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AN AMADORI COMPOUND FROM TOBACCO*

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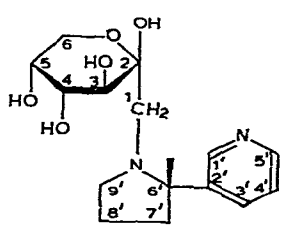
ABSTRACT

The Amadori compound 1-deoxy-1-[(*S*)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructopyranose has been isolated from flue-cured lamina of Delhi tobacco and its structure established from the results of hydrolysis, g.l.c.–m.s. of its trimethylsilyl derivative, and ^{13}C-n.m.r. spectroscopy. The significance of the compound in relation to tobacco quality is discussed.

INTRODUCTION

The interaction of sugars and amino acids yields *N*-glycosylamino acids which then rearrange to form 1-amino-1-deoxy-D-ketose derivatives. Such Amadori compounds have been isolated from liver[1]. Investigations of Amadori compounds isolated from tobacco[2–5], soya sauce[6,7], and tea[8] have been reported and their role in browning[9] and flavour[10–12] has been recognised.

Tobacco contains several pyridine alkaloids, of which the major is nicotine. However, the Cherry Red strain of Bright Yellow tobacco contains nornicotine [(*S*)-2-(3-pyridyl)pyrrolidine] as the principal alkaloid. During our studies of the



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carbohydrates of Canadian tobacco (*Nicotiana tabacum*, Delhi 76), significant amounts of a reducing component were noted, which, on the basis of its chromatographic behaviour, could have been misidentified[13,14] as a C_3 or C_4 sugar, erythrose, quinovose, or possibly "tabakose", but is in fact the Amadori compound 1-deoxy-1-[(*S*)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructopyranose (**1**). A similar compound has also been isolated from Cherry Red tobacco[15]. We now report on the isolation and characterisation of this Amadori product.

RESULTS AND DISCUSSION

Extraction of a large batch of cured tobacco powder [*Nicotiana tabacum* (Delhi 76), cured lamina, third harvest] with hot, aqueous 80% ethanol furnished material which, after removal of bound lipids (4.2%), yielded solids of low molecular weight (44.4%) and meal residue (45.4%). Paper chromatography (p.c.) of the fraction of low molecular weight revealed substantial amounts of a fast-moving, reducing component. Attempts to isolate this compound by chromatography on charcoal–Celite or by t.l.c. after acetylation and deacetylation failed because of the instability of the product under alkaline conditions.

However, preparative p.c., using two solvent systems, yielded a product which, on extraction with chloroform, gave a solid (~1.4% of the 80% ethanolic extract or ~0.72% of the cured tobacco). Following crystallisation from chloroform, these yields were 0.8% and 0.4%, respectively. The product {m.p. 67–70°, $[\alpha]_D^{25}$ −94° (water)} was shown to have structure **1**.

The hygroscopic crystals were homogeneous in p.c. and paper electrophoresis (p.e.) and gave a bluish purple colour with ninhydrin and a brown colour with aniline hydrogen phthalate. Partial hydrolysis gave [p.c. and p.e. (acetate buffer)] nornicotine, and complete hydrolysis produced, in addition, 5-(hydroxymethyl)-2-furaldehyde.

G.l.c. (on 5% SE-52) of the trimethylsilylated derivative prepared from the uncrystallised, chloroform-soluble material gave two partially separated peaks in the ratio ~1:1.1 [*T* 0.93 and 0.96, relative to that of octa-*O*-(trimethylsilyl)sucrose]. The trimethylsilylated crystalline material gave (on 3% OV-225) two well-separated peaks (*T* 1.25 and 1.40 relative to that of methyl arachidate). The ratio of 1:2.5 recorded 2 h after silylation changed to a constant value of 1:8.2 after 24 h. G.l.c.–m.s. of the minor peak (*T* 1.25) showed a base peak *m/z* 437 and a number of fragments (5–77%) (Table II). The major peak (*T* 1.40) showed a base peak *m/z* 161 and fragments ≤5%. Both components showed fragments with *m/z* 598 (M^+), 583 ($M^+ - 15$), and 437 ($M^+ - 161$), indicative of a sugar moiety with four Me_3SiO groups. The presence of fragment ions with *m/z* 437 and 257 was further indicative of tetra-*O*-(trimethylsilyl)-D-fructose[16], and those with *m/z* 161 and 147 showed the presence of 1-methylidene-2-(3-pyridyl)pyrrolidinium and 2-(3-pyridyl)pyrrolidinium ions. The relative abundance[17] of the ions with *m/z* 217 and 319 in the mass spectrum of the minor peak suggested the sugar moiety to be furanoid.

TABLE I

^{13}C-N.M.R. DATA FOR **1**, ITS RELATED COMPOUNDS, AND D-FRUCTOSE[a]

	1		*Nornicotino-β-*D-*fructofuranose*[15]	*Theanino-β-*D-*fructopyranose*[8]	D-*Fructose*[19]			*Nicotine*[b]
	20 MHz	*70 MHz*			*β-Pyranose*	*α-Furanose*	*β-Furanose*	
C-1	60.01	60.19	67.6	63.94	64.7	63.8	63.6	
C-2	97.79	98.08	97.7	95.35	99.1	105.5	102.6	
C-3	70.50	70.62	70.5	68.97	68.4	82.9	76.4	
C-4	70.02	70.14	70.0	69.38	70.5	77.0	75.4	
C-5	71.40	71.66	71.4	69.94	70.0	82.2	81.6	
C-6	64.20	64.18	64.2	62.72	64.1	61.9	63.2	
C-1′	149.75	149.81	149.81					149.8
C-2′	138.14	138.1	138.2					139.1
C-3′			138.1					134.4
C-4′	125.68	125.67	125.6					123.3
C-5′	149.35	149.15	149.3					148.8
C-6′	69.09	68.72	69.1					68.8
C-7′	33.58	34.11	33.6					35.7
C-8′	23.72	23.97	23.7					22.8
C-9′	56.94	56.86	56.9					56.9

[a]Solutions in D_2O (internal Me_4Si). [b]Bruker, ^{13}C-Data Bank, Vol. 1.

TABLE II

MASS-SPECTRAL DATA

m/z *(relative abundance, %)*		*Elemental composition*
Minor peak (T *1.25*)	*Major peak* (T *1.40*)	
598 (trace, $M^{+\cdot}$)	598 (trace, $M^{+\cdot}$)	$C_{27}H_{54}N_2O_5Si_4$
583 (10)	583 (3)	$C_{26}H_{51}N_2O_5Si_4$
437 (100)	437 (4)	$C_{17}H_{31}O_5Si_4$
347 (12)	347 (1)	
319 (13)	319 (5)	
303 (20)	303 (3)	
257 (32)	257 (2)	$C_{11}H_{21}O_3Si_2$
217 (30)	217 (5)	$C_9H_{21}O_2Si_2$
205 (15)	205 (4)	
191 (21)	191 (1)	
161 (77)	161 (100)	$C_{10}H_{13}N_2$
147 (46)	147 (10)	$C_9H_{11}N_2$
117 (5)	117 (5)	

The structure of the sugar moiety in the major component was established on the basis of ^{13}C-n.m.r. data. Both the crystalline and uncrystallised compound showed fourteen major and three minor signals (δ 75.55, 78.91, and 81.68). The assignments for the major signals together with reported data for related compounds are shown in Table I. These data, especially the chemical shift (δ 98.08) for C-2 (the resonance of C-2 of fructofuranose occurs further downfield by 4–6 p.p.m.), indicated the fructose moiety in the major component to be pyranoid. The other assignments (Table I) are in agreement with those reported by Koiwai *et al.*[15], except for the signal at δ 67.6 assigned to C-1 of the fructose moiety. We did not observe a resonance at this position, and have assigned to it a chemical shift of δ 60.19. Support for this assignment is provided by the C-1 resonance of the β-D-fructosyl group (other than that in the sucrose end-group) in the spectra of grass levans, *etc.*, which occurs[18] at δ 61.3. The signals for C-3′ and C-2′ of the nornicotine moiety were unresolved at δ 138.10 at 70 MHz, although they have been assigned[15] values of δ 138.1 and 138.2.

The $[\alpha]_D$ value of **1** indicates the fructopyranose moiety to be β. The close proximity of the minor signals (δ 75.55, 78.91, and 81.68) to those reported[19] for C-3,4,5 of β-D-fructofuranose (Table I) suggested that the major product is 1-deoxy-1-[(*S*)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructopyranose (**1**) which contains ~12% of the β-D-fructofuranose analogue as an impurity. Based on the foregoing evidence, the structure **1** was assigned to the major Amadori compound. The evidence presented also casts doubt on the identity of the Amadori compound reported by Koiwai *et al.*[15] as a fructofuranose derivative.

The nornicotino-D-fructose content (0.72%) of *Nicotiana tabacum* (Delhi 76) corresponds to a bound (*S*)-nornicotine content of 0.34% compared to 0.43% for

the Cherry Red strain of Bright Yellow tobacco[15]. The Delhi figure (Dr. Rosa, personal communication) of 2.75–3% of total alkaloid with 95% of nicotine suggested a (non-nicotine) alkaloidal content of 0.135–0.15% only. The nornicotine content of 0.34%, on the basis of present findings, is more than double these figures, and the nornicotino-D-fructose content is almost of the same order of magnitude as in the Cherry Red variety[15]. Natural back mutation[20] of nicotine-type tobacco to Cherry Red has been known to occur to the extent of 0.8% in a generation, and results in a crop of lower quality. It is possible that the Delhi Crop may be undergoing such a mutation.

The high content of bound nornicotine reflects unfavourably on tobacco quality, especially taste[14], and is indicative of an associated health hazard. The known presence of secondary amines and oxides of nitrogen in tobacco smoke has led to the notion that interaction between these compounds could lead to nitrosamines and help explain the tumorigenic activity of tobacco smoke in laboratory animals. The nitroso derivatives of the smoke components piperidine, pyrrolidine, methylaniline, anabasine, and nornicotine are carcinogenic in laboratory animals[14].

EXPERIMENTAL

General. — I.r. spectra were recorded with a Beckman IR 120A spectrophotometer. G.l.c.–m.s. was performed with (*a*) a Kratos MS-50 mass spectrometer coupled to a Perkin–Elmer Sigma 3GC gas chromatograph and an Incos O 2400 mass data system [operational details: m.s. 70 eV, inlet and source temperatures 250°; g.l.c., column (6 ft × 0.25 in.) of 3% of OV-225 on Chromosorb WHP (80–100 mesh), temperature programme (4°/min) 150→250°]; or (*b*) a combined Finnigan 3100D GC/MS system (operational details: 70 eV, direct probe inlet, inlet and source temperatures of 250°). Routine g.l.c. was performed with a Pye 104 Gas Chromatograph, with flame-ionisation detectors, dual glass columns (5 ft × 0.125 in., packed with 3% OV-225), temperature programme (4°/min) 150→250°, and a nitrogen flow-rate of 45 ml/min. ^{13}C-N.m.r. spectra were recorded at 37° with a Varian CFT-20 spectrometer (20 MHz, 10-mm tube) in the pulsed F.t. mode with complete proton decoupling. Chemical shifts (p.p.m.) were recorded relative to that of external Me_4Si, using D_2O as a field-frequency lock signal. More-accurate values were obtained on a CχP-300 instrument (70 MHz, 10-mm tube, 20°). Chemical shifts were measured relative to that of internal Me_4Si in a sealed, concentric tube (o.d. 5 mm). Spectra were obtained for solutions in D_2O (~30 mg/ml). Optical rotations were measured with a Perkin–Elmer 141 polarimeter.

Descending p.c. was performed on Whatman No. 1 paper with the organic phases of *A*, 1-butanol–acetic acid–water (4:1:5); *B*, 1-butanol–pyridine–water (10:3:3); *C*, ethyl acetate–pyridine–water (8:2:1); and *D*, 1-butanol–pyridine–water (6:4:3). Preparative p.c. was performed on Whatman Nos. 17 and 3MM papers. Paper electrophoresis[21] was performed on Whatman No. 3MM paper in *A*, 0.2M borate buffer (pH 10); and *B*, 0.2M acetate buffer (pH 5); at 800 V for 2–3 h. Detection

was effected with *A*, aniline hydrogen phthalate; *B*, naphthoresorcinol–trichloroacetic acid[22]; *C*, alkaline ferricyanide[1]; *D*, alkaline silver nitrate[23]; and *E*, 0.3% ninhydrin in ethanol at 70–80°. Concentrations were carried out at 35° on a rotary evaporator. Melting points were determined on an Electrothermal apparatus and are uncorrected.

Isolation and reactions of the Amadori compound. — Tobacco powder [Delhi 76, cured lamina, third harvest (1010 g, 966 g dry-matter basis)] was extracted with boiling, aqueous 80% ethanol (solid–liquid ratio, 1:50). The hot mixture was filtered and the residue was subjected to 10 further extractions, when only a negligible amount of solid was detected in an aliquot of the filtrate. The combined filtrates were concentrated (to 5 L), shaken with methanol (12.5 L) and chloroform (6.25 L), and then stored for 18 h. More chloroform (6.25 L) and water (6.25 L) were added, and shaking and settling produced two phases. The chloroform phase was concentrated, yielding lipid material (40.4 g). The methanol–water phase, on concentration to dryness, yielded the fraction (434.8 g) of lower molecular weight.

A portion (20 g) of the latter material was fractionated on sheets of Whatman No. 17 paper (23 × 57 cm, ~150 mg/sheet, 4 days, solvent *A*), to yield the fast-moving component (FC, 1 g). FC (0.55 g) was purified by p.p.c. on 32 sheets of Whatman No. 3MM paper (23 × 57 cm, ~20 mg/sheet, solvent *B*). Elution of the appropriate zones with methanol–water (1:1) yielded the Amadori product (184 mg), a solution of which in warm chloroform (20 mL) was filtered and then concentrated, to yield a fluffy solid (160 mg). Crystallisation of a portion (65 mg) from chloroform (2–3 mL) gave the product as fine, white needles, which were removed by filtration, washed with ether, and dried, to give **1** (30 mg), m.p. 67–70°, $[\alpha]_D^{25}$ −94° (*c* 1, water), which was homogeneous in p.c. (R_{Glc} 1.91, 3.35, 4.55, and 2.1; solvents *A–D*), M_{Glc} 0.52 (buffer *A*), and m_{GlcN} 0.72 (buffer *B*). The colour reactions to reagents *A–E* were brown, yellow with a reddish tinge, very pale green, black, and bluish purple, respectively.

Partial hydrolysis of FC (2 mg) in 0.05M sulfuric acid (0.2 mL) for 10 min at 100°, followed by neutralisation ($BaCO_3$) and filtration, gave unhydrolysed material (R_F 0.27, solvent *A*) and a component which gave a bluish purple colour with spray (*E*) and had R_F 0.48 (solvent *A*) and M_{GlcN} 1.22 (buffer *B*). Nornicotine showed identical behaviour.

Samples (2 mg) of FC were hydrolysed with 0.05M (1 and 2 h) and 0.1M (1 and 2 h) sulfuric acid (0.2 ml) at 100°. Each hydrolysate was neutralised ($BaCO_3$), filtered, and concentrated. P.c. showed that hydrolysis was complete in 0.1M acid after 2 h. FC (10 mg) was hydrolysed with 0.1M sulfuric acid (1 ml) at 100° for 2 h. The hydrolysate was treated as described above. P.c. revealed a ninhydrin-positive component (R_F 0.48, solvent *A*) and a component having an R_F value (0.80) and colour reaction (sprays *A* and *B*) identical with those of 5-(hydroxymethyl)-2-furaldehyde. The hydrolysis products isolated by p.c. gave mass spectra identical to those given by *S*-nornicotine and 5-(hydroxymethyl)-2-furaldehyde.

Crystalline **1** (2 mg) was treated with pyridine (0.05 ml), *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (0.05 ml) and a 2:1 mixture of hexamethyldisilazane and

chlorotrimethylsilane in pyridine (0.05 ml) for 24 h. G.l.c. of the reaction mixture showed two peaks (T 1.25 and 1.40) relative to that of methyl arachidate. The g.l.c.–m.s. data of the trimethylsilyl derivatives are summarised in Table II.

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