Isolation, Purification, and Stereochemistry of Simmondsin

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Highly purified simmondsin was isolated on a preparative scale from jojoba meal by continuous extraction with acetone followed by a two-step preparative column chromatographic procedure. The stereochemistry of the isolated compound was further elaborated by means of X-ray crystallography of simmondsin crystals and by two-dimensional NMR spectroscopy.

INTRODUCTION

Simmondsia chinensis is an evergreen shrub that grew originally in the Sonora Desert (United States). The seeds contain a liquid wax with properties similar to those of whale sperm oil. A high-protein residue, jojoba meal, remains after the oil is removed. However when used as a livestock feed ingredient, toxicity is of concern (Booth et al., 1974; Verbiscar et al., 1980). The effects seem to be especially related to the inhibition of food intake by simmondsin and other related cyanomethylenecyclohexyl glycosides (Elliger et al., 1973, 1974; Cokelaere et al., 1992). Different procedures have been described to "detoxify" the meal (Verbiscar et al., 1980, 1981). The toxicity of the mentioned compounds, however, has never been fully proved nor have the physiological mechanisms by which simmondsin and analogous products inhibit food intake. For animal studies, larger amounts of highly purified simmondsin are needed. This paper deals with the isolation of highly purified simmondsin from jojoba meal on a preparative scale and the study of its stereochemistry by means of X-ray crystallography and two-dimensional NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Plant Material. Jojoba meal was supplied by EMEC Agro Industries (Antwerp, Belgium). Raw extraction material (extraction residue of jojoba meal by a nonpublished procedure) was supplied by Laboratoires d'O (St. Martens-Latem, Belgium).

Extraction of Simmondsin. Jojoba meal (1 kg) was first extracted with hexane (5 L) to eliminate any remaining residues of oil. The deoiled meal was extracted with acetone for 12 h by means of a Soxhlet apparatus. The solvent in the extract was evaporated, and 40 g of a brown residue was obtained. This residue can be replaced by the raw extraction material supplied by Laboratoires d'O. The residue was dissolved in 200 mL of methanol and adsorbed on 100 g of silica gel (0.2–0.5 mm). The methanol was removed under vacuum and the silica gel put on top of a silica gel column (length 30 cm, 6 cm i.d.), containing a suspension of 0.5 kg of silica gel (0.040–0.063 mm) in chloroform. The column was first eluted with 1 L of chloroform and the organic solvent discarded, followed by elution with acetone; 100-mL fractions were collected. TLC was used to monitor the elution of simmondsin and analogous compounds. The first fractions contain mainly simmondsin 2′-ferulate, followed by mixtures of decreasing proportions of simmondsin to simmondsin analogues. Fractions rich in simmondsin (>80%) were collected and filtered over activated carbon, resulting in a light yellow solution. After evaporation of the acetone, the crude residue (29 g) was further purified on another silica gel column (60 cm × 6 cm i.d.), containing a suspension of 1 kg of silica gel (0.040–0.063 mm) in chloroform. A solution of the unpurified simmondsin in methanol was first adsorbed on silica gel (0.2–0.5 mm) and, after evaporation of the solvent, put on top of the column. Elution was performed with 10 L of a mixture of methanol and chloroform (5/95 v/v). The eluates were conducted through a UV cell for direct monitoring of eluting compounds and the corresponding fractions collected separately. All peaks were examined for the presence of simmondsin and analogues by HPLC. The fractions containing only simmondsin were collected, and the solvent was evaporated in vacuum. The residue (22 g) was crystallized from ethyl acetate/methanol (1:3). Separately, for the X-ray crystallographic study, smaller amounts of simmondsin were recrystallized from ethyl acetate.

Thin-Layer Chromatography. Thin-layer chromatography was performed on silica gel plates (Polygram Sil G/UV254, Machery-Nagel, Germany) using a mixture of methanol and chloroform (20/80 v/v) as a solvent. The spots were visualized by spraying the plates with 1-naphthol reagent. This reagent was prepared by adding 10.5 mL of a 15% ethanolic solution of 1-naphthol to a mixture of 40.5 mL of ethanol, 4 mL of water, and 6.5 mL of sulfuric acid. After spraying, the plates were heated at 100 °C in an oven for 5 min. Simmondsin and analogous compounds showed as violet spots.

HPLC. High-performance liquid chromatography was performed with a Merck Hitachi 6200 pump. Samples were injected into a Rheodyne injector (Model 7125) (Berkeley, CA) supplied with a 20-μL injector loop. A stainless steel C_{18} (7-μm particle size) column was used (25 cm × 0.4 cm i.d.) (E. Merck, Darmstadt, Germany). The flow rate of the solvent, a mixture of methanol and water (10/90 v/v), was 1.0 mL/min. The column eluate was monitored at wavelength 220 nm with a Hitachi Model L-3000 photodiode array detector.

Spectroscopy. *Nuclear Magnetic Resonance Spectroscopy.* ^{1}H and ^{13}C NMR spectra and 2-D spectra were recorded with a Bruker AC 400-MHz spectrometer at 400 and 100 MHz with CD_3OD as solvent. Chemical shifts are referenced to tetramethylsilane. The coupling constants of the ^{1}H NMR spectra were established by classic spin-decoupling techniques. Two-dimensional NMR spectra were recorded in absolute value and in long-range ^{13}C–^{1}H correlated spectra mode.

Mass Spectrometry. Electron impact mass spectrometry at an ionization potential of 70 eV was performed with an HP 5995A mass spectrometer. Simmondsin was introduced into the ion source by a direct insert probe (DIP). The ion source temperature was 280 °C, and the probe temperature was raised from room temperature to 280 °C at 60 °C/min. Liquid surface-assisted secondary ion mass spectrometry (L-SIMS) was performed with a Kratos Concept 1H instrument using a 7-keV Cs beam. Simmondsin was dissolved in glycerol on the probe tip.

X-ray Analysis. A multifaceted crystal with approximate dimensions 0.3 × 0.4 × 0.6 mm was used. The space group was C_2. A Siemens *P*4 diffractometer equipped with a graphite monochromator was used for intensity data collection in the $\omega - 2\theta$ mode (θ_{max} = 58°). Lattice parameters were determined at

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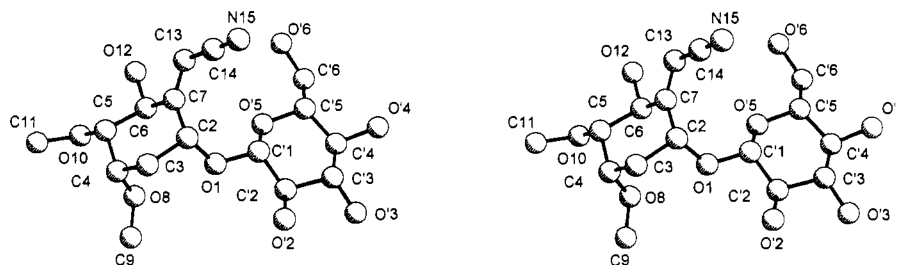

Figure 1. PLUTO stereoplot from the X-ray crystallographic structure of simmondsin.

273 K from a least-squares analysis of the setting angles of 24 reflections in the range $23° \le 2\theta \le 45°$. An empirical absorption correction was applied (minimum transmission 0.080, maximum transmission 0.101). A total of 3203 reflections was measured ($0 \le h \le 23, 0 \le k \le 10, -16 \le l \le 16$) with 1549 unique ($R_{int}$ = 0.043) and 1492 with $I \ge 3\sigma(I)$. Three standard reflections monitored every hour showed a small decay of 0.4%. The structure was solved by direct methods and refined using the NRCVAX system (Gabe et al., 1989). All non-hydrogen atom positions were refined anisotropically and the hydrogen atoms isotropically. With weights $w = (\sigma^2(F) + 0.0001F^2)^{-1}$, the model converged to R = 4.9%, R_w = 7.4%, and S = 5.3 (largest Δ/σ = 0.8). The maximum and minimum residual electron densities in the final difference Fourier synthesis are 0.68 and −0.24 e Å^{-3}, respectively. The molecular illustrations are produced with PLUTO (Motherwell and Clegg, 1978). All geometry calculations were performed with NRCVAX and PARST (Nardelli, 1983). Crystallographic calculations were done on an IBM AT compatible personal computer.

RESULTS AND DISCUSSION

Isolation Procedure. The described method allows the isolation of pure simmondsin from jojoba meal on a preparative basis in an acceptable yield (1.8% calculated on the meal). Previous studies using gradient column chromatography as described by Booth et al. (1974) have demonstrated that this method results in rather poor yields of simmondsin because of the many crystallization steps necessary to obtain a pure product. A much better yield was seen with an isocratic separation system applied on a crude extract obtained by previous fractionation on a silica gel column. The described method can be scaled up to extract and purify large amounts of simmondsin from jojoba meal. The large volumes of acetone used in this method can be recovered easily during the concentration step. This also applies for mixtures of chloroform/methanol after the analysis of their composition by gas chromatography and adaptation of the ratio of compounds prior to their reuse. This permits the production of large amounts of simmondsin at a relatively low cost. The purity of simmondsin was checked by both TLC and HPLC procedures. TLC showed only one violet spot and the HPLC analysis only one peak.

Stereochemistry. *X-ray Diffraction Analysis.* Simmondsin was obtained as colorless crystals from ethyl acetate. Crystal data: $C_{16}H_{25}O_9N$, M_w = 375.37 monoclinic, space group $C_2(C_2^3)$, No 5, a = 20.458(3), b = 9.079(2), c = 14.334(4) Å, β = 127.946 (16)°, V = 2099.5(8) Å^3, Z = 4, D_c = 1.32, D_m = 1.33, μ (Cu Kα, λ = 1.5418 Å) 0.91 mm^{-1}.

A stereo PLUTO plot with the atomic numbering scheme is shown in Figure 1. The pyran ring adopts a slightly twisted chair conformation with puckering parameters: Q_t = 0.559(7) Å, θ_2 = 171.6(7)°, ϕ_2 = 143.8(5)° (Cremer and Pople, 1975). Asymmetry parameters following Nardelli (1983) show a twofold axis through the midpoint of the C′1–O′5 and the C′3–C′4 bonds and approximate mirror planes through the vertices C′1, C′2, and C′3. The cyclohexyl ring has the expected chair conformation with a normal puckering amplitude Q_t = 0.553(8) Å and ϕ_2 = 15.9(5)°, θ_2 = 171.8(8)°. Endocyclic torsion angles ($\omega_{i,j}$) about the bonds between atoms i and j follow: $\omega_{2,3}$ = 47.3(8)°, $\omega_{3,4}$ = −52.6(8)°, $\omega_{4,5}$ = 57.8(8)°, $\omega_{5,6}$ = −59.2(8)°, $\omega_{6,7}$ = 57.2(8)°, $\omega_{7,2}$ = −50.8(8)° in the pyran ring and $\omega_{1,2}$ = 57.51(7)°, $\omega_{2,3}$ = −50.5(8) °, $\omega_{3,4}$ = 47.1(8)°, $\omega_{4,5}$ = −51.9(8)°, $\omega_{5,6}$ = 62.3(7)°, $\omega_{5,1}$ = −65.1(7)°. The cyanomethylene group has the E disposition and is syn to the axial β-glucose moiety as was already indirectly proved by chemical methods (Elliger et al., 1974).

Table I. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data for Simmondsin[a]

	δ	mult	J	J	C	δ
H_1	4.88	t	J_{1-6} = 3.5	J_{1-7} = 4.0	C_1	76.8
					C_2	166.4
H_3	4.72	dd	J_{3-4} = 9.0	J_{3-10} = 2.0	C_3	70.8
H_4	3.13	dd	J_{4-3} = 9.0	J_{4-5} = 3.0	C_4	86.4
H_5	3.90	q	J_{5-4} = 3.0	J_{7-5} = 4.0	C_5	76.5
H_6	1.69	ddd	J_{6-1} = 3.5	J_{6-5} = 3.5	C_6	32.1
H_7	2.50	dt	J_{7-1} = 4.0	J_{7-6} = 15.0		
					CN	117.6
H_{10}	5.70	d	J_{10-3} = 2.0		C_{10}	95.2
H_8(3)	3.43	s			C_8	58.2 or 58.5
H_9(3)	3.47	s			C_9	58.5 or 58.2
$H_{1'}$	4.38	d	$J_{1'-2'}$ = 7.8		$C_{1'}$	104.1
$H_{2'}$	3.22	dd	$J_{2'-1'}$ = 7.8	$J_{2'-3'}$ = 9.0	$C_{2'}$	74.6
$H_{3'}$	3.35	dd	$J_{3'-2'}$ = 9.0	$J_{3'-4'}$ = 9.0	$C_{3'}$	78.2
$H_{4'}$	3.28	dd	$J_{4'-3'}$ = 9.0	$J_{4'-5'}$ = 9.0	$C_{4'}$	71.5
$H_{5'}$	3.22	ddd			$C_{5'}$	78.2
$H_{6'}$	3.65	dd	$J_{6'-5'}$ = 5.2	$J_{6'-7'}$ = 12.0	$C_{6'}$	62.8
$H_{7'}$	3.82	dd	$J_{7'-6'}$ = 12.0	$J_{7'-5'}$ = 2.2		

[a] The numbering of the carbon atoms corresponds to the numbering of the bound hydrogens.

NMR Spectroscopy. Studies of ^{1}H and ^{13}C NMR (Table I) and the two-dimensional spectrum confirm the structure obtained by X-ray diffraction. The presence of the two methoxy substituents is proved by the two singlets at, respectively, δ 3.43 (3 H) and 3.47 (3 H). The presence of the β-glucose moiety was proved by the presence of the signals between δ 3.30 and 3.85. The signal at δ 4.38 (1 H, d, J = 7.8 Hz) is attributed to the equatorial anomeric proton of glucose, proving β-linkage at the anomeric center. The signals at δ = 3.65 (1 H, dd) and 3.82 (1 H, dd) are the methylene protons of glucose. The signals at δ = 3.22, 3.28, 3.35, and 3.22 (Table I) are the four axial protons of glucose as proved by the observed diaxial coupling constants of 9 Hz. The other signals are attributed to the cyanomethylenecyclohexyl part of the molecule. The coupling constant of 9 Hz between H_3 and H_4 confirms a

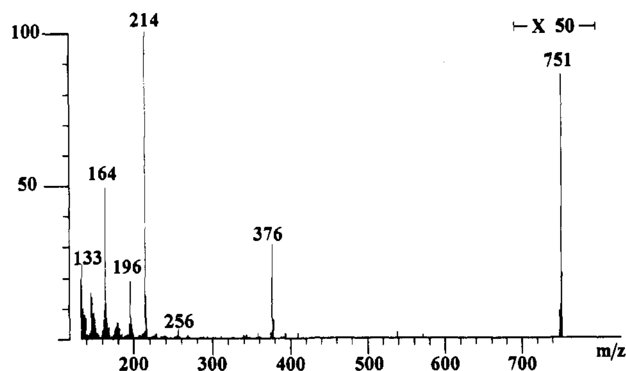

Figure 2. L-SIMS mass spectrum of simmondsin.

diaxial relationship. H_5, on the other hand, shows an equatorial coupling constant of 3 Hz with H_4.

The NMR results correspond with those of Elliger et al. (1973). However, by using a 400-MHz apparatus, high resolution of the different resonance patterns is obtained and no more previous derivatization or chemical transformation of the molecule is needed to elucidate the structure.

The chemical shift values of the carbons signals (Table I) could be unambiguously attributed to the mentioned carbon atoms by the described two-dimensional NMR spectroscopy.

Mass Spectrometry. Electron impact ionization mass spectrometry of the underivatized compound did not show a mass spectrum due to the thermal lability of the compound. The ionization technique with cesium ions results in a spectrum (Figure 2) with an intense molecular ion + 1 at m/z 376. Simmondsin minus glucose plus oxygen results in the fragment m/z 214, glucose minus oxygen results in the fragment ion m/z 164, and m/z 133 represents the cesium ion. A weak fragment ion is seen at m/z 751 corresponding to 2M + 1, a feature not uncommon to the used method.

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