Isolation of Glycolipids from the Surface Lipids of *N. bigelovii* and Their Distribution in *N. bigelovii* and *N. glutinosa*

Toshiake Matsuzaki, Yasuhiro Shinozaki, Shizuo Suhara, Masaki Ninomiya, Hitoshi Shigematsu and Akira Koiwai

Tobacco Science Research Laboratory and *Life Science Research Laboratory Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

Received June 5, 1989

Sucrose esters and glucose esters consisting of short chain fatty acids are characteristic of tobacco plants. These glycolipids are thought to be the precursors of short chain fatty acids found abundantly in tobacco smoke, especially in oriental tobacco smoke. These lipids also have biological activity against insects and plants. In a previous report we isolated from *N. glutinosa* two types of sucrose esters, i.e., (2,3,4-tri-O-acyl)-α-D-glucopyranosyl-(3-O-acetyl)-β-D-fructofuranoside (M1) and (2,3,4-tri-O-acyl)-α-D-glucopyranosyl-β-D-fructofuranoside (M2). These lipids inhibited tobacco and barnyardgrass seed germination and growth. There are some other surface lipids that inhibit tobacco seed germination and growth, such as the lipids of *N. bigelovii*, *N. glutinosa* species of section Repandae, *N. cavicola*, and *N. miersii*. Preliminary studies showed that *N. bigelovii* had more apolar glycolipids than those found in *N. glutinosa*, as well as the same Rf compounds as M1 and M2. In addition, some *Nicotiana* species contained more than one type of glycolipids and others did not. It is very interesting from the chemotaxonomical point of view to study the distribution of glycolipids in *Nicotiana* species. This report deals with the study of surface lipids of *N. bigelovii*, and the distribution of glycolipids in the surface lipids of 57 *Nicotiana* species.

Tobacco plants (57 *Nicotiana* species) were grown in soil in 120 cm² pots in a greenhouse at 28°C. Leaf surface lipids were extracted from 3-month-old plants by dipping leaves for 10 sec in chloroform. After concentration in vacuo, each extract was dissolved into an appropriate volume of chloroform and kept at -20°C until use. Plants of *N. bigelovii* were grown in soil in 200 cm² pots for the large scale extraction.

The stored leaf surface lipids of *N. bigelovii* were chromatographed on a silica gel (Wakogel C300) column and separated into four fractions by successive elution with chloroform, chloroform-acetone (1:1, v/v), acetone, and methanol. The weight percentages of each fraction were 48.2, 50.6, 0.9, and 0.3%. Each eluate was tested by the tobacco seed germination assay and the inhibitory activity was found in the fractions eluted with chloroform and chloroform-acetone (1:1, v/v). The inhibitory compounds in the fractions were further purified by silica gel column chromatography. Three inhibitory glycolipids, I, II, and III, were obtained. The Rf values of I, II, and III were 0.76, 0.42, and 0.29, respectively, when developed by HPTLC (Merck Art 5642) with the solvent system of chloroform-acetone-acetic acid (30:70:1, v/v/v). Mild alkaline hydrolysis of either I, II, or III gave D-glucose, acetic acid, methyl propionic acid, 2-methyl butanoic acid, 3-methyl pentanoic acid, and 4-methyl pentanoic acid. The fatty acid compositions of I, II, and III are shown in Table I.

The IR spectra of either I, II, or III showed absorptions around 3450 cm⁻¹ (OH proton) and 1745 cm⁻¹ (ester carbonyl). Further purification of I, II, and III was done by reverse phase HPLC (YMC A-314 ODS, 1 ml/min, methanol-acetonitrile-water 8:2:1, v/v/v) and glycolipids I-1 ~ I-5, II-1 ~ II-5, and III-1 ~ III-7 were obtained. The ¹H-NMR spectrum of I-1 ~ I-5 indicated the presence of α and β-D-glucopyranose ring systems, respectively. The structure of I-1 ~ I-5 was estimated as 2,3,4-tri-O-acyl-α,β-D-glucopyranosyl by ¹H-NMR and ¹³C-NMR (i.e., ¹H-NMR of I-5, 500 MHz Bruker, δ ppm α anomer; 5.50 (d, J = 3.6 Hz1), 4.85 (dd, J = 3.7, 10.2 Hz2), 5.66 (t, J = 9.9 Hz3), 5.00 (t, 9.9 Hz4), 4.06 (m, Hz5), 3.69 (dd, J = 2.2, 12.8 Hz6), 3.57 (dd, J = 4.4, 12.1 Hz6); β anomer; 4.72 (d, J = 8.1 Hz1), 4.9 (dd, J = 8.1, 10.1 Hz2), 5.36 (t, J = 9.7 Hz3), 5.04 (t, J = 9.7 Hz4), 3.54 (m Hz5), 3.75 and 3.57 (overlapped with β anomer H6), ¹³C-NMR of I-5, 125 MHz Bruker, δ ppm α anomer; 90.23 (C1), 69.09 (C2), 69.04 (C3), 68.87 (C4), 71.59 (C5), 61.24 (C6) β anomer; 95.9 (C1), 73.49 (C2), 71.59 (C3), 69.66 (C4), 74.74 (C5), 61.24 (C6). It is a tendency that the longer retention time the collecting sample had, the longer fatty acids were esterified in the molecules.

The ¹H-NMR spectra of II-1 ~ II-5 from δ 3.0 ppm to δ 6.0 ppm were almost identical with that of M1 isolated from *N. glutinosa* (¹H-NMR of II-5, measured in CDCl₃).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>I (%)</th>
<th>II (%)</th>
<th>III (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>2.5</td>
<td>25.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Methyl-propionic</td>
<td>15.2</td>
<td>7.3</td>
<td>29.4</td>
</tr>
<tr>
<td>2-Methyl butylic</td>
<td>31.1</td>
<td>25.3</td>
<td>34.3</td>
</tr>
<tr>
<td>3-Methyl pentanoic</td>
<td>50.6</td>
<td>41.6</td>
<td>31.5</td>
</tr>
<tr>
<td>4-Methyl pentanoic</td>
<td>0.6</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Table II. Distribution of Glycolipids in 57 *Nicotiana* Species by Thin Layer Chromatography

<table>
<thead>
<tr>
<th>Section (Nicotiana)</th>
<th>Species</th>
<th>Glycolipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I*</td>
</tr>
<tr>
<td>Paniculatae</td>
<td>glauca</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>paniculata</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>knightiana</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>solanifolia</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>benavidesii</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>raimondii</td>
<td>–</td>
</tr>
<tr>
<td>Rustica</td>
<td>rustica</td>
<td>+</td>
</tr>
<tr>
<td>Tomentosae</td>
<td>tomentosa</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>tomentosiformis</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>otophora</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>setchelli</td>
<td>+ + ++</td>
</tr>
<tr>
<td></td>
<td>glutinoso</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>kawakamii</td>
<td>–</td>
</tr>
<tr>
<td>Genuinae</td>
<td>tabacum</td>
<td>–</td>
</tr>
<tr>
<td>Undulatae</td>
<td>undulata</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>arentii</td>
<td>–</td>
</tr>
<tr>
<td>Trigonophyllae</td>
<td>trigonophylla</td>
<td>–</td>
</tr>
<tr>
<td>Alatae</td>
<td>sylvestris</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>langsdorffii</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>alata</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>forgetiana</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>bonariensis</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>longiflora</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>plumbaginifolia</td>
<td>–</td>
</tr>
<tr>
<td>Repandae</td>
<td>repanda</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>stocktonii</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>nesophila</td>
<td>–</td>
</tr>
<tr>
<td>Noctiflorae</td>
<td>noctiflora</td>
<td>–</td>
</tr>
<tr>
<td>Acuminatae</td>
<td>acuminata</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>pauciflora</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>attenuata</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>miersii</td>
<td>+ + + + **</td>
</tr>
<tr>
<td></td>
<td>corymbosa</td>
<td>–</td>
</tr>
<tr>
<td>Bigelovinæ</td>
<td>bigelovii</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>clevelandii</td>
<td>+ + +</td>
</tr>
<tr>
<td>Nudicaules</td>
<td>nudicaulis</td>
<td>+ + **</td>
</tr>
<tr>
<td>Suaveolentes</td>
<td>benthamiana</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>umbratica</td>
<td>+ + + + **</td>
</tr>
<tr>
<td></td>
<td>cavicola</td>
<td>+ + + + **</td>
</tr>
<tr>
<td></td>
<td>debneyi</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>gossei</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>amplexicaulis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>maritima</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>velutina</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>hesperis</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>occidentalis</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>simulans</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>megalosiphon</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>rotundifolia</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>excelsior</td>
<td>+ +</td>
</tr>
<tr>
<td></td>
<td>suaveolens</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>ingulba</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>exigua</td>
<td>–</td>
</tr>
</tbody>
</table>
Table II. Distribution of Glycolipids in 57 Nicotiana Species by Thin Layer Chromatography

<table>
<thead>
<tr>
<th>Section</th>
<th>Glycolipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species (Nicotiana)</td>
<td>I*</td>
</tr>
<tr>
<td>goodspedia</td>
<td>-</td>
</tr>
<tr>
<td>rosulata</td>
<td>-</td>
</tr>
<tr>
<td>fragrans</td>
<td>-</td>
</tr>
<tr>
<td>africana</td>
<td>+</td>
</tr>
</tbody>
</table>

-, none; ±, trace; +, small; ++, moderate; +++, high.

* Lipids (1 mg) were developed with chloroform-acetone-acetic acid (30:70:1, v/v/v) by thin layer chromatography (Merck Art 5642); I, glucose ester (Rf 0.76); II, sucrose ester (Rf 0.42); III, sucrose ester (Rf 0.29).

** Different Rf than I, II, or III.

after shaking with small amounts of D$_2$O, δ ppm 5.66 (d, J = 3.6 g1), 5.49 (t, J = 10.0 g3), 5.25 (t, J = 7.9 f3), 4.94 (t, J = 10.0 g4), 4.84 (dd, J = 3.6, 10.4 g2) 4.40 (t, J = 8.0 f4), 4.10 (m, f5), 3.85, 3.74 (dd, dd f6), 3.61 3.51 (m, overlapped with f1 and g6). The $^{13}$C-NMR spectrum of II-5 also indicated the presence of sucrose carbons and the chemical shift of these carbons also coincided with the results of M1 (δ ppm, 89.5 (g1), 70.7 (g2), 69.1 (g3), 68.8 (g4), 71.2 (g5), 61.4 (g6), 64.0 (f1), 104.0 (f2), 78.7 (f3), 72.0 (f4), 82.5 (f5), 61.6 (f6), measured in CDCl$_3$ after shaking with small amounts of D$_2$O). The carbons of g6, f1, f4, and f6 were separated doublets in CDCl$_3$ solution after shaking with D$_2$O/H$_2$O (cal. 1: 1 mol). Therefore, the structure of II-1 ~II-5 was estimated as (2,3,4-O-triacyl)-α-ᴅ-glucopyranosyl-(3-0-acyl)-β-ᴅ-fructofuranoside.

The glycolipids III-1 ~III-7 could not be separated further by reverse phased HPLC developed with the solvent of methanol-acetonitrile-water. The $^{1}$H-NMR and $^{13}$C-NMR showed that III-1 ~III-7 were mixtures of M2$^{35}$ types of sucrose esters ((2,3,4-tri-O-acyl)-α-ᴅ-glucopyranosyl-β-ᴅ-fructofuranoside) obtained from N. glutinosa and another unidentified types of acyl sucrose.

Tabel II shows the distribution of glycolipids in 57 Nicotiana species. Lipids were developed with chloroform-acetone-acetic acid (30:70:1, v/v/v, Merck Art 5642) and a glucose ester and two different sucrose esters were found at Rf 0.72, Rf 0.42, and Rf 0.29, respectively. The results indicate that Nicotiana species were tentatively classified as follows; 1. species having no glycolipids in the surface lipids (Nicotiana species of section Paniculatae, Undulatae, and Repandae, N. sylvestris, N. corymbosa, and several Nicotiana species of section Suaveolentes), 2. species having glucose esters mainly (N. acuminate and N. pauciflora), 3. species having sucrose esters mainly (N. glutinosa, N. tomentosa, N. plumbanginifolia, N. tomentosiformis, N. tabacum, and N. longiflora), 4. species having both glucose esters and sucrose esters (N. otophora, N. setchellii, N. trigonophylla, N. bigelovii, and N. clevelandii). These classification would give some clues to consider the botanical classification of the genus Nicotiana chemically. For instance, N. sylvestris has no glycolipids, but N. tomentosiformis has. N. tabacum, a commercial tobacco plant, is known to have arisen after hybridization of N. sylvestris and N. tomentosiformis.$^{7,8}$ This suggest the idea that glycolipid-forming genes in surface tissues of N. tabacum might come from N. tomentosiformis, not from N. sylvestris. Another chemotaxonically interesting point is that species such as those in the section Paniculatae, Undulatae, or Repandae have no glycolipids and are classified as one group ("section"). This classification has a correlation with the botanical classification of section Paniculatae, section Undulatae, and section Repandae. However, chemotaxonically classification by surface glycolipids in other species does not always correspond to botanical classifications.

In a previous report,$^{5}$ we studied the effects of 54 Nicotiana surface lipids on tobacco seed germination and growth. Among them, several species such as N. repanda, N. stocktonii, N. nesophila, N. attenuata, N. miersii, N. caviola, N. bigelovii, and N. occidentalis had strong inhibitory activity. It was shown that these species except for N. section Repandae had glycolipids with a high ratio, and three types of glycolipids obtained from N. bigelovii were involved in the plant inhibitory activity. Therefore, glycolipids contained in the surface lipids of Nicotiana species might also be involved in the inhibitory activity as well as acylnornicotines in the species of N. section Repandae.

References

4) R. A. Heckman, M. F. Dube, D. Lynn and J. M.
6) B. A. Burke, G. Goldsby and J. B. Mudd, Phytochemistry, 26, 2567 (1987).