Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 13, pp. 1511-1516, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00260-0

Synthesis and Reactions of Nitroso Sulphamethoxazole with Biological Nucleophiles : Implications for Immune Mediated Toxicity.

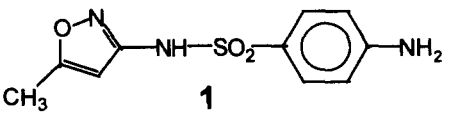
Dean J. Naisbitt, Paul M. O'Neill, Munir Pirmohamed and B. Kevin Park*.

Dept. of Pharmacology and Therapeutics, The University of Liverpool, PO Box 147, Liverpool L69 3BX, U.K. Fax No.: (+44) 151 794 5540, E mail: bkpark@liv.ac.uk

Abstract : Sulphamethoxazole hydroxylamine (SMX-NHOH) and nitroso sulphamethoxazole (SMX-NO) were prepared by a modified literature procedure. SMX-NO produced a complex set of unstable intermediates with sulphur nucleophiles, but did not react with amino containing compounds. No reactions were observed between sulphamethoxazole (SMX) / SMX-NHOH and the nucleophiles used in this study. Thus antigens formed from N-oxidation of SMX are likely to be unstable *in vivo*.

Introduction

Sulphamethoxazole (SMX) **1** belongs to a family of drugs known as the sulphonamides. Sulphonamide therapy is associated with a variety of idiosyncratic reactions (fever, lymphadenopathy, skin rash, hepatitis, nephritis, and blood dyscrasias) which occur in 2-3% of the population.[1]



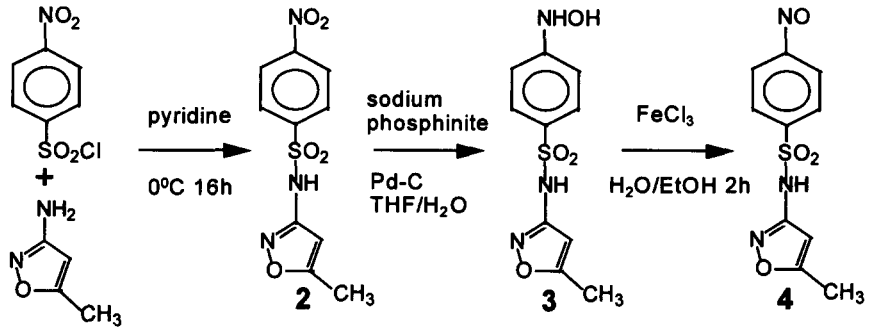
1

Recently, increased administration of sulphonamides for the treatment of infectious complications occurring in immunosuppressive disorders such as AIDS has led to a renewed interest in the mechanism of drug toxicity.[2] The pathogenesis of adverse reactions to the drug may be associated with hapten formation and a subsequent immune response. Covalent binding of reactive metabolites to nucleophilic groups on cellular proteins could result in such a response.[3] It has been postulated that sulphamethoxazole hydroxylamine (SMX-NHOH) and nitroso sulphamethoxazole (SMX-NO) are involved in SMX toxicity,[4] although there is little direct evidence indicating that either metabolite is the ultimate immuno-toxin. Alternatively, reactive metabolites may undergo conjugation with glutathione (GSH) which is the most abundant cellular thiol, constituting greater than 90% of non-protein thiols.[5] GSH is known to protect cells against toxicity from oxidative metabolites either by conjugation or by reduction reactions.

The aim of this study was to synthesise SMX-NHOH **3** and SMX-NO **4** and investigate their reactivity towards biological nucleophiles. In particular, we investigated whether stable adducts were formed in these reactions. The reaction pathways were monitored by NMR and LC-MS.

Chemistry

In this study a modification of Johnstone's method was utilised to synthesise SMX-NHOH **3** and SMX-NO **4** (Scheme 1).[6] NO_2 SMX **2** was prepared via a nucleophilic addition of 4-nitrobenzenesulphonyl chloride to 3-amino 5-methylisoxazole as previously described.[7] The reaction was terminated after 24h. The resulting brown precipitate was recrystallized from a toluene : ethyl acetate mixture (3:1 v/v).



Scheme 1. Sulphamethoxazole hydroxylamine and nitroso sulphamethoxazole synthesis.

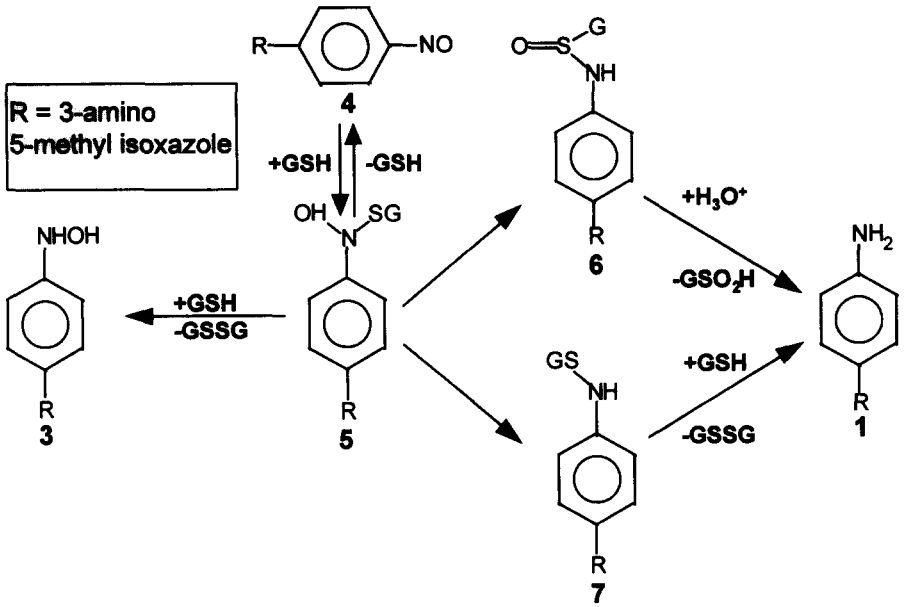
Johnstone has previously synthesised many aromatic hydroxylamines by nitro reduction using a stirred mixture of sodium phosphinite in water / THF (5% palladium on carbon catalyst).[6] SMX-NHOH **3** was prepared similarly, using an excess of THF to enhance the solubility of NO_2 SMX **2**. The reaction was monitored by TLC until all the starting material had reacted. Ether extraction produced a crude product of greater than 85% yield. This was recrystallised from chloroform, obtaining a product of greater than 99% purity when analysed by HPLC, elemental analysis, mass spectroscopy, and NMR.[8] Initial attempts at synthesising SMX-NO **4**, using $FeCl_3$ in H_2O proved unsuccessful. Consideration of known syntheses revealed that compounds with electron releasing groups in the *-para* position produced yields in excess of 80%, whereas electron withdrawing groups *-para* to the hydroxylamine functionality generally gave reduced yields. The reaction time was increased from 30 min., and followed by TLC. The reaction reached completion two hours later and after recrystallisation from chloroform, the product was found to be greater than 99% pure (by HPLC) and in yields of greater than 85%.[9]

Results and Discussion.

The complex reaction profile of nitroso benzene with biological thiols including glutathione (GSH) has been well documented (Scheme 2).[10] These reactions, although well explored, produced little direct evidence identifying an adduct with the stability to act as a stable drug conjugate required for hapten-mediated drug toxicity.[11]

Previously the reactions of SMX-NO **4** and GSH (at physiological concentrations and in excess), have been analysed in aqueous conditions by HPLC.[4] From these studies the formation of two labile intermediates was defined; the semi-mercaptal **5**, which quickly decomposed in the presence of excess of GSH, and the

sulphinamide **6**, which although more stable is known to be reduced back to the parent amine under acidic conditions.[10] We initially repeated these experiments under identical conditions using LC-MS analysis.[12]



Scheme 2. Proposed reactions involved in the reduction of nitroso SMX by GSH. Intermediates are 5. semimercaptal, 6. sulphinamide, 7. sulphenamide.

The reactions were followed over a period of 20h. SMX **1** and SMX-NHOH **3** were identified from authentic standards, whereas the semi-mercaptal **5** and sulphinamide **6** intermediates were identified from their molecular ion peak on the mass spectra and the retention times quoted previously.[7] The semi-mercaptal was formed initially; it then rearranged to the sulphinamide and SMX itself. The products formed and an example of the traces obtained using 0.1mM GSH are presented in figure 1. Products of reduction, SMX-NHOH and SMX, and products of thiol addition, semi-mercaptal and sulphinamide were detected as parent ions. The concentration of semi-mercaptal declined with time and coincided with increased formation of SMX and sulphinamide. With the addition of excess GSH, formation of the sulphinamide metabolite was reduced and reduction to SMX occurred at a greater rate (results not shown).

Two major difficulties were encountered with the use of LC-MS. First we were unable to measure the concentration of SMX-NO present throughout the reaction because it only produces a very weak molecular ion. Secondly, absolute quantification of the amount of products formed is not possible. To overcome these problems, the reactions of SMX-NHOH and SMX-NO with GSH were analysed by NMR. The reactions were followed using two concentrations of GSH (1:1 and 3:1, GSH : metabolite).

When using the higher masses required for NMR analysis, aqueous conditions (D_2O) could not be utilised because of solubility problems, and d_6 DMSO was therefore used as the solvent. NMR analysis has the

advantage of allowing measurement of the NO intermediate and hence a more complete quantitative reaction profile was analysed over a period of 96h.

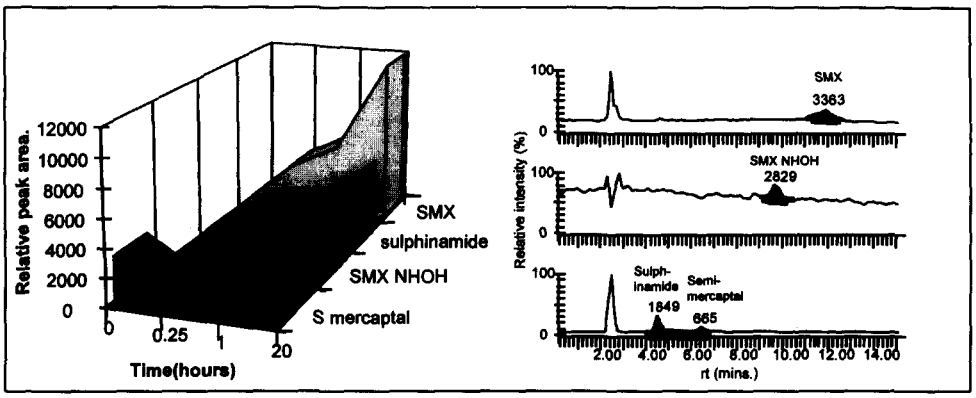


Figure 1. Ratio of product formation / disappearance in the reaction of 0.05mM SMX-NO with 0.1mM GSH under aqueous conditions, when followed by LC-MS and a molecular ion spectrum showing the retention times of the intermediates.

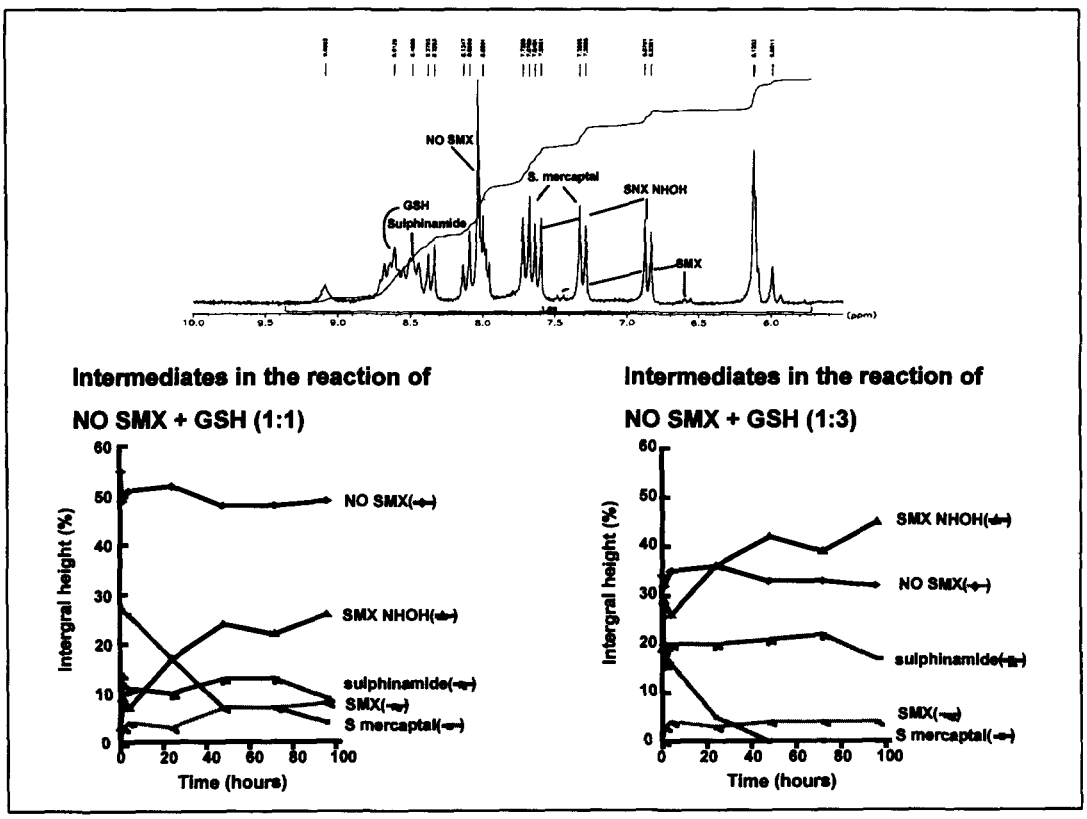
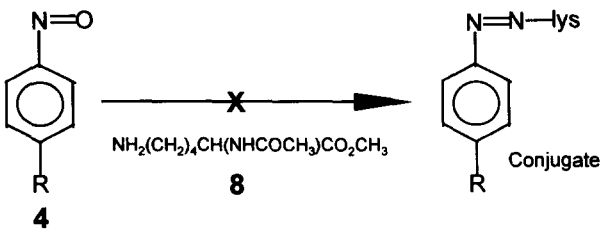


Figure 2. The reaction profile of SMX-NO and GSH in DMSO, when followed by NMR spectroscopy and an example of a spectrum obtained.

The amount of each intermediate formed was quantified by measurement of the integral peak height from each spectrum throughout the reaction period. Figure 2 shows the ratio of products formed and an example of a typical spectrum. SMX-NO, SMX-NHOH, NO_2-SMX, SMX, GSH and GSSG peaks were identified from NMR analysis of standard compounds, thus allowing analysis of the semi-mercaptal and sulphinamide metabolites by examining their chemical shifts and from previous studies involving the reaction of GSH with substituted nitro nitroso benzenes.[10] The assignment of integrals corresponding to the sulphinamide was difficult since the peaks had similar chemical shifts to GSH and GSSG. In order to assign the signals from the sulphinamide product, an NMR was run on a mixture of oxidised and reduced GSH, an assignment was performed subsequently. The results indicate that the initial reaction involving GSH occurs instantaneously, and the amount of SMX-NO remaining was dependent upon GSH concentrations. Little reduction of SMX-NHOH was obtained under these conditions.

Similar results were obtained from the reaction of SMX-NO with mercaptoethanol. Identical conditions were used as in the reactions with GSH, and a similar time course was obtained. The spectra were easily analysed as mercapto-ethanol has no protons between 7.5 and 8.5 on the NMR spectrum.

The reaction of SMX-NHOH with GSH and mercaptoethanol was also analysed over a 96h period. No reduction of SMX-NHOH was obtained throughout the reaction.



Scheme 3. Hypothesised conjugate structure from the reaction of SMX-NO and lysine.

To investigate the reactions further, we considered the structure of human serum albumin (HSA) to identify other groups on proteins which may be sufficiently nucleophilic to react with SMX-NO. Various amino acids within HSA contain free amine groups, which could react with nitroso compounds forming diazo products. In our study we utilised protected lysine **8** (Scheme 3) which is one of the major constituents of HSA (greater than 10% of the total amino acid content by mass). Identical reactions conditions were used to analyse the reaction of lysine with SMX-NO **4** and SMX-NHOH **3**. After a period of 96h, NMR analysis revealed no reaction in either case. Following this, we studied the reactions of aniline, a more chemically reactive amine than lysine and after heating, under deuterated acetic acid catalysis, no product was observed.

From the results obtained, we have identified the formation of unstable intermediates in the reaction of SMX-NO with biological thiols under aqueous and non aqueous reaction conditions. The semi-mercaptal

intermediate is broken down under physiological conditions in the presence of excess thiol to give SMX-NHOH or the parent amine, both of which do not possess the chemical reactivity to become covalently bound to proteins. SMX-NHOH is known to undergo further metabolism *in vivo* by O-acetyltransferase enzymes, products formed have not been investigated, although a recent study suggests they may be protein and DNA reactive.[13] In contrast, the sulphinamide is stable in both aqueous and non-aqueous conditions, although it is likely to undergo hydrolysis to the parent amine under the physiological conditions of antigen processing (pH 5.5 and excess GSH).[14] Immune toxicity mediated by a drug hapten requires the formation of a covalent bond which is stable *in vivo.* The current work suggests that although SMX-NO may react with sulphydryl nucleophiles in proteins, the products formed are likely to be unstable. Consistent with this conclusion, in a parallel study we found no evidence for hapten formation *in vivo,* after administration of either SMX or SMX-NHOH to the rat.

Acknowledgements: We thank the Wellcome Trust (DJN) for a studentship. BKP is a Wellcome Principal Fellow and PON is a Roche Research Lecturer. These studies were also supported by a joint MRC / North West Regional Health Authority Project grant. The authors also thank Dr. J.L. Maggs for his assistance with LC-MS.

References and notes

1. Mandell, G.L.; Sande, M.A.; *Goodman and Gilmans, The Pharmacological Basis of Therapeutics;* Gillman, A.G.; Goodman, L.S.; Rall, T.W.; Murad, R., Ed.; Macmillan Publishing Co.: New York, **1985;** pp 1095-1114,
2. Gordin, F.M.; Simon, G.L.; Wofsy, C.B.; Mills, L. *Ann. Int. Med.* **1984**, *100*, 495
3. Park, B.K.; Coleman, J.W.; Kitteringham, N.R. *Biochem. Pharmacol.* **1987**, *36*, 581
4. Cribb, A.E.; Miller, M.; Leeder, J.S.; Hill, J.; Spielberg, S.P. *Drug Metab. Disp.*, **1991**, *19*, 900
5. Larsson, A.; Orrenius, S.; Holmgren, A.; Mannervik, B. *Biochemical, Physiological, Toxicological and Clinical Aspects*, Raven Press: New York, **1983**
6. Johnstone, R.A.; Telford, R.P.; Entwistle, I.D.; Gilkerson,T. *Tetrahedron*, **1978**, *34*, 213
7. Rieder, M.J.; Uetrecht, J.; Shear, N.H.; Spielberg, S.P. *J. Pharmacol. Exp. Ther.*, **1988**, *244*, 724
8. **3:** ^{1}H-NMR (d^6-DMSO, 200MHz) δ 9.08 (s, 1H, NHOH), 8.73 (s, 1H,NHOH), 7.61 (d, 2H, J=8.80 Hz, Ar-H), 6.85 (d, 2H, J-8.80Hz, Ar-H), 6.11 (s, 1H, Ar-H), 2.33 (s, 3H, CH_3). Anal. Calcd. for $C_{10}H_{11}N_3O_4S$: C, 44.61; H, 4.12; N, 15.61. Found C, 44.55; H, 4.11; N, 15.63. FABMS m/z 270 (MH^+), HRMS calcd for above, 269.04703; found 269.04732.
9. **4:** ^{1}H-NMR (d^6-DMSO, 200MHz) δ 8.19-8.04 (m, 4H, Ar-H), 6.18 (s, 1H, Ar-H), 2.31 (s, 1H, Ar-H). Anal. Calcd. for $C_{10}H_9N_3O_4S$: C, 44.94; H, 3.39; N, 15.73. Found C, 45.20; H, 3.42; N, 15.75. FABMS m/z 268 (MH^+), HRMS calcd for above, 267.03138; found 267.03167.
10. Ellis, M.K.; Hill, S.; Foster, P.M.D. *Chem-Biol. Interactions*, **1992**, *82*, 151
11. Park, B.K.; Kitteringham, N.R. *Drug Metab. Rev.*, **1990**, *22*, 81
12. Maggs, J.L.; Williams, D.; Pirmohamed, M.; Park, B.K. *J. Pharmacol. Exp. Ther.*,**1995**, *275*, 1463
13. Nakamura, H.; Uetrecht, J.; Cribb, A.E.; Miller, M.A.; Zahid, N.; Hill, J.; Josephy, P.D.; Grant, D.M.; Spielberg, S.P. *J. Pharmacol. Exp. Ther.*, **1995**, *275*, 1099
14. Kalish, R.S. *J. Am. Acad. Dermatol.* **1995**, *32*, 640

(*Received in Belgium* 9 *April* 1996; *accepted* 4 *June* 1996)