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INSECT INHIBITORY STEROIDAL SACCHARIDE ESTERS FROM *PHYSALIS PERUVIANA*[1]

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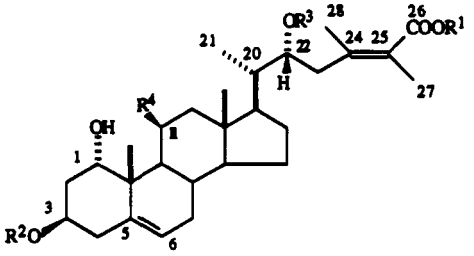
ABSTRACT.—Leaves of *Physalis peruviana* gave glycoside esters derived from (24*E*,22*R*)-1α,22-dihydroxy-3β-(β-D-glucopyranosyloxy)-ergosta-5,24-dien-26-oic acid and from (24*E*,22*R*)-1α,3β,11β,22-tetrahydroxyergosta-5,24-dien-26-oic acid. These compounds reduced the growth of *Helicoverpa zea* larvae to 50% of control size at concentrations of 5–110 ppm in artificial diets, but no toxic effect was observed in a standard brine shrimp bioassay.

Physalis peruviana L. (Solanaceae), native to tropical South America, but naturalized in warmer regions worldwide (1), has found use in traditional medicine. Physiological activity of this plant is likely to be due to the presence of a series of steroidal lactones termed withanolides (2), and a number of these substances have also been reported to be significant defensive agents against insects (3,4). We have examined *P. peruviana* in this respect using as bioassay subjects, larvae of the corn earworm, *Helicoverpa zea* (syn. *Heliothis zea* Boddie), an insect that is an economic pest of numerous crops including the solanaceous plants, tobacco and tomato, and have shown that chromatographic fractions from its leaves are strongly inhibitory to this insect (5). We feel that *P. peruviana* may provide a valuable source of resistance when transferred by intergeneric hybridization into other plant species. Our earlier work led to the identification of glucosides of perulactone (a modified withanolide) and its isomer as defensive agents having activity against *H. zea* in the 150 mg/kg (ppm) range (6); additionally, two new types of steroidal glycoside esters that were about four times more active were identified as **1** and **2** (7). The present paper describes the isolation and structural identification of an additional seven compounds of the latter type, certain of which possess still higher activity against *H. zea*.

RESULTS AND DISCUSSION

Further fractionation of preparative hplc fractions from the MeOH extract (7) of *P. peruviana* leaves gave two sets of glycoside esters. Compounds **3–7** are spectrally very similar to the C-26 esters, **1** and **2**, reported earlier, having glucosylation at position-3, but they possess hydroxyl- instead of acetoxyl-functionalization at C-22 as shown by the absence of the ^{1}H-nmr signal near δ 5.16 and the appearance of new signals in the complex region near δ 3.8 ppm. The small shift in position for the signal of Me-21 to about 1.02 ppm in these compounds now causes this methyl resonance to appear partly superimposed upon that of Me-19. Changes in the ^{13}C-nmr spectra of **3–7** include the appearance of a new peak at δ 72.5 for C-22 and the absence of the formerly observed signal for the acetoxylated carbon near 76 ppm as well as typical shifts to lower field for the adjoining C-20 and C-23 by ca. 3 ppm. The lowered chromatographic retention of **3–7** on reversed-phase material compared to **1** and **2** is a consequence of the increased hydrophilicity of the side-chain caused by the free hydroxyl group at C-22. Compounds **9** and **10** which bear an additional hydroxyl substituent on the steroid nucleus at position-11, but which are not glucosylated at the 3-position, are also eluted before **1** and **2**.

[1]Communication No. 2 on this topic. For Part 1 see Waiss *et al.* (7).



1 R^1=glc-(1→4)-glc-6″-Ac-(1→2)-glc; R^2=glc; R^3=Ac; R^4=H
1a R^1=glc-(1→4)-glc-(1→2)-glc-6′-Ac; R^2=glc; R^3=Ac; R^4=H
2 R^1=glc-6‴-Ac-(1→4)-glc-6″-Ac-(1→2)-glc; R^2=glc; R^3=Ac; R^4=H
2a R^1=glc-(1→4)-glc-6″-Ac-(1→2)-glc-6′-Ac; R^2=glc; R^3=Ac; R^4=H
3 R^1=R^2=glc; R^3=R^4=H
4 R^1=glc-(1→2)-glc; R^2=glc; R^3=R^4=H
5 R^1=glc-(1→2)-glc-6′-Ac; R^2=glc; R^3=R^4=H
5a R^1=glc-(1→2)-glc-6′-Ac; R^2=R^3=R^4=H
6 R^1=glc-(1→4)-glc-(1→2)-glc-6′-Ac; R^2=glc; R^3=R^4=H
6a R^1=glc-(1→4)-glc-(1→2)-glc-6′-Ac; R^2=R^3=R^4=H
7 R^1=glc-(1→4)-glc-6″-Ac-(1→2)-glc-6′-Ac; R^2=glc; R^3=R^4=H
7a R^1=glc-(1→4)-glc-6″-Ac-(1→2)-glc-6′-Ac; R^2=R^3=R^4=H
8 R^1=Me, R^2=glc; R^3=R^4=H
9 R^1=glc; R^2=R^3=H; R^4=OH
10 R^1=glc-(1→2)-glc-6′-Ac; R^2=R^3=H; R^4=OH
12 R^1=glc-(1→4)-glc-6″-Ac-(1→2)-glc-6′-Ac; R^2=R^4=H; R^3=OAc

Compounds **3**–**7**, which contain only β-linked glucose, show characteristic ^{1}H-nmr signals for glycosides esterified at an anomeric position (7,8). Thus, doublets associated with the esterified position (J=8 Hz) were present near δ 5.65 for **4**–**7** and at δ 5.55 for **3** in addition to the resonances for normal anomeric protons which occurred at positions between 4.3 and 4.7 ppm. The resonance of the anomeric proton of the single esterified glucose in **3** appears at slightly higher field than the signal of H-1′ of the di- and triglucosyl esters which show the influence of further glycosidic substitution at position-2′. Compounds **3**–**7** are converted into **8** by methanolytic removal of the entire esterified glycosidic moiety with dilute NaOMe under conditions not affecting normal glycosidic linkages, as had previously been done for **1** (7), showing that in all cases one glucosyl unit is attached at position-3. Solvated crystals of **8**, from MeOH, were satisfactory for X-ray crystallographic analysis (Figure 1), and allowed us to unequivocally define the absolute stereochemistry of the steroidal portion of compounds **1**–**7**. This confirmed our previous assignment of trans-geometry to the 24,25-double bond as well as the *R* configuration of position-22. Linkages of the esterified di- and triglucosides were established by modified Hakomori methylation (9) followed by hydrolysis to the methylated glucoses and conversion to the peracetyl aldonitrile (PAAN) derivatives (10) for glc. Under the methylation conditions any acetate groups were removed, and their positions were methylated. Compounds **4** and **5** gave PAAN's of 3,4,6-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose, indicative of 1″→2′ linkage for the glucoses of these diglucosyl esters. Compounds **6** and **7** additionally gave the PAAN of 2,3,6-tri-*O*-methylglucose; after partial deglucosylation of these materials with β-glucosidase (see below), the remaining esterified diglucosides yielded the PAAN's of 3,4,6-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylglucose. These results showed that the glucose units within the triglucosyl moieties of **6** and **7** were attached 1‴→4″ and 1″→2′.

Upon β-glucosidase treatment of **5**–**7**, rapid hydrolysis of the 3-*O*-glucosyl substituent occurred to give glycosylated derivatives **5a** and **6a** which still retain one acetate

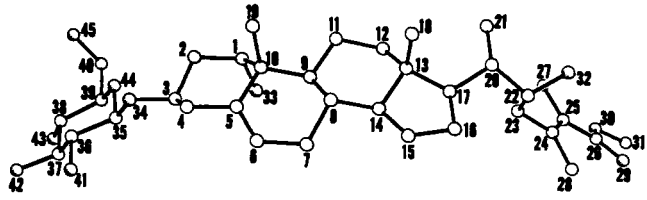


FIGURE 1. Perspective view of compound **8** with crystallographic numbering scheme. Open bonds represent double bonds, and shaded circles represent oxygen atoms.

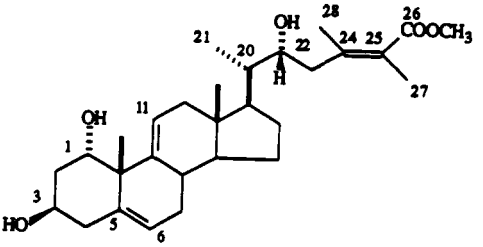
group as well as a diacetyl derivative, **7a**, thus confirming that the position-3 sugar in these compounds bears no substituent. Extended incubation removed the terminal glucose from **6a** yielding an acetoxydiglucoside, but trisaccharide ester **7a** did not undergo enzymatic hydrolysis to a diacetoxydiglucoside; instead slow hydrolytic deacetoxylation took place to give **6a** which then lost glucose. Diglucoside esters, such as that obtained from **4**, were unaffected by the enzyme even when no acetyl group was present. As in the examples of **1** and **2** (7), ^{1}H-nmr signals at δ 4.0–4.3 for the nonequivalent protons of CH_2- groups (partly overlapping in **6** and **7**) indicated that acetylation always occurred at position-6 of the sugars, and this was confirmed by correlated -CH_2OAc signals near δ_C 65, whereas nonacetylated -CH_2OH carbons appeared at about 2 ppm higher field. Mass spectral information allows identification of the position for the acetylated sugar(s) in favorable cases since successive loss of glucosyl units may occur from the parent ion produced by "soft ionization" methods such as the liquid secondary ion (lsims) and fast-atom bombardment (fab) techniques (11). Protonated molecular ions are favored by incorporation of the sample into pure glycerol matrix; however, use of NaI/glycerol, which was frequently necessary in order to obtain sufficient intensity of high mass peaks, gave almost exclusive formation of $[M+Na]^+$. In our experiments, we observed that the latter ionic species lost their esterified saccharide moieties almost entirely as intact blocks and could not be used in establishing details of substitution. Monodeglucosylated derivatives, **5a**, **6a**, and **7a**, are especially useful for the purpose of observing sequential loss of sugar units since this can occur only from the side-chain ester in these compounds, thereby avoiding ambiguity. Thus, for **5a**, loss of H_2O and one glucosyl unit (without loss of acetyl) from the protonated molecular ion shows that the acetate group must be attached to the remaining glucose (position-6′). Similarly, the monoacetyl triglucoside ester **6a** shows loss of H_2O and two unsubstituted glucose units, and the diacetyl triglucoside ester **7a** loses H_2O and one glucose from the parent ion indicating that acetylation occurs at position-6′ and at positions-6′ and -6″, respectively. The monoacetyl diglucoside resulting from prolonged enzymatic treatment of **6a** was identical to **5a**.

It can be seen that the locations for glucose acetoxylation in triglucoside esters **6** and **7** are different from those assigned earlier (7) for **1** and **2**. However, the ^{1}H- and ^{13}C-nmr sugar signals for the corresponding compound in each pair are very similar in position and appearance; and this strongly indicates that both sets of mono- and diacetates have a common substitution pattern. Original assignments had been based on indirect evidence which included the inability of β-glucosidase to effect side-chain hydrolysis of **2**, a result that now appears not necessarily only to be a consequence of acetoxylation upon the terminal sugar, but rather is an effect which occurs also when the central sugar bears an acetate as in **7**. To examine this point further, we determined the mass spectrum of compound **12** (obtained earlier by β-glucosidase action on **2**) under conditions favoring sequential loss of sugar units. Loss of H_2O and one glucose from the protonated molecular

ion occurred (the same result as from **7a**), showing that the terminal glucose of **12** is non-acetoxylated, and that we had incorrectly assigned the positions of the substituents in the parent compound. The correct location of acetate units is given by structure **2a**, a result in accord with the nmr comparison. Compound **1** was no longer available for mass spectral examination; however, the above nmr information indicates that its structure should be revised to **1a**.

The mono- and diglucosyl esters, **9** and **10**, have steroidal systems similar to those of **1–7**, but an additional hydroxyl substituent is present. This can be located at position-11β, as the ^{1}H-nmr spectra of both **9** and **10** show shifts of about +0.22 ppm for methyls -18 and -19, a value in excellent agreement with that reported for 11β-hydroxy steroids in the 5α-series (12). This is the only position in which a hydroxyl group can induce a shift of this magnitude upon both angular methyl groups. Also, the ^{13}C-nmr spectra of **9** and **10** are in accord with hydroxylation at position-11, with appropriate shifts for this position as well as adjoining ring positions. Again, simultaneous effects occur upon methyls-18 and -19 (ca. +2.4 and 3.0 ppm respectively, compared to non-hydroxylated examples), and this can only be induced by a hydroxyl group at position-11β (13). Further evidence for a position-11 hydroxyl is provided by elimination of this group during acidic methanolysis of **10** to give **11**. Methyls -18 and -19 now give signals at δ_H 0.69 and 1.21, shifted as reported for $\Delta^{9,11}$-androstanes (12) and for cholesta-4,9(11)-dien-3α-ol (14). Compound **10** was shown to be a 1″→2′ diglucoside by methylation analysis as above, and the position of its anomeric ester signal in the ^{1}H-nmr spectrum was δ 5.65 compared to 5.54 for **9**, an effect of glycosylation at 2′ which was also present in the respective ^{13}C-nmr spectra. The position of acetoxylation was established at 6′ by mass spectral fragmentation analogous to that of **5a**.

Artificial diets (15) containing the test compounds at several levels were presented to larval *H. zea*, and their growth was determined after a 10-day period. The most active substance was the 11-hydroxy diglucoside ester, **10**, which reduced the weight of larvae to 50% of control values (ED_{50}) at a dietary concentration of 5.4 ppm (6.3×10^{-6} moles/kg). The triglucoside esters with 3-*O*-glucosyl substitution, **6** and **7**, had ED_{50}s of 15 and 50 ppm (1.3 and 4.2×10^{-5} mole/kg), respectively, which may be compared to 35 ppm (ca. 3×10^{-5} mole/kg) for both **1a** and **2a** which are analogous compounds having acetoxylation at position-22. Monoglucoside ester, **3**, had an ED_{50} of 85 ppm, the corresponding diglucoside esters **4** and **5** had respective ED_{50}s of 64 and 22 ppm, and the least active ester was the 11-hydroxy monoglucoside ester, **9**, at 110 ppm (1.7×10^{-4} mole/kg). By comparison, 4β-hydroxywithanolide E, which is found in *P. peruviana* at concentrations (dry basis) of over 2000 mg/kg, has an ED_{50} of about 250 ppm. The glycosylated modified withanolide, perulactone 3-*O*-β-D-glucoside, present at about 600 mg/kg, had an ED_{50} of 150 ppm. Allowing for the H_2O content of fresh leaves, it can been seen that these substances as well as compounds **6** and **10** are present in a sufficiently high concentration to contribute strongly toward plant resistance, especially



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if their effects are additive as is the likely case. For all compounds of the present study there is no clear structure-activity relationship, but the most striking difference is that between **9** and **10**, which show about a 27-fold difference in activity. Their structures differ only by a single acetoxy glucose unit, and their polarities (as estimated by chromatographic partitioning between the stationary and mobile phases) are very similar. There are no obviously reactive groups, so the differences in insect inhibitory effect are governed by very subtle factors. It is likely that growth inhibition is a consequence of feeding deterrence, leading to semi-starvation of the animals. We observed that these compounds are not lethal over the range of concentrations evaluated; for example, **10** was tested at 10 times the ED_{50}, and all animals lived. Growth was 4% of control weight at this level, and even at 20 times the ED_{50}, 9 of 10 larvae remained alive with average growth at only 1% of control. This is consistent with the behavior of *H. zea* on fresh *P. peruviana* leaves where the larvae search and sample without settling down to feed. These leaves show a fine pattern of "shotgun" holes instead of the usual serrated feeding zones on preferred hosts where a large amount of plant material has been ingested.

We examined the potential toxicity of the glycoside esters upon brine shrimp, with a standard bioassay that has been developed for cytotoxicity screening (16) using concentrations of 1000 ppm for **6**, **7**, and **10**. Compound **4** was soluble only to the extent of 500 ppm, and **5** and **9** were solubility limited at 250 ppm. For all test compounds the survival of shrimp was nearly 100%, showing that the substances are not generally toxic, a result in agreement with the survival of insect larvae. It is important to emphasize here that when feeding can be suppressed, toxicity is not a sufficient criterion in screening for plant protection as insect resistance is conferred equally well by deterrence. It is critically important to use the appropriate experimental subject, i.e., the targeted pest, for bioassays in screening sources of resistance.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra and specific rotations were determined on Perkin-Elmer 237 and 241 instruments, respectively, and uv spectra were taken using a Hewlett-Packard 8451 spectrophotometer. ^{1}H-Nmr spectra were obtained on Varian EM-390 and Nicolet NT-200 spectrometers at 90 and 200 MHz, respectively, and ^{13}C-nmr spectra were taken at 50 MHz on the latter instrument. Nmr assignments were facilitated by ^{1}H-^{1}H and ^{13}C-^{1}H 2D correlation techniques and by comparison with spectra of known compounds (7,17). Sugar signal assignments are indicated by primed numbers starting with the ester-bound glucose. Ei (70 ev) mass spectra and liquid secondary ion (lsims) mass spectra (glycerol or glycerol plus NaI matrices) were determined on a VG micromass 7070 HS instrument. Glc analyses of methylated glucose peracetyl aldonitrile derivatives were on a Hewlett-Packard model 5880 gas chromatograph using a 0.53 mm×15 m DB-17 fused silica column at 170° with He flow of 5 ml/min^{-1}. Initial lc was on Sephadex LH-20 followed by prep. hplc on Rainin Dynamax and Microsorb C-18 columns with detection by uv at 215 and 232 nm, and the activity of fractions was followed by bioassay.

PLANT MATERIAL.—Seeds of *Physalis peruviana* were obtained locally, and a voucher specimen is at the State of California Division of Plant Industry, Analysis and Identification Branch, Sacramento, CA (G.D. Barbe, 1990). Plants were greenhouse grown, and leaves were harvested at intervals and freeze-dried.

BIOASSAYS.—Solutions were evaporated onto cellulose powder (5% of final diet wt.) at various dietary levels for combination with modified Berger-diet premix (15). The test diets were divided into 10 portions, placed in individual plastic cups, and neonate larvae of *H. zea* were added. The insects were maintained at 26° for ten days, and their weights were then compared with those of controls grown on diets containing as additive only 5% cellulose powder.

Brine shrimp bioassays (16) were run on solutions of test compounds in 5 ml of brine solution for 24 h, and mortality was compared to that of control solutions without additive.

EXTRACTION, FRACTIONATION, AND DERIVATIZATION.—Finely ground dry leaf material, 2000 g, was extracted and given preliminary purification as previously described (7). Prep. hplc separation on Dynamax C-18 (41.2 mm×25 cm) using a gradient of 25 to 40% aqueous CH_3CN at 40 ml min^{-1} with injection

volumes of 4 ml each containing 1.0 g of material yielded active fractions: Fr. 3, 420–510 ml (containing **4** and **6**) and Fr. 4, 510–620 ml (containing **3**, **5**, **7**, **9**, and **10**). Fraction 4, when isocratically rechromatographed on the same column with 35% CH_3CN/H_2O gave zones containing **3**, **5**, and **7** (225–290 ml) and **9** and **10** (290–310). Final separations were on Microsorb C-18 using the conditions shown below.

Enzymatic hydrolyses.—Typically, solutions of β-glucosidase (EC 3.2.1.21, Sigma G 0395) were prepared in 0.1 M pH 5 acetate buffer at a concentration of 1.0 mg/ml. The glycoside (1.0 mg/ml) was then added, and progress of hydrolysis at 35° was followed by hplc. Removal of glucose at position-3 was complete within 2 h, but hydrolysis of the terminal glucose of C-26 esterified triglucoside moieties required much longer. In these cases, additional 1.0 mg/ml portions of enzyme were added at twice daily intervals for up to three days. Diglucoside (sophoroside) units esterified to C-26 did not undergo hydrolysis by β-glucosidase in any of the cases examined. Product isolation was carried out by addition of XAD-2 resin (1.0 ml per mg of glycoside hydrolyzed) and stirring for 1 h followed by filtration of the resin. After an H_2O rinse, the resin was treated with MeOH to displace adsorbed materials which were then separated by hplc.

Basic methanolysis of the glycoside esters.—Compounds **3**–**7**, 10 mg, were allowed to remain 4 h in 0.3 ml of methanolic 0.1 M NaOMe. The solutions were diluted with 0.5 ml of 40% CH_3CN and chromatographed directly on Microsorb C-18, 10×250 mm, 40% CH_3CN, 40–45 ml to give **8**. Mp 252–253° (MeOH), spectroscopically identical to previously reported values (5).

Sugar linkage analysis.—Permethylation of glycosides was carried out by a modified Hakomori procedure using NaOH/DMSO and CH_3I (9). After extractive workup with aqueous $CHCl_3$ and evaporation, the samples were heated at 100° with 2 M trifluoroacetic acid and dried under nitrogen at 80°. Preparation of peracetyl aldonitrile derivatives was done in pyridine by first warming with hydroxylamine HCl at 90° for 20 min followed by Ac_2O for 20 min (10).

Compound **3**.—Microsorb C-18, 41.2×250 mm, 25% aqueous CH_3CN, 880–940 ml, amorphous solid, 12 mg. $[\alpha]^{25}D$ −21° (*c*=0.4, MeOH); ir ν max (KBr) 1710, 1625 cm^{-1}; uv λ max (MeOH) 230 (log ε max 4.00) nm; ^1H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, *J*=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.94 (d, *J*=1 Hz, H_3-27), 2.07 (d, *J*=1 Hz, H_3-28), 3.14 (t, *J*=8 Hz, H-2″), 4.05 (m, H-3), 4.37 (d, *J*=8 Hz, H-1″), 5.52 (m, H-6), 5.55 (d, *J*=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^-$ 785 (28); other ions at *m/z* 605 (18) [MH−H_2O−glc]$^+$, 443 (67) [MH−H_2O−2 glc]$^-$, 425 (90) [MH−2 H_2O−2 glc]$^+$, 407 (100) [MH−3 H_2O−2 glc]$^+$. $C_{40}H_{65}O_{15}$ requires 785.

Compound **4**.—Microsorb C-18, 21.4×250 mm, 24% aqueous CH_3CN, 205–225 ml, amorphous solid, 104 mg. $[\alpha]^{25}D$ −34° (*c*=0.5, MeOH); ir ν max (KBr) 1710, 1625 cm^{-1}; uv λ max (MeOH) 234 (log ε max 4.12) nm; ^1H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, *J*=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.97 (br s, H_3-27), 2.11 (d, *J*=1 Hz, H_3-28), 4.05 (m, H-3), 4.38 (d, *J*=8 Hz, H-1‴), 4.62 (d, *J*=8 Hz, H-1″), 5.52 (m, H-6), 5.65 (d, *J*=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^+$ 947 (16); other ions at *m/z* 785 (12) [MH−glc]$^+$, 767 (5) [MH−H_2O−glc]$^-$, 623 (8) [MH−2 glc]$^+$, 605 (18) [MH−H_2O−2 glc]$^+$, 443 (71) [MH−H_2O−3 glc]$^+$, 425 (86) [MH−2 H_2O−3 glc]$^+$, 407 (100) [MH−3 H_2O−3 glc]$^-$. $C_{46}H_{75}O_{20}$ requires 947.

Compound **5**.—Microsorb C-18, 41.2×250 mm, 25% aqueous CH_3CN, 940–1030 ml, amorphous solid, 75 mg. $[\alpha]^{25}D$ −26° (*c*=0.5, MeOH); ir ν max (KBr) 1720 br, 1625 cm^{-1}; uv λ max (MeOH) 234 (log ε max 4.06) nm; ^1H nmr (CD_3OD) δ 0.77 (s, H_3-18), 1.01 (d, *J*=7 Hz, H_3-21), 1.03 (s, H_3-19), 1.94 (br s, H_3-27), 2.05 (s, OAc), 2.10 (br s, H_3-28), 4.05 (m, H-3), 4.14 (dd, *J*=12 and 5 Hz, 1/2H_2-6′), 4.26 (br d, *J*=12 Hz, 1/2H_2-6′), 4.38 (d, *J*=8 Hz, H-1‴), 4.65 (d, *J*=8 Hz, H-1″), 5.52 (m, H-6), 5.66 (d, *J*=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^+$ 989 (20); other ions at *m/z* 827 (6) [MH−glc]$^+$, 809 (5) [MH−H_2O−glc]$^+$, 647 (12) [MH−H_2O−2 glc]$^-$, 605 (16) [MH−H_2O−glc−glc Ac]$^+$, 443 (66) [MH−H_2O−2 glc−glc Ac]$^+$, 425 (100) [MH−2 H_2O−2 glc−glc Ac]$^+$, 407 (98) [MH−3 H_2O−2 glc−glc Ac]$^+$. $C_{48}H_{73}O_{21}$ requires 989.

β-Glucosidase treatment of **5** for 4 h gave **5a** which was isolated by adsorption on XAD-2 resin followed by hplc: Microsorb C-18, 10×250 mm, 30% aqueous CH_3CN, 65–72 ml. ^1H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, *J*=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.94 (br s, H_3-27), 2.04 (s, OAc), 2.09 (d, *J*=1 Hz, H_3-28), 3.19 (t, *J*=8 Hz, H-2″), 4.13 (dd, *J*=12 and 5 Hz, 1/2 H_2-6′), 4.25 (br d, *J*=12 Hz, 1/2 H_2-6′), 4.65 (d, *J*=8 Hz, H-1″), 5.49 (m, H-6), 5.65 (d, *J*=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^+$ 827 (34); other ions at *m/z* 809 (5) [MH−H_2O]$^-$, 647 (10) [MH−H_2O−glc]$^+$, 443 (56) [MH−H_2O−glc−glc Ac]$^-$, 425 (40) [MH−2 H_2O−glc−glc Ac]$^-$, 407 (25) [MH−3 H_2O−glc−glc Ac]$^-$. $C_{42}H_{67}O_{16}$ requires 827.

Compound **6**.—Microsorb C-18, 21.4×250 mm, 24% aqueous CH_3CN, 276–300 ml, amorphous solid, 317 mg. $[\alpha]^{25}D$ −16° (*c*=0.5, MeOH); ir ν max (KBr) 1715 br, 1620 cm^{-1}; uv λ max (MeOH) 234 (log ε max 4.01) nm; ^1H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, *J*=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.93 (br s, H_3-27), 2.06 (s, OAc), 2.08 (br s, H_3-28), 4.05 (m, H-3), 4.28 (d, *J*=8 Hz, H-1‴), ca. 4.3 (m, obscured,

H_2-6′), 4.38 (d, J=8 Hz, H-1‴), 4.68 (d, J=8 Hz, H-1″), 5.52 (m, H-6), 5.65 (d, J=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^+$ 1151 (12); other ions at *m/z* 1133 (2) [MH−H_2O]$^+$, 971 (4) [MH−H_2O−glc]$^+$, 647 (6) [MH−H_2O−3 glc]$^+$, 605 (7) [MH−H_2O−2 glc−glc Ac]$^+$, 443 (30) [MH−H_2O−3 glc−glc Ac]$^+$, 425 (52) [MH−2 H_2O−3 glc−glc Ac]$^+$, 407 (49) [MH−3 H_2O−3 glc−glc Ac]$^+$. $C_{54}H_{87}O_{26}$ requires 1151.

β-Glucosidase treatment of **6** for 16 h gave **6a** which was isolated by adsorption on XAD-2 resin followed by hplc: Microsorb C-18, 10×250 mm, 30% aqueous CH_3CN, 42–46 ml. ^{1}H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, J=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.93 (br s, H_3-27), 2.05 (s, OAc), 2.09 (d, J=1 Hz, H_3-28), 4.25 (dd, J=12 and ca. 5 Hz, partly obscured, 1/2 H_2-6′), 4.28 (d, J=8 Hz, H-1‴), 4.35 (br d, J=12 Hz, 1/2 H_2-6′), 4.66 (d, J=8 Hz, H-1″), 5.49 (m, H-6), 5.64 (d, J=8 Hz, H-1′); lsims (glycerol *m/z* [MH]$^+$ 989 (100); other ions at *m/z* 971 (10) [MH−H_2O]$^+$, 809 (8) [MH−H_2O−glc]$^+$, 647 (28) [MH−H_2O−2 glc]$^+$, 443 (87) [MH−H_2O−2 glc−glc Ac]$^+$, 425 (63) [MH−2 H_2O−2 glc−glc Ac]$^+$. $C_{48}H_{77}O_{21}$ requires 989. After 5 days, β-glucosidase gave about 50% conversion of **6a** into **5a**, identical to material obtained above. Methylation linkage analysis was performed on this material.

Compound **7**.—Microsorb C-18, 41.2×250 mm, 25% aqueous CH_3CN, 1030–1105 ml, amorphous solid, 60 mg. [α]^{25}D −17° (*c*=0.5, MeOH); ir ν max (KBr) 1720 br, 1625 cm^{-1}; uv λ max (MeOH) 234 (log ε max 4.02) nm; ^{1}H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, J=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.93 (br s, H_3-27), 2.05 (s, OAc), 2.09 (br s, H_3-28 and OAc), 4.05 (m, H-3), ca. 4.1-4.5 (complex H_2-6′, H_2-6″, H-1‴ and H-1⁗), 4.68 (d, J= 8 Hz, H-1″), 5.54 (m, H-6), 5.66 (d, J=8 Hz, H-1′); lsims (glycerol+NaI) *m/z* [M+Na]$^+$ 1215 (62), [MH]$^+$ 1193 (14); other ions at *m/z* 1013 (14) [MH−H_2O−glc]$^+$, 851 (15) [MH−H_2O−2 glc]$^+$. $C_{56}H_{88}O_{27}$+Na requires 1215. $C_{56}H_{89}O_{27}$ requires 1193.

β-Glucosidase treatment of **7** for 14 h gave **7a** which was isolated by adsorption on XAD-2 resin followed by hplc: Microsorb C-18, 10×250 mm, 30% aqueous CH_3CN, 50–54 ml. ^{1}H nmr (CD_3OD) δ 0.76 (s, H_3-18), 1.01 (d, J=7 Hz, H_3-21), 1.02 (s, H_3-19), 1.93 (br s, H_3-27), 2.05 (s, OAc), 2.08 (br s, H_3-28 and OAc), ca. 4.12 (dd, J=12 and 6 Hz, 1/2 H_2-6″), 4.25 (dd, J=12 and 4 Hz, 1/2 H_2-6′), ca. 4.33 (m, obscured, H_2-6′), 4.33 (d, J=8 Hz, H-1‴), 4.46 (br d, J=12 Hz, 1/2 H_2-6′), 4.67 (d, J=8 Hz, H-1″), 5.50 (m, H-6), 5.65 (d, J=8 Hz, H-1′); lsims (glycerol) *m/z* [MH]$^+$ 1031 (42); other ions at *m/z* 1013 (8) [MH−H_2O]$^+$, 851 (15) [MH−H_2O−glc]$^+$, 647 (14) [MH−H_2O−glc−glc Ac]$^+$, 443 (78) [MH−H_2O−*glc*−2 *glc* Ac]$^+$, 425 (59) [MH−2 H_2O−glc−2 glc Ac]$^+$. $C_{50}H_{79}O_{22}$ requires 1031. If hydrolysis was allowed to proceed over a 7 day period with twice daily addition of enzyme, a chromatographic peak corresponding to the monoacetyl triglucoside, **6a**, appeared and increased to a steady state level of about 15% of the total material for most of this time before decreasing. The nonenzymatic product was converted to **5a** by action of the enzyme, and this monoacetyl diglucoside was subjected to methylation linkage analysis. It was observed that a small amount of completely deacetylated material also had been formed by the end of the incubation period.

Compound **9**.—Microsorb C-18, 21.4×250 mm, 25% aqueous CH_3CN, 260–275 ml, mp 210–211° (MeOH-H_2O), 55 mg. [α]^{25}D −9.4° (*c*=0.5, MeOH); ir ν max (KBr) 1705, 1625 cm^{-1}; uv λ max (MeOH) 232 (log ε max 3.97) nm; ^{1}H nmr (CD_3OD) δ 0.98 (s, H_3-18), 1.03 (d, J=7 Hz, H_3-21), 1.24 (s, H_3-19), 1.93 (d, J=1 Hz, H_3-27), 2.06 (d, J=1Hz, H_3-28), 3.68 (dd, J=12 and 5 Hz, 1/2 H-6′), 4.04 (m, H-1), 4.35 (m, H-11), 5.36 (m, H-6), 5.54 (d, J=8 Hz, H-1′); ^{13}C nmr, see Table 1; lsims (glycerol+NaI) *m/z* [M+Na]$^+$ 661.38. $C_{34}H_{54}O_{11}$+Na requires 661.356.

Compound **10**.—Microsorb C-18, 21.4×250 mm, 25% aqueous CH_3CN, 300–320 ml, amorphous solid, 90 mg [α]^{25}D −19° (*c*=0.5, MeOH); ir ν max (KBr) 1720 br, 1625 cm^{-1}; uv λ max (MeOH) 234 (log ε max 3.97) nm; ^{1}H nmr (CD_3OD) δ 0.97 (s, H_3-18), 1.04 (d, J=7 Hz, H_3-21), 1.25 (s, H_3-19), 1.93 (br s, H_3-27), 2.05 (s, OAc), 2.10 (d, J=1 Hz, H_3-28), 3.20 (t, J=8 Hz, H-2″), 4.05 (m, H-1), 4.14 (dd, J=12 and 4 Hz, 1/2 H_2-6′), 4.26 (br d, J=12 Hz, 1/2 H_2-6′), 4.38 (m, H-11), 4.64 (d, J=8 Hz, H-1″), 5.37 (m, H-6), 5.65 (d, J=8 Hz, H-1′); ^{13}C nmr, see Table 1; lsims (glycerol+NaI) *m/z* [M+Na]$^+$ 865.45, lsims (glycerol) *m/z* [MH]$^+$ 843 (10); other ions at *m/z* 825 (4) [MH−H_2O]$^+$, 807 (2) [MH−2 H_2O]$^+$, 645 (6) [MH−2 H_2O−glc]$^+$, 441 (16) [MH−2 H_2O−glc−glc Ac]$^+$. $C_{42}H_{66}O_{17}$+Na requires 865.420, $C_{42}H_{67}O_{17}$ requires 843.

Compound **11**.—To a solution of methanolic 1 M HCl, prepared by addition of 0.25 ml AcCl to 5.0 ml MeOH, was added 20 mg of **10**, and the mixture was refluxed 3 h. After evaporation, the product was purified on Microsorb C-18, 10×250 mm to give 8.0 mg of **11**, ret'n. vol 33–36 ml, mp 177–180° (MeOH). Ir ν max ($CHCl_3$) 3600, 3550 br, 3010, 1710 br, 1625 cm^{-1}; ^{1}H nmr ($CDCl_3$) δ 0.69 (s, H_3-18), 1.02 (d, J=7 Hz, H_3-21), 1.21 (s, H_3-19), 1.93 (d, J=1.4 Hz, H_3-27), 2.00 (d, J=1.4 Hz, H_3-28), 3.74 (s, OMe), 3.90 (br d, J=12 Hz, H-22), 4.04 (7 lines, J=6 Hz, H-3), 4.21 (br s, H-1), 5.62 (br d, J=6 Hz, H-6), 5.72 (br d, J=6 Hz); ^{13}C nmr, see Table 1; eims *m/z* M$^+$ 472.3173 (78), [M−H_2O]$^+$ 454 (43), [M−2 H_2O]$^+$ 436 (9), [M−$C_7H_{12}O_2$]$^+$ 344 (14), [$C_7H_{12}O_2$]$^+$ 128 (100), [$C_7H_{12}O_2$−MeOH]$^+$ 96 (54). $C_{29}H_{44}O_5$ requires 472.3189.

TABLE 1. ^{13}C-Nmr Data[a] for Compounds **9–11**.

Carbon	Compound			Carbon	Compound		
	9	**10**	**11**		**9**	**10**	**11**
1	73.8[b]	73.8[b]	72.9	1′	95.6	93.9	—
2	39.1	39.1	36.5	2′	73.8[b]	82.1	—
3	66.4	66.5	67.3	3′	78.1[d]	77.6[c]	—
4	41.5	41.6	41.5[b]	4′	71.1	70.7	—
5	140.5	140.5	136.0	5′	78.7[d]	75.3	—
6	123.7	123.7	124.3	6′	62.3	64.9	—
7	33.4	33.5	31.1	1″	—	105.2	—
8	29.8	29.9	34.6	2″	—	75.8	—
9	46.6	46.6	142.3	3″	—	78.2[c]	—
10	42.90[c]	43.0[b]	45.2	4″	—	71.3	—
11	68.6	68.7	119.0	5″	—	78.7[c]	—
12	50.2	50.2	41.6[b]	6″	—	62.3	—
13	42.88[c]	42.9[b]	41.5[b]	OAc(Me)	—	20.9	—
14	59.7	59.8	52.4	OAc(CO)	—	172.9	—
15	25.5	25.6	25.3	OMe	—	—	51.4
16	28.4	28.5	27.6				
17	54.9	54.9	53.1				
18	14.6	14.6	11.4				
19	23.0	23.1	26.7				
20	44.2	44.3	41.5				
21	12.9	12.9	12.0				
22	72.5	72.5	71.2				
23	37.4	37.8	36.0				
24	148.8	150.5	143.0				
25	124.8	124.5	125.8				
26	169.6	168.7	170.1				
27	15.9	15.9	15.9				
28	22.1	22.3	21.2				

[a]In ppm for CD_3OD solutions of **9** and **10** and $CDCl_3$ for **11**.
[b–d]Values within columns may be interchanged.

Mass spectral data for compound **12**.—Lsims (glycerol) *m/z* $[M+Na]^+$ 1095 (6), $[MH]^+$ 1073 (2); other ions at *m/z* 1055 (2) $[MH-H_2O]^+$, 893 (5) $[MH-H_2O-glc]^+$, 689 (6) $[MH-H_2O-glc-glc\ Ac]^+$, 485 (15) $[MH-H_2O-glc-2\ glc\ Ac]^+$, 443 (82) $[MH-H_2O-Ac-glc-2\ glc\ Ac]^+$, 425 (75) $[MH-2\ H_2O-Ac-glc-2\ glc\ Ac]^+$. $C_{52}H_{80}O_{23}$+Na requires 1095, $C_{52}H_{81}O_{23}$ requires 1073.

CRYSTAL STRUCTURE OF COMPOUND **8**.[2]—$C_{35}H_{56}O_{10}\cdot CH_3OH$, monoclinic space group $P2_1$, a=7.709 (1), b=10.644(1), c=21.848(3) Å, $\angle\beta$=93.41(1)°. U=1789.4 Å^3, Z=2, D_c=1.24 g cm^{-3}, F(000)=727.9, μ(Cu-Kα)=7.03 cm^{-1}; final R=0.038 (425 parameters), R_w=0.043 for 3549 unique reflections, average parameter shift is ±0.03 σ, and difference Fourier synthesis excursions are within ±0.2 Å^{-3}. Small colorless, tetragonal crystals of fair quality were obtained from MeOH by slow evaporation.

DATA COLLECTION AND STRUCTURAL REFINEMENT.—Intensity data were collected in the range of $3° \leq 2\theta \leq 114°$ on a Nicolet R3 diffractometer with graphite monochromatized Cu-Kα radiation (λ=1.5418 Å) by the θ–2θ scan technique with variable scan speed (4–30° min^{-1}) at room temperature. The intensity data were corrected for background and Lorentz-polarization effects (18), but not for absorption. The crystal structures were solved by direct methods and refined by a "blocked cascade" full-matrix least squares procedure with the SHELXTL (19) program package. The function minimized was $[\Sigma\omega(|F_o|-|F_c|)^2]$, where $\omega=[\sigma^2|F_o|+0.001\ |F_o|^2]^{-1}$. Unique reflections with the criteria of ($|F_o| \geq 3\sigma|F_o|$) were included in the

[2]Atomic coordinates, bond lengths and bond angles, anisotropic thermal parameters for the non-hydrogen atoms, positional parameters for the hydrogen atoms, and observed-calculated structure factors have been deposited at the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK.

structure refinement calculation. Scattering factors were from "International Tables for X-ray Crystallography (20); those of oxygen were corrected for anomalous dispersion. A secondary extinction correction (0.0050) was included in the final cycles of refinement to minimize the discrepancy between observed and calculated structure factors of the most intense reflections, which led to a small improvement in R-index. Positions of all non-hydrogen atoms were refined anisotropically, and all hydrogen positions were estimated, but verified in subsequent difference Fourier maps and included at invarient idealized values in the respective structure-factor calculation. The absolute configuration of the structure was determined by applying Hamilton's statistical method (21) of comparing the R-values for the two enantiomeric structures; the ratio of the two final R_w values is 1.0026, which has a significance level better than 0.5%.

CHARACTERISTICS OF STRUCTURE.—The absolute configuration of **8** is: 1*S*, 3*R*, 8*S*, 9*S*, 10*R*, 13*S*, 14*S*, 17*R*, 20*S*, 22*R*, 35*R*, 36*R*, 37*S*, 38*S*, and 39*R*. The molecular structure and absolute configuration of **8** with the numbering scheme used in the X-ray investigation is shown in Figure 1. The crystal structure consists of solvent of crystallization; one molecule of MeOH per formula unit. Each MeOH molecule [C(46) and O(47)] forms two relatively strong intermolecular hydrogen bonds [O(47)...O(33), 2.74 Å and O(47)...O(32) 2.79 Å] with the two hydroxy groups on the steroidal portion of the host molecule enhancing a compact and stable molecular packing in the crystal.

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