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Mechanism of Simmondsin Decomposition during Sodium Hydroxide Treatment

M. VAN BOVEN,*,† M. LAGA,† S. LEONARD,† R. BUSSON,‡ R. HOLSER,§ E. DECUYPERE,# G. FLO,⊥ S. LIEVENS,⊥ AND M. COKELAERE⊥

Laboratory of Toxicology and Food Chemistry, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium; Laboratory of Medicinal Chemistry, Katholieke Universiteit Leuven, Rega Institute, B-3000 Leuven, Belgium; New Crops Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604, Laboratory of Physiology and Immunology of Domestic Animals, Katholieke Universiteit Leuven, B-3001 Heverlee, Belgium; and Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, Universitaire Campus, B-8500 Kortrijk, Belgium

Jojoba seed meal shows appetite-suppressing activity due to the presence of simmondsin. This pharmacological activity disappears with treatment of the meal with sodium hydroxide. To elucidate this mechanism of inactivation, the reaction of simmondsin in 1 N NaOH at 20 °C was monitored as a function of time. The end products of the reaction as well as intermediates were isolated and identified. The half-life of simmondsin was ∼60 min with D-glucose and 2-hydroxy-3-methoxyphenylacetonitrile as reaction end products. The reaction mechanism could be elucidated by the isolation of isosimmondsin and a simmondsin lactone derivative. Those compounds were isolated and purified by a combination of column chromatography and HPLC and identified mainly by HRMS and NMR spectroscopy.

KEYWORDS: ***Simmondsia chinensis*; jojoba seed meal; simmondsin decomposition; isosimmondsin; permethylsimmondsin**

INTRODUCTION

The seeds of the jojoba plant (*Simmondsia chinensis* Link Schneider), an evergreen shrub native to the Sonoran desert, produce a liquid wax commonly referred to as jojoba oil. The seed flour, which remains after the oil has been removed, contains ∼30% protein. However, when used as an animal food ingredient, the meal causes food intake reduction and growth retardation. The inhibitory effect on food intake is mainly due to the presence of simmondsin and simmondsin 2′-ferulate as described by Booth et al. (*1*) and Cokelaere et al. (*2*, *3*).

To be used as an animal feed ingredient, the previously mentioned antinutritional compounds have to be inactivated or eliminated from the meal. For that reason, several authors have described methods for the inactivation or elimination of simmondsin and simmondsin ferulate from jojoba meal. The inactivation can be achieved by chemical methods such as exposing the meal to ammonia or ammonia and hydrogen peroxide (*4*) or to sodium hydroxide at room temperature (*5*). The reaction with ammonia or ammonia and hydrogen peroxide results in the formation of simmondsin amide (Radziszewski reaction). Detailed knowledge of the chemical behavior of simmondsin during the Abbott treatment is still lacking. It was the aim of the present work to elucidate the mechanism of this reaction at room temperature. This knowledge can enhance the processing conditions of jojoba seed meal or lead to possible interesting new simmondsin derivatives. Refluxing simmondsin with 2 N NaOH, as described by Elliger et al. (*6*, *7*), results in the formation of 2-hydroxy-3-methoxyphenylacetonitrile and a mixture of α- and β-D-glucose.

MATERIALS AND METHODS

Materials and Reagents. Simmondsin [(7*Z*)-(1*R*,3*S*,4*R*,5*S*)-2-(cyanomethylene)-3-hydroxy-4,5-dimethoxycyclohexyl β-D-glucopyranoside] was prepared from jojoba seed meal obtained from EMEC Agro Industries (Antwerp, Belgium). A CHRIST LOC-1 apparatus (Vanderheyden, Belgium) was used for lyophilization of reaction mixtures neutralized with an equimolar quantity of hydrochloric acid or samples collected from HPLC. Melting points were measured with a Reichert−Wien microscope or with a Reichert−Jung apparatus (system Kofler) for glucose. All solvents used were of analytical grade from Merck (Darmstadt, Germany). Tri Sil was obtained from Pierce (Rockford, IL).

Permethylation of Simmondsin. Permethylation was performed according to the procedure described by Ciucanu and Kerek (*8*). Finely powdered NaOH (80 mg) and methyl iodide (0.4 mL) were added to a solution of 30 mg of simmondsin in 2 mL of Me_2SO at 25 °C. The

* Author to whom correspondence should be addressed (telephone +32 16 323439; fax +32 16 323405; e-mail maurits.vanboven@pharm.kuleuven.ac.be).
† Laboratory of Toxicology and Food Chemistry.
‡ Laboratory of Medicinal Chemistry.
§ New Crops Research.
# Laboratory of Physiology and Immunology of Domestic Animals.
⊥ Interdisciplinary Research Center.

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mixture was sonicated for 10 min, diluted with water (5 mL), and extracted with $CHCl_3$ (2 × 5 mL). The chloroform was washed with water (2 × 20 mL) and dried by means of Na_2SO_4. A colorless oil was obtained after evaporation of the organic solvent.

Degradation of Simmondsin in 1 N NaOH. Simmondsin (100 mg) was dissolved in 1 mL of 1 N NaOH. After 30 min or 1, 2, 4, 8, 16, 32, and 48 h of reaction at 20 °C, 50 μL aliquots were withdrawn from the reaction mixture and neutralized to pH 7 with 50 μL of 1 N HCl. The neutralized samples were submitted to TLC and HPLC analysis as described under below.

Isolation of Reaction Intermediates. To isolate reaction intermediates, the same reaction was done on a preparative scale. Simmondsin (10 g) was dissolved in 100 mL of 1 N NaOH; after 2 h, the reaction was stopped by neutralization with 1 N HCl. White crystals developed and were filtered. The filtrate was lyophilized, and the obtained white residue was sonicated with 100 mL of methanol; sodium chloride was filtered off, and the methanol fraction was submitted to column chromatography.

Column Chromatography. The methanol fraction was adsorbed on 40 g of silica gel (0.040−0.063 mm, Merck) and separated by chromatography on a 100 cm × 5.5 cm i.d. silica gel (700 g) column, using a mixture of ethyl acetate, 1-propanol, and water (25:25:5, v/v/v) as mobile phase. Fractions of 50 mL were collected and monitored by TLC. The fractions showing one spot on TLC were collected and after evaporation under reduced pressure used for GC-FID, GC-MS, mass spectrometry, and ^1H and ^{13}C NMR spectroscopy. Unresolved mixtures were submitted to HPLC for further separation and isolation of pure compounds. After elution of the column with 2 L of the above solvent mixture, the column was further eluted with 1 L of methanol.

Thin-Layer Chromatography. Reaction mixtures and column eluates were monitored by TLC on 4 × 8 cm silica gel plates (Polygram SIL G/UV 254, Macherey-Nagel, Germany) with a mixture of chloroform and methanol (80:20, v/v) as eluate. The plates were first examined under short- and long-wavelength UV illumination and then sprayed with the 1-naphthol spray. The spray was prepared by adding 10.5 mL of a 15% ethanol 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 they had been sprayed with the reagent, the plates were heated in an oven at 100 °C for 5 min; glucosides and heterosides developed as visible spots.

HPLC. The analysis of simmondsin and the isolation of simmondsin reaction products were performed by HPLC (*9*) with a 10 cm × 4.6 mm i.d. Chromolith RP-18 column (Merck) with a mixture of acetonitrile and water (2:98, v/v) as eluant at 2.0 mL/min. The eluates were monitored by both an L-3000 diode array detector (Merck-Hitachi) and an R401 differential refractometer (Waters). Eluates containing a single peak were lyophilized and used for GC-FID, GC-MS, HRMS, and NMR spectroscopy.

Mass Spectrometry. High-resolution mass spectrometry (HRMS) was performed with a qTof2 quadrupole/orthogonal acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a mixture of 2-propanol and water (1:1, v/v) at 3 μL/min.

GC-FID. A Chrompack 9000 gas chromatograph equipped with a flame ionization detector was used for the analysis of silylated reaction mixtures or isolated compounds after silylation. Separations were made using a 40 m × 0.2 mm i.d., 0.33 μm CP Sil-24 glass capillary column with a chemically bonded cross-linked phenyl (50%) methyl silicone (Chrompack, Middelburg, Belgium). Samples of 1 μL were injected by means of a split injector, 1:100. To protect the column, a special insert glass liner was used. Injector and detector temperatures were 320 °C. Helium was used as carrier gas at 25 cm/s (set at 60 °C). Solutions were injected at 100 °C; the oven temperature was kept at 100 °C for 2 min and programmed to 295 °C at 10 °C/min and kept at 295 °C for 20 min. Data acquisition and processing were done with a Merck-Hitachi 2500 chromatointegrator.

Preparation of Trimethylsilyl Derivatives for Gas Chromatography. To 1 mg of the isolated compounds or to 1 mg reaction mixtures was added 0.5 mL of Tri-Sil reagent. After 30 min of reaction at 40 °C, the samples were ready for GC-FID and GC-MS analyses.

Gas Chromatography—Mass Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5890 series II gas chromatograph. The chromatograph was equipped with a Hewlett-Packard 5971A mass selective detector and with an electron impact ion source (70 eV), a quadrupole mass filter, an electron multiplier detector, and a 30 m × 0.25 mm i.d., 0.25 μm HP-1 column (cross-linked methylsilicone). The carrier gas was He set at 20 cm/s. Extracts were introduced by splitless injection at 70 °C. The oven temperature was kept at 70 °C for 2 min and programmed to 100 °C at 35 °C/min and from 100 to 280 °C at 10 °C/min. The oven temperature was then maintained at 280 °C for 30 min.

Nuclear Magnetic Resonance Spectroscopy. ^1H and ^{13}C NMR spectra were recorded at 499.3 and 125.6 MHz, respectively, on a Varian Unity 500 spectrometer. Samples were dissolved in D_2O and measured at 22 °C. Chemical shifts are reported in parts per million (ppm, δ) using the residual HOD signal set at 4.70 ppm as reference for the ^1H spectra and external dioxane set at 67.4 ppm for the ^{13}C NMR spectra. The coupling patterns in the ^1H NMR spectra were elucidated by homonuclear decoupling techniques and especially by 1D-TOCSY spectra (*t*otal *c*orrelation *s*pectroscop*y*). First-order analysis was used for extraction of the coupling constants. Peak assignments were based on 2D correlation experiments such as COSY (homonuclear *c*orrelation *s*pectroscop*y*) or inverse detected GHSQC (*g*radient *h*eteronuclear *s*ingle-*q*uantum *c*oherence).

RESULTS AND DISCUSSION

Simmondsin Stability in 1 N NaOH. The concentration of simmondsin after 30 min or 1, 2, 4, 8, 16, 32, and 48 h of reaction in 1 N NaOH was determined by HPLC. From the peak areas of different known concentrations of simmondsin (t_R = 20.29 min), the amount of simmondsin present in the reaction mixtures was calculated. The half-life of simmondsin in the circumstances described was ∼60 min.

Monitoring the reaction by TLC gave information about the presence of glucosides along with their relative importance as a function of time. After 2 h of reaction time, five separated spots could be observed on TLC monitoring: three spots with 254 nm UV absorption (a spot with R_f = 0.92, a spot with R_f = 0.52 corresponding with simmondsin, and a spot with R_f = 0.47). After the plates had been sprayed with the 1-naphthol reagent, four violet spots were detected: a spot corresponding with simmondsin (R_f = 0.52), a spot with R_f = 0.47 corresponding with the already mentioned UV absorbing compound, and two UV transparent components with R_f 0.33 and 0.14. After 48 h of reaction time, only two end products could be detected by means of TLC: a UV-absorbing component with R_f = 0.92 and a 1-naphthol-positive product with R_f = 0.14. The product with R_f = 0.14 was identified by GC-FID and GC-MS as a mixture of α- and β-glucose and the product with R_f 0.92 as 2-hydroxy-3-methoxyphenylacetonitrile. Because the composition of the reaction mixture after 2 h of reaction showed high concentrations of reaction intermediates, this reaction period was chosen for isolating reaction products on a preparative scale.

Starting with 10 g of simmondsin and neutralization of the reaction mixture after 2 h of reaction time resulted in the formation of white crystals with mp 104 °C (yield = 72%). The NMR and MS data of the isolated compound (**4**) were identical with the corresponding data of previously identified 2-hydroxy-3-methoxyphenylacetonitrile (*10*). The resulting filtrate was submitted to column chromatography.

Column Chromatography. Column eluates were monitored by TLC. The first eluting compound was simmondsin (**1**) followed by a mixture of two unresolved compounds (**2** and **3**). This mixture was further separated by HPLC. After elution

Table 1. ^{1}H and ^{13}C NMR Data for Simmondsin (**1**), Isosimmondsin (**3**), Simmondsin Lactone Derivative (**2**), and Permethylsimmondsin (**5**)

	simmondsin (**1**)			isosimmondsin (**3**)			lactone derivative (**2**)			permethylsimmondsin (**5**)		
	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)
1′	103.96 (CH)	4.54 (d)	80	100.96 (CH)	4.685 (d)	7.3	103.93 (CH)	4.37 d)	7.8	99.43 (CH)	4.56 (d)	7.6
2′	73.97 (CH)	3.30 (dd)	8.0/9.2	74.91 (CH)	3.50–3.30		74.93 (CH)	3.18 (m)		83.49 (CH)	3.08 (m)	nd
3′	76.95 (CH)	3.48 (t)	9,2	77.99 (CH)	3.50–3.30		78.16 (CH)	3.29 (t)	8.8	86.67 (CH)	3.158 (t)	6.3
4′	70.37 (CH)	3.38–3.46	nd	71.42 (CH)	3.50–3.30		71.43 (CH)	3.35 (t)	8.8	79.04 (CH)	3.176 (t)	6.8
5′	77.07 (CH)	3.38–3.46	nd	78.33 (CH)	3.50–3.30		77.78 (CH)	3.18 (m)		74.74 (CH)	3.25 (m)	nd
6′	61.41 (CH_2)	3.85 (dd)	1.9/12.6	62.67 (CH_2)	3.85 (dd)	1.5/12.2	62.52 (CH_2)	3.73 (dd)	2.0/11.7	70.99 (CH_2)	3.56 (m)	nd
		3.76 (dd)	4.4/12.6		3.64 (dd)	5.8/12.2		3.59 (dd)	5.4/11.7		3.52 (om)	
1	77.35 (CH)	4.95 (t)	3.7	150.91 (C)			72.86 (CH)	4.87 (dd)	1.8/3.9	149.35 (C)		
2	165.09 (C)			109.25 (C)			168.82 (C)			111.60 (C)		
3	70.05 (CH)	4.70 (dd)	2.0/9.7	69.50 (CH)	4.28 (d)	4.9	83.91 (CH)	5.40 (dd)	2.0/9.8	77.80 (CH)	4.06 (d)	2.7
4	85.07 (CH)	3.28 (dd)	3.3/9.7	82.50 (CH)	3.48 (dd)	2.3/4.8	88.06 (CH)	3.16 (dd)	3.0/9.8	76.01 (CH)	3.64 (om)	
5	75,52 (CH)	4.03 (q)	3.5	75.93 (CH)	3.79 (ddd)	2.2/4.9/7.7	77.31 (CH)	3.94 (q)	2,9	75.05 (CH)	3.61 (om)	
6	31.01 (CH_2)	2.57 (dt)	3.6/15.9	28.83 (CH_2)	2.72 (dd)	4.9/17.1	32.31 (CH_2)	2.74 (ddd)	1.8/2.9/16.1	26.86 (CH_2)	2.41 (dd)	9.6/16.1
		1.75 (dt)	3.7/15.9		2.47 (dd)	7.7/17.1		1.59 (ddd)	3.0/3.9/16.1		2.68 (dd)	5.2/16.0
7	96.27 (CH)	5.79 (d)	$J_{7,3}$= 2.0	15.67 (CH_2)	3.58 (d)	17.6	116.83 (CH)	6.07 (d)	$J_{7,3}$ = 2.0	24.87 (CH)	4.10 (q)	7.2
					3.25 (d)	17.6						
8	118.02 (CN)			119.63 (CN)			175.22 (CO)			121.87 (CN)		
4-OMe	58.44 (CH_3)	3.45 (s)		58.65 (CH_3)	3.48 (s)		58.63 (CH_3)	3.5 (s)		60.80	3.63 (s)	
5-OMe	58.09 (CH_3)	3.42 (s)		57.29 (CH_3)	3.42 (s)		58.63 (CH_3)	3.470 (s)		60.80	3.58 (s)	
Glc-OMe										60.32	3.501(s)	
Glc-OMe										59.16	3.36 (s)	
Glc-OMe										58.23	3.52 (s)	
Glc-OMe										57.26	3.53 (s)	
Glc-OMe										56.68	3.42(s)	
7-Me										19.17	1.45 (d)	

of the mixture, the column was eluted with 1 L of methanol. GC-FID and GC-MS of the methanol fraction showed the presence of α- and β-glucose. Following slow evaporation of the methanol fraction only crystals of β-glucose (mp 159 °C and $[\alpha]_D$ +18.6°) were formed, proving the D-form of the sugar.

HPLC of Unresolved Mixture. The HPLC procedure allowed isolation of ~10 mg of the two constituents, with t_R values of 3.74 min (**3**) and 10.04 min (**2**), after 30 injections of the mixture isolated by column chromatography. The peak with t_R = 3.74 min corresponded to the spot with R_f = 0.33 and showed no UV absorption. The peak with with t_R = 10.04 min corresponded to the spot with R_f = 0.47 and showed a UV maximum at 217 nm. The t_R for simmondsin under the same conditions was 20.29 min. Solvents from these two collected peaks were removed by lyophilization, and the residues from the two peaks were used for GC-FID, GC-MS, NMR, and HRMS analyses.

Identification of the Compound with t_R = 3.74 min as [2-(Cyanomethyl)-3-hydroxy-4,5-dimethoxycyclohex-2-enyl β-D-Glucopyranoside] (3). GC of the silylated HPLC compound with t_R = 3.74 min (**3**) resulted in a single peak with t_R = 28.19 min eluting just after simmondsin with t_R = 27.60 min. GC-MS showed a spectrum without the presence of a molecular ion (out of range of the apparatus). The fragmentation pattern was, however, completely different from the pattern of simmondsin. Inspection of the fragment ions resulted in very minimal structural information.

The measured mass of the $(M + Na)^{\bullet+}$ ion of the isolated compound was 398.1422, corresponding to $C_{16}H_{25}NO_9Na$, the molecular formula of simmondsin.

The ^{1}H and ^{13}C NMR data of the isolated compound are shown in **Table 1**. All of the protons were readily assigned with the help of 2D-COSY and GHSQC and especially by the use of selective 1D-TOCSY experiments to separate the glucosyl protons from the aglycon protons, and, from the ^{13}C NMR data, the presence of a β-glucopyranosyl moiety as in simmondsin was clear.

The aglycon part shows, in comparison to the aglycon portion of simmondsin, some important differences. The diagnostic ^{13}C absorptions of the isolated compound in relation to the corresponding values of simmondsin were C1 (77.35 → 150.91), C2 (165.09 → 109.25), and C7 (96.27 → 15.67). These significant differences correspond to drastic changes in the chemical environment of the carbon atoms. Moreover, the ^{1}H simmondsin absorptions for H1 at δ 4.95 and for H7 at δ 5.79, each integrating for one proton, disappear and are replaced by two signals at δ 3.58 and 3.25 for H7. All of these changes indicate an isomerization of the double bond from the 2,7- to the 1,2-position. Long-range correlations with the quaternary carbons in the GHMBC spectrum between C1 and H1′/H3/H6/H7 and between C2 and H3/H4/H6/H7 gave conclusive evidence of the 1,2-double-bond position. Simultaneously with the previously described isomerization, a conformational flip of the puckered part of the now half-chair aglycon occurs, as is clearly visible from the relatively small $J_{3,4}$ and the typically large ay−ax value for $J_{5,6a}$.

The absence of a UV maximum is consistent with deconjugation of the double bond.

Because the present product had the same molecular weight and elemental composition as simmondsin, the name isosimmondsin was coined.

Identification of the Compound with t_R = 10.04 min (2). GC-FID and GC-MS analyses of the silylated HPLC compound with t_R = 10.04 min resulted in five early eluting compounds, formed by thermal decomposition of the compound at this high injection temperature. No structural information could be obtained from these results except that simmondsinamide shows the same thermolability when analyzed by GC under the same circumstances. However no simmondsinamide could be detected during the reaction.

The $(M + Na)^{\bullet+}$ mass obtained for the isolated compound was 399.1263, corresponding to $C_{16}H_{24}O_{10}Na$. Obviously, in this compound the simmondsin nitrogen is replaced by oxygen with elimination of one hydrogen from the simmondsin molecule.

The ^{1}H and ^{13}C NMR data of the isolated compound are shown in **Table 1** and were readily assigned. The presence of two separate moieties was confirmed by TOCSY experiments

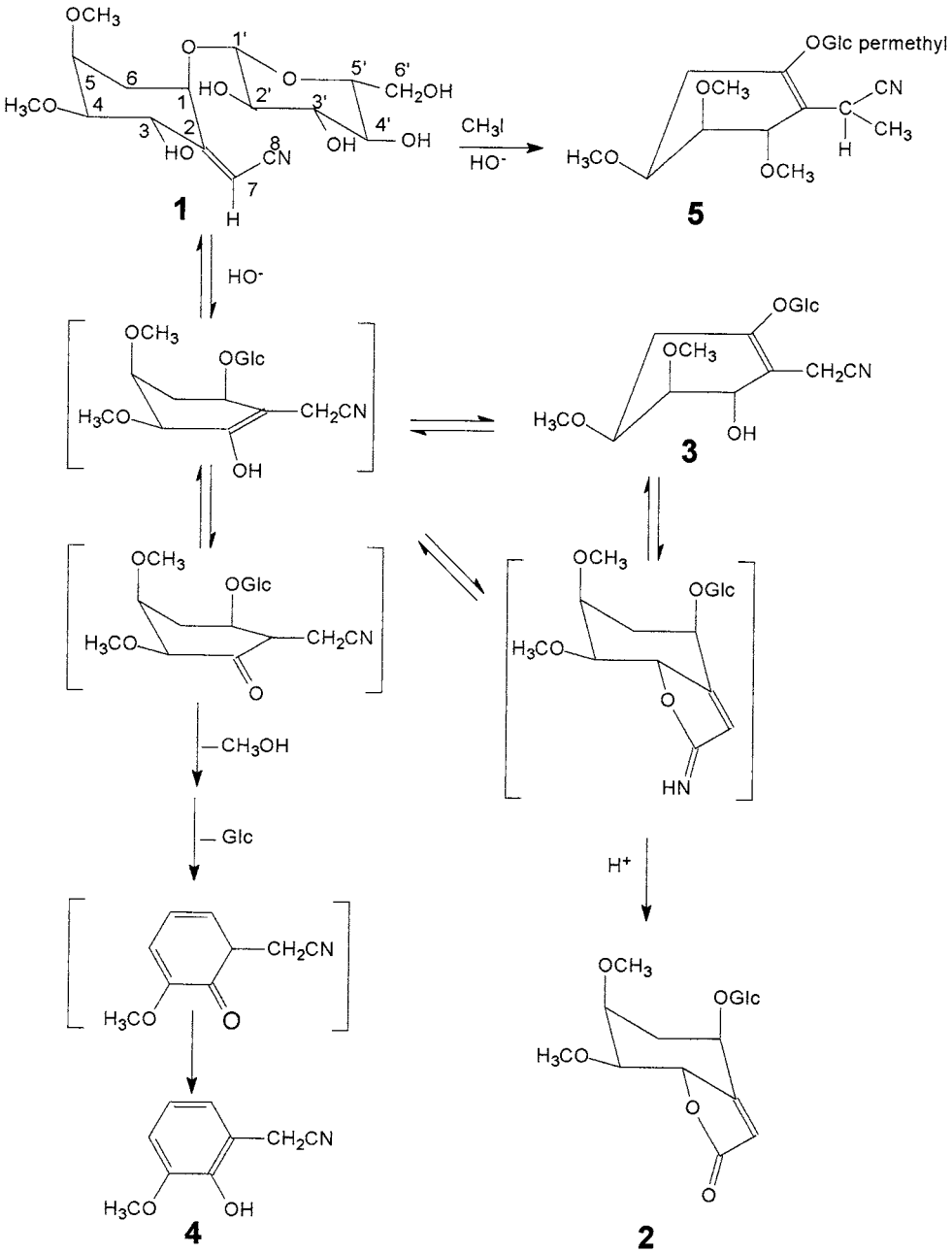

Figure 1. Proposed reaction mechanism of simmondsin degradation in 1 N NaOH.

and, from the ^{13}C NMR data, the presence of a β-glucopyranosyl moiety as in simmondsin was clear. The data for the aglycon part, on the other hand, showed important differences in comparison with the simmondsin spectrum. The CN absorption for simmondsin at δ 118.00 was replaced by a new absorption at δ 175.20, suggesting a carboxyl absorption. The C3 signal at δ 70.05 for simmondsin was shifted to δ 83.00, and the C7 absorption was shifted from δ 70.05 in simmondsin to δ 83.91; the other ^1H and ^{13}C aglycon absorptions were similar to the corresponding signals in simmondsin. All of these data indicate transformation of the nitrile into a lactone by interaction with the 3-OH group. The mechanism of the formation of the lactone is not yet proved, but the product probably originates (**Figure 1**) from hydrolysis of the primary intermediate imino ester compound, the latter being formed by 3-OH attack on the nitrile function after *Z*/*E* isomerization via isosimmondsin. During cyclization, the conformation of the aglycon ring flips back and the double bond as present in isosimmondsin reisomerizes to the 2−7-position as in simmondsin itself.

Permethylsimmondsin (5). The permethylation of simmondsin according to Ciucanu (*8*) resulted in one compound (yield = 99 ± 2%) with t_R = 26.03 min. GC-MS showed a molecular ion at *m*/*z* 459. Complete etherification of the five simmondsin hydroxyl groups should result in a molecular weight of 445. This difference with the result obtained means that another methyl group is incorporated into the simmondsin molecule. The mass spectrum does not allow localization of this methyl group, but the structure of the isolated compound could be elucidated by NMR spectroscopy (**Table 1**). The NMR data clearly show the presence of the sixth methyl group at δ 19.17 on C7 in the α-position to the nitrile function. The C7−C2 double bond isomerized as in isosimmondsin to the C2−C1 position, changing the multiplicity of the H6 protons from a double triplet to a double doublet. As in isosimmondsin, this

isomerization simultaneously causes the same conformational flip as indicated by the 2.7 Hz J_{3-4} coupling; this coupling corresponds with a diequatorial conformation instead of a diaxial one as for simmondsin. The isolated compound was stable to the alkaline hydrolysis described for simmondsin.

Permethylated simmondsin (**5**) was tested for its food intake reducing ability in the same way as other simmondsin derivatives (*11*). It was not possible on a statistical basis to distinguish between simmondsin and permethylsimmondsin for their anorexic effects (unpublished results).

Mechanism for the Reaction of Simmondsin in Alkaline Medium. The proposed mechanism for the reaction of simmondsin in alkaline media with the formation of isosimmondsin, the simmondsin lactone derivative, 2-hydroxy-3-methoxyphenylacetonitrile, and D-glucose is represented in **Figure 1**.

The reaction could be initiated by the abstraction of the axially oriented allylic proton H3 from simmondsin by the strong base with the isomerization of the 2,7-double bond to the 2,3-position. The double bond of this intermediate can either isomerize from the 2,3-position to the 1,2-position with simultaneously a conformational strain-releasing flip of the puckered part of the now half-chair, resulting in the isolated compound **3**, which we name isosimmondsin, or tautomerize to an as yet unisolated ketone isomer (**Figure 1**). This ketone isomer can easily undergo aromatization because of the weak acid axial α-proton necessary for the elimination of methanol and glucose. The proposed mechanism is confirmed by the stability of permethylsimmondsin to alkaline hydrolysis. Because we assume that for the alkaline degradation the 3-hydroxy function is necessary, methylation of this hydroxyl makes the proposed pathway impossible.

Free rotation of the cyanomethyl group in the 2,3-enol isomer or in isosimmondsin brings the nitrile function very close to the hydroxyl group. In this way attack of the 3-OH group on the nitrile function and reisomerization of the double bond can result in an intermediate iminoester. The imino ester could, however, not be isolated, obviously due to direct hydrolysis of the labile imino ester intermediate and transformation into the corresponding lactone during neutralization of the reaction mixture. Indirect proof for this pssibility was obtained by the transformation of isosimmondsin by 1 N HCl into the lactone described. In the alkaline medium of the reaction the imino ester is in equilibrium with isosimmondsin, which transforms completely into 2-hydroxy-3-methoxyphenylacetonitrile by the pathway indicated in **Figure 1**. The driving force for the base-induced instability of simmondsin is most probably the relief of the 1,3-diaxial steric interaction between the *O*-glucosyl and the 5-OMe substituent upon abstraction of the allylic 3-H proton and isomerization of the double bond.

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