N-Nitrosation of Myosmine Yields HPB (4-Hydroxy-1-(3-pyridyl)-1-butanone) and NNN (*N*-Nitrosonornicotine)

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N-Nitrosonornicotine (NNN) is formed by synthetic or biological *N*-nitrosation of the tobacco alkaloid nornicotine. Following metabolic activation of NNN, DNA and protein adducts are formed releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), an actual biomarker to differentiate between tobacco smokers and passive smokers. NNN and HPB can be prepared in a new one-step reaction by *N*-nitrosation of the nicotinoid myosmine which has been found not only in tobacco but also in nut products. The reaction was tested also in human gastric juice. The formation rate of NNN and HPB depends on the pH value in the reaction solutions. This is important under the aspect of myosmine uptake by humans from other biological sources and subsequent biological activation. The new reaction pathway indicates that human exposure to nicotinoid nitrosation products seems to be not restricted exclusively to tobacco.

Keywords: *Myosmine; N-nitrosation; nicotinoids; tobacco; human gastric juice*

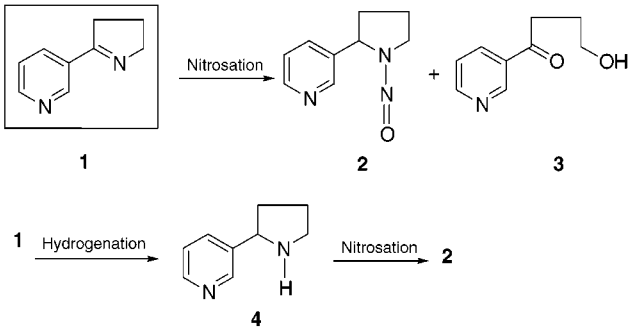
INTRODUCTION

Myosmine (**1**, 3-(1-pyrrolin-2-yl)pyridine) is known as a typical alkaloid in tobacco plants and was detected also in nut and nut products (Zwickenpflug et al., 1998) (Scheme 1). To date, it was of minor interest compared to other tobacco alkaloids such as nicotine (**7**) and nornicotine (**4**) which play an important role in the discussion on the carcinogenic risk assessment of tobacco products (Hoffmann et al., 1974; Hecht, 1998). *N*-Nitrosation of **4** and **7** yields *N*-nitrosonornicotine (NNN, **2**) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **8**). Metabolic activation of both **2** and **8** leads to formation of DNA and protein adducts which release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **3**) upon hydrolysis (Scheme 2). Therefore, determination of the hemoglobin adduct of **3** by GC−MS is used as a biomarker of uptake and activation of **2** and **8** in tobacco users and nonsmokers exposed to environmental tobacco smoke (ETS) (Hecht, 1998). However, as shown by Branner et al. (1998) and Carmella et al. (1990) this adduct cannot serve as a dose-dependent biomarker of **2** and **8** uptake. Whereas the reaction mechanism of *N*-nitrosation from **7** has been investigated in detail (Hecht et al., 1978; Caldwell et al., 1993), no reports are available on *N*-nitrosation of **1** or its interaction with biological systems. Therefore **1** was nitrosated in different buffer solutions and in human gastric juice in order to look for pathways similar to activation from **4** and **7**.

MATERIALS AND METHODS

All nicotinoid derivatives were tested in comparison to standard substances by GC−MS, UV spectra, and retention times on HPLC-DAD (Hecht et al., 1981; Duffield et al., 1965)

Scheme 1. *N*-Nitrosation and Hydrogenation of Myosmine (1)



Nitrosation of 1 in Buffer Solutions and in Human Gastric Juice. 1 (0.3 mM) was dissolved in different buffer solutions. The solutions were prepared at pH 9.0 (0.01 M $Na_2B_4O_7 \cdot 10H_2O$), pH 7.5 (0.02 M Na_2HPO_4 + 0.08 M KH_2PO_4), and pH 5.5 (0.06 M Na_2HPO_4 + 0.06 M KH_2PO4). N_2O_3 was generated by reaction of $NaNO_2$ with glacial acetic acid. $NaNO_2$ (3 mM) was treated with 30−40 mL of acid in a separate three-neck flask and the released N_2O_3 transferred under a N_2 stream through a Teflon tube fitted with glass pipets in the different buffer solutions containing **1**. After a reaction time of 1 h at 37 °C the buffer solutions were extracted three times with 20 mL of $CHCl_3$. The organic phases were separated, dried over Na_2SO_4, concentrated, and analyzed by TLC and/or HPLC. Gastric juice with pH 2.0 was collected from men undergoing endoscopic clinical investigation; 250 μL of gastric juice was incubated with 100 μL of a solution containing 3 mM $NaNO_2$ and with 50 μL of a solution containing 0.3 mM **1** at 37 °C for 30 min. The gastric juice was not further treated or characterized. Aliquots of the sample material were analyzed directly by HPLC−DAD.

TLC Cleanup and Separation of the *N*-Nitrosation Reaction Solution. TLC separation was executed on analytical Kieselgel F254 plates using $CHCl_3$/EtOH (9:1 v/v). For comparison standard solutions of **3**, **1,** and **2** were cochromatographed with aliquots of the reaction solutions. The relevant

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Scheme 2. Proposed Reaction Pathways of *N*-Nitrosation of Myosmine (1) and Nicotine (7)

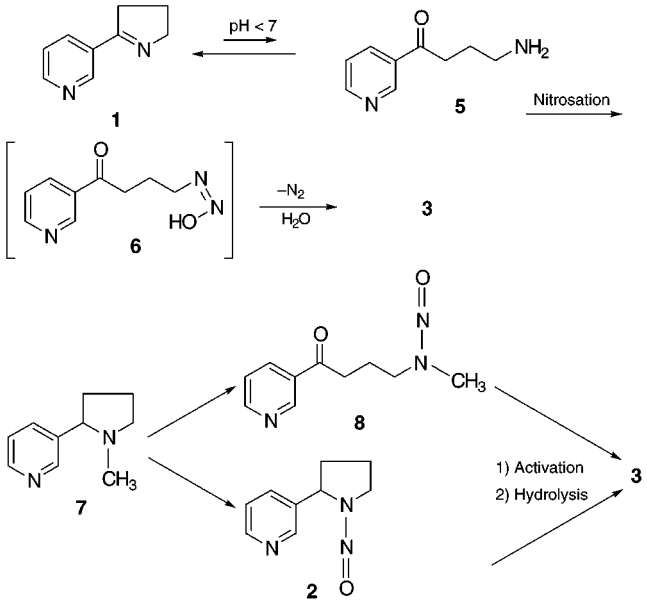

Table 1. *N*-Nitrosation of Myosmine (1) at Different pH Values

pH	yield of **1** (%)	yield of **2** (%)	yield of **3** (%)
9	90	nd	10
7.5	45	15	40
5.5	5	20	75

spots of the reaction solutions were removed from the plates and analyzed for further characterization by HPLC−DAD.

HPLC−DAD Measurements. HPLC−DAD analyses were performed with a Gynkotek Chromeleon system using a LiChrospher 100 RP-18 125 × 4 mm 5 μm column equipped with a 4 × 4 mm 5 μm precolumn (Merck). The column was operated at a flow rate of 0.7 mL/min with a gradient using CH_3CN/0.02 mM phosphate buffer (pH 6.5). After an initial hold for 3 min at 100% buffer, CH_3CN was linearly increased over 37 min from 0% to 30% and in 2 min to 50%. After a hold for 2 min CH_3CN lowered down to 0% in 2 min. The operating wavelength of the diode array system for spectra recording was adjusted to 270 nm with a bandwidth of 140 nm (200−340 nm). The chromatograms were recorded at a wavelength of 233 and 254 nm. The retention time for **3** was observed at 29.4 ± 0.6 min, for **2** at 34 ± 0.6 min, and for **1** at 41 ± 0.7 min. Previously the standard solutions from **1**−**3** were tested by GC−MS and compared with literature data (not shown). The UV spectra of **1**−**3** recorded by DAD were identical to those reported by Hu et al. (1979) and Hecht et al. (1981).

RESULTS AND DISCUSSION

Yields from *N*-nitrosation of **1** in different buffer solutions are shown in Table 1. When **1** is directly *N*-nitrosated with N_2O_3 at low pH, **3** is the main reaction product and **2** is found in minor amounts (Figure 1). The pH value has an influence on the formation of compounds **2** and **3** with the greatest yield of both products at pH 5.5. The *N*-nitrosation in the fasting human gastric juice was tested with and without $NaNO_2$ addition. In the presence but not in the absence of $NaNO_2$, formation of **2** and **3** was observed (Figure 1). These results indicate that *N*-nitrosation in gastric juice may depend on pH as well as on $NaNO_2$ concentration. Therefore, the extent to which **2** and **3** are formed is difficult to predict because the actual pH and N_2O_3 concentration in the gastrointestinal lumen have to be considered.

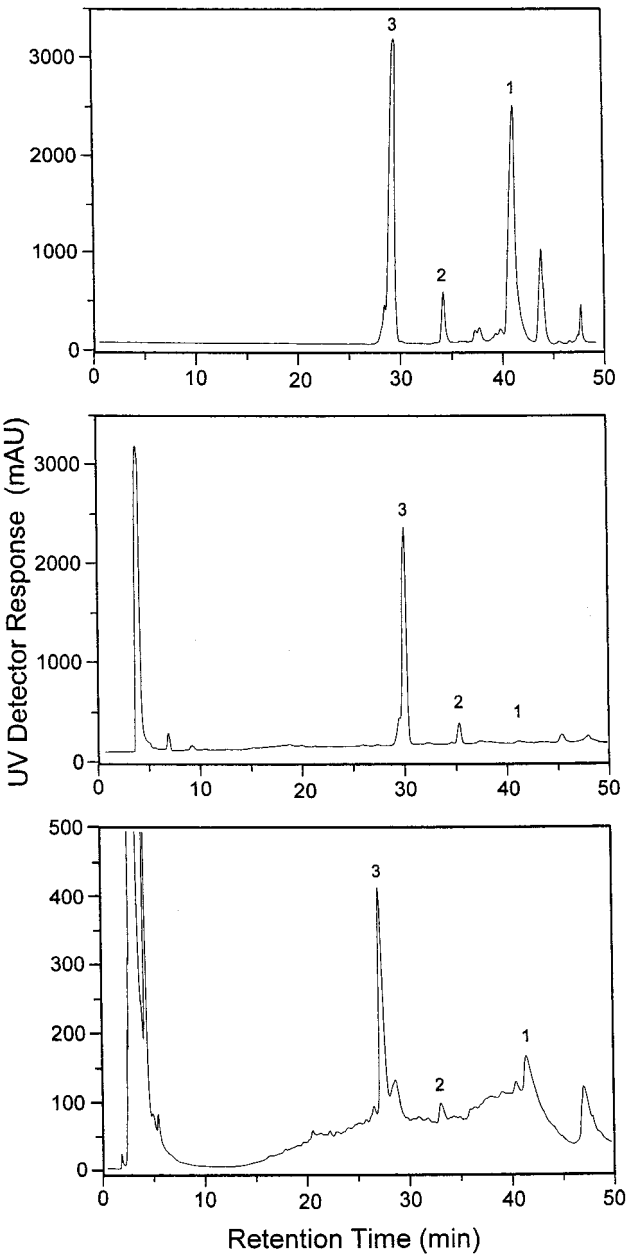

Figure 1. The upper trace represents HPLC−DAD chromatograms of a standard mixture from HPB (**3**), NNN (**2**), and myosmine (**1**). The middle trace shows the products from *N*-nitrosation reaction solution at pH 5.5. The lower trace shows the products after incubation of human gastric juice with $NaNO_2$ and **1**.

As **1** underlies pH-dependent ring-chain tautomerism (Brandänge and Rodriguez, 1983) two reaction pathways of **1** with $NaNO_2$ can be proposed, based on the available quantities of primary amines **5** or imines **1** (Scheme 2). Under acidic conditions hydrolysis of **1** to the corresponding amino ketone form **5** under ring opening is favored. Thus, the primary amine **5** is nitrosated releasing the unstable diazotate **6** which may decompose in situ directly to **3**. *N*-Nitrosation of the imine structure **1** favors the formation of **2**. This process may be influenced by the polarity in the carbon−nitrogen double bond of the pyrrolidine ring of **1**. Further studies are necessary to explain the whole mechanism of **1** nitrosation in detail. At present the preparation of **2** is described as a *N*-nitrosation product of **4** (Scheme 1) (Hu et al., 1979). Synthesis of **4** starts from the ethylnicotinate and involves three steps in-

cluding compound **1** as intermediate. The reaction mechanism of nicotinoid synthesis by this way is described as α-aroylpyrrolidone rearrangement (Späth and Bretscheider, 1928; Korte and Schulze-Steinen, 1962). Subsequent *N*-nitrosation using $NaNO_2$/HCl yields **2** in sufficient amounts (Hu et al., 1979). The synthesis of **3** was performed in a similar way using butyrolactone instead of pyrrolidone as the condensating component (Hecht et al., 1981). For synthesis, direct *N*-nitrosation of **1** offers new possibilities in the preparation of **2** and **3** in a one-step reaction without isolating **4**. Consequently, the presence of **2** in tobacco products may result not only from nitrosation of **4** and **7** but also from nitrosation of **1**. In nut products containing **1** the same activation pathway yielding **2** and **3** must be considered as well. Furthermore in biological systems the formation of adducts of **3** may not be restricted to metabolic activation of **2** and **8** but can also result from *N*-nitrosation of **1**. Whereas endogenous *N*-nitrosation of **7** is reported to be without any relevance, this reaction may be expected to occur for **1** at the same high extent as for **4** (Carmella et al., 1997). Therefore, in the actual discussion on tobacco-specific *N*-nitrosamines (TSNA) background contamination in humans, these new aspects should be taken into account.

In summary, a new pathway to synthesize the well-known nicotinoid derivatives **2** and **3** in a one-step reaction from **1** is presented. This reaction was verified also in human gastric juice. Therefore, the so-called tobacco-specific nitrosamine **2** seems to be not restricted to tobacco exclusively. The difference between the real uptake of nicotinoids from tobacco and the background contamination resulting from other non-tobacco-specific nicotinoid sources demands further elucidation.

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