SYNTHESIS OF THE METABOLITE *N*-HYDROXY-DESFERRIOXAMINE B

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N-Hydroxy-desferrioxamine B (**5**), a postulated metabolite of the microbial product desferrioxamine B (**1**), has been prepared by reduction of the intermediate oxime **6** with sodium cyanoborohydride. The oxime was obtained by selective oxidation of desferrioxamine B with hydrogen peroxide and a catalytic amount of sodium tungstate dihydrate. The iron complex derived from **5** enabled definite proof of the structure of one of four metabolites of desferrioxamine B found in urine samples of patients treated with this drug.

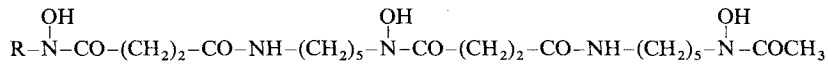
The secondary metabolite desferrioxamine B (**1**) was originally isolated from *Streptomyces pilosus* in 1960 by BICKEL *et al.*[1] The iron containing siderophore ferrioxamine B plays an important role in iron transport in microbes, plants and animals.[2]

In the form of the methanesulfonate salt, the chelator desferrioxamine B (**1**) has been used as a drug to treat patients with transfusional iron overload or acute iron intoxication.[3] Another important therapeutical application of desferrioxamine B is the removal of aluminum from patients suffering from impaired renal function and undergoing chronic haemodialysis treatment.[4,5] Recently it has been shown that the sustained administration of desferrioxamine B slowed the clinical progression of the dementia associated with ALZHEIMER's disease.[6] Furthermore desferrioxamine B has been proposed as a promising drug for the treatment of malaria because of its inhibitory activity against plasmodia *in vitro* and *in vivo*.[7~10] In a recent clinical trial it was shown that the administration of desferrioxamine B reduced significantly the adverse effects of reperfusion injury.[11,12]

Although desferrioxamine B (**1**) has been firmly established as a safe drug in chelation therapy, various toxic side effects have been reported, especially in patients treated with high doses.[13~16] The hypothesis that the toxicity could be due to the depletion of other essential metal ions such as zinc or copper by the chelator has not been substantiated by experimental evidence.[17] Since the side effcets are observed more frequently in patients with limited iron overload, it has been speculated that the toxic effects of the drug could be linked to the formation of certain metabolites.[18] It is well known that ferrioxamine B, the iron complex, is quite stable towards metabolic degradation whereas the free ligand is rapidly metabolized. In view of the potentially more widespread use of desferrioxamine B for the treatment of diseases which are not associated with chronic iron overload, such as ALZHEIMER's disease or malaria, it is imperative to carefully investigate the metabolism of desferrioxamine B and to evaluate the toxic potential of the metabolic products.

In 1964, KEBERLE[19] reported three metabolites in the urine of patients treated with the drug and he assigned structure (**2**) to the main metabolite in which the aminomethylene group of desferrioxamine B (**1**) is replaced by a carboxy group. The synthesis of this major metabolite (**2**) has been described recently by DIONIS *et al.*[20] Several high-performance liquid chromatography methods for the determination and

Fig. 1. Metabolites of desferrioxamine B.



1 R = $H_2N-(CH_2)_5$
2 R = $HOOC-(CH_2)_4$
3 R = $HOOC-(CH_2)_2$
4 R = $H_3C-(CH_2)_3$
5 R = $HO-NH-(CH_2)_5$

quantification of desferrioxamine B and metabolites as their iron(III) complexes have been developed.[18,21~23] Recently SINGH *et al.*[22] published an HPLC method that enabled the authors to separate four metabolites (**2**~**5**) from the urine of treated patients (Fig. 1). Structures of the identified metabolites (**2**~**5**) were suggested based on their fast atom bombardment mass spectra. All detected metabolites (**2**~**5**) are formed by a metabolic degradation at the primary amino group of desferrioxamine B. For the first time the hydroxylamine analogue of desferrioxamine B (**5**) was described as a metabolite of this iron chelator.

In this paper we describe the synthesis of *N*-hydroxy-desferrioxamine B (**5**) to provide a definite proof of the proposed structure of this metabolite. In addition, the availability of reference samples of the synthetic intermediate **6** will allow investigations of the role of the oxime in the metabolic degradation pathway of desferrioxamine B.

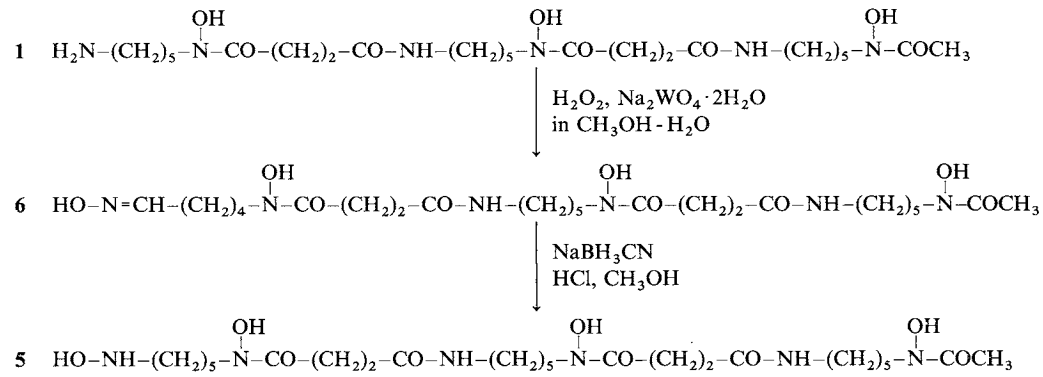
The most widely used synthetic approach for the conversion of an amine to a hydroxylamine proceeds *via* the formation of a Schiff base with an aromatic aldehyde, followed by an oxidation of the intermediate imine to an oxaziridine and subsequent acid-catalyzed hydrolysis in the presence of hydroxylamine to the desired hydroxylamine derivative. The Schiff base of desferrioxamine B with *p*-methoxybenzaldehyde could be prepared, but the following oxidation to the corresponding oxaziridine failed due to poor solubility of the imine of desferrioxamine B in anhydrous solvents.

Considering the very low solubility of desferrioxamine B base (**1**) another synthetic strategy was chosen. Desferrioxamine B base was oxidized to the corresponding oxime (**6**) by heating a suspension of the base with 3 equivalents of hydrogen peroxide and a catalytic amount of sodium tungstate dihydrate in methanol-water, 58:42 (v/v) under an argon atmosphere at 50°C overnight (Scheme 1).[24] The use of this solvent mixture resulted in optimal conversion. The excess of hydrogen peroxide was destroyed by stirring the reaction mixture with catalytic amounts of platinum on activated charcoal under an inert atmosphere. The oxime (**6**) was crystallized from water at a pH-value of 2.5 in 50% yield.

The reduction of the oxime of desferrioxamine B (**6**) with sodium cyanoborohydride and 2 N HCl in methanol proceeded under an argon atmosphere to *N*-hydroxy-desferrioxamine B (**5**) (Scheme 1).[25] The product was purified by preparative HPLC on reversed phase C_{18}. The pure product could only be isolated in moderate yield (24%), presumably as a result of an autooxidative degradation reaction.

For the preparation of the iron complexes of the oxime of desferrioxamine B (**7**) and *N*-hydroxy-desferrioxamine B (**8**) an efficient ligand-exchange reaction was employed.[26] Both the oxime of desferrioxamine B (**6**) and *N*-hydroxy-desferrioxamine B (**5**) were stirred with a slight excess of ferric acetylacetonate in a biphasic mixture consisting of ethyl acetate and water at room temperature. The water soluble ferric complex began to form instantaneously. The extent of the reaction was monitored using

Scheme 1. Synthesis of *N*-hydroxy-desferrioxamine B.



analytical HPLC which indicated a quantitative formation of the oxime of ferrioxamine B (**7**) as well as of the *N*-hydroxy-ferrioxamine B (**8**) within 2 hours. The aqueous layer was separated from the organic phase and lyophilized. By this procedure pure iron complexes were obtained without contamination by inorganic salts.

The iron complex of the synthetically prepared *N*-hydroxy-desferrioxamine B was compared with the authentic metabolite isolated from urine samples of patients treated with desferroxamine B (**1**) by HPLC-analysis. Identical HPLC-retention times and instability patterns could be confirmed (personal communication from Prof. HIDER). In addtion both samples showed the $(M+H)^+$-peak of 630 in the fast atom bombardment spectra.

The formation of the metabolite *N*-hydroxy-desferrioxamine B (**5**) in biological systems is not surprising: *N*-hydroxylation of primary amines is a common bioconversion reaction.[27] The limited stability of *N*-hydroxy-desferrioxamine B is due to autooxidation to the corresponding nitroso and nitro derivatives, a well known degradation process of hydroxylamines.

The structure of the recently described new metabolite of desferrioxamine B has now been corroborated by chemical synthesis of *N*-hydroxy-desferrioxamine B (**5**), which was subsequently converted into the iron(III) complex. This synthetic reference material was shown to be identical to the authentic metabolite, isolated from the urine of patients, by analytical HPLC and FAB-MS. Moreover the chemical synthesis of *N*-hydroxy-desferrioxamine B provides access to larger quantities of the metabolite for further pharmacokinetic studies of the metabolism of desferrioxamine B (**1**) and for toxicological investigations.

Experimental

^{1}H NMR spectra were recorded at 360 MHz. FAB mass spectra were determined on a VG ZAB-HF spectrometer (VG Analytical, Manchester, UK). Melting points are uncorrected. Desferrioxamine B base was obtained from the Pharmaceutical Division of Ciba-Geigy. 30% H_2O_2, the peroxide test strips, ferric acetylacetonate, CH_3OH (pro analysis) and CH_3CN (HPLC grade) were purchased from E. Merck. $Na_2WO_4 \cdot 2H_2O$, $NaBH_3CN$, platinum on activated charcoal and trifluoroacetic acid were obtained from Fluka (Buchs, Switzerland). Reaction progress and purification steps were monitored by analytical reversed-phase HPLC. The stationary phase Lichrocart HPLC-cartridge (Lichrospher 100RP-18, 5 μm, 4 × 125 mm) was purchased from E. Merck. Mobile phase A consisted of 2.2 mM KH_2PO_4 in H_2O adjusted to pH 3.0 with H_3PO_4 (about 0.3 mM), mobile phase B was prepared by mixing 80% CH_3CN with 20% of mobile phase A. The flow rate of the mobile phase was 1.5 ml/minute. The wavelength for detection was set to

220 nm. 20 μl was the injection volume for each sample. A gradient was run from 0% mobile phase B to 40% mobile phase B in 10 minutes and from 40% mobile phase B to 100% mobile phase B in 2 minutes.

Oxime of Desferrioxamine B (**6**)

To a stirred suspension of 6.0 g (10.7 mmol) desferrioxamine B base (**1**) in 70 ml of CH_3OH and 50 ml of H_2O, 0.21 g (0.64 mmol) $Na_2WO_4 \cdot 2H_2O$ in 1 ml of H_2O and 5 ml (48.9 mmol) of 30% H_2O_2 were added under an argon atmosphere. The reaction mixture was heated to 50°C and stirred overnight. The progression of the reaction was monitored by analytical HPLC (see above). The unreacted H_2O_2 was destroyed by careful addition of 2~3 portions of 0.1 g 10% platinum on activated charcoal under a N_2 atmosphere until no H_2O_2 could be detected in the reaction solution by peroxide test strips. This degradation of H_2O_2 should be done in a ventilated hood in view of the pyrophoric properties of the reaction mixture. The peroxide free reaction mixture was concentrated under vacuum to a volume of 30 ml, the pH value of the solution was adjusted to 2.5 with 2 N HCl and the oxime of desferrioxamine B (**6**) was crystallized at 4°C. Yield 50%. MP 107.8~108.8°C; ^{1}H NMR (DMSO) δ 1.20~1.30 (4H, m), 1.33~1.43 (6H, m), 1.45~1.58 (6H, m), 1.97 (3H, s), 2.10 (2H, m), 2.27 (4H, t, J=8 Hz), 2.58 (4H, t, J=8 Hz), 2.95~3.06 (4H, m), 3.42~3.52 (6H, m), 6.62 (0.5H, t, J=6 Hz), 7.28 (0.5H, t, J=6 Hz), 7.7~8.18 (2H, m), 9.52~9.67 (3H, m), 10.33 (0.5H, s), 10.71 (0.5H, s); IR ν_{max}(KBr) cm^{-1} 3420, 3310, 3150, 2930, 2860, 1650, 1620, 1565, 1460, 1425, 1395; FAB-MS m/z 575 $(M+H)^+$.

Anal Calcd for $C_{25}H_{46}N_6O_9$(2%H_2O): C 51.21, H 8.13, N 14.33, O 26.33.
Found: C 51.18, H 7.88, N 14.30, O 26.29.

N-Hydroxy-desferrioxamine B Trifluoroacetate (**5**)

A suspension of 0.5 g (0.87 mmol) oxime of desferrioxamine B (**6**) and 0.055 g (0.87 mmol) $NaBH_3CN$ in 50 ml of CH_3OH under argon atmosphere was prepared and its pH value maintained at 3.0 by addition of *ca.* 2 ml of 2 N HCl-CH_3OH, 50:50 (v/v). After 35 minutes no further change of the pH value was observed and the reaction mixture was stirred for another 5 hours under an argon atmosphere. The conversion of the oxime to the hydroxylamine was monitored by HPLC-analysis as described above. The solvent was removed under vacuum and the residue purified by preparative HPLC on a reversed phase C_{18}-column (Lichroprep RP-18, 15~25 μm) with 0.1% aqueous trifluroacetic acid as mobile phase A and a mixture of 80% acetonitrile and 20% mobile phase A as mobile phase B. The conical column used had a diameter of 26~49 mm and a length of 230 mm. The flow rate of the mobile phase was 20 ml/minute. A gradient was run from 5% phase B to 15% phase B in 20 minutes, from 15% phase B to 28% phase B in 80 minutes and from 28% phase B to 100% phase B in 10 minutes. Detection wavelength was set to 235 nm. After purification *N*-hydoxy-desferrioxamine B trifluoroacetate (**5**) was obtained in 24% yield. In order to avoid autooxidation the product was stored under argon. ^{1}H NMR (DMSO) δ 1.19~1.30 (6H, m), 1.31~1.41 (6H, m), 1.46~1.62 (6H, m), 1.97 (3H, s), 2.22~2.33 (4H, m), 2.53~2.65 (4H, m), 2.95~3.05 (4H, m), 3.07 (2H, t, J=8 Hz), 3.43~3.53 (6H, m), 7.75~7.84 (2H, m), 9.61~9.77 (3H, m), 10.65 (0.5 H, s), 11.05 (0.5H, s); IR ν_{max}(KBr) cm^{-1} 3420, 3310, 3150, 2930, 2860, 1680, 1620, 1565, 1460, 1425, 1395; FAB-MS m/z 577 $(M+H)^+$.

Anal Calcd for $C_{27}H_{49}F_3N_6O_{11}$(2%H_2O): C 46.01, H 7.23, N 11.92.
Found: C 46.22, H 7.22, N 11.71.

Oxime of Ferrioxamine B (**7**)

A solution of 200 mg (0.348 mmol) of oxime of desferrioxamine B (**6**) in 40 ml of H_2O was added at room temperature to a solution of 140 mg (0.396 mmol) of iron(III)-acetylacetonate in 50 ml of ethyl acetate. The mixture was stirred for 2 hours. The aqueous phase was separated from the organic layer, washed with four 50 ml portions of ethyl acetate and lyophilized. Yield 90%. FAB-MS m/z 628 $(M+H)^+$.

N-Hydroxy-ferrioxamine B (**8**)

A solution of 2.5 mg (0.0036 mmol) *N*-hydroxy-desferrioxamine B (**5**) in 1 ml of H_2O was added at room temperature to a solution of 1.4 mg (0.0040 mmol) iron(III)-acetylacetonate in 1.25 ml of ethyl acetate. The mixture was stirred for 2 hours. The aqueous phase was separated from the organic layer,

washed with four 1 ml portions of ethyl acetate and lyophilized. Yield 90%. FAB-MS m/z 630 $(M+H)^+$.

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