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Syntheses of isotope-labeled tobacco-specific nitrosamines and their metabolites

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We report here on the syntheses of three deuterium-labeled tobacco-specific nitrosamines namely [2,4,5,6-d_4]nitrosonornicotine([2,4,5,6-d_4]NNN), 4-(methylnitrosamino)-1-(3-[2,4,5,6-d_4]pyridyl)-1-butanone ([2,4,5,6-d_4]NNK), and 4-(methylnitrosamino)-1-(3-[2,4,5,6-d_4]pyridyl)-1-butanol ([2,4,5,6-d_4]NNAL). A metabolite of NNK and myosmine, 4-hydroxy-1-(3-[2,4,5,6-d_4]pyridyl)-1-butanone, was also synthesized. The synthetic strategy reported here is similar to that reported in the literature for the preparation of corresponding unlabeled compounds. The commercially available [2,4,5,6-d_4]ethylnicotinate was used as starting material. During the course of these syntheses [2,4,5,6-d_4]myosmine and [2,4,5,6-d_4]nornicotine were obtained as stable intermediates. These isotope-labeled compounds are useful internal standards for quantification of TSNA and their metabolites in smokers in molecular epidemiological studies.

Keywords: synthesis; isotope label; tobacco-specific nitrosamines

Introduction

Cigarette smoking is the single most preventable cause of cancer deaths in America, as it is responsible for cancers of the lung, head and neck, bladder, pancreas, and kidney; it has also been implied as a possible cause of other cancers.[1,2] Lung cancer is the leading cause of mortality in men and women. In the USA, smoking contributes to >80% of all deaths from lung cancer in men and in women.[3,4] Cigarette smoke contains more than 60 different carcinogenic compounds including tobacco-specific nitrosamines (TSNA). TSNA are formed from nicotine and related tobacco alkaloids and are believed to play a significant role in the development of cancer in humans who consume tobacco products.[5–9]

Seven TSNA have been identified, which are formed by *N*-nitrosation of nicotine or minor tobacco alkaloids.[10] In 1974 Hoffmann *et al*. isolated *N*′-nitrosonornicotine (NNN) from cigarette smoke.[11] NNN causes esophageal and nasal cavity tumors in rats; lung tumors in mice; tracheal tumors in hamsters; and nasal tumors in mink.[12] In addition to NNN, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent carcinogen in laboratory animals that, independent of the route of administration, induces primarily lung adenocarcinoma.[13,14] Both tobacco smoke and unburned tobacco contain substantial amounts of NNK.[15,16] Tobacco smoking is the only known etiologic agent that causes pancreatic cancer; in addition to the induction of lung adenocarcinoma, NNK and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) induce cancer of the pancreas in rats.[17] Co-administration of NNK with NNN by swabbing in the rat causes oral tumors.[18] Collectively, literature data provide a strong support for the contribution of NNN and NNK to the development of several tobacco-related cancers. In fact, the International Agency for Research on Cancer now classified NNN and NNK in group 1 as carcinogenic to humans.[16]

Numerous studies have quantified NNAL and its glucuronic acid conjugates as well as cotinine and its *N*-glucuronic acid conjugate in biological fluids (urine, blood) of smokers, non-smokers exposed to environmental tobacco smoke as well as smokeless tobacco users. Methods for the quantification of nicotine, cotinine, and NNAL in human toe nails have also been reported.[19] Studies described above that were aimed at accurate quantification of metabolites derived from tobacco carcinogens required the availability of isotope-labeled internal standards. For example, [pyridine-d4] NNAL has been used as an internal standard, but detailed description of its synthesis has not been given.[20] In addition, the unlabeled 5-methyl-N′-nitrososonornicotine was used as an internal standard for the quantification of total urinary NNN.[21] However, isotope-labeled internal standards are required for accurate quantification of TSNA and metabolites in biological fluids of subjects who use tobacco products.

For better mechanistic information, such isotope-labeled TSNA have been used in metabolism and tumorigenicity studies.[22,23] Jalas *et al*. reported *in vivo* tumorigenicity study and *in vitro* metabolism study of stereospecifically deuterium-substituted methylene carbon of NNK and NNAL, respectively.[23] Lao *et al*. reported an accurate and sensitive high-performance liquid chromatography (HPLC) Electrospray ionization-mass spectroscopy ESI-MS/MS method using pyridine-d4-substituted pyridyloxobutyl-DNA adducts from the lung and the liver of rats treated with NNK.[22] NNK on metabolic activation by α-

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hydroxylation yields adducts with DNA and globin via unstable intermediates. On hydrolysis, these adducts release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB); the minor tobacco alkaloid myosmine is easily nitrosated to yield NNN and HPB.[24] Myosmine was the first structurally identified tobacco alkaloid besides nicotine. Besides tobacco, myosmine has also been detected in other plants and in nuts and nut products.[25] Formation of HPB as a metabolite of myosmine is attributed to its α-hydroxylation, followed by ring opening.[24] HPB can be quantified by chemical transformation to its pentafluorobenzoate derivative using gas chromatography negative ion chemical ionization mass spectrometry.[26–28] Quantification of released HPB was also achieved by using di-deuterated [4,4-d_2]HPB as an internal standard.[26,27,29] We were the first to report on the presence of tobacco-derived compounds in human pancreatic juice using gas chromatography-mass spectrometry analytical technique and d_4-NNK as an internal standard.[30]

As mentioned above our group and others have employed certain pyridine deuterium-substituted TSNAs as internal standards for the quantification of TSNAs in biological fluid of smokers; however, the detailed synthesis and complete spectral data were not reported. Therefore, in the present article, we report on the synthesis of three TSNAs and metabolites, namely [2,4,5,6-d_4]NNN, [2,4,5,6-d_4]NNK, [2,4,5,6-d_4]NNAL, and [2,4,5,6-d_4] HPB. [2,4,5,6-d_4]Nornicotine and [2,4,5,6-d_4]myosamine were obtained as an intermediate during the course of this synthesis.

Results and discussion

Synthesis of [2,4,5,6-d_4]NNN (**4**) is summarized in Scheme 1. The key intermediate in this synthesis was [2,4,5,6-d_4]nornicotine (**3**), which was obtained in two steps. Condensation of [2,4,5,6-d_4]ethylnicotinate with 1-vinyl-2-pyrrolidinone followed by mild acidic hydrolysis gave an open chain compound followed by decarboxylation, and cyclization gave [2,4,5,6-d_4]myosmine (**2**) in moderate yield. [2,4,5,6-d_4]Myosmine (**2**) on further reduction with $NaBH_4$ gave the key intermediate [2,4,5,6-d_4]nornicotine (**3**) in 92% yield. Compound **3** was nitrosated at pH 4 to give [2,4,5,6-d_4]NNN (**4**) in 57% yield as an oil.

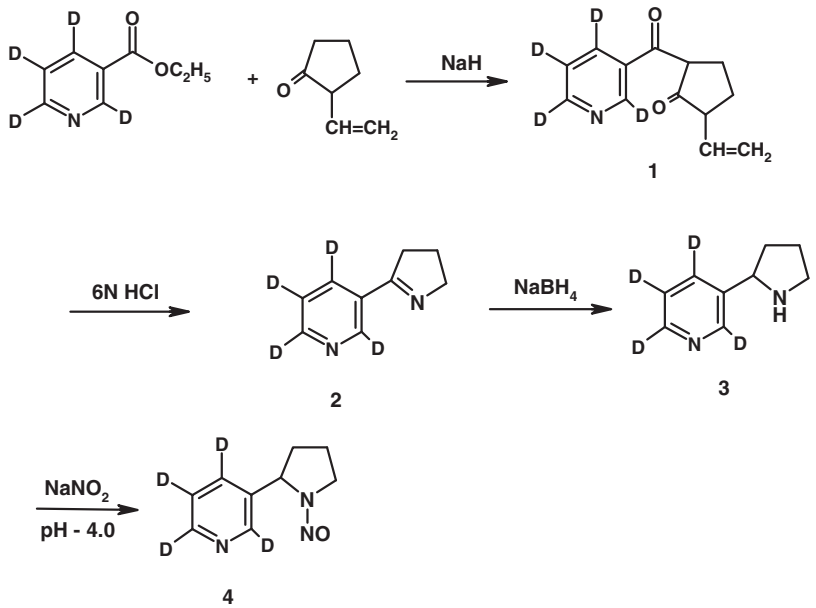
The synthesis of [2,4,5,6-d_4]NNK (**7**) and [2,4,5,6-d_4]NNAL (**8**) is summarized in Scheme 2. The synthesis is identical to that reported in the literature for the preparation of unlabeled NNK and NNAL.[31–33] Condensation of [2,4,5,6-d_4]ethylnicotinate with 1-methyl-2-pyrrolidinone in the presence of NaH produced lactam **5** in 41% yield. Lactam **5** under acidic hydrolysis condition followed by nitrosation at pH 4 gave [d_4] NNK **7** in 44% yield. Reduction of [2,4,5,6-d_4]NNK (**7**) with $NaBH_4$ furnished quantitative yield of [2,4,5,6-d_4]NNAL (**8**). In a previous report the syntheses of tobacco alkaloids with deuterium in the pyridine ring have been described. Direct deuterium exchange reaction reportedly gave relatively less stable alkaloids as compared with those with deuterated pyrrolidine ring.[34] More stable deuterated metabolites of nicotine and TSNAs were prepared from 3-bromopyridine.[35]

The synthesis of [2,4,5,6-d_4] HPB (**9**) is summarized in Scheme 3. Condensation of [2,4,5,6-d_4]ethylnicotinate and γ-butyrolactone was accomplished by using sodium methoxide in benzene. The lactone was hydrolyzed in 6 N HCl at room temperature without isolation. The desired compound was purified on silica gel column to give a moderate yield of the pure keto alcohol (**9**). The structures of compounds were confirmed by proton nuclear magnetic resonance (NMR) and by MS analysis and final purity was confirmed by reverse phase HPLC.

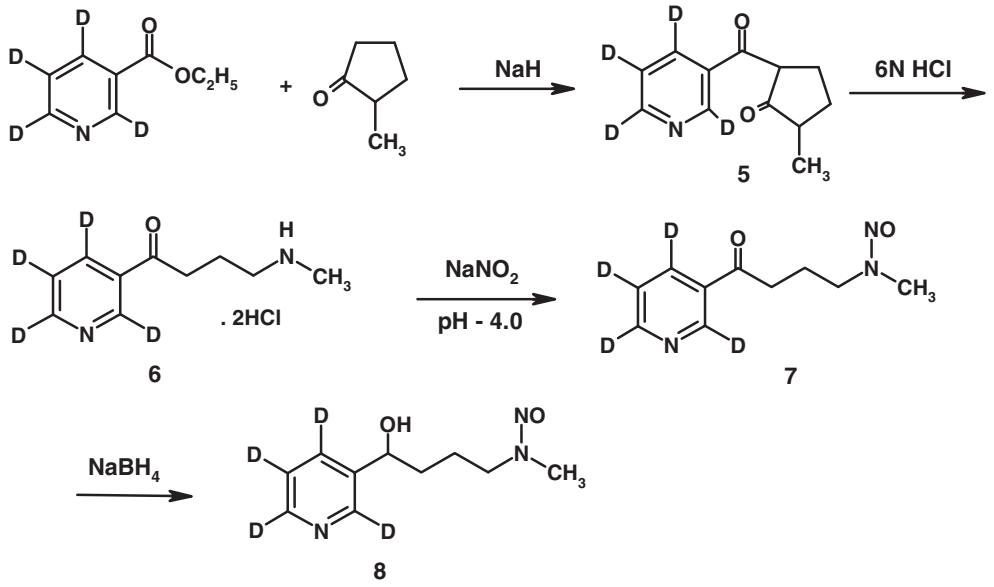
Experimental

General

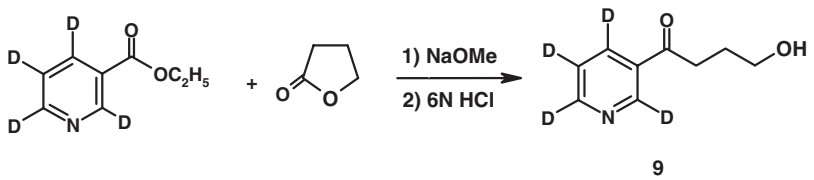
Melting points were recorded on a Fisher-Johnson melting point apparatus and are uncorrected. Unless stated otherwise, proton NMR spectra were recorded in $CDCl_3$ using Bruker AM 360WB, Bruker 500 MHz, and Varian 300 MHz instruments. The chemical shifts are reported in ppm downfield from Tetramethylsilane TMS. MS were run on a Hewlett-Packard Model 5988A instrument and on a Finnigan Mat95 instrument at the University of Minnesota and at the proteomic facility in Penn State Cancer Institute at the Penn State College of Medicine,



Scheme 1



Scheme 2



Scheme 3

Hershey, PA. Thin-layer chromatography was done on aluminum-supported, pre-coated silica gel plates (EM Industries, Gibbstown, NJ). Ethyl [2,4,5,6-d_4]nicotinate was purchased from Cambridge Isotope Laboratories (Andover, MA). All other starting materials were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

Synthesis

1-Vinyl-3-([2,4,5,6-d_4]pyridinoyl)-2-pyrrolidinone (1) and [2,4,5,6-d_4]myosmine (2).

Under nitrogen atmosphere, sodium hydride (38.76 mmol, 1.98 g of 60% suspension in mineral oil) was washed twice with hexane and resuspended in dry toluene (100 mL). To this suspension, a solution of ethyl [2,4,5,6-d_4]nicotinate (5.0 g, 32.3 mmol) and 1-vinyl-2-pyrrolidinone (3.59 g, 32.3 mmol) in dry toluene (100 mL) was added dropwise with stirring. The mixture was then refluxed for 48 h, cooled to room temperature and poured over cold 2 N HCl (100 mL). The toluene layer was separated and the aqueous layer was adjusted to pH 4 and extracted with chloroform (4×100 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated to give **1** (2.41 g, 34%) as an oil; MS m/e, 221 (M^{+1}, 90), 195 (M^+-CO, 100), 128 (30). Lactam **1** was used in a subsequent step without further purification.

A solution of lactam **1** (2.0 g, 9.1 mmol) in 6 N HCl was heated to reflux for 14 h. The reaction mixture was cooled and poured into ice and the pH of the solution was adjusted to 10. The aqueous layer was extracted with $CHCl_3$ (4×20 mL) and the combined organic layers were dried ($MgSO_4$), filtered, and evaporated to yield the crude product, **2**. Purification was accomplished on a silica gel column by eluting with $CHCl_3$:MeOH (9:1) to give **2** (0.71 g, 52%) as an oil. ^{1}H NMR δ 2.02 and 2.06 (t, 2H, C–CH_2–C, $J = 7.48$ Hz, $J = 7.29$ Hz), 2.93 (tt, 2H, = C–CH_2, $J = 8.05$ Hz, $J = 2.08$ Hz), 4.06 (tt, 2H, N–CH_2, $J = 7.41$ Hz, $J = 2.09$ Hz), MS m/z (relative intensity) 150 (M^+, 80), 122 (100), 109 (30), 82 (30).

[2,4,5,6-d_4]Nornicotine (3)

To a mixture of **2** (0.3 g, 2.0 mmol) in methanol (10 mL) and glacial acetic acid (1 mL), $NaBH_4$ (0.11 g, 3.0 mmol) in MeOH (5 mL) was added over 5 min. The reaction mixture was stirred at room temperature for 2 h and at 50°C for 1 h. The mixture was quenched with H_2O. The reaction mixture was then stripped of the methanol and concentrated to 10 mL. It was then treated with HCl (2 N) and stirred at 50–60°C for 1 h to decompose the complex. The mixture was adjusted to pH 10 with NaOH solution and extracted with $CHCl_3$ (3×20 mL). Combined organic fractions were dried ($MgSO_4$), filtered, and evaporated to give a crude product, which was chromatographed over silica gel using $CHCl_3$:MeOH (9:1) as an eluent to give **3** (0.28 g, 92%) as an oil; ^{1}H NMR δ 1.70–1.78 (m, 1H, 2–CH_2), 1.90–2.01 (m, 2 H, CH_2), 2.25–2.28 (m, 1H, 3–CH_2), 3.07–3.12 (m, 1H, N–CH_2), 3.23–3.27 (m, 1H, N–CH_2), 4.22 (t, 1H, CH, $J = 8.03$ Hz); MS m/z (relative intensity) 153 (M^{+1}, 100).

[2,4,5,6-d_4]N′-Nitrosonornicotine (4).

A cold solution of nornicotine (**3**) (0.2 g in 2 mL of H_2O, 1.32 mmol) was adjusted to pH 4 with aqueous 2 N HCl solution. To this mixture, a solution of $NaNO_2$ (0.16 g in 0.5 mL H_2O, 2.4 mmol) was added dropwise at 0°C. The mixture was stirred at room temperature for 8 h. The reaction mixture was extracted at pH 5 with $CHCl_3$ (3 × 20 mL). Then, the aqueous layer was adjusted to pH 8 with 2 N NaOH solution and extracted with $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ layers were dried over $MgSO_4$, filtered, and evaporated. The crude product was purified on a silica gel column using $CHCl_3$:MeOH (9:1) as an eluent to give **4** (0.14 g , 57%) as an oil; ^{1}H NMR δ 2.05–2.51 (m, 3H), 2.52–2.2.60 (m, 1H), 3.68–3.74 (m, 0.8H), 3.86–3.91 (m, 0.8H), 4.41–4.43 (m, 0.4H), 5.25 (t, 0.3H, J = 7.0 Hz), 5.70 (t, 0.7H, J = 6.5 Hz); MS m/z (relative intensity) 181 (M^+, 30), 151 (M-NO, 100), 137 (10), 123 (25), 82 (50).

1-Methyl-3-([2,4,5,6-d_4]pyridinoyl)-2-pyrrolidinone (5).

The lactam **5** was prepared in a similar manner as reported for lactam **1**. Purified lactam **5** was obtained by chromatography on silica gel column and by eluting with EtOAc as a yellow oil (41%). ^{1}H NMR δ 2.20–2.31 (m, 1H, C–CH_2-), 2.67–2.75 (m, 1H, C–CH_2-), 2.86 (s, 3H, N–CH_3), 3.35–3.51 (m, 1H, N–CH_2–C), 3.56–3.63 (m, 1H, N–CH_2–C), 4.42 and 4.44 (d, 1H, CH, J = 5.2 Hz).

4-Methylamino-1-(3-[2,4,5,6-D]pyridyl)-1-butanone dihydrochloride (6).

A solution of lactam **5** (2.2 g, 10.6 mmol) in 40 mL of 6 N HCl was heated at reflux with stirring for 48 h. The reaction mixture was cooled and adjusted to pH 12 with concentrated NaOH (reaction mixture was kept below 0°C during the addition). The alkaline solution was extracted with chloroform (3 × 100 mL). The combined organic layers were extracted with 2N HCl solution (4 × 100 mL). The aqueous extract was concentrated to give 1.85 g of hydrochloride **6** that was recrystallized from ethanol, m.p., 178–180°C. Dihydrochloride salt **6** was used in a subsequent step without further characterization.

4-(Methylnitrosamino)-1-(3-[2,4,5,6-D_4]pyridyl)-1-butanone ([2,4,5,6-d_4]NNK) (7).

A cold solution of hydrochloride **6** (1.50 g in 30 mL of H_2O, 5.91 mmol) was adjusted to pH 4 with aqueous 2 N NaOH solution. To this mixture a solution of $NaNO_2$ (0.69 g in 5 mL H_2O, 10 mmol) was added dropwise at 0°C. The mixture was stirred at room temperature for 8 h. The reaction mixture was extracted at pH 5 with $CHCl_3$ (3 × 20 mL). Then, the aqueous layer was adjusted to pH 8 with 2 N NaOH solution and extracted with $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ layers were dried over $MgSO_4$, filtered, and evaporated. The crude product was purified on a silica gel column using EtOAc as an eluent to give **7** (0.55 g, 44%) as a low melting solid, m.p. 62–64°C. ^{1}H NMR δ 1.97 (t, 0.2H, Z–CH_2–C, J = 6.93 Hz), 2.01 (t, 0.2H, Z–CH_2–C, J = 7.12 Hz), 2.22 (t, 0.8H, E–CH_2–C, J = 6.85 Hz), 2.26 (t, 0.8H, E–CH_2–C, J = 6.81 Hz), 2.95 (t, 0.4H, Z–CO–CH_2, J = 6.88 Hz), 3.07 (t, 1.6H, E–CO–CH_2, J = 6.87 Hz), 3.09 (s, 2.5H, E–N–CH_3), 3.72 (t, 0.4H, Z–N–CH_2, J = 7.21 Hz), 3.80 (s, 0.5H, Z–N–CH_3), 4.27 (t, 1.6H, E–N–CH_2, J = 6.81 Hz); MS m/z (relative intensity) 211 (M^+, 3) 181 (M-NO, 70), 150 (10), 122 (10), 110 (100), 82 (100).

4-(Methylnitrosamino)-1-(3-[2,4,5,6-d_4]pyridyl-1-butanol ([2,4,5,6-d_4]NNAL) (8).

To a stirring solution of **7** (0.4 g, 1.9 mmol) in methanol (15 mL), $NaBH_4$ (0.14 g, 3.8 mmol) was added portionwise over a period of 10 min. The reaction mixture was stirred at room temperature for an additional 2 h and then concentrated to dryness. The residue was triturated with H_2O and the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). Combined organic layers were dried ($MgSO_4$), filtered, and evaporated to dryness to give crude product **8**, which was purified by silica gel column chromatography eluting with $CHCl_3$: MeOH (9:1) as an eluent to give **8** (0.38 g, 94%) as a low melting solid; ^{1}H NMR δ 1.65–1.91 (m, 4H), 3.02 (s, 2.5H, E–N–CH_3), 3.55–3.65 (m, Z–N–CH_2), 3.73 (s, 0.5H, Z–N–CH_3), 4.15–4.21 (m, E–N–CH_2), 4.78–4.81 (m, 1H, CH); MS m/z (relative intensity) 214 (M^{+1}, 3) 185 (M-NO, 10), 165 (10).

4-Hydroxy-1-(3-[2,4,5,6-d_4]pyridyl)-1-butanone (HPB) (9).

To a stirring suspension of sodium methoxide (9.4 mmol) in benzene (5 mL) at room temperature, a solution of γ-butyrolactone (0.67 g, 7.8 mmol) and ethyl[2,4,5,6-d_4]nicotinate (1.18 g, 7.6 mmol) in 2 mL of benzene was added dropwise. The mixture was stirred at room temperature for 1 h and allowed to stand overnight. Water (10 mL) was then added and the mixture extracted three times with $CHCl_3$ to remove organic soluble impurities. The aqueous layer was acidified to pH 2 with 6 N HCl and stirred for 1 h at room temperature. The acidic mixture was adjusted to pH 10 with NaOH and extracted with ethyl acetate (3 × 20 mL). Combined organic layers were dried, filtered, and evaporated to yield the crude product, which was purified by flash chromatography on silica gel using chloroform/MeOH gradient (up to 10% of MeOH). The keto-alcohol **9** was obtained as an oil that solidified gradually into colorless crystals (0.28 g, 60%); ^{1}H NMR δ 3.78 (t, 2H, CH_2OH), 3.17 (t, 2H, –CO–CH_2–), 2.06 (m, 2H, –C–CH_2–C); MS m/z (relative intensity) 170 (M^{+1}, 10), 152 (8), 138 (30), 110 (45), 84 (100).

Conclusion

In summary, deuterium-labeled TSNA namely [2,4,5,6-d_4]NNN, [2,4,5,6-d_4]NNK, [2,4,5,6-d_4]NNAL, and [2,4,5,6-d_4] HPB were synthesized. During the course of this work [2,4,5,6-d_4]myosmine and [2,4,5,6-d_4]nornicotine were obtained as intermediates. These isotope-labeled compounds are useful as internal standards in molecular epidemiological studies for quantification of carcinogens and their metabolites in humans who use tobacco products.

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