Isolation of 1-(1'-2'S-Nornicotino)-1-deoxy- β -D-fructofuranose and Its Formation in Flue-curing Process of Tobacco Leaves

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Received November 10, 1978

1-(1'-2'S-Nornicotino)-1-deoxy- β -D-fructofuranose was first isolated from flue-cured leaves of Cherry Red tobacco, *Nicotiana tabacum*, cv. Bright Yellow. Its structure was established spectrometrically and synthetically. This substance was shown to be formed from nornicotine during flue-curing. Its smoking effect was mild.

A number of pyridine alkaloids are present in tobacco leaves. In the courses of our phytochemical studies on tobacco alkaloids, we have noticed the presence of a fairly large quantity of an isatin- and BrCN-positive alkaloid in the flue-cured leaves of Cherry Red strain of Bright Yellow tobacco variety in which nornicotine is contained as a principal alkaloid. Its presence has also been reported for the cured tobacco leaves of DB 101 CR by Stephens and Weybrew.¹⁾ Nornicotine type tobacco is generally considered to be light but has a disagreeable taste. Natural back mutation of nicotine-type tobacco to Cherry Red tobacco has been known to occur in such a high frequency as 0.8% in a generation²⁾ and do at a much higher rate in a certain strain. In most cases nornicotine type tobacco contaminates normal nicotine type tobacco crops, which leads to the lowering of the quality of the whole tobacco crops. It is important for nornicotine type tobacco to determine the relationship between the smoking quality and the characters of the said substance which is characteristically abundant.

Some reactions between amino or imino compounds and sugars have been known to take place during the dehydration and storage processes of tobacco leaves. The flue-curing process in tobacco production involves these process. The reaction products are partly responsible for the non-enzymatic browning of

foods and flavor formation.³⁾ Tomita *et al.*⁴⁾ have isolated Amadori rearrangement compounds of several combination products of hexose and amino acids from cured tobacco leaves. However, the complete structures of these compounds have not been established. In this study we isolated an Amadori rearrangement compound of the combination product of nornicotine and glucose from Cherry Red tobacco leaves, physicochemically established its structure, and examine its effect on the smoking flavor.

EXPERIMENTAL

Method. The following spectrometers were used: JASCO Model IR-G infrared spectrometer, Hitachi EPS-3T spectrometer, JEOL JNM-PS-100 NMR spectrometer (100 MHz) with sodium 3-trimethylsilylpropanesulfonate as an internal standard for recording ¹H-NMR spectra, JNM-FX-100 NMR spectrometer with D₂O in dioxane as an internal standard for recording ¹³C-NMR spectra, JASCO DIP-181 digital polarimeter for optical rotation, Hitachi RMU-7 mass spectrometer, Hitachi RM 50-GC for recording GC-MS spectra, and JMS-D 100 FD for FD-MS spectra. Descending paper chromatography was carried out on Whatman No. 1 paper in a solvent mixture of *tert*-amyl alcohol-0.2 N sodium acetate (1:1, v/v). The paper was impregnated with sodium acetate solution and dried before use. The chromatogram were sprayed with *p*-aminobenzoic acid-BrCN for pyridine, isatin for pyrrolidine, and aniline hydrogen phthalate for reducing sugar. Melting points are uncorrected.

Curing of tobacco leaves. *Nicotiana tabacum*, cv. Bright Yellow, Cherry Red strain TR 261 was grown in the field in the usual manner. Leaves of the 8th to 9th position on the stalk from the top of the plants were harvested in the morning and hung in a curing barn. Ten leaves of each position on the stalk were sampled at a time from the barn at the designated intervals, lyophilized, and pulverized for analysis.

Isolation of U-A. One and a half kg of flue-cured leaves of Cherry Red tobacco were extracted three times with 80% aqueous methanol at room temperature. The extract was concentrated *in vacuo*, and the residue was dissolved in 3 liters of water. After removal of insoluble substances the solution was passed through a Dowex 50 $\times$ 4 ion exchange resin (H⁺) column (15 $\times$ 90 cm). The column was washed with 5 liters of 0.5 N sodium citrate (pH 6.9) to remove amino acids and eluted successively with 3.5 liters of 0.5 N NH₄OH to obtain alkaloids. U-A which was positive with both BrCN and isatin was eluted in the first 1.5 liters. The eluate was immediately concentrated to dryness *in vacuo* at 40°C. Reddish resin (10.3 g) was obtained by treatment with activated charcoal in anhydrous ethanol. One g of this resin was chromatographed on an AVICEL column (2.8 $\times$ 150 cm) with *tert*-amyl alcohol saturated with 0.2 N sodium acetate. Tenth to 26th fractions (15 ml/fraction) were combined and shaken with a slightly acidic aqueous solution. The aqueous layer was passed through a Dowex 50 $\times$ 4 resin (H⁺) column (2.8 $\times$ 20 cm), washed with water, and eluted with 1 liter of 2 N NH₄OH. The eluate was immediately concentrated to dryness *in vacuo* and dried over P₂O₅, yielding 0.5 g of orange resin. Upon standing in a refrigerator for a day after addition of a small volume of chloroform, colorless crystals like cotton fibre were obtained. Crystals were recrystallized from chloroform-ether to give 150 mg of white powder. The powder was further purified with HPLC (2 ml/min flow of 50% methanol, μ Bondapak C₁₈ column, UV₂₅₄ detector). The U-A fraction was concentrated to dryness *in vacuo*, and crystallized in the same procedure as above, giving 80 mg of white crystals, mp 66~68°C (decomp.).

Trimethylsilylation of U-A. One mg of U-A was trimethylsilylated in an 1 ml REACTI vial by adding 50 μ l of BSTFA [bis(TMS)trifluoroacetamide, Tokyo Kasei] and successively 50 μ l of TMS-HT (a mixture of hexamethylsilazane and trimethylchlorosilane in pyridine, Tokyo Kasei). Trimethylsilylation was completed after an hour. The reaction mixture was subjected to GC-MS.

Acetylation of U-A. U-A was acetylated with acetic anhydride in pyridine at room temperature or at 0°C. In either case a dark red-brown sirup was resulted.

This sirup was subjected to preparative HPLC (2 ml/min flow of 50% methanol, μ -Bondapak C₁₈ column). Among the several peaks obtained the fourth peak (main peak) was collected and subjected to MS.

Hydrolysis of U-A. i) Twenty mg of U-A were hydrolyzed with 2 ml of N HCl for 4 hr at 100°C. The hydrolyzate was filtered to remove white precipitate. No original compound was detected in the hydrolyzate. The excess HCl was removed by evaporation to dryness *in vacuo*, and the solution was made alkaline. The base was extracted with ether and subjected to GC-MS. Recovery of nornicotine was determined by UV. ii) Five mg of U-A were dissolved in 20 ml of a strong alkaline solution and steam-distilled with N HCl solution. The base was extracted with ether from the alkaline solution, and subjected to GC-MS. iii) Twenty mg of this compound were dissolved in 0.5 ml of water and the solution was adjusted to pH 6. To the solution was added 0.1 ml of a snail (*Helix pomatia*) digestive fluid (Industrie Biologique, France). The solution was stood for a day at room temperature, boiled shortly to precipitate protein, and subjected to Dowex 50(H⁺) ion exchange column chromatography. The adsorbed base was eluted with 0.5 N NH₄OH and extracted with chloroform. The extract was subjected to GC-MS.

Synthesis of 1-(1'-2'-S-nornicotino)-1-deoxy-D-fructose. Three g of (S)-nornicotine isolated from tobacco leaves and 3 g of D-glucose were boiled in 50 ml of methanol for 2 hr and after addition of 200 mg of malic acid for additional 2 hr. The resultant red-colored solution was diluted with a double portion of water and passed through a Dowex 50 (H⁺) column; the column was washed with distilled water. The reaction product on the column was eluted with 2 liters of 2 N NH₄OH and the eluate was immediately concentrated to dryness *in vacuo*. The residue was repeatedly washed with *n*-hexane until no precipitate appeared with silicotungstic acid in the washings. On addition of chloroform the residue was once dissolved and soon after that voluminous fiber-like crystals appeared. The crystals were recrystallized twice from chloroform-ether, yielding 1.68 g of white powder. Further purification was made with HPLC.

Quantitative determination of U-A in tobacco leaves. One g of powdered dry tobacco leaves was extracted with 30 ml of 50% ethanol three times at 50°C for 30 min. The extract were combined, concentrated and made up to 50 ml. The solution was filtered with a filter paper and passed through a Dowex 50 $\times$ 4 (H⁺) column (1 $\times$ 15 cm). The column was washed with 100 ml of water and eluted with 100 ml of 2 N NH₄OH. The eluate was concentrated to dryness *in vacuo* and the residue was made to 5 ml with 0.15 M sodium

borate buffer (pH 8.0). The solution was analyzed with a strong anion-exchange resin column. Alternatively, the sample was analyzed by HPLC. The analytical sample solution for HPLC was prepared by dissolving the residue in 50% methanol solution instead of the borate buffer solution.

Evaluation of smoking quality. Nicotine-free tobacco shreds obtained from tobacco grafted on tomato were sprayed with 0.01~0.5% alcoholic solution of U-A. Test cigarettes were prepared from their leaves.

RESULTS AND DISCUSSION

Identification

This substance was contained around 1% of dry weight in nornicotine type flue-cured tobacco. U-A was so easy to be degraded that it had to be quickly isolated. U-A was positive with *p*-aminobenzoic acid-BrCN, isatin and aniline-hydrogen phthalate, suggesting the presence of pyridine, pyrrolidine and reducing sugars, respectively. Precise FD-MS: m/e 311.1607 ($M^+ + 1$), $C_{15}H_{23}N_2O_5$.

Anal. found: C, 57.72; H, 7.10; N, 9.06; O, 25.61% calcd. for $C_{15}H_{23}N_2O_5$: C, 58.05; H, 7.15; N, 9.03; O, 25.78. $UV_{max}^{0.03N\ HCl}$ nm (ϵ): 260 (53,940); $\lambda_{max}^{methanol}$ 262. IR ν_{max}^{KBr} cm^{-1} : 810 and 715 (3-substituted pyridine). 1H -NMR $\delta_{Me_4Si}^{D_2O}$: 8.60 (s, 1H, 2-H of pyridine), 8.55 (d, 1H, 6-H of pyridine), 7.75 (d, 1H, 4-H of pyridine), 7.30 (q, 1H, 5-H of pyridine), 1.04~4.00 (m, 14H). These information indicated the presence of 3-substituted pyridine without conjugated double bond or carbonyl. Hydrolysis of U-A with HCl gave slightly racemized (*S*)-nornicotine ($[\alpha]_D^{25} -70^\circ$, cf. pure (*S*)-nornicotine, $[\alpha]_D^{25} -88^\circ$; recovery of nornicotine, 80% in HCl hydrolysis, 60% in alkaline hydrolysis). On hydrolysis with the snail digestive fluid D-glucose (56% recovery, by glucose oxidase method) and nornicotine were recovered, but not stoichiometrically. Thus it consisted of a nornicotine and a 6-

carbon sugar moiety. U-A was negative to the test of secondary amines with sodium nitropruside and acetaldehyde, indicating that the sugar moiety combines with pyrrolidine-*N* of nornicotine. This substance was finally identified as a nornicotine-sugar condensate by spectrometrical comparison with the compound prepared from D-glucose and (*S*)-nornicotine. The structure of the sugar moiety was decided from the following informations. In MS spectrometry m/e 419.1802 ($M^+ - 15$), 437 ($M^+ - 161$), and 2-(3-pyridyl)-1-methylidenepyrrolidium of the trimethylsilylated one indicated the presence of four OH groups. The abundant presence of m/e 217,⁶⁾ 437 and 161 in the MS of the trimethylsilylated one, the degree of unsaturation,⁵⁾ and IR absorption (785 and 915 cm^{-1})⁷⁾ supported that the sugar moiety had a furanoside structure. ^{13}C -NMR $\delta_{Me_4Si}^{D_2O}$ spectra gave no signal other than fifteen signals [δ (ppm): 149.7 (d, $-CH=$), 149.3 (d, $-CH=$), 138.2 (s, $>C=$), 138.1 (d, $-CH=$), 125.6 (d, $-CH=$), 97.7 (s, $-C-$), 71.4 (d, $>CH-$), 70.5 (d, $>CH-$), 70.0 (d, $>CH-$), 69.1 (d, $>CH-$), 67.6 (t, $-CH_2-$), 64.2 (t, $-CH_2-$), 56.9 (t, $-CH_2-$), 33.6 (t, $-CH_2-$), 23.7 (t, $-CH_2-$)], indicating that it has a single anomeric structure. Signals of 149.7, 149.3, 138.2, 138.1, 125.6, 69.1, 56.9, 33.6 and 23.7 are ascribed to those of nornicotine carbon. The remaining signals 97.7 (s, $-C-$), 71.4 (d, $>CH-$), 70.5 (d, $>CH-$), 70.0 (d, $>CH-$), 67.6 (t, $-CH_2-$), 64.2 (t, $-CH_2-$), indicated the sugar moiety has fructofuranoside structure with exo-methylene and were assigned to 2C, 5C, 3C, 4C, 1C, and 6C,^{6,7)} respectively. The anomeric chemical shift of C-2 supported β -form (cf. α -anomer shifts to upper field more than 4 ppm⁷⁾). An exact identity of the 1H -NMR spectra with those of the synthetic one from D-glucose indicated that it belongs to D-series. Consequently, U-A was confirmed to be 1-(1'-2'*S*-nornicotino)-1-deoxy- β -D-fructofuranoside, which implies that U-A is an Amadori rearrangement compound of *N'*-D-glucosyl-2'*S*-nornicotine. Reduction of 2, 6-dichlorophe-

FIG. 1. Ion Exchange Column Chromatogram of Tobacco (CR) Extract.

The analytical sample was prepared as described in the text. JEOL JLC-3BC; JEOL LCR-3 anion exchange resin column: 1st buffer, 0.11 M Na-borate (pH 7.5); 2nd buffer, 0.25 M Na-borate (pH 9.6). Flow rate: buffer, 0.51 ml/min; orcinol- H_2SO_4 , 1.02 ml/min.

FIG. 2. HPLC of Tobacco (CR) Extract.

The analytical sample was prepared as described in the text. Waters ALC 201 HPLC; μ -Bondapak C_{18} (4×300 mm); 50% methanol solution containing 0.025 N NH_4HCO_3 , 2 ml/min flow.

nolindophenol and *o*-dinitrobenzene in a dilute alkaline solution at room temperature took place very slowly, suggesting that furanoside structure is rather stably retained.

Formation

The isolated compound was shown to be slightly racemized $[[\alpha]_D^{25} - 83.8^\circ$ (589 nm) ($c = 1.08$, water); *cf.* synthetic one $[\alpha]_D^{25} - 97.85^\circ$ ($c = 0.96$, water)], which reflects the mode of formation of nornicotine in tobacco leaves.¹⁰⁾ The U-A content as determined by HPLC increased during curing, and the most extensive increase was observed in the midrib of the drying stage when lamina had been dried.

On the other hand, the content of nicotine decreased. The nornicotine content increased first and then decreased with the increase of U-A content. These changes suggest that U-A is formed non-enzymatically from glucose and nornicotine, both of which are actively formed from starch and nicotine, respectively, during flue-curing of tobacco leaves. A large amount of malic acid appearing during curing of tobacco leaves seems to be favorable for promoting the condensation of glucose and nornicotine and the subsequent Amadori-rearrangement. U-A was found more or less in any flue-cured tobacco leaves.

FIG. 3. Changes in U-A, Nicotine and Nornicotine Contents in Cherry Red Tobacco Leaves during Curing.

U-A ($\times$ --- $\times$) was determined as described in the text. Nicotine ($\bigcirc$ --- $\bigcirc$) and nornicotine ($\triangle$ --- $\triangle$) were determined by GC of the ether extract from the alkaline solution of tobacco extract using isoquinoline as an internal standard. Temperature (—).

Stability

The isolated U-A degraded with browning in the crystalline state on standing in the air, probably by Strecker degradation, and gave several UV absorptive peaks in HPLC. Observation of m/e 943, 923, 642 and 621 ($2M^+ + 1$) in FD-MS indicates that U-A is easy to polymerize. In cured leaves U-A stayed unchanged for a long time at room temperature, probably due to the acidic condition. Cherry Red leaves have been known to become red-brown in the drying processes of tobacco as indicated by its name. This compound must be partly responsible for the coloration of Cherry Red tobacco leaves. In tobacco leaves, other secondary-amine alkaloids such as anabasine and anatabine are commonly present. However, their condensation with glucose are a little difficult under the same condition as for the synthesis of U-A.¹¹⁾

Smoking effect

It was reported that nornicotine type tobacco has an unpleasant taste.¹² However, the iso-

lated compound gave rather an improving effect on taste. This compound showed almost no effect on respiration, heart rate and bradycardia of rabbit in a inhalation experiment of the mist of this compound, or on the contraction of *Branchiura sowerbyi* in the test solution as compared with nicotine.^{13,14)} Smoking effect was mild as imagined by these physiological observations.

Acknowledgment. We are grateful to the Research Institute, Sankyo Co. for the measurement of FD-MS, and Nihon Denshi Co. for the measurement of ^{13}C -NMR.

REFERENCES

- 1) R. L. Stephens and J. A. Weybrew, *Tobacco Sci.*, **3**, 48 (1959).
- 2) E. A. Wernsman and D. F. Matzinger, *ibid.*, **14**, 34 (1970).
- 3) M. Amadori, *Atti accad. Lincei*, **2**, 337 (1925); *ibid.*, **9**, 68, 226 (1929).
- 4) H. Tomita, M. Noguchi and E. Tamaki, *Agric. Biol. Chem.*, **29**, 515 (1965).
- 5) D. C. DeJongh, T. Radford, J. O. Hribar, S. Hanessian, M. Bieber, G. Dawson and C. C.

- Sweeley, *J. Amer. Chem. Soc.*, **91**, 1728 (1969).
- 6) R. George, S. Ritche, N. Cyr, B. Korsch, H. J. Koch and A. S. Perlin, *Can. J. Chem.*, **53**, 1424 (1975).
- 7) A. S. Perlin, N. Cyr, H. J. Koch and B. Korsch, *Ann. New York Acad. Sci.*, **222**, 935 (1973).
- 8) R. Kuhn and L. Birkofer, *Ber.*, **71**, 621 (1938).
- 9) F. Feigle and V. Anger, *Microchim. Acta*, **1**, 1381 (1939).
- 10) T. Kisaki and E. Tamaki, *Arch. Biochem. Biophys.*, **94**, 252 (1961).
- 11) Unpublished.
- 12) J. M. Moseley and C. H. Rayburn, *11th Tobacco Chemist's Research Conference*, New Haven, Connecticut, Oct. 9~10 (1957); *Tobacco*, **145**, No. 16, 22 (1957).
- 13) Unpublished.
- 14) M. Asano, C. Okubo, K. Miyazaki and R. Miwa, *Kokyu to Junkan*, **33**, 27 (1975).
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