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Uptake of NO-releasing drugs by the P2 nucleoside transporter in trypanosomes

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Abstract

Nitric oxide ($NO^{\bullet}$) has been identified as a principal regulatory molecule of the immune system and the major cytotoxic mediator of activated immune cells. $NO^{\bullet}$ can also react rapidly with a variety of biological species, particularly with the superoxide radical anion $O_2^{\bullet-}$ at almost diffusion-limited rates to form peroxynitrite anion ($ONOO^-$). $ONOO^-$ and its proton-catalyzed decomposition products are capable of oxidizing a great diversity of biomolecules and can act as a source of toxic hydroxyl radicals. As a consequence, a strategy for the development of molecules with potential trypanocidal activities could be developed to increase the concentration of nitric oxide in the parasites through $NO^{\bullet}$-releasing compounds. In this way, the rate of formation of peroxynitrite from $NO^{\bullet}$ and $O_2^{\bullet-}$ would be faster than the rate of dismutation of superoxide radicals by superoxide dismutases which constitute the primary antioxidant enzymatic defense system in trypanosomes. The adenosine transport systems of parasitic protozoa, which are also in certain cases implicated in the selective uptake of active drugs such as melarsoprol or pentamidine, could be exploited to specifically target these $NO^{\bullet}$-releasing compounds inside the parasites. In this work, we present the synthesis, characterization and biological evaluation of a series of molecules that contain both a group which would specifically target these drugs inside the parasites via the purine transporter, and an $NO^{\bullet}$-donor group that would exert a specific pharmacological effect by increasing NO level, and thus the peroxynitrite concentration inside the parasite.

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- Nitric oxide
- Peroxynitrite
- Thionitrite
- *S*-nitrosothiol
- Adenosine

Introduction

South-American and African trypanosomiasis, in particular African sleeping sickness, a disease caused by parasitic protozoa of the *Trypanosoma brucei* subgroup, remains a major public health problem, and there is now a great need to develop drugs to replace those to which these parasites have become resistant (1). Protection against active oxygen species is provided in part by an enzymatic defense system which is essential for the survival of aerobic organisms and differs according to species. Both mammalian and protozoan enzymatic systems have in common superoxide dismutases (SODs) that catalyze the dismutation of superoxide radical into hydrogen peroxide and oxygen.

In addition to SODs, the protective mammalian enzymes are various hydroperoxidases such as glutathione peroxidase, catalase, and other hemoprotein peroxidases. In the absence of catalase, the antioxidant defense system in trypanosomes is weak, and essentially based on the presence of a spermidine-glutathione conjugate named trypanothione, whose oxidative form is regenerated in its reduced dithiol form by an NADPH-dependent flavoprotein, trypanothione reductase (2). As a consequence, most of the trypanosomes, and parasitic protozoans in general, are susceptible to free oxygen radical-induced oxidative stress and do not tolerate a high concentration of oxygen. This vulnerability to reactive oxygen species could be exploited to design new drugs with trypanocidal activity.

Nitric oxide ($NO^{\bullet}$), a key messenger implicated in a wide range of biological processes including cardiovascular (3-5) and neuronal (6) systems, also plays a critical role of protection against parasitic infections as a regulatory molecule and cytotoxic mediator of the immune system (7,8). For example, macrophages from *Trypanosoma brucei brucei*-infected mice have been shown to produce high levels of nitric oxide (9) and a number of reports have demonstrated that *in vitro* cytotoxicity against the intracellular form of leishmanias is mediated by $NO^{\bullet}$ (10-12). Although few physiologic target molecules of NO have been clearly identified, its role in the protective mechanisms would occur through inactivation of critical enzymes and nitrosation of thiols and other nucleophilic residues (13-16). $NO^{\bullet}$ can also react rapidly with a variety of radical species, like superoxide radical anion $O_2^{\bullet -}$. While $O_2^{\bullet -}$ itself is not an efficient oxidizing agent, together with $NO^{\bullet}$ it can produce the more powerful oxidizing peroxynitrite anion $ONOO^-$ at an almost diffusion-limited rate (6.7 nM/s) (17) which depends on the concentrations of both radicals. Peroxynitrite and its proton-catalyzed decomposition products are capable of oxidizing a great diversity of biomolecules (18,19) including heme-containing proteins such as hemoglobin and myeloperoxidase, seleno-proteins such as glutathione peroxidase, DNA or lipids within the cell, or nitrating and nitrosating phenolic compounds such as tyrosines of certain proteins like SODs, and can act as a source of toxic hydroxyl radicals. Peroxynitrite anion, like $NO^{\bullet}$, seems to play a major role in the protective mechanisms of the host against parasitic infections, and, for example, has been shown to be highly cytotoxic against *Trypanosoma cruzi* epimastigotes, the causal agent of Chagas' disease, inactivating two key enzymes for their energetic metabolism, i.e., succinate dehydrogenase and NADH-fumarate reductase (20,21).

Most of parasitic protozoa are unable to synthesize purines *de novo* and consequently must use specific transporters to obtain them from the hosts for their survival. The African trypanosome *Trypanosoma brucei brucei* that invades the central nervous system causing the fatal neurologic disorder known as sleeping sickness, possesses two adenosine transporter systems: a P1 type which also transports inosine, and a P2 type which is also able to transport adenine. Both systems have been shown to be implicated in the selective uptake of trivalent melaminophenyl arsenical drugs (22) such as melarsen oxide 1a and melarsoprol 1b (Figure 1), which are still the only drugs of choice for the treatment of the late stage of human African trypanosomiasis, and of pentamidine 2 (23), one of the most frequently administered drugs in the treatment of the early stage of the disease. Moreover, the nitroheterocyclic compound megazol 3 (Figure 1), that has been shown to be active against many microorganisms including *Trypanosoma cruzi*, might also act as a substrate for carrier protein P2, although the passive diffusion process would remain the major route of entry (Barrett MP, Fairlamb AH, Rousseau B, Perié J and Chauvière G, unpublished results). In view of the analo-

gies between adenine (or adenosine), benzamidine and melamine, it has been hypothesized that the amidine motif (N=C-NH_2) is the real structural feature for specific P2-transporter recognition and uptake (24).

Trypanosoma equiperdum, a non-tsetse-transmissible strain, possesses a transport system similar to that of *T. brucei*, comprising two adenosine transporters, P1 and P2, the latter also transporting adenine and the melaminophenyl arsenical drug cymelarsen 1c (25) (Figure 1).

In the present paper, we describe the synthesis of a series of drugs 7a-7c which contain both a group that would specifically target these drugs into the parasite via the P2-transporter, and an NO•-donor group that would exert a specific pharmacological effect by increasing the level of NO, and thus the peroxynitrite concentration inside the parasite. The uncommon stability of *S*-nitroso-*N*-acetylpenicillamine (SNAP) as a solid (26) or in solution suggests that penicillamine derivatives should be good canditates for new stable *S*-nitrosothiols and could function as useful nitric oxide-releasing compounds. We describe here the effect of melaminyl thionitrite 7a on adenosine transport by *T. equiperdum*.

Results

Synthesis and stability of thionitrites 7a-c

The general synthetic route to thionitrites 7a-c is shown in Figure 2. Penicillamine 4 was activated for coupling with amines by conversion into 3-acetamido-4,4-dimethylthietan-2-one 5 (27), which was prepared directly from the racemate of penicillamine by reaction with acetic anhydride in pyridine (40% yield). Reaction of thietanone 5 with the amino melaminyl derivative 8 (synthesized in two steps from 2-chloro-4,6-diamino-1,3,5-triazine and 4-aminoacetanilide, Figure 3), with 4-aminobenzamidine (commercially available) or with the adenine derivative 9 (synthesized in two steps from adenine and 2-bromo-1[(*tert*-butyloxycarbonyl) amino]ethane, Figure 4) gave the thiols 6a-c, respectively. No protection was required for amidine function for these coupling reactions. The thionitrites 7a-c were then obtained as hydrochloride salts under mild con-

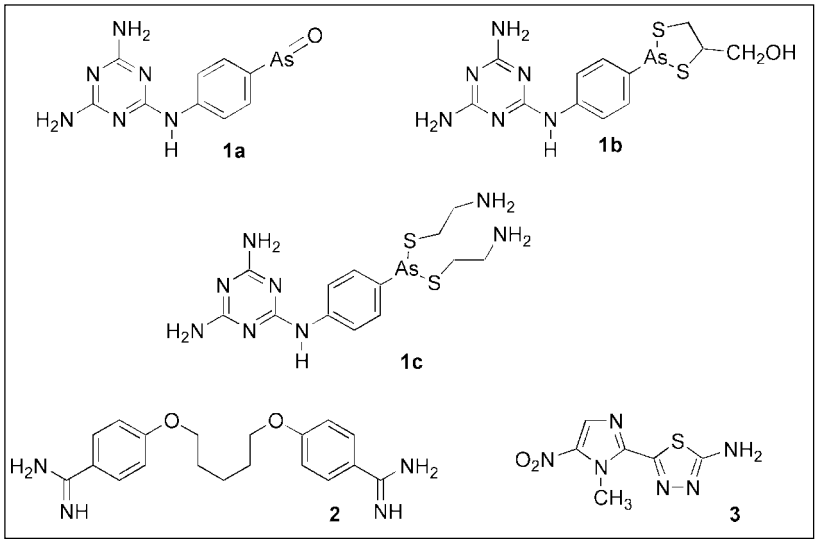

Figure 1 - Structures of melarsen oxide (1a), melarsoprol (1b), cymelarsen (1c), pentamidine (2) and megazol (3).

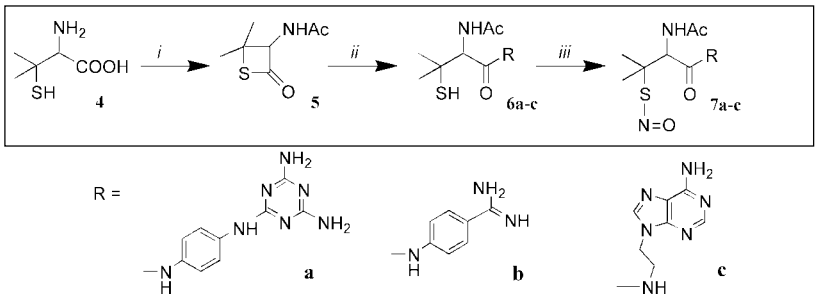

Figure 2 - Synthesis of compounds 5, 6 and 7. The reaction conditions were: *i*, acetic anhydride; pyridine (0°C for 30 min, and then at room temperature (RT for 15 h). *ii*, 7a: 8, DMF (RT for 20 h). 7b: 4-aminobenzamidine, NaOH 1 M; chloroform (RT for 2 h). *iii*, 7c: 9, NaOH 1 M; chloroform (RT for 2 h).

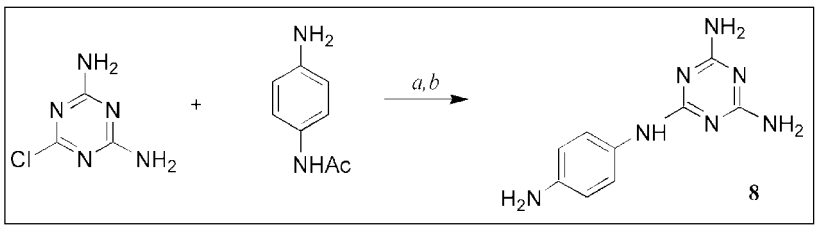

Figure 3 - Synthesis of compound 8. *a*, 1 eq NaOH; H_2O (100°C, 3 h). *b*, 1.2 M HCl (100°C, 2 h).

ditions by electrophilic nitrosation of the corresponding parent thiols 6a-c with sodium nitrite in acid solution at room temperature (28).

Like SNAP compounds, 7a-c were stable as solids and presented a green color when in solution in organic or aqueous media which characterizes the presence of an *S*-nitroso group. They were fully characterized by mass spectrometry, $^{1}H/^{13}C$-NMR and UV-visible spectroscopies. Decomposition studies were carried out by UV-visible spectroscopy by measuring the disappearance of the characteristic absorbance at 340 nm ($\varepsilon \cdot 800$ M/cm). 7a-c decomposed slowly in sodium phosphate buffers, pH 7.5, with a half-life between 2 and 3 h, comparable to the half-life of SNAP under the same conditions.

Biological evaluation

Cymelarsen 1c, SNAP and thionitrite 7a were tested on *T. equiperdum* E1 for their ability to inhibit the uptake of [2-^{3}H]adenosine via the transporter P2 in the presence of saturating concentration of inosine which was required to inhibit P1 transporter. The compounds were also tested for their *in vitro* toxicity on the same strain (LD_{100} after 18 h). The data reported in Table 1 show that the melaminyl derivative 7a efficiently inhibits adenosine transport in *T. equiperdum* in the presence of inosine, suggesting a specific interaction with the P2 transporter with a K_i of 0.5 µM which is equal to the K_M values of adenosine that enters the parasite through both adenosine transporters P1 (K_M = 0.6 µM) and P2 (K_M = 0.7 µM). In comparison, the uptake of cymelarsen via P2 is less efficient (K_i = 41 µM) and SNAP has no affinity for this transporter (K_i >100 mM).

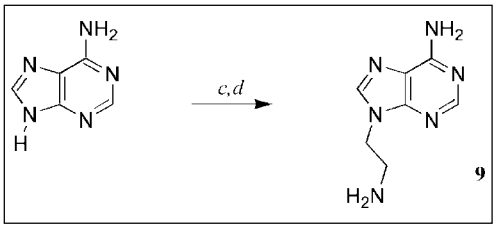

Figure 4 - Synthesis of compound 9. *c*, 2-Bromo-1-[(tert-butyloxycarbonyl)amino]ethane, K_2CO_3, $Bu_4N^+I^-$; DMF (RT, room temperature for 16 h). *d*, Trifluoroacetic acid; CH_2Cl_2 (RT for 4 h).

Table 1 - Inhibition of adenosine transport by *T. equiperdum* in the presence of inosine and *in vitro* toxicity of the same strain.

Nd, Not determined.

	K_i	LD_{100}
Adenosine	0.7 µM (K_M)	nd
Cymelarsen 1a	41 µM	nd
SNAP	>100 mM	125 µM
7a	0.5 µM	250 µM

Discussion

Chemical reagents that release NO• under physiological conditions are good candidates to mimic the activity of NO-synthase (29), an NADPH-dependent flavo-hemoprotein that produces NO• from L-arginine in many types of cells by a two-step oxidation reaction. A potential therapeutic application lies in their possible use as vasodilators or as drugs for the treatment of angina, and a chemical application is their use as a depot for NO• gas which is difficult to handle due to its high reactivity and toxicity as a free radical. Naturally occurring thionitrites like *S*-nitroso-albumine (30) or *S*-nitrosoglutathione (31) are currently postulated to be carriers of NO•, but synthetic *S*-nitrosothiols are usually relatively unstable and spontaneously decompose in solution to yield quantitatively NO• and the corresponding thiyl radical, which dimerizes to give the disulfide, as primary products (32-35). In spite of the presence of amino groups, the three thionitrites of this study, like the well-known SNAP, exhibit significant stability in the solid form and in solution to permit full structural characterization or a therapeutic use, and are capable to slowly generate NO• under physiological conditions with half-life times of several hours.

Conversion of penicillamine into its corresponding thietanone for coupling with

amino compounds is a convenient and general method that can be carried out either in organic solvents or in biphasic systems. The nitrosation reactions to obtain the thionitrites required the investigation of various methods. Only sodium nitrite under acidic conditions gave a satisfactory result as nitrosating agent, and no diazotization/deamination reactions were observed. As shown by UV-visible spectroscopy, the final compounds decomposed in neutral or basic aqueous solutions within a few hours, i.e., at a rate of decomposition comparable to that of SNAP, and were highly stable under acidic conditions.

The thionitrite 7a, which possesses a P2 recognition motif, strongly inhibits the adenosine uptake by the transporter P2. Despite a specific interaction with this transporter, these data do not indicate if compound 7a is really transported by P2. Under the same conditions, the melaminophenyl arsenical drug cymelarsen that has been shown to enter through the trypanosomal P2 transporter, has a higher K_i value, and SNAP does not compete with adenosine for transport. Both compound 7a and SNAP present a weak *in vitro* antiparasitic activity against *Trypanosoma equiperdum* which was quantitatively related to the amount of NO produced by the decomposition of these thionitrites, thus suggesting the direct toxic effect of $NO^{\bullet}$ on trypanosomes. However, this activity may also be due to the relatively rapid homolytic breakdown of the thionitrites under physiological conditions with half-life times of 2.5 h.

In summary, we have prepared a series of molecules with potential trypanocidal activities that contain both a P2-transporter recognition motif and a group that acts as an NO donor. Preliminary affinity data indicate a specific interaction of compound 7a with P2. The toxicity of this melaminyl compound could be attributed mainly to its ability to yield nitric oxide, but its rapid decomposition might in part explain its poor *in vitro* activity. The biological activities of compounds 7b and 7c are under study.

References

1. Kuzoe FA (1993). Current situation of African trypanosomiasis. *Acta Tropica*, 54: 153-162.
2. Fairlamb AH, Blackburn P, Ulrich P, Chait BT & Cerami A (1985). Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science*, 227: 1485-1487.
3. Furchgott RF & Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, 288: 373-376.
4. Palmer RM, Ferrige AG & Moncada S (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, 327: 524-526.
5. Ignarro LJ, Buga GM, Wood KS, Byrns RE & Chaudhuri G (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences, USA*, 84: 9265-9269.
6. Nathan C (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB Journal*, 6: 3051-3064.
7. Hibbs Jr JB, Vavrin Z & Taintor RR (1987). L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *Journal of Immunology*, 138: 550-565.
8. Stuehr DJ & Nathan CF (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *Journal of Experimental Medicine*, 169: 1543-1555.
9. Mabbott NA, Sutherland IA & Sternberg JM (1995). Suppressor macrophages in *Trypanosoma brucei* infection: nitric oxide is related to both suppressive activity and lifespan *in vivo*. *Parasite Immunology*, 17: 143-150.
10. Green SJ, Meltzer MS, Hibbs Jr JB & Nacy CA (1990). Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *Journal of Immunology*, 144: 278-283.
11. Liew FY, Li Y & Millott S (1990). Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. *Journal of Immunology*, 145: 4306-4310.
12. Green SJ, Nacy CA & Meltzer MS (1991). Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. *Journal of Leukocyte Biology*, 50: 93-103.
13. Lancaster Jr JR & Hibbs Jr JB (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proceedings of the National Academy of Sciences, USA*, 87: 1223-1227.
14. Drapier JC & Hibbs Jr JB (1986). Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition in-

volves the iron-sulfur prosthetic group and is reversible. *Journal of Clinical Investigation*, 78: 790-797.
15. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ & Loscalzo J (1992). *S*-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proceedings of the National Academy of Sciences, USA*, 89: 444-448.
16. Becker K, Savvides SN, Keese M, Schirmer RH & Karplus PA (1998). Enzyme inactivation through sulfhydryl oxidation by physiologic NO-carriers. *Nature Structural Biology*, 5: 267-271.
17. Huie RE & Padmaja S (1993). The reaction of NO with superoxide. *Free Radical Research Communications*, 18: 195-199.
18. Koppenol WH (1998). The basic chemistry of nitrogen monoxide and peroxinitrite. *Free Radical Biology and Medicine*, 25: 385-391.
19. Squadrito GL & Pryor WA (1998). Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radical Biology and Medicine*, 25: 392-403.
20. Denicola A, Rubbo H, Rodríguez D & Radi R (1993). Peroxynitrite-mediated cytotoxicity to *Trypanosoma cruzi*. *Archives of Biochemistry and Biophysics*, 304: 279-286.
21. Rubbo H, Denicola A & Radi R (1994). Peroxynitrite inactivates thiol-containing enzymes of *Trypanosoma cruzi* energetic metabolism and inhibits cell respiration. *Archives of Biochemistry and Biophysics*, 308: 96-102.
22. Carter NS & Fairlamb AH (1993). Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature*, 361: 173-176.
23. Carter NS, Berger BJ & Fairlamb AH (1995). Uptake of diamine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. *Journal of Biological Chemistry*, 270: 28153-28157.
24. Tye CK, Kasinathan G, Barrett MP, Brun R, Doyle VE, Fairlamb AH, Weaver R & Gilbert IH (1998). An approach to use an unusual adenosine transporter to selectively deliver polyamine analogues to trypanosomes. *Bioorganic and Medicinal Chemistry Letters*, 8: 811-816.
25. Barrett MP, Zhang ZQ, Denise H, Giroud C & Baltz T (1995). A diamine-resistant *Trypanosoma equiperdum* clone contains a P2 purine transporter with reduced substrate affinity. *Molecular and Biochemical Parasitology*, 73: 223-229.
26. Field L, Dilts RV, Ravichandran R, Lenhert PG & Carnahan GE (1978). An unusually stable thionitrite from *N*-acetyl-D,L-penicillamine; X-ray crystal and molecular structure of 2-(acetylamino)-2-carboxy-1,1-dimethylethyl thionitrite. *Journal of the Chemical Society, Chemical Communications*, 249-250.
27. Al-Zaidi SMR, Crilley MM & Stoodley RJ (1983). Studies related to thietan-2-ones. Part 1. Conversion of penicillamine into DL-2-methylpenicillamine using thietan-2-one-based chemistry. *Journal of the Chemical Society. Perkin Transactions I*, 2259-2265.
28. Moynihan HA & Roberts SM (1994). Preparation of some novel *S*-nitroso compounds as potential slow-release agents of nitric oxide *in vivo*. *Journal of the Chemical Society. Perkin Transactions I*, 797-805.
29. Griffith OW & Stuehr DJ (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annual Review of Physiology*, 57: 707-736.
30. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR & Loscalzo J (1992). Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proceedings of the National Academy of Sciences, USA*, 89: 7674-7677.
31. Singh SP, Wishnok JS, Keshive M, Deen WM & Tannenbaum SR (1996). The chemistry of the *S*-nitrosoglutathione/glutathione system. *Proceedings of the National Academy of Sciences, USA*, 93: 14428-14433.
32. Roy B, du Moulinet d'Hardemare A & Fontecave M (1994). New thionitrites: synthesis, stability, and nitric oxide generation. *Journal of Organic Chemistry*, 59: 7019-7026.
33. Askew SC, Barnett DJ, McAninly J & Williams DLH (1995). Catalysis by Cu^{2+} of nitric oxide release from *S*-nitrosothiols (RSNO). *Journal of the Chemical Society. Perkin Transactions II*, 741-745.
34. Williams DLH (1996). The mechanism of nitric oxide formation from *S*-nitrosothiols (thionitrites). *Journal of the Chemical Society, Chemical Communications*, 1085-1091.
35. Petit C, Hoffmann P, Souchard JP & Labidalle S (1997). Synthesis and characterization of new aromatic thionitrites. *Phosphorus, Sulfur, and Silicon and the Related Elements*, 129: 59-67.