147. New Saponins from *Phytolacca dodecandra* l'HERIT

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(9.V.84)

Summary

Three bidesmosidic saponins (**1–3**) have been isolated from the methanolic extract of the berries of *Phytolacca dodecandra* l'HERIT *(Phytolaccaceae)* by a combination of rotation locular counter-current chromatography and column chromatography on reversed phase *(RP-8)* with MeOH/H_2O. The structures have been established by ^{1}H-NMR, ^{13}C-NMR, FAB-MS, and D/CI-MS, as well as on the basis of acidic and basic hydrolyses. The monodesmosidic saponins **1a–3a** obtained after partial hydrolysis with a base exhibit strong molluscicidal activity against the schistosomiasis-transmitting snail *Biomphalaria glabrata*. Saponins **1–3**, **1a**, and **3a** are reported for the first time, whereas **2a** has been identified previously in the aqueous extract of *P. dodecandra* berries.

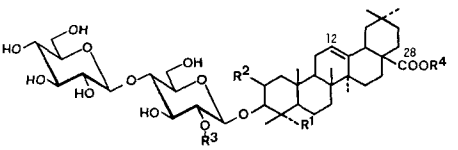
Introduction. – The rising costs of chemotherapy and of synthetic molluscicides have led to an increasing interest in plants and plant-derived compounds which are lethal to the intermediate host of schistosomiasis (bilharzia). The use of plants with molluscicidal properties appears to be a simple and inexpensive technology for the control of the snail vector. *Phytolacca dodecandra* l'HERIT *(Phytolaccaceae)* is one of the most promising plants. Water extracts of the berries have been evaluated in both laboratory and field trials [1–3]. The discovery of the remarkable molluscicidal properties of *P. dodecandra*, used in Ethiopia as a soap substitute, was made in 1965 by *Lemma* [4]. He observed dead snails in rivers where people washed their clothes with dried fruits. Subsequently, the water extract was studied by *Parkhurst et al.* [5] who isolated very potent monodesmosidic saponins formed of oleanolic acid and of various glycosidic chains.

In the course of our systematic screening studies on compounds with molluscicidal activity from medicinal plants [6] [7], we undertook the reinvestigation of *P. dodecandra* in order to improve the extraction procedure and to develop a method for the quantitative determination of the saponins. The dried berries were extracted by the classical method using solvents of increasing polarity, namely petroleum ether, $CHCl_3$, MeOH, and H_2O. The extracts obtained showed no molluscicidal activity against *Biomphalaria glabrata* snails, whereas direct water extraction of the fruits yielded a highly potent

solution. The MeOH extract, composed of numerous bidesmosidic saponins, was investigated. In the present paper, we report on the isolation and structure elucidation of three new saponins.

Results. – Dried berries (48 g) obtained from plants cultivated in Ethiopia (strain 1733) were extracted successively with petroleum ether, $CHCl_3$, MeOH, and H_2O. A part of the MeOH extract (8 g) was submitted in two portions to rotation locular counter-current chromatography (RLCC) [8] with the solvent system AcOEt/EtOH/H_2O 40:20:40 in the ascending mode. The eluate was monitored by TLC and collected in six fractions: *I* (200 mg), *II* (400 mg), *III* (440 mg), *IV* (470 mg), *V* (1370 mg), and *VI* (1520 mg). These fractions were further separated by reversed-phase chromatography (see *Exper. Part*). Pure saponin **1** (50 mg) was obtained from *Fraction III*, **2** (540 mg) from *Fraction IV* and *V*, and **3** (45 mg) from *Fraction V*.

Acidic hydrolysis of saponins **1–3** afforded glucose and as aglycones bayogenin, oleanolic acid, and hederagenin, respectively, identified by MS and ^{1}H-NMR and by comparison with authentic samples. Basic hydrolysis of **1–3** yielded glucose and the monodesmosidic saponins **1a**, **2a**, and **3a**, respectively. The mol.wt. of the saponins



Compound	R^1 (C(23))	R^2	R^3	R^4
1	CH_2OH	OH	H	Glc
1a	CH_2OH	OH	H	H
2	CH_3	H	Glc	Glc
2a	CH_3	H	Glc	H
3	CH_2OH	H	Glc	Glc
3a	CH_2OH	H	Glc	H

was established by the combination of desorption/chemical-ionization (D/CI) MS [9] and fast atom bombardment (FAB) MS [10]. The MS (D/CI) of **1** (reactant gas NH_3, negative-ion mode) showed a quasi-molecular ion ($[M - H]^-$) at *m/z* 973 and a signal at *m/z* 955 after loss of H_2O. Additional signals were observed at *m/z* 811 ($[(M - H)\text{-}162]^-$) and *m/z* 649 ($[(M - H)\text{-}324]^-$), corresponding to the loss of one and two glucosyl moieties, respectively.

The quasi-molecular ion of **1** was not obtained in the positive-ion spectrum, but the following signals were observed: *m/z* 830 ($[(M + NH_4)\text{-}162]^+$), *m/z* 668 ($[(M + NH_4)\text{-}324]^+$) and *m/z* 506 ($[(M + NH_4)\text{-}486]^+$), corresponding to the loss of one, two, and three glucosyl units. These results suggest that one sugar moiety is attached at C(28) and is very easily eliminated. This is confirmed by the spectrum of **1a** where identical signals are observed, the quasi-molecular ion appearing at *m/z* 830 ($[M + NH_4]^+$). Thus, **1** is a bidesmosidic saponin with three hexose units, one glucose being attached to the aglycone by an ester linkage. Compound **1a** is a monodesmosidic bayogenin-diglucoside.

The MS (FAB) of **2** (matrix thioglycerol, negative-ion mode) shows quasi-molecular ions at *m/z* 1139 ($[M + Cl]^-$) and *m/z* 1103 ($[M - H]^-$). Signals at *m/z* 941 ($[(M - H)\text{-}162]^-$), 779 ($[(M - H)\text{-}324]^-$), 617 ($[(M - H)\text{-}486]^-$), and 455 ($[(M - H)\text{-}648]^-$) correspond to the subsequent loss of four glucosyl moieties. The partially hydrolyzed saponin **2a** shows a similar spectrum with one hexose unit less: quasi-molecular

ions are observed at *m/z* 977 ($[M + Cl]^-$) and *m/z* 941 ($[M - H]^-$); other signals are present at *m/z* 779, 617, and 455. Compound **2** is a bidesmosidic oleanolic acid-tetraglucoside with one sugar unit linked to the aglycone at C(28), whereas **2a** is the corresponding prosapogenin with three glucosyl moieties attached at C(3). Compounds **3** and **3a** show similar fragmentation patterns to **2** and **2a**, respectively, with a shift of 16 *u* resulting from the substitution of a CH_3-group attached on C(4) of the aglycone by CH_2OH.

The interglycosidic linkages as well as the position of attachment of the sugar chains to the aglycones were established by ^{13}C-NMR spectroscopy. In all the saponins, a sugar chain is linked to the aglycone at C(3), the ^{13}C-NMR signal of which appears at 83.2 ppm for **1** and **1a**, at 89.1 ppm for **2** and **2a**, and at 82.1 ppm for **3** and **3a**. These chemical shifts are in agreement with values reported from the literature for olean-12-ene-derived triterpene-3-*O*-glycosides [6] [11]. The free COOH-group is observed at 180 ppm (monodesmodic saponins **1a–3a**) whereas, when esterified with a glucosyl moiety, the chemical shift is 176 ppm (bidesmosidic saponins **1–3**). The β-D-pyranosyl configuration of glucose was established by ^{1}H- and ^{13}C-NMR.

Table. *^{13}C-NMR Chemical Shifts of Saponins* **1a** *and* **2a** *(sugar moieties).* Pertinent shifts discussed in the text are shown in italics.

Saponin	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
1a inner sugar moiety	104.8[a])	74.8	*76.8*	*81.3*	*76.2*	62.1[b])
terminal sugar moiety	105.2[a])	75.0	78.2	71.6	78.4	62.5[b])
2a inner sugar moiety	104.8[c])	*81.6*	*74.8*	*81.2*	77.1	62.8[d])
terminal sugar moiety	104.9[c])	76.2	77.8	71.5	78.0	62.2[d])
terminal sugar moiety	105.5[c])	76.6	78.2	71.8	78.4	62.3[d])

[a])[b])[c])[d]) Signals may be interchangeable.

The ^{13}C-NMR data of saponins **1a** and **2a**, which allow the determination of the interglycosidic linkages, are summarized in the *Table*. The chemical shifts of the C-atoms of the inner β-D-glucopyranosyl moiety of compound **1a** clearly indicate that the terminal sugar unit is attached at position 4. The C(4)-signal is shifted downfield by 9.7 to 81.3 ppm; the C(3) and C(5)-signals are shifted upfield by 1.4 and 2.2 ppm, respectively, whereas the other C-atoms remain almost unaffected. These results are in agreement with the glycosylation rule previously established by *Konishi et al.* [12]. Namely, when a OH-group of a sugar is glycosylated, the α-C-atoms are shifted downfield by 6 to 9 ppm, while β-C-atoms are observed at higher field. The spectrum of saponin **1** shows six additional signals for the β-D-glucopyranosyl unit esterified on the COOH-group. Compound **2a** is also a triglucoside, but with all three sugars branched at position 3 of oleanolic acid. From the ^{13}C-NMR data *(Table)*, it can be seen that two of the β-D-glucopyranose moieties are terminal with shifts similar to those of the C-atoms of the terminal sugar of **1a**. The third β-D-glucopyranose unit (inner moiety) however, has chemical shifts for C(2) and C(4) at 81.6 and 81.2 ppm, respectively, these downfield shifts indicating that it is substituted by terminal glucose units at positions 2 and 4. The ^{13}C-NMR signals arising from the sugar moiety of saponin **3a** have shifts very similar to those of the sugar moiety of **2a**, indicating the same branched sugar unit.

Thus, the structure of **1a** is established as 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]bayogenin and saponin **2a** is identical to 3-*O*-[2′,4′-di-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl]oleanolic acid or oleanoglycotoxin-A, previously isolated from the aqueous extract of *P. dodecandra* by *Parkhurst et al.* [5]. Compounds **2a** and **3a** are the prosapogenins of **2** and **3**, respectively tetraglucosides of oleanolic acid and hederagenin, with one glucosyl unit linked to the aglycone at C(28).

Discussion. – Although several phytochemical studies of *P. dodecandra* berries have been published [5] [14], it has been possible to isolate the three previously unreported bidesmosidic saponins **1–3** from the MeOH extract. These glycosides showed no molluscicidal activity, but after treatment with a base afforded the monodesmosidic saponins **1a–3a** which killed *Biomphalaria glabrata* snails within 24 h at concentrations of 12, 6, and 12 ppm, respectively. Compound **2a** was previously identified in the aqueous extract of *P. dodecandra* berries among other highly active monodesmosidic saponins [5]. Direct water extraction afforded **1a–3a**, and only traces of **1–3** could be detected. The genuine bidesmosidic saponins are easily hydrolyzed during the aqueous extraction procedure. The monodesmosidic saponins so formed are artefacts since they are absent in the MeOH extract.

The saponin composition of *P. dodecandra* berries appears to be very complex and is formed of numerous glycosides of oleanolic acid, hederagenin, and bayogenin. Several other bidesmosidic saponins could be isolated. Their structure elucidation is currently in progress. We undertook also a systematic phytochemical investigation of berries originating from different cultivated strains which may vary in their saponin content.

Financial support has been provided by the *Swiss National Science Foundation*, the *Parasitic Diseases Programme* of the *World Health Organisation* and the *UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases*. Prof. *Teklemariam Ayele*, Addis Ababa, Prof. *H. Kloos* and Dr. *Ch. B. Lugt*, are gratefully acknowledged for providing plant material. Thanks are also due to Dr. *K. E. Mott*, WHO, for helpful advice and to the following who carried out spectral measurements: Prof. *W. J. Richter*, *Ciba-Geigy*, Basel (FAB-MS), Mr. *D. Chollet*, *Zyma*, Nyon (D/CI-MS) and Prof. *J. Lauterwein*, University of Lausanne (NMR).

Experimental Part

General Remarks. Melting points (m.p.) were determined on a *Kofler* block and are uncorrected. TLC were carried out on silica gel precoated Al sheets *(Merck)* with $CHCl_3$/MeOH/H_2O 58:37:5. Detection was with *Godin* reagent [15]. For column chromatography, a *Lobar-Lichroprep-RP-8* column (40–63 μm; i.d. 2.5 × 27 cm; *Merck*) equipped with a *Duramat-80* pump (*Chemie und Filter*, Regensdorf) and a *Sephadex-LH-20* column (i.d. 2.5 × 25 cm; *Pharmacia Fine Chemicals*) were used. Rotation locular counter-current chromatography (RLCC) was achieved on a *Tokyo Rikakikai* apparatus (Tokyo, Japan) with 16 tubes, each divided by a centrally perforated PTFE disc into 37 loculi. The flow rate was 45 ml/h, the rotation speed 60–70 rpm and the slope 20°. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a *Bruker-WP-360* apparatus at 360 MHz and 90.52 MHz, resp., in (D_5)pyridine as solvent and TMS as internal standard. Desorption/chemical ionization (D/CI) MS were recorded on a *Ribermag-R10-10B* quadrupole. Fast-atom-bombardment (FAB) MS were obtained on a *ZAB-1S* spectrometer. The target was bombarded with 5-keV Xe-atoms; samples were suspended in thioglycerol. GC analyses were carried out on a *Hewlett-Packard-5790* chromatograph using a 12.5 × 0.2 mm capillary column of cross-linked dimethylsilicone; the temp. required for the analysis of per(trimethylsilyl)-sugar derivatives was 165° for 2 min, then increased at 2°/min.

Acidic Hydrolysis. The saponin (2 mg) in MeOH (1 ml) was refluxed in 4N (10 ml) HCl for 4 h. The aglycone was extracted with AcOEt and identified by TLC on silica gel with (i-Pr)$_2$O/acetone 75:30. The aq. layer was adjusted to pH 6 with $NaHCO_3$. After evaporation to dryness, the sugars were extracted with pyridine from the residue and analyzed by TLC on silica gel with AcOEt/MeOH/H_2O/AcOH 95:15:15:20; detection with *p*-anisidine phthalate, and by GC as per(trimethylsilyl) derivatives, prepared by adding 60 μl of hexamethyldisilazane/trimethylchlorosilane 2:1 to 100 μl of the pyridine solution.

Basic Hydrolysis. The saponin (30 mg) in MeOH (5 ml) was refluxed in 0.5N aq. KOH (20 ml) for 1 h. The mixture was adjusted to pH 6 with aq. HCl and then extracted with 2 × 25 ml of BuOH; the org. phase was washed with 20 ml of H_2O, and evaporated to dryness. The partially hydrolyzed saponin was purified on a *Sephadex-LH-20* column eluted with MeOH.

Isolation. Dried berries (48 g) of *Phytolacca dodecandra* l'HERIT collected in Ethiopia (strain 1733) were extracted successively with petroleum ether (2 × 500 ml), $CHCl_3$ (2 × 500 ml), MeOH (3 × 500 ml), and finally H_2O (2 × 300 ml). The MeOH extract (13 g) was further studied. A first separation was carried out by RLCC with AcOEt/EtOH/H_2O 40:20:40 in the ascending mode with two portions of 4 g of extract. The eluate was collected into six fractions: *I* (200 mg), *II* (400 mg), *III* (440 mg), *IV* (470 mg), *V* (1370 mg), and *VI* (1520 mg). Chromatography of *Fraction III* on a *Lobar-RP-8* column with MeOH/H_2O 65:35 yielded compound **1**, purified by a second chromatographic separation under the same conditions and then on a *Sephadex-LH-20* column with MeOH: 50 mg of pure **1**. *Fraction IV* was submitted to reversed-phase chromatography on a *Lobar-RP-8* column with MeOH/H_2O 65:35 to give **2** (240 mg; further purified on a *Sephadex-LH-20* column with MeOH). Four separations of *Fraction V* (each time 300 mg) on a *Lobar-RP-8* column afforded **2** (300 mg; further purified on a *Sephadex-LH-20* column with MeOH) and a mixture of 2 other saponins. Chromatography of this mixture on a *Sephadex LH-20* column with MeOH and then on a *Lobar-RP-8* column with MeOH/H_2O 55:45 yielded 45 mg of pure **3**.

28-O-(β-D-Glucopyranosyl)-3-O-[O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]bayogenin (= β-D-Glucopyranosyl-3β-[(O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl)oxy]-2β,23-dihydroxyolean-12-en-28-oate; **1**; $C_{48}H_{78}O_{20}$): white powder, m.p. 240–250° (dec.). ^{13}C-NMR (90.52 MHz; (D_5)pyridine): δ's of the aglycone and the diglucosyl moiety at C(3) identical to those of **1a**; δ's of the β-D-glucopyranosyl ester unit: 95.7 (C(1)), 74.1 (C(2)), 79.1 (C(3)), 71.2 (C(4)), 78.9 (C(5)), 62.0 (C(6)). MS (D/CI; NH_3, negative ions): 973 ($[M - H]^-$), 955 ($[(M - H)\text{-}18]^-$), 811 ($[(M - H)\text{-}162]^-$), 649 ($[(M-H)\text{-}324]^-$). MS (D/CI; NH_3, positive ions): 830 ($[(M + NH_4)\text{-}162]^+$), 668 ($[(M + NH_4)\text{-}324]^+$), 506 ($[(M + NH_4)\text{-}486]^+$).

Acidic hydrolysis of **1** afforded D-glucose and an aglycone identified as *bayogenin (= 2β,3β,23-trihydroxyolean-12-en-28-oic acid):* ^{1}H-NMR (360 MHz, (D_6)acetone): 0.93 (*s*, CH_3); 1.04 (*s*, CH_3); 1.06 (*s*, CH_3); 1.07 (*s*, CH_3); 1.28 (*s*, CH_3); 1.42 (*s*, CH_3); 3.66 (*AB*, J_{AB} = 9.5, CH_2OH); 3.74 (*d*, *J* = 3.6, H–C(3)); 4.17 (*m*, H–C(2)); 5.38 (*t*, *J* = 3, H–C(12)). MS (D/CI; CH_4, positive ions): 489 ($[M + H]^+$), 487, 471, 453, 435, 391, 248.

3-O-[O-β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl]bayogenin (= 3β-[O-β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl)oxy]-2β,23-dihydroxyolean-12-en-28-oic Acid; **1a**; $C_{42}H_{68}O_{15}$) was obtained after basic hydrolysis of **1**: white powder, m.p. 250–260° (dec.). ^{13}C-NMR (90.52 MHz, (D_5)pyridine): aglycone: 15.0 (C(24)), 17.3 (C(25)), 17.6 (C(26)), 18.1 (C(6)), 23.8 (C(11)), 23.8 (C(30)), 24.1 (C(16)), 26.3 (C(27)), 28.4 (C(15)), 31.0 (C(20)), 33.4 (C(22)), 33.1 (C(7)), 33.3 (C(29)), 34.3 (C(21)), 37.1 (C(10)), 40.0 (C(8)), 42.1 (C(14)), 42.4 (C(18)), 42.8 (C(4)), 44.1 (C(19)), 46.5 (C(17)), 46.7 (C(1)), 47.9 (C(9)), 48.6 (C(5)), 65.6 (C(2)), 70.6 (C(23)), 83.2 (C(3)), 122.9 (C(12)), 144.9 (C(13)), 180.1 (C(28)); sugar moieties: see *Table*. MS (D/CI; NH_3, positive ions): 830 ($[M + NH_4]^+$), 813 ($[M + H]^+$), 668 ($[(M NH_4)\text{-}162]^+$), 651 ($[(M + H)\text{-}162]^+$), 506 ($[(M + NH_4)\text{-}324]^+$), 489 ($[(M + H)\text{-}324]^+$).

β-D-Glucopyranosyl 3-O-[2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl]oleanolate (= β-D-Glucopyranosyl 3β-[(2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl)oxy]olean-12-en-28-oate; **2**; $C_{54}H_{88}O_{23}$): white powder, m.p. 248–252° (dec.). ^{13}C-NMR (90.52 MHz; (D_5)pyridine): δ's of the aglycone and the 3 sugar units at C(3) identical with those of **2a**; δ's of the β-D-glucopyranosyl ester moiety: 95.8 (C(1)), 74.1 (C(2)), 79.3 (C(3)), 71.2 (C(4)), 78.9 (C(5)), 62.2 (C(6)). MS (FAB; thioglycerol, negative ions): 1139 ($[M + Cl]^-$), 1103 ($[M + Cl]^-$), 941 ($[(M - H)\text{-}162]^-$), 779 ($[(M - H)\text{-}324]^-$), 617 ($[(M - H)\text{-}486]^-$), 455 ($[(M - H)\text{-}648]^-$).

Acidic hydrolysis of **2** afforded oleanolic acid and D-glucose.

3-O-[2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl]oleanolic Acid (= 3β-[(2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl)oxy]olean-12-en-28-oic Acid; **2a**; $C_{48}H_{78}O_{18}$) was obtained after partial (basic) hydrolysis of **2**; white powder, m.p. 230–240° (dec.). ^{13}C-NMR (90.52 MHz, (D_5)pyridine): δ's of the aglycone correspond to those previously described for oleanolic acid [11]; sugar signals: see *Table*. MS (FAB; thioglycerol, negative ions): 977 ($[M + Cl]^-$), 941 ($[M - H]^-$), 579 ($[(M - H)\text{-}162]^-$), 617 ($[(M - H)\text{-}324]^-$).

Acetylation of **2a**: Overnight, **2a** (12 mg) was stirred with 2 ml of anh. pyridine/Ac_2O 1:1. Addition of H_2O induced the precipitation of the peracetate, m.p. 156–158°.

3-O-[2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl]-28-O-(β-D-glucopyranosyl)hederagenin (= β-D-Glucopyranosyl 3β-[(2',4'-Di-O-(β-Glucopyranosyl)-β-D-glucopyranosyl)oxy]-23-hydroxyolean-12-en-28-oate; **3**; $C_{54}H_{88}O_{24}$): white powder, m.p. 250–255° (dec.). ^{13}C-NMR (90.52 MHz, (D_5)pyridine): δ's of the aglycone and the 3 sugar units at C(3) identical with those of **3a**; δ's of the β-D-glucopyranosyl ester moiety: 95.7 (C(1)), 74.1 (C(2)), 79.1 (C(3)), 71.4 (C(4)), 78.9 (C(5)), 62.0 (C(6)). MS (FAB; thioglycerol, negative ions): 1155 ($[M + Cl]^-$), 1119 ($[M-H]^-$), 957 ($[(M - H)\text{-}162]^-$), 795 ($[(M - H)\text{-}324]^-$), 633 ($[(M - H)\text{-}486]^-$), 471 ($[(M - H)\text{-}648]^-$).

3-O-[2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl]hederagenin (= 3β-[(2',4'-Di-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic Acid; **3a**; $C_{48}H_{78}O_{19}$) was obtained after basic

hydrolysis of **3**: white powder, m.p. 245–250° (dec.). ^{13}C-NMR (90.52 MHz, (D_5)pyridine): δ's of the aglycone correspond to those previously described [11]; inner sugar moiety: 103.6 (C(1')), 82.3 (C(2')), 74.7 (C(3')), 80.5 (C(4')), 76.7 (C(5')), 62.8 (C(6')); terminal sugar moieties: 104.7 (C(1")), 76.0 (C(2")), 77.9 (C(3")), 71.7 (C(4")), 78.1 (C(5")), 62.2 (C(6")), 105.3 (C(1"')), 76.7 (C(2"')), 78.2 (C(3"')), 71.6 (C(4"')), 78.3 (C(5"')), 62.4 (C(6"')).

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