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Short Communications

Enzymatic preparation of [U-^{14}C]-4-chloronitrosobenzene[1]

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Summary. [U-^{14}C]-4-Chloroaniline (**1**) was converted in good yield to [U-^{14}C]-4-chloronitrosobenzene (**3**) by oxidation with H_2O_2 in the presence of chloroperoxidase.

In recent years, aromatic C-nitroso compounds have been a subject of considerable interest to biochemical toxicologists. Mammalian metabolism of aromatic amines and nitro compounds is known in many cases to produce the nitroso oxidation state as an intermediary metabolite[2]. The toxicity of aromatic amine and nitro compounds, including mutagenic properties, is probably the result of metabolic production of the nitroso and similar reactive metabolites. In our own work we have found that nitroso aromatics will react under physiological conditions to produce hydroxamic acids[3,4], which are also known to be toxic metabolites. A convenient route to radiolabeled nitroso compounds of high specific activity and purity was necessary to continue our studies. We now report the development of such a method, which is based on a previously-reported enzymatic oxidation.

We could find no literature precedence for the preparation of ^{14}C-labeled nitroso aromatic compounds. The most general procedure for the synthesis of aryl nitroso compounds involves reduction of the corresponding nitro compound selectively to the hydroxylamine oxidation state, followed by mild oxidation of the hydroxylamine to the nitroso compound[5]. ^{15}N-Labeled nitrosobenzene was synthesized in 38% yield by such a procedure; however, the sequence employed 16 mmoles of starting material[6]. It is our experience that such a reduction-oxidation sequence is not adaptable to the preparation of nitroso compounds with high specific activity from μmole quantities of starting material. A second but less common method for the synthesis of aryl nitroso compounds is the direct oxidation of an arylamine to the nitroso compound by use of peracids[7]. This chemical oxidation generally results in poor yields with the azo, azoxy and nitro compounds being major contaminants.

The unique ability of the fungal enzyme, chloroperoxidase [E.C. 1.11.1.10], to catalyze peroxide oxidation of arylamines to the nitroso oxidation state was previously described by us[8,9]. This enzymatic oxidation proceeds through initial production of the arylhydroxylamine (e.g. **2**), which in most cases is more rapidly oxidized to the nitroso compound than is the starting arylamine. We have found that this enzymatic oxidation can be scaled-up to the degree that makes it useful for μmolar preparative reactions. The only labeled substrate investigated was [U-^{14}C]-4-chloroaniline (**1**); however, prior studies with unlabeled arylamine substrates indicate that the method should be of general value[9], except for those arylamines possessing considerable steric hindrance about the amine functional group[8].

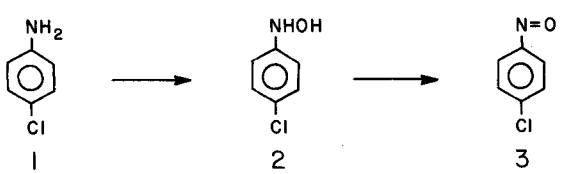
The general method involves incubation of enzyme, H_2O_2 and the substrate arylamine at a concentration consistent with its solubility in aqueous buffer (generally < 0.5 mM). Only EtOH was investigated as a solvent for addition of the

arylamine. If the final concentration of EtOH exceeded 0.5% (v/v), then the yield of **3** was significantly decreased. The initial concentration of peroxide is limited to 2 mM, since higher concentrations greatly increase the rate of H_2O_2-dependent enzyme inactivation[9]. The buffer pH must be in the range 3-6[8], and reaction temperature between 22 and 30 °C. The enzyme is present at a concentration of about 1 unit/ml, although this is a highly flexible parameter that is best optimized by preliminary cold runs. In the case of **1**, readdition of H_2O_2 and the enzyme after an initial 5-min reaction period was necessary to complete the conversion of **1** and **2** to **3**. This was necessitated both from the effect of enzyme inactivation and from the catalatic properties of chloroperoxidase[8], the latter effect being particularly pronounced as the concentration of the arylamine decreased during the reaction. 5 min after the 2nd treatment the amount of **3** present was about 80-90% of theory as determined by HPLC analysis. Extraction of the reaction with pentane completely removes the product **3**, while leaving most of the unreacted **1** and **2** in the aqueous phase. Evaporation of solvent must be carried out with an efficient apparatus, since nitroso aromatics often possess a high vapor pressure. Recovery studies with **3** indicated that even a micro Kuderna-Danish concentrator gave only 75% retention of the product upon 15-fold concentration of the solvent pentane. The radiochemical purity of **3** following the extraction clean-up was generally about 95%, with impurities consisting of 4-chloronitrobenzene (3 mol%), starting material **1** (1 mol%) and 4,4′-dichloroazoxybenzene (0.6 mol%). The overall yield of **3** was in the range of 52-60% for the 95% radiochemical purity product as determined from four separate runs. Unlabeled **3** was not employed as a carrier to increase the overall recovery of labeled **3**, since our needs were for a product with high specific activity. Highly pure **3** (> 99 mol%) was readily prepared by use of HPLC fractionation, although a considerable decrease in yield occurred. The stability of labeled **3** having a sp.act. of 6.3 mCi/mmole was moderate when stored at −20 °C as an ethanolic solution. HPLC analysis indicated about 2% decomposition of **3** after such storage for 30 days.

Prior to our investigation of chloroperoxidase as a synthetic method, all attempts to prepare labeled **3** in acceptable yields through the use of conventional methods failed. The success in employing this enzyme for the synthesis of **3** is attributable to the high percent conversion of **1** to **3** in the reaction mixture. This minimizes the need for extensive purification procedures and the loss of product associated with such procedures. This enzymatic method should be generally applicable to the preparation of many other nitroso aromatics on a micro scale.

Experimental. High-pressure LC analyses were conducted with a Waters Associates component system consisting of a Model 6000A pump, model U6K injector and model 440 dual wavelength (λ 254 and 313) absorbance detector. The HPLC column was a μBondapak C_{18} (3.9 mm × 30 cm) and the solvents were aq. methanol under isocratic conditions at a flow rate of 1.5 ml/min. UV-spectra were obtained on a Beckman Model 35 spectrophotometer and liquid scintillation counting was done in Aquasol (New England Nuclear) with a Searle Analytic 92 counter. Unlabeled 4-chloronitrosobenzene (**3**) was prepared from 4-chloronitrobenzene by standard methods[5], and purified by steam distillation followed by recrystallization from 95% EtOH. [U-^{14}C]-4-Chloroaniline (**1**) was prepared from [U-^{14}C] aniline sulfate (Midwest Research Institute) by the method of Attar et al.[10], then purified by chromatography on EM silica gel 60 with hexane/$CHCl_3$ (1:1) and $CHCl_3$. Analysis of the radiolabeled **1** indicated a sp.act. of 6.3 mCi/mmole and radiochemical purity of 99%. Chloroperoxidase (purified grade) was obtained from Sigma Chemical Co. and enzyme activity assayed by the standard method[8]; note: 1 standard unit = 4070 Sigma units.

Synthesis and purification of [U-^{14}C]-4-chloronitrosobenzene (3). To 100 ml of 0.05 M, pH 4.5, KH_2PO_4 buffer at 25 °C in a 250 ml sep. funnel was added [U-^{14}C]-**1** (2.7 mg, 21 μmoles, 135 μCi) as a solution in 0.5 ml of 95% EtOH. To this was added 0.10 ml (0.2 mmole) of a 2 M aqueous solution of H_2O_2, followed by 100 units[8] of chloroperoxidase. After 5 min the addition of H_2O_2 and enzyme was repeated and the reaction allowed to proceed for another 5 min, then the reaction was extracted once with 35 ml of pentane. The pentane extract was washed with 10 ml of H_2O, dried (Na_2SO_4) and combined with 2 ml of EtOH in a micro Kuderna-Danish concentrator (Supelco Co.). The solvent was reduced in volume to 2.0 ml by heating in a water bath at 70 °C. Comparison of authentic **3** to aliquots of the product in ethanolic solution by HPLC with 60% MeOH as solvent, and by UV-spectrophotometry in MeOH indicated the yield of **3** to be 1.78 mg (60%); λ max 314 (ε 11,400) and 289 nm (ε 9900); spectrum identical to authentic **3**. HPLC analysis indicated impurities to be **1** (16 μg, 1 mol%), 4-chloronitrobenzene (59 μg, 3 mol%) and 4,4′-dichloroazoxybenzene (20 μg, 0.6 mol%).



Sequential oxidation of 4-chloroaniline (**1**) to 4-chlorophenylhydroxylamine (**2**) and 4-chloronitrosobenzene (**3**).

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