Beiträge zur Tabakforschung International · Volume 12 · No. 3 · February 1984 DOI: 10.2478/cttr-2013-0532

The Methylation of Nornicotine to Nicotine, a Minor Biosynthetic Pathway in *Nicotiana tabacum**

by Edward Leete

Natural Products Laboratory, Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, U.S.A.

SUMMARY

It has been well established that nornicotine is formed in *Nicotiana* species by the demethylation of nicotine. However, the reverse reaction has not been unequivocally substantiated. This problem has been examined by feeding (*RS*)-[2′-^{14}C]nornicotine to *Nicotiana tabacum* plants. Most of the administered nornicotine was recovered unchanged, but a small amount of activity was detected in the nicotine. Activity was also found in myosmine. The nicotine was degraded and found to have essentially all its radioactivity located at the C-2′ position, indicative of a direct synthesis from nornicotine. It has thus been established that the methylation of nornicotine to nicotine does occur in the tobacco plant, but it is not considered to be a major pathway for the biosynthesis of nicotine.

ZUSAMMENFASSUNG

Obwohl Nornicotin in der Spezies *Nicotiana* erwiesenermaßen auf dem Wege der Demethylierung aus Nicotin entsteht, ist der Nachweis für die umgekehrte Reaktion bisher nicht eindeutig erbracht worden. Diese Frage wurde untersucht, indem Pflanzen der Spezies *Nicotiana tabacum* (*RS*)-[2′-^{14}C]Nornicotin zugeführt wurde. Wenn das markierte Nornicotin auch zum größten Teil unverändert wiederaufgefunden wurde, ließ sich doch eine kleine Menge der Radioaktivität im Nicotin feststellen. Auch im Myosmin wurde Radioaktivität gefunden. Beim Abbau des Nicotin wurde praktisch die gesamte Radioaktivität an dem C-Atom der Position 2′ gefunden, was auf eine Synthese direkt aus dem Nornicotin schließen läßt. Wenngleich auf diese Weise nachgewiesen wurde, daß eine Methylierung des Nornicotin zu Nicotin in der Tabakpflanze stattfindet, wird doch nicht angenommen, daß das Nicotin hauptsächlich auf diesem Syntheseweg in der Pflanze entsteht.

RÉSUMÉ

Bien qu'il soit connu que la nornicotine est formée dans les espèces de *Nicotiana* par la déméthylation de la nicotine, la réaction inverse n'a cependant pas été prouvée de façon incontestable. Ce problème a été étudié en alimentant des plantes de tabac avec de la (*RS*)-[2′-^{14}C]-nornicotine, au moyen d'une mèche. La majeure partie de la nornicotine marquée au ^{14}C fut récupérée sans avoir subi de changement, cependant une certaine radioactivité était discernée dans la nicotine. La radioactivité est aussi apparue dans la myosmine. Par dégradation de la nicotine on a déterminé que la radioactivité se situe au niveau C−2′, ce qui indique une synthèse directe à partir de la nornicotine. On a donc prouvé que la méthylation de la nornicotine en nicotine se produit dans la plante de tabac, mais cela n'est pas considéré comme la voie prépondérante de la biosynthèse de la nicotine.

* Contribution No. 187 from this laboratory and Part 48 in the series: Tobacco Alkaloids and Related Compounds. This investigation was supported by a research grant (GM-13246) from the National Institutes of Health, U. S. Public Health Service.

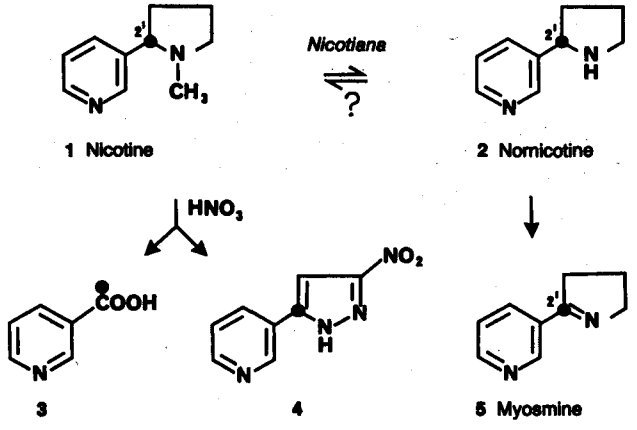
Received: 7th September 1982 — accepted: 14th April 1983.

INTRODUCTION

It has been shown that the main route for the formation of nornicotine (compound 2) in *Nicotiana* species is by the demethylation of nicotine (compound 1). This reaction, illustrated in Figure 1, occurs in both the healthy growing plant (1 — 3) and as a post-harvest transformation during the curing of tobacco (4 — 7). However, the mechanism of this demethylation is still obscure (8). Demethylation of nicotine also occurred in a cell-suspension culture of *N. tabacum* (9). The question as to whether nicotine can be formed in tobacco by the methylation of nornicotine has not been unequivocally answered. The levels of radioactivity found in nicotine and nornicotine after a short-term exposure to $^{14}CO_2$ are not consistent with any significant nornicotine → nicotine conversion (10). Some experiments have indicated that this conversion may occur to a limited extent (11 — 15), but the results are ambiguous. For example when a mixture of [^{15}N]nornicotine and L-[methyl-^{14}C]methionine was fed to *N. tabacum*, the enrichment of the resultant nicotine with ^{15}N was very low (~ 0.02 % excess) compared with the specific incorporation of the ^{14}C (13). In none of these previous experiments was the direct conversion of nornicotine to nicotine confirmed by degradations to establish the specific labeling of the resultant nicotine.

We have now investigated this hypothetical conversion by feeding [2′-^{14}C]nornicotine to intact *N. tabacum* plants and excised leaves of this species.

Figure 1. Biological and chemical transformations of nicotine and nornicotine.



EXPERIMENTAL

General Methods

A Nuclear Chicago Mark II liquid scintillation counter was used for assay of the radioactive compounds, using dioxane-ethanol as the solvent with the usual scintillators (16). Assays were carried out in duplicate and were reproducible to 5 %.

(RS)-[2′-^{14}C]Nornicotine

This compound was prepared from [carboxyl-^{14}C]-nicotinic acid (Radiochemical Centre, Amersham) as previously described (17), via myosmine. The nornicotine was finally purified by thin-layer chromatography (TLC) on silica gel PF-254 (Merck) developing with a mixture of chloroform, ethanol and concentrated ammonia (90:10:1). In this system, nornicotine, nicotine, and myosmine have R_f values of 0.15, 0.60, and 0.75, respectively. No radioactivity was detected in the purified nornicotine at positions coincident with nicotine or myosmine. The method of synthesis also precludes any contamination with radioactive nicotine.

Feeding of (RS)-[2′-^{14}C]nornicotine to N. tabacum and Isolation of the Alkaloids

Details of the three feeding experiments are given in Table 1. In experiments 1 and 2 the (*RS*)-[2′-^{14}C]-nornicotine (148 mg, 1 mmol, 6.18×10^7 d.p.m.) was dissolved in water (25 ml) containing acetic acid (0.12 ml). This solution was divided equally between 10 *N. tabacum* plants (2 months old, containing 6—8 leaves) growing in soil in a greenhouse. The feeding was carried out by the wick method, the cotton wicks being inserted into the stems of the plants about 10 cm above the ground level. There was no evidence of injury to the plants at the site of insertion of the cotton wicks. The small beakers into which the cotton wicks were placed were replenished daily with water. After 2 or 8 days the plants were harvested and were macerated in a Waring Blendor with a mixture of chloroform and concentrated ammonia as previously described (18). The residual activity in the beakers was negligible (0.004 % in experiment 2). The alkaloids were separated by TLC as previously described (18). The nicotine was purified as its diperchlorate, crystallizing (from ethanol) until material of constant specific activity was obtained. The amount of myosmine was estimated by UV spectroscopy, diluted with inactive alkaloid and purified by sublimation, and conversion to its dipicrate. The nornicotine obtained from the mixture of alkaloids was purified by distillation, and converted to its dipicrate. In experiment 3, excised leaves from mature *N. tabacum* plants (4 months old) were spread out on a bench in a greenhouse and painted with a solution of the (*RS*)-[2′-^{14}C]-nornicotine dissolved in dilute acetic acid. The leaves were allowed to dry at room temperature under natural light and then extracted with chloroform and aqueous ammonia as previously described.

Degradation of the Labeled Nicotine

The following degradation is the one carried out on the nicotine obtained from experiment 2. Nicotine diperchlorate (330 mg), 9.1×10^3 d.p.m./mmol, was heated

Table 1. Feeding of (*RS*)-[2′-^{14}C]nornicotine to *N. tabacum* and activities of the resultant alkaloids.

	Experiment 1 (wick feeding to intact plant)	Experiment 2 (wick feeding to intact plant)	Experiment 3 (painting on excised leaves)
(*RS*)-[2′-^{14}C]Nornicotine fed:			
weight (mg)	148	148	288.6
total activity (d.p.m.)	6.18×10^7	6.18×10^7	1.205×10^8
specific activity (d.p.m./mmol)	6.18×10^7	6.18×10^7	6.18×10^7
Duration of feeding (days)	2	8	28
Weight of plants (g)	1200 (fresh weight)	1600 (fresh weight)	56 (dry weight)
Activity of crude alkaloids	5.21×10^7	4.20×10^7	3.76×10^7
Recovery of activity fed	84 %	68 %	31 %
Activity in aqueous NH_3 layer	1.95×10^6	4.43×10^6	8.1×10^6
Recovery of activity fed	3.1 %	7.2 %	6.7 %
Nicotine: weight (mg)	373	520	24.5
Diperchlorate (d.p.m./mmol)	4.8×10^3	9.1×10^3	
Dipicrate (d.p.m./mmol)	4.7×10^3	9.0×10^3	3.62×10^5
Absolute incorporation *	0.018 %	0.047 %	0.045 %
Specific incorporation **	0.008 %	0.015 %	0.59 %
Nornicotine: weight (mg)	104	91	102
Dipicrate (d.p.m./mmol)	5.73×10^7	5.54×10^7	1.72×10^7
Absolute incorporation	65 %	55.1 %	9.84 %
Specific incorporation	92.7 %	89.6 %	27.8 %
Myosmine: weight (mg)	not isolated	0.25	not isolated
Dipicrate (d.p.m./mmol)		1.57×10^7	
Specific incorporation		25.4 %	

* Specific incorporation = specific activity of the isolated alkaloid / specific activity of the administered nornicotine.
** Absolute incorporation = total activity in the isolated alkaloid / total activity administered to the plant.

on a steam bath with concentrated nitric acid (10 ml) and sodium nitrite (0.2 g) for 18 h. The reaction mixture was worked up as previously described (19) affording 3-nitro-5-(3′-pyridyl)pyrazole (11 mg), 8.9×10^3 d.p.m. / mmol, and nicotinic acid (41 mg), 8.9×10^3 d.p.m. / mmol. The nicotinic acid was heated with calcium oxide yielding pyridine which was collected and assayed as its picrate (0.3×10^3 d.p.m. / mmol). A similar degradation was carried out on the nicotine obtained from experiment 2. Nicotine dipicrate (3.62×10^5 d.p.m./mmol) afforded on oxidation 3-nitro-5-(3′-pyridyl)pyrazole (3.65×10^5 d.p.m. /mmol) and nicotinic acid (3.50×10^5 d.p.m. / mmol).

RESULTS AND DISCUSSION

A small but significant amount of radioactivity was detected in the nicotine isolated from *N. tabacum* plants which had been fed (*RS*)- [2′-^{14}C]nornicotine for 8 days (experiment 2). In view of this low activity the nicotine was purified by repeated crystallization of its diperchlorate from ethanol. Confirmation of its radiochemical purity was obtained by preparation of its dipicrate, having the same specific activity. More convincing evidence in favor of the radiochemical integrity of the nicotine was obtained by carrying out an oxidation with nitric acid to yield 3-nitro-5-(3′-pyridyl)pyrazole (compound 4) having essentially the same specific activity as the nicotine. It has been shown (19) that this pyrazole derivative is not formed from nornicotine, which was a possible contaminant of the labeled nicotine. Furthermore, the other product from this oxidation, nicotinic acid (compound 3), was found to have $>$ 96 % of its activity on its carboxyl group. This result indicates that the labeled nicotine, isolated from the plant, was labeled specifically at the C-2′ position showing that there had been a direct methylation of the nornicotine to afford nicotine. The amount of methylation after 8 days of feeding was quite low (0.047 % absolute incorporation). A similar degree of conversion (0.045 %) was obtained in experiment 3 in which the excised leaves were allowed to metabolize nornicotine for 4 weeks. In this experiment the total recovery of activity was much lower, possibly due to volatilization of the alkaloids from the leaves during the drying process. In the other experiments the recovery of the activity was quite good, indicating that the nornicotine is metabolically fairly stable in the intact healthy plant.

These results probably indicate that the enzyme responsible for the methylation of putrescine to *N*-methylputrescine (putrescine *N*-methyltransferase) which has been isolated from tobacco roots (20) is not completely specific, and is able to catalyze, to a small extent, the transfer of a methyl group from *S*-adenosyl-L-methionine to nornicotine to afford nicotine. It was previously found (20) that this enzyme is highly specific for putrescine. *N*-Methylputrescine was methylated to a limited extent, but no radioactivity was transferred from *S*-adenosyl-L-[methyl-^{14}C]methionine to 1,3-diaminopropane, cadaverine, nornicotine, Δ^1-pyrroline, or ornithine in a 30-minute incubation with the purified enzyme. The methylation of nornicotine to nicotine in the tobacco plant is apparently a very slow process. In experiment 1 in which the feeding was carried out for only 2 days there was only a 0.018 % conversion of the nornicotine to nicotine, with a 65 % recovery of the activity in the nornicotine. Slight discrepancies between the activity on the crude alkaloids and that ultimately recovered on the purified alkaloids simply are a function of losses which occur during the isolation and purification of the individual alkaloids.

The dehydrogenation of the administered nornicotine to myosmine (compound 5) (isolated in experiment 2) is in accord with previous work (3, 15). The labeled myosmine was degraded as previously described (3) and found to have essentially all its activity at the C-2′ position.

REFERENCES

1. Dawson, R. F.: On the biogenesis of nornicotine and anabasine; J. Am. Chem. Soc. 67 (1945) 503—504.
2. Kisaki, T., and E. Tamaki: Phytochemical studies on the tobacco alkaloids, III. Observations on the interconversion of DL-nicotine and DL-nornicotine in excised tobacco leaves; Arch. Biochem. Biophys. 94 (1961) 252—256.
3. Leete, E., and M. R. Chedekel: Metabolism of nicotine in *Nicotiana glauca;* Phytochemistry 13 (1974) 1853—1859.
4. Wada, E.: Conversion of nicotine to nornicotine in cherry red tobacco during flue-curing; Arch. Biochem. Biophys. 62 (1956) 471—475.
5. Weybrew, J. A., T. J. Mann and E. L. Moore: Nicotine conversion in cherry redness; Tob. Sci. 4 (1960) 190—193.
6. Stepka, W., and L. J. Dewey: Conversion of nicotine to nornicotine in harvested tobacco, Fate of the methyl group; Plant Physiol. 36 (1961) 592—597.
7. Tso, T. C.: Physiology and biochemistry of tobacco plants; Dowden, Hutchinson and Ross, Inc., Stroudsburg, Pennsylvania, 1972, pp. 242—244.
8. Leete, E.: Biosynthesis and metabolism of the tobacco alkaloids, chapter 3; in: Alkaloids, Chemical and biological perspectives, Vol. 1, edited by S. W. Pelletier, John Wiley and Sons, Inc., 1983, pp. 85—152.
9. Barz, W., M. Kettner and W. Hüsemann: On the degradation of nicotine in *Nicotiana* cell suspension cultures; Planta Med. 34 (1978) 73—78.
10. Alworth, W. L., and H. Rapoport: Biosynthesis of the nicotine alkaloids in *Nicotiana glutinosa*, Interrelationships among nicotine, nornicotine, anabasine, and anatabine; Arch. Biochem. Biophys. 112 (1965) 45—53.
11. Tso, T. C., and R. N. Jeffrey: Biochemical studies on tobacco alkaloids, I. The fate of labeled tobacco alkaloids supplied to *Nicotiana* plants; Arch. Biochem. Biophys. 80 (1959) 46—56.
12. Tso, T. C.: Nornicotine as a precursor of nicotine in *Nicotiana* plants; Bot. Bull. Acad. Sin. (Taipei) 4 (1963) 75—79.
13. Ladesic, B., and T. C. Tso: Biochemical studies on tobacco alkaloids, VI. Biosynthesis of nicotine through nornicotine; Phytochemistry 3 (1964) 541—545.
14. Mizusaki, S., T. Kisaki and E. Tamaki: Phytochemical studies on the tobacco alkaloids, IX. Question for nornicotine as a precursor of nicotine; Agric. Biol. Chem. 29 (1965) 719—723.
15. Kisaki, T., and E. Tamaki: Phytochemical studies on the tobacco alkaloids, X. Degradation of the tobacco alkaloids and their optical rotatory changes in tobacco plants; Phytochemistry 5 (1966) 293—300.
16. Friedman, A. R., and E. Leete: Biosynthesis of the *Nicotiana* alkaloids, IX. The non-random incorporation of acetate-2-^{14}C into the pyridine ring of anabasine; J. Am. Chem. Soc. 85 (1963) 2141—2144.
17. Hu, M. W., W. E. Bondinell and D. Hoffmann: Synthesis of carbon-14-labelled myosmine, nornicotine and *N*′-nitrosonornicotine; J. Labelled Compd. 10 (1974) 79—88.
18. Leete, E., and S. A. Slattery: Incorporation of [2-^{14}C]- and [6-^{14}C]nicotinic acid into the tobacco alkaloids, Biosynthesis of anatabine and α,β-dipyridyl; J. Am. Chem. Soc. 98 (1976) 6326—6330.
19. Leete, E., and H. V. Isaacson: Mechanism of the formation of 3-nitro-5-(3′-pyridyl)pyrazole from nicotine; J. Indian Chem. Soc. 55 (1978) 1125—1127.
20. Mizusaki, S., Y. Tanabe, M. Noguchi and E. Tamaki: Phytochemical studies on tobacco alkaloids, XIV. Occurrence and properties of putrescine *N*-methyltransferase in tobacco roots; Plant Cell Physiol. 12 (1971) 633—640.

Author's address:

Natural Products Laboratory,
Department of Chemistry, University of Minnesota,
Minneapolis, Minnesota, 55455, U. S. A.