. Conscious of this and of the oxidative instability of tetrahydropterins, over 20 years ago at Strathclyde, we synthesised a family of so-called blocked dihydropterins which contained geminal alkyl groups at C7 and thus were not susceptible to aromatisation.[15,16] At that time, the biochemistry of nitric oxide was unknown; the significance of nitric oxide as now well understood led us to consider that some of our compounds might be able to mimic the properties of tetrahydrobiopterin and activate NOSs. 6-Acetyl-7,7-dimethyl-7,8-dihydropterin **3** was selected for study.



Dihydropterin **3** is a stable compound soluble in water and polar, hydrogen bonding organic solvents, and substantially more so than tetrahydrobiopterin and other pterins that lack the blocked dihydro structural feature. Compound **3** was prepared by methods previously described[15,16] by substitution of 2-amino-6-chloro-5-nitropyrimidin-4(3*H*)-one with 2-amino-2-methylpentan-3-one (36%) and subsequent reductive cyclisation to the corresponding 6-ethyl pterin (94%); oxidation of the 6-ethyl group to the 6-acetyl group was achieved with air passed through a solution of the precursor 6-ethyl pterin in *n*-butanol–acetic acid–water (52%). For studies with purified, tetrahydrobiopterin free nNOS, **3** was reduced in methanol solution acidified with dilute aqueous hydrochloric acid with sodium cyanoborohydride to afford the racemic 5,6,7,8-tetrahydropterin, **4** (87%) which was purified by HPLC and used within two days of purification. Provided that hydrochloric acid was used for acidification, it was possible to reduce the C–N double bond without reduction of the carbonyl group. If methanol was acidified by acetic acid, partial reduction of the C6 acetyl group also occurred.

The evaluation of **3** in cells used two cell types. First, macrophages which produce iNOS, cultured from mouse bone marrow were investigated. For NO assay, macrophages were assessed both without pretreatment and after preincubation with 2,4-diamino-6-hydroxypyrimidine, a known inhibitor of the first enzyme in the biosynthesis of tetrahydrobiopterin, thereby allowing for competition between the dihydropterin **3**, and as will be seen from the following, its tetrahydro derivative **4**, and endogenously synthesised tetrahydrobiopterin. The dihydropterin **3** was added to macrophages as a solution in DMSO to give a final concentration of 1 μM. Nitric oxide production was determined by assay of aliquots from the reaction mixture using the Griess method determining total nitrite plus nitrate.[17] Under the assay conditions used after 72 h, there was no significant difference between the quantity of nitric oxide produced by undepleted cells and in the presence or absence of **3** (Table 1). However, when tetrahydrobiopterin depleted cells were studied, clear evidence for nitric oxide production was obtained. Similar experiments were carried out in endothelial cells, which produce eNOS, cultured from porcine pulmonary artery with a similar outcome, namely that **3** supported nitric oxide production in cells depleted of endogenous tetrahydrobiopterin. These experiments lead to two important conclusions: dihydropterin **3** is able to penetrate cell membranes and is able to provide an alternative to the natural cofactor, tetrahydrobiopterin, for the production of nitric oxide by those cells.

The functional evaluation of **3** was carried out using rat aortic rings. Rings of rat aorta were maintained in physiological salt solution at 37 °C and gassed with 95% O_2/5% CO_2. They were incubated for 6 h either with the inhibitor of GTP cyclohydrolase-1 2,4-diamino-6-hydroxypyrimidine (10 mM) to deplete endogenous tetrahydrobiopterin, or with vehicle. The aortic rings were then preconstricted with phenylephrine at its EC_{50} of 1.2×10^{-7} M. Consistent with the observations in endothelial cells and macrophages, the dihydropterin **3** relaxed aortic rings that had been depleted of tetrahydrobiopterin but had no effect on rings that had not been depleted. At 0.3 mM, the dihydropterin **3** produced a maximal relaxation of 24.2 ± 7.8%, which compares with 0 ± 5% for the vehicle control (0.3 mM NH_4OH in the tissue experiments). The vasorelaxation was blocked by incubation with L-NAME, an inhibitor of nitric oxide synthase (3×10^{-4} M),[5] and the residual relaxation in the presence of both **3** and L-NAME was 5.4 ± 2.7%. In these experiments, the EC_{50} of dihydropterin **3** was 10 nM; under the same conditions, the natural cofactor, tetrahydrobiopterin **1**, caused no relaxation but a slightly increased contraction.

Together the results in cells and tissues pose a mechanistic question. Dihydropterin **3** is clearly effective and

Table 1. Nitric oxide production in macrophages and endothelial cells promoted by **3**

Cell type	NO produced (μM)	
	Vehicle	Vehicle + 3
Macrophage untreated	2.1 ± 0.3	1.7 ± 0.4
Macrophage BH_4 depleted	4.1 ± 1.0	6.2 ± 0.5
Endothelial BH_4 depleted	8.5 ± 0.8	11.4 ± 1.0

The concentration of **3** in the macrophage experiments was 1 μM and in the endothelial cell experiments, 30 μM.