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# Contribution of Nicotine and Nornicotine toward the Production of *N*′-Nitrosonornicotine in Air-Cured Tobacco (*Nicotiana tabacum*)

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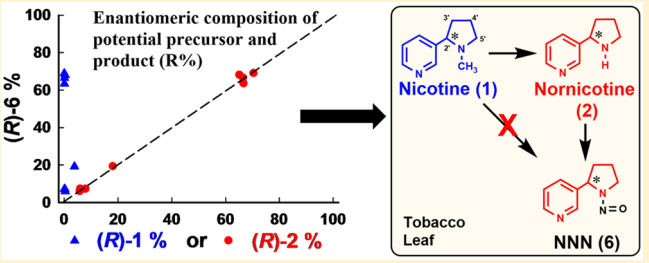
Supporting Information

**ABSTRACT:** *N*′-Nitrosonornicotine (**6**) is a potent and organ-specific carcinogen found in tobacco and tobacco smoke in substantial amounts. Nicotine (**1**) and nornicotine (**2**) are proposed to be the precursors of **6** in tobacco. Since **1** can be rapidly demethylated to **2** in tobacco, to distinguish between the direct formation of **6** from these potential precursors is difficult. A gas chromatography/thermal energy analyzer method using two columns in series was developed to separate the enantiomers of **6**, *N*′-nitrosoanabasine (**7**), and *N*′-nitrosoanatabine (**8**). Tobacco lines with different combinations of three nicotine demethylases inhibited were grown in the field. Air-cured leaves were analyzed for the enantiomeric composition of four main alkaloids and their corresponding tobacco-specific nitrosamines. The percentage of (*R*)-**6** of total **6** varied from 7% to 69% in mutant lines. The measured **6** had the same enantiomeric composition as **2**, rather than **1**, even when the level of **2** was reduced to 0.6% of **1** in a triple mutant line. The pattern of the enantiomeric composition of **1**, **2**, and **6** demonstrated that the direct formation of **6** from **1**, if it occurs, is negligible in air-cured tobacco. Since (*S*)-**6** is more highly carcinogenic than its *R* form, the reduction of (*S*)-**2** should be a priority for the reduction of **6**.




Tobacco-specific nitrosamines (TSNAs) are a class of toxicants formed by nitrosation of tobacco alkaloids. The four main TSNAs identified in tobacco are *N*′-nitrosonornicotine (**6**; NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (**5**; NNK), *N*′-nitrosoanabasine (**7**; NAB), and *N*′-nitrosoanatabine (**8**; NAT). These are nitrosated products of the corresponding alkaloid and mostly formed during the tobacco curing process (Figure 1).[1,2] Compound **6** generally occurs in greater quantities than the others and is considered the second most potently carcinogenic of the TSNAs. The compound is formed primarily during tobacco curing,[3] and its levels may be influenced by endogenous factors such as genotypes,[2,4,5] tobacco types,[6] different positions of the leaf on the stalk or within a single leaf,[7] or external factors such as the use of nitrogen fertilizer,[5,8,9] curing conditions,[2] storage conditions,[10,11] and nitrite scavengers.[12]

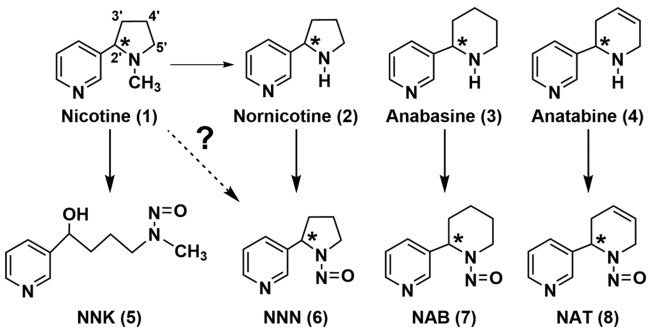



**Figure 1.** Overview of the nitrosation reactions leading to the TSNA formation in tobacco leaf. All compounds were quantified in this study. The carbon with an asterisk (*) is the asymmetric carbon atom.

The presence of **6** in tobacco (*Nicotiana tabacum* L., Solanaceae) was first suggested by Druckrey and Preussmann in 1962 and then confirmed in 1974 in tobacco smoke.[13,14] Since then, many researchers have endeavored to study the formation mechanisms of **6** (Table S1, Supporting Information). On the basis of feeding assays, Hecht and his colleagues proposed that both nicotine (**1**) and nornicotine (**2**) are alkaloid precursors for **6**.[15] Compound **2** has been suggested as the major precursor based on the nitrosation rate in vitro.[16,17] The major role that **2** may play in the formation of **6** was confirmed by suppression of nicotine demethylase activities through RNA interference (RNAi) technology.[18] Since **1** can be rapidly demethylated to **2** in tobacco, to distinguish between the formation of **6** directly from **1** or through **2** is difficult technically.

Compound **6** can induce tumors of the oral cavity, esophagus, and nasal mucosa when administered to rats.[19] This is a chiral compound, and the carcinogenic potential of the two enantiomers of **6** is different, with the *S* enantiomer having a greater carcinogenicity than the *R* enantiomer.[20−23] Balbo et al. reported 14 times more oral cavity tumors in rats treated

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Table 1. Nicotine (1), Nornicotine (2), and *N*′-Nitrosonornicotine (6) Levels in Air-Cured Lamina Affected by Different Nicotine Demethylase Mutations[a]

| | | treatment | | | 1 | 2 | 6 |
|---|---|---|---|---|---|---|---|
| | | *E4* | *E5* | *E10* | mg g$^{-1}$ | mg g$^{-1}$ | $\mu$g g$^{-1}$ |
| 2010 | *E4E5E10* | + | + | + | 16.2 ± 3.5 | 24.80 ± 2.28 | 12.41 ± 3.75 |
| | *e4E5E10* | − | + | + | 29.7 ± 7.7 | 0.88 ± 0.22 | 1.16 ± 0.68 |
| | *E4e5E10* | + | − | + | 11.5 ± 1.7 | 20.86 ± 2.35 | 8.99 ± 3.42 |
| | *E4E5e10* | + | − | − | 15.0 ± 2.1 | 19.30 ± 4.00 | 10.18 ± 3.33 |
| | *e4e5E10* | − | − | + | 31.7 ± 2.5 | 0.90 ± 0.11 | 1.65 ± 0.50 |
| | *e4E5e10* | − | + | − | 37.6 ± 3.8 | 0.95 ± 0.11 | 1.59 ± 0.54 |
| | *E4e5e10* | + | − | − | 14.0 ± 0.5 | 18.34 ± 1.95 | 13.63 ± 3.91 |
| | *e4e5e10* | − | − | − | 44.2 ± 1.2 | 0.48 ± 0.04 | 0.75 ± 0.11 |
| 2011 | TN 90 LC[b] | +[s] | + | + | 70.5 ± 8.5 | 1.86 ± 0.12 | 0.96 ± 0.26 |
| | *E4E5E10* | + | + | + | 11.8 ± 3.5 | 44.99 ± 8.20 | 8.40 ± 3.03 |
| | *e4E5E10* | − | + | + | 50.1 ± 5.2 | 1.63 ± 0.15 | 1.20 ± 0.38 |
| | *E4e5E10* | + | − | + | 8.8 ± 4.6 | 42.55 ± 2.68 | 9.60 ± 1.84 |
| | *E4E5e10* | + | − | − | 2.9 ± 0.9 | 43.10 ± 4.92 | 8.16 ± 1.42 |
| | *e4e5E10* | − | − | + | 52.1 ± 3.8 | 1.44 ± 0.21 | 0.72 ± 0.34 |
| | *e4E5e10* | − | + | − | 61.0 ± 8.5 | 1.53 ± 0.12 | 1.52 ± 0.37 |
| | *E4e5e10* | + | − | − | 9.6 ± 4.6 | 41.01 ± 2.73 | 10.32 ± 3.16 |
| | *e4e5e10* | − | − | − | 63.1 ± 3.9 | 0.40 ± 0.03 | 0.44 ± 0.14 |

[a]Tobacco mutants with different demethylase genes silenced were grown in the field for two years. The air-cured laminas were analyzed for alkaloid and TSNA level (see Table S2 in the Supporting Information for the rest of the alkaloid and TSNA data). All results are the average of three (2010) or four (2011) replicates ± standard deviation. Each replicate consists of five middle leaves, one from each of five plants. [b]TN 90LC is a commercial variety and used as control only in 2011.

with (*S*)-**6** when compared to the incidence in rats treated with (*R*)-**6**.[22] Since (*S*)-**6** was found to be the predominant form of the compound in tobacco products,[24] the *S* form accounts for the greater degree of harmfulness.

Compounds **1**, **2**, and **6** are all chiral, with a stereocenter at the 2′-position (Figure 1). Recently, it was demonstrated that the enantiomeric composition of **2** varies in nicotine demethylase mutant lines, while the composition of **1** remained unchanged.[25,26] To determine the role of **1** in the formation of **6**, the enantiomeric composition was compared among **6**, **1**, and **2** in tobacco mutant lines, but no sign of involvement of **1** in the formation of **6** in tobacco was evident.

## RESULTS AND DISCUSSION

**Effects of Individual Demethylases on *N*′-Nitrosonornicotine (6).** Through RNAi-mediated suppression of nicotine demethylation, **2** has been shown to be the major precursor for **6**.[18] The relative contribution of each of the three demethylases toward the **2** contents has been evaluated.[27] To evaluate the effects of each nicotine demethylase on the formation of **6**, a burley line (with a high propensity for nicotine demethylation), along with seven derived mutant lines, was grown in the field at Rocky Mount, North Carolina (2010), and Lexington, Kentucky (2011). The middle leaves were sampled after being air-cured and analyzed for alkaloids and TSNAs (Table 1 and Table S2, Supporting Information). TN 90LC is a commercial variety and was added to the 2011 experiment.

Alkaloid and TSNA production is sensitive to the environment. Although the general effects of the three demethylase genes on alkaloids and TSNAs were similar between the two years, the average concentration levels were different in the alkaloids and TSNAs. Besides environmental effects, it is obvious that nicotine demethylase has significant effects on the content of **6**. During the two-year experiment, the content of **6** in mutant lines ranged from 0.44 $\mu$g g$^{-1}$ (triple mutant line) to 13.63 $\mu$g g$^{-1}$ (double mutant line with a functional *E4* gene). The range of content of **6** in this study was comparable with a previous report,[18] in which the same parent and its derived RNAi lines were used and **6** ranged from 0.19 to 12.32 $\mu$g g$^{-1}$.

Nitrosation formation from secondary amines is generally much more rapid than that from tertiary amines. In this study, it was found the nitrosation reaction rate of **1** is 20- to 200-fold lower than the **2** nitrosation (Figure S1, Supporting Information). The nitrosation reaction occurred in the decreasing order **2** > **4** > **3** > **1**. These results are consistent with previous aqueous results showing nitrosation of **2** is more rapid than that of **3**.[17] Therefore, the nitrosation reaction in air-cured tobacco is much slower for tertiary amines than for secondary amines.

Among the three demethylases, CYP82E4 accounts for 96% of the **2** and 90% of the **6** formed subsequently (see the Experimental Section for the calculations). CYP82E5v2 and CYP82E10 accounted for 2% and 5% of **2** and **6**, respectively (Table 1). These results confirmed the major role played by nicotine demethylases in the formation of **6**, as proposed by Lewis et al.[27] Since the three nicotine demethylases are associated with about 95% of the **6** in the air-cured leaf, the question was posed as to the contribution of **1** and **2** to the accumulation of **6** present in a triple mutant line (*e4e5e10*). The answer found may facilitate the further reduction of **6**.

**Development of a Simple Quantification Method for Enantiomers of TSNAs.** To further explore the precursor for the remaining 5% of **6** in the triple mutant line (*e4e5e10*), the enantiomeric compositions of **1**, **2**, and **6** were measured. The hypothesis was that the composition of **6** would reflect the composition of its alkaloid precursor. Carmella and his colleagues used this strategy before by comparing the **6** from in vitro nicotine demethylation with that in tobacco.[28]

Methods for enantiomeric separation of **6** by capillary electrophoresis[29] and gas chromatography/thermal energy analyzer (GC/TEA)[28] have been reported before. Since the


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content of **6** has been reduced significantly in mutant lines, a more sensitive method for TSNA enantiomer measurement was modified and developed from a previously published method.[28] The major change in the method was that instead of single chiral column, a serial combination of a chiral column and a nonpolar column (Zebron ZB-5 in this study) was installed in the GC, and therefore the step of collecting the HPLC elute was avoided. All the enantiomers of **6**, **7**, and **8** could be baseline-separated by this method (Figure 2). Samples

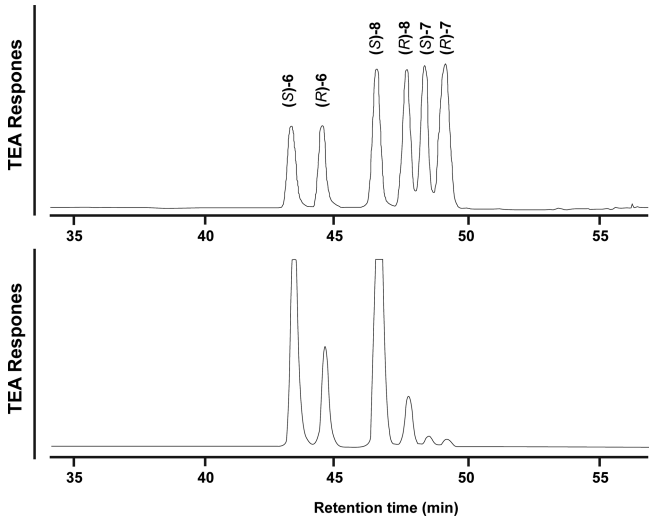


**Figure 2.** (Top) Gas chromatography of standard solution (mixtures of racemic *N*′-nitrosonornicotine (**6**), *N*′-nitrosoanabasine (**7**), and *N*′-nitrosoanatabine (**8**)) and (bottom) a TSNA extract from reference cigarette 2R1 tobacco. The enantiomers of three TSNAs are eluted sequentially: (*S*)-**6** (43.6 min), (*R*)-**6** (44.7 min), (*S*)-**8** (46.6 min), (*R*)-**8** (47.7 min), (*S*)-**7** (48.4 min), (*R*)-**7** (49.1 min). These two chromatographs were converted from printed chromatograms using the software Adobe Illustrator CS4.

2R1 and 1R4F were two reference cigarette tobaccos used as controls to validate the improved method (Table 2). Similar amounts of **2**, **6**, and **8** in 2R1 were measured as described by Carmella et al.[28] The composition of **6** in the reference cigarette tobacco was consistent with the composition of cigarette tobacco and smokeless tobacco products, which ranged from 34% to 43% of *R* form for total **6**, as reported by Stepanov et al.[24] When compared with previous methods, the present newly developed method involves a simpler procedure, a better separation, and lower compound loss during measurement.

**Nicotine (1) Is Not Involved in the Formation of *N*′-Nitrosonornicotine (6) in the Tobacco Leaf.** After development of the above method, all the mutant lines from the 2011 growing season were analyzed for their alkaloid and TSNA composition. Not only the total contents (Table 1) but mutations in nicotine demethylase levels created a population containing variable enantiomeric compositions of **1**, **2**, and **6** (Table 2). Despite the large variation in the levels of **2** spanning from 0.44 to 10.32 $\mu g\ g^{-1}$ and the (*R*)-**2** portion from 5.9% to 66.6% of the total amount of **2**, in all samples the enantiomeric composition of **6** was similar to that of **2**. Also in the triple mutant line, the enantiomeric composition of **6** was the same as the enantiomeric composition of **2**, suggesting that even for the residual **6** formed in the triple mutant line, **2** is the major contributing precursor alkaloid. On the basis of the enantiomeric composition of **1**, **2**, and **6**, no evidence was found for the involvement of **1** in the formation of **6** in air-cured tobacco leaf.

During the treatment of wastewater from a cigarette factory, compound **6** is also formed from **2**, not from **1**, as in tobacco leaf.[30] Therefore, to further reduce the level of **6** in the triple nicotine demethylase mutant line, future investigators should continue to focus on reducing the level of **2** and nitrosation agents (nitrate and nitrite).

Compounds **7** and **8** consist of approximately 40% and 15% of the *R* form, respectively, and the composition was not affected by the mutation of nicotine demethylase genes (Table 2). These two substances have the same enantiomeric composition as **3** and **4** from previous reports,[31−33] suggesting that there is no enantiomeric composition change in air-cured tobacco leaf during or after the nitrosation reaction of the respective secondary amine alkaloids. Therefore, compounds **6**,

**Table 2. Enantiomeric Compositions of Nicotine (1), Nornicotine (2), NNN (6), NAB (7), and NAT (8) in Air-Cured Mutant Lamina from a 2011 Field Trial**[a]

| | treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *E4* | *E5* | *E10* | (*R*)-**1**% | (*R*)-**2**% | (*R*)-**6**% | (*R*)-**8**% | (*R*)-**7**%[b] |
| 2R1 | | | | 0.5 | 30.0 | 29.5 | 15.2 | 41.3 |
| 1R4F | | | | 0.3 | 29.0 | 26.6 | 16.5 | 40.5 |
| TN 90LC | $+^s$ | + | + | 0.0 ± 0.0 | 66.7 ± 2.2 | 63.5 ± 2.6 | 15.5 ± 0.6 | 42.4 |
| parent | + | + | + | 0.1 ± 0.1 | 6.0 ± 0.7 | 7.3 ± 1.3 | 17.3 ± 3.8 | 39.9 |
| *e4E5E10* | − | + | + | 0.0 ± 0.0 | 70.5 ± 3.5 | 69.0 ± 3.2 | 18.4 ± 2.7 | 41.7 |
| *E4e5E10* | + | − | + | 0.1 ± 0.0 | 6.5 ± 0.4 | 7.0 ± 0.2 | 15.6 ± 1.4 | 41.0 |
| *E4E5e10* | + | − | − | 0.4 ± 0.3 | 5.9 ± 0.2 | 6.0 ± 1.0 | 14.7 ± 1.2 | 36.3 |
| *e4e5E10* | − | − | + | 0.4 ± 0.0 | 65.2 ± 8.3 | 68.1 ± 0.4 | 17.1 ± 1.1 | 40.8 |
| *e4E5e10* | − | + | − | 0.1 ± 0.0 | 66.6 ± 3.6 | 66.5 ± 1.2 | 15.4 ± 0.4 | 44.8 |
| *E4e5e10* | + | − | − | 0.2 ± 0.1 | 8.0 ± 0.4 | 7.3 ± 1.1 | 13.5 ± 0.8 | 37.5 |
| *e4e5e10* | − | − | − | 3.8 ± 0.3 | 18.1 ± 2.5 | 19.3 ± 3.2 | 16.4 ± 0.9 | 40.8 |

[a]Tobacco mutants with different demethylase genes silenced were grown in the field in 2011. The air-cured laminas were analyzed for the enantiomeric composition of alkaloids and TSNAs. Two reference cigarette tobaccos, 2R1 and 1R4F, were used as controls. TN 90LC has a functional *CYP82E4* gene, but the gene expression is suppressed ($+^s$). All results are the average of four replicates ± standard deviation. Each replicate consists of five middle leaves, one from each of five plants. Generally, a higher percentage of the *R* form of NNN was found in tobacco containing a functional *CYP82E4* gene. [b]Samples were combined to get a quantifiable response for (*R*)-7%.



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7, and **8** are all formed from secondary amine alkaloid nitrosation.

The enantiomeric composition of TSNAs in cut filler and cigarette smoke of the reference cigarette 3R4F was analyzed. The *R*% of compounds **6**, **7**, and **8** was 26%, 40%, and 16% in the cut filler, and that in cigarette smoke was 27%, 44%, and 19%, respectively. Therefore, the **6** in cigarette smoke is either the nitrosation product of nornicotine or the direct transfer from the cut filler.

**Nicotine (1) May Not Be the Direct Precursor of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (5) in Tobacco Leaf.** Our knowledge on the mechanism of the formation of **5** and **6** (Figure 1) comes mainly from the study of the in vitro reaction between **1** and sodium nitrite.[34] Since it has been demonstrated in this study that **1** is unlikely the direct precursor for **6** in tobacco, it is suspected that this compound may not serve as a direct precursor in the formation of **5** in vivo. Pseudooxynicotine, as an autoxidation product of **1**, is another possible candidate of the precursor for **5**.[35] The dependence of **5** on pseudoxynicotine in tobacco supports the role of the latter compound in the formation of **5**.[1] However, this was not found in another study, in which pseudoxynicotine and **5** were measured in 50 air-cured burley and 44 flue-cured tobacco samples from different countries.[36] Visualization of the pseudoxynicotine and **5** data from the paper of Land and Vuarnoz suggests there is no significant correlation between pseudoxynicotine and compound **5** in tobacco (Figure S2, Supporting Information), which could be due to variable nitrite levels in the samples.[36] Therefore, additional investigations are needed to determine whether pseudoxynicotine serves as a direct alkaloid precursor in tobacco.

**Relationship between *N*′-Nitrosonornicotine (6) and Its Potential Precursors.** Many studies concerning the relationship between **6** and its potential precursors have been reported (Table S1, Supporting Information). There are large variations in these reported results, and some of them contradict one another. The reasons for this could be due to many uncontrolled factors in these studies that influence the formation of compound **6**: alkaloid content, nitrosating agents, the environment, culture practices, and differences in nicotine demethylation during curing.

Also examined was the correlation between **6** and its putative precursors in the nicotine demethylase mutant (Figure S3, Supporting Information). After perturbation of the demethylation of **1**, this alkaloid in air-cured leaves exhibits a significantly negative correlation with **6** ($r$ = −0.84; Figure S3A, Supporting Information), and **2** has a significantly positive correlation with **6** ($r$ = 0.91; Figure S3B, Supporting Information), as was expected. There was no significant correlation between nitrate, nitrite, and **6** found in this study.

**Competition of Four Alkaloids for the Nitrosation Reaction.** Besides the effects on compound **6**, CYP82E4 also has a great influence on the level of the other three TSNAs. In this study, it was found that a dramatic increase of **2** (which increased over 10-fold, Table 1) changed the relationship between the other three alkaloids and their corresponding nitrosation products (Figure 3). Whenever there was a functional CYP82E4 present in the tobacco, levels of compounds **5**, **7**, and **8** were not influenced by their corresponding precursors, which is different from the situation when the CYP82E4 function is inhibited in tobacco (Figure 3). Lewis et al. found that the RNAi-mediated suppression of nicotine demethylation increased the levels of **5**, consistent with

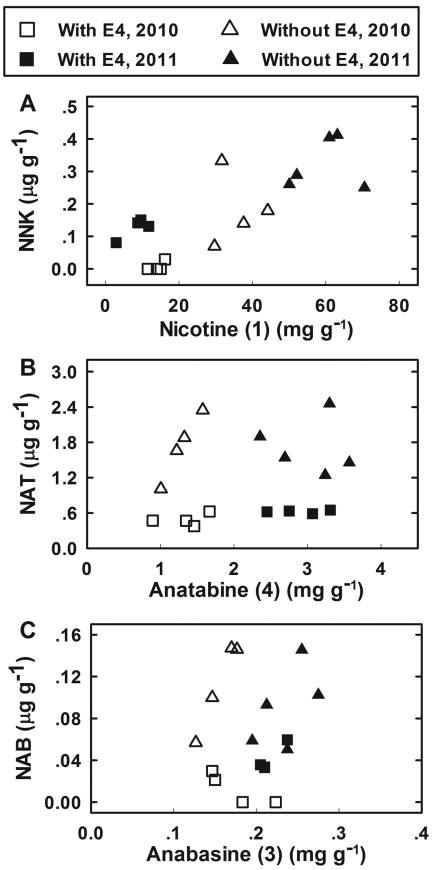


**Figure 3.** Relationships between nicotine (**1**), anatabine (**4**), and anabasine (**3**) and their corresponding nitrosation products associated with nicotine demethylase CYP82E4 in air-cured burley tobacco leaf. Lower NNK (**5**), NAT (**8**), and NAB (**7**) were associated with tobacco containing functional CYP82E4 (square vs triangle). Each data point is the average of three (2010) or four (2011) replicates. Each replicate consists of five middle leaves, one from each of five plants.

the results in the present study.[18] It is proposed that this could be due to the competition among **2** and the three other main alkaloids in tobacco leaves for nitrosating agents. However, there were no significant differences in the nitrite and nitrate contents in air-cured tobacco leaf (Table S3, Supporting Information), which are potential sources of nitrosating agents. The nitrosation competition phenomenon found in nicotine demethylase mutant lines may not apply to commercial tobacco varieties.

**Further Changes of the Composition of *N*′-Nitrosonornicotine (6).** Due to the enantioselectivity of three demethylases, the mutant tobacco lines with different combinations of nicotine demethylase gene suppressed were found to contain different compositions of **2** and **6**. A tobacco line with the mutation in CYP82E4 (*e4E5E10*) was shown to have dramatically reduced (*S*)-**2**[26] and (*S*)-**6** levels, but not those of (*R*)-**2**[26] and (*R*)-**6** (Figure 4). Further loss of function of the other two demethylases in *e4e5e10* reduces the (*R*)-**2** and (*R*)-**6** levels, but not for (*S*)-**2** and (*S*)-**6**. Currently, most seeds of commercial tobacco varieties, such as TN 90LC, come from parent plants that have been screened for low nicotine demethylation.[37] These varieties contain similar concentration levels of **2** and **6** to those found in *e4E5E10*. Previous investigators have incorporated the triple mutant phenotype


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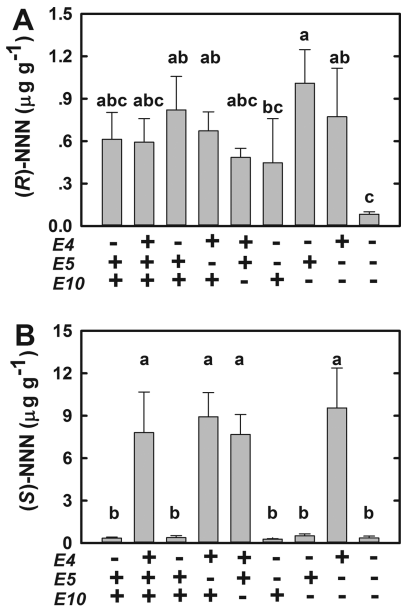


**Figure 4.** *N*′-Nitrosonornicotine (**6**) enantiomer accumulation in air-cured mutant lamina from a 2011 field trial. Each data point is an average of four bulk samples, and each bulk sample consists of five middle leaves, one from each of five plants. The error bars represent standard deviations, and +/− below the *x*-axis indicates the presence/absence of a functional demethylase gene. The data were analyzed with one-way ANOVA by Sigmaplot 12.0. Values with different letters are significantly different (Holm−Sidak test, $p < 0.05$).

into commercial tobacco varieties,[38,39] so there should be another lowering in the levels of **6** in tobacco in the future. Compared with current commercial varieties, the triple mutant lines reduce the levels of **6**, but mainly (*R*)-**6** relative to (*S*)-**6**. Since the *S* form is the more carcinogenic,[20−23] future endeavors should focus on the specific reduction of (*S*)-**2**.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Nicotine (**1**) and nornicotine (**2**) were purified and separated by thin-layer chromatography on silica gel 60 F254 plates (EMD Chemicals Inc.). The alkaloid contents were measured by gas chromatography coupled with a flame ionization detector (PerkinElmer Autosystem XL with Prevent). The enantiomeric composition of compound **1** was measured by high-performance liquid chromatography equipped with ultraviolet detector (PerkinElmer series 200) on a Chiralcel OD-H column (Chiral Technologies Inc.). The quantification and enantiomeric separation of TSNAs were performed by gas chromatography (HP 5890 GC, Hewlett Packward) coupled with a thermal energy analyzer (TEA model 543, Thermedics Inc.).

**Plant Material.** TN 90LC is a widely used commercial burley tobacco cultivar and has been selected from the TN90 cultivar as a low nicotine converter.[37] The burley tobacco breeding line DH98-325-6 with a very high nicotine demethylation capacity was used as parent to develop genetic stocks possessing different combinations of knockout mutations in the *CYP82E4*, *CYP82E5 v2*, and *CYP82E10* genes.[27] In this report, small letters represent a homozygous knockout mutation and capital letters represent a homozygous functional gene. For example, *e4E5E10* represents the mutant line with a homozygous knockout mutation in the *CYP82E4* gene and functional *CYP82E5 v2* and *CYP82E10* genes.

The burley parent line (DH98-325-6) along with seven derived mutant lines were grown at the Upper Coastal Plain Research Station (Rocky Mount, NC) during 2010 and also at Spindletop Research Farm (Lexington, KY) in 2011, both using standard production and curing practices for burley tobacco. TN 90LC was added to the 2011 experiment. After curing, the fourth leaf from the top of each stalk was sampled and used to quantify the alkaloid contents and TSNAs and their enantiomeric composition. All the samples were oven-dried (55 °C) and ground to pass through a 1 mm sieve.

**Alkaloid Quantification and Separation of Enantiomers of Nicotine (1) and Nornicotine (2).** The procedure of quantification of the four tobacco main alkaloids and of separation of the enantiomers of **1** and **2** has been described in a previous study.[26] Briefly, ground tobacco leaves were extracted by methyl *tert*-butyl alcohol and aqueous sodium hydroxide. A portion of the methyl *tert*-butyl alcohol extract was injected into a GC/FID instrument, and quantification of the alkaloids was determined against alkaloid standards and a quinoline internal standard. Contribution of nicotine demethylase CYP82E4 to the contents of **2** and **6** is calculated as follows: $V_x = (C_{x,E4E5E10} - C_{x,\ e4E5E10})/\ C_{x,E4E5E10}$. $V_x$ represents the contribution of nicotine demethylase CYP82E4 to the accumulation of compound *x* (**2** or **6**), $C_{x,E4E5E10}$ represents the concentration of compound *x* (**2** or **6**) in the parent line *E4ESE10*, and $C_{x,e4E5E10}$ represents the concentration of compound *x* (**2** or **6**) in mutant line *e4E5E10*.

The remainder of methyl *tert*-butyl alcohol extract was used for the analysis of the enantiomeric composition of **1** and **2**. The chlorophyll in the methyl *tert*-butyl alcohol extract was removed through an acid wash, and **1** and **2** in the extract were separated and purified by thin-layer chromatography. Purified **1** was further separated into its *R* and *S* forms by HPLC/UV on a Chiralcel OD-H column. Compound **2** was methylated to **1** by incubating with formic acid and formaldehyde at 110 °C.[26] The enantiomeric composition of **1** and **2** was calculated from the peak areas of the two isomers with absolute amounts of the isomers calculated based on the total amount of each alkaloid present and their *R*/*S* ratio.

**Measurement and Enantiomeric Separation of TSNAs.** Measurements of compounds **5**−**8** were performed according to method 1 of Morgan et al.[40] Ground tobacco leaves were extracted with methylene chloride and aqueous sodium hydroxide. The methylene chloride extracts were filtered, and an aliquot of the extracts was injected into the GC/TEA for quantification of TSNAs. The rest of the solution was used for the measurement of the enantiomeric composition of TSNAs.

On the basis of a previous report, a method was developed with baseline separation of the three chiral TSNAs and with similar pretreatment.[28] Briefly, the residual extracts mentioned above were cleaned and concentrated using extraction buffer (20 mM ascorbic acid, 54.8 mM citric acid, and 90.4 mM sodium hydrogen phosphate) and the use of a SEP-Pak Plus silica gel solid-phase extraction cartridge. Differing from the previous report in which further HPLC purification was required, the eluate containing the TSNAs was directly injected into the GC/TEA for the separation of enantiomers (Figure 2). For this purpose, a tandem column arrangement was installed in the GC/TEA. The first was a Cyclosil-B column (30 m × 250 μm × 0.25 μm, J & W Scientific), and the second was a Zebron ZB-5 column (30 m × 530 μm × 1.5 μm, Phenomenex) connected via a standard column connector. The carrier gas was He, and the injection temperature 225 °C. The column oven temperature program was 60 °C for 2 min, 20 °C $min^{-1}$ to 180 °C, held for 10 min, 0.5 °C $min^{-1}$ to 203 °C, increased at 6 °C $min^{-1}$ to 215 °C, and then held for 10 min. The enantiomeric composition of **6**−**8** was calculated from the peak areas of the two isomers with absolute amounts of the isomers calculated based on total amount of each TSNA present and their *R*/*S* ratio.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00678.

Figure S1 Nitrosation reactions in nicotine demethylase mutant lines; Figure S2 Relationship between pseudooxynicotine and total NNK in tobacco for 50 air-cured


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burley and 24 flue-cured tobacco samples; Figure S3 Correlation relationships between NNN and its putative precursors influenced by nicotine demethylase CYP82E4 in air-cured lamina based on two-year studies; Table S1 Correlations between NNN and its putative precursors (nicotine, nornicotine, nitrite, and nitrate) from the literature; Table S2 Alkaloid and TSNA concentrations in cured mutant lamina from 2010 and 2011 field trials; Table S3 Nitrite and nitrate levels in cured mutant lamina from 2011 field trial; Experiment procedure: Measurement of nitrate and nitrite (PDF)

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### Notes

The authors declare no competing financial interest.

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