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NARCISSIFLORINE, NARCISSIFLORININE AND NARCISSIFLORIDINE, THREE TRITERPENE SAPONINS FROM *ANEMONE NARCISSIFLORA*

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Key Word Index—*Anemone narcissiflora*; Ranunculaceae; narcissiflorine; narcissiflorinine; narcissifloridine; saponins; oleanolic acid derivatives.

Abstract—Narcissiflorine, narcissiflorinine and narcissifloridine, three new saponins, have been isolated from the ethanolic extract of *Anemone narcissiflora* (Ranunculaceae). The structural elucidation of narcissiflorine, narcissiflorinine and narcissifloridine has showed them to be [α-L-arabinofuranosyl-(1 → 4)-β-D-glucuronopyranosyl-(1 → 3)]-3β-hydroxy-olean-12-en-28-oic acid, [α-L-arabinofuranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucuronypyranosyl-(1 → 3)]-3β-hydroxy-olean-12-en-28-oic acid and [α-L-arabinofuranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)]-3-β-hydroxy-olean-12-en-28-oic acid, respectively.

INTRODUCTION

Several saponins [1–8] have been isolated from various species of the genus *Anemone* but complete structures of very few saponins have been elucidated [5–9]. Oleanolic acid, betulinic acid, sitosterol, β-amyrin and olean-3-*O*-α-L-rhamnopyransoyl-Δ^{12}-en-28-oic acid from *A. narcissiflora* have already been reported [9]. The present investigation deals with the isolation and structural elucidation of three new triterpene saponins, narcissiflorine, narcissiflorinine and narcissifloridine.

RESULTS AND DISCUSSION

Narcissiflorine, $C_{41}H_{64}O_{13}$, gave all the tests of saponins [10] and on hydrolysis with 7% H_2SO_4 yielded oleanolic acid (IR, ^{1}H NMR, MS) [11–14], D-glucuronic acid and L-arabinose (co-PPC). The sugars were present in equimolar proportion (1:1) as revealed by colorimetric estimation [15], and the genin content was 59.5% (quantitative hydrolysis) against 59.68% calculated for 1 unit of oleanolic acid and 2 units of sugars per molecule of narcissiflorine. Therefore, it was concluded that narcissiflorine contains 1 mol each of oleanolic acid, D-glucuronic acid and L-arabinose.

The saponin was not hydrolysed with 5N NH_4OH which is a specific reagent for the hydrolysis of sugar esters [16], indicating that sugars were not present in an ester combination with the COOH group at C-28. This led to the conclusion that both sugars were linked as a bioside unit to the hydroxyl at C-3.

The sequence of the sugar moieties in narcissiflorine was determined by partial hydrolysis of the saponin whereupon prosapogenin PS_1 was isolated and the presence of L-arabinose was confirmed in the hydrolysate by co-PPC. Hydrolysis of prosapogenin PS_1 yielded oleanolic acid and D-glucuronic acid, indicating that L-arabinose was the end sugar and D-glucuronic acid was directly linked to the genin.

On treatment with CH_2N_2 narcissiflorine formed a dimethyl ester (**1**) which on reduction with $LiAlH_4$ gave a glycoside (**2**). On hydrolysis the glycoside (**2**) yielded erythrodiol, D-glucose and L-arabinose. The erythrodiol content was *ca* 60% and the ratio of D-glucose to L-arabinose, determined colorimetrically [15] was 1:1. Since narcissiflorine contained D-glucuronic acid, which was not present in the glycoside (**2**), it may be concluded that the D-glucose unit present in **2** resulted from D-glucuronic acid during reduction of **1**. Partial hydrolysis of glycoside **2** yielded another prosapogenin, PS_2. Acid hydrolysis of PS_2 yielded a genin content of 73% and a D-glucose content of 27% (by difference). Permethylation of PS_2 followed by hydrolysis and co-PPC examination of the hydrolysate showed the presence of 2:3:4:6-tetra-*O*-methyl-D-glucose indicating that C-1 of the glucose unit was linked to the C-3 of the genin and the D-glucose moiety was present as a pyranoside. Glycoside **2** was permethylated and hydrolysed to give 2,3,6-tri-*O*-methyl-D-glucose and 2,3,5-tri-*O*-methyl-L-arabinose. Release of 2,3,5-tri-*O*-methyl-L-arabinose clearly suggested that the arabinose unit in **2** was present as a furanoside leaving its anomeric OH for glycosidic linkage. Since the structure of PS_2 had already been established, the formation of 2,3,6-tri-*O*-methyl D-glucose can only be explained by assuming that C-4 of the glucose moiety in the pyranose form was involved in the formation of a glycosidic linkage with C-1 of L-arabinose. The size of the rings and the nature of glycosidic linkages in **2** were also supported by periodate oxidation [17].

Hydrolysis of narcissiflorine with the enzyme diastase yielded only L-arabinose as free sugar indicating that L-arabinose was linked to glucuronic acid through an α-linkage and glucuronic acid was linked to the aglycone through a β-linkage. The observed molecular rotation, $[M]_D$, for **1** was in good agreement with that calculated for the above configuration on the basis of Klyne's rule [18–20] (Table 1). Hence, narcissiflorine is [α-L-

Table 1. $[M]_D$ values for narcissiflorine dimethyl ester (1) and narcissiflorinine dimethyl ester

Substance	$[\alpha]_D^{26}$ in MeOH	$[M]_D$
Dimethyl ester of oleanolic acid β-D-glucuronopyranoside	+12	+79
α-Methyl-L-arabinoside	−125	−205
α-Methyl-L-rhamnoside	−62	−111
Calculated for **1** (+79 − 205)		−126
Observed for **1**		−122
Calculated for narcissiflorine dimethyl ester (+79 − 111 −205)		−237
Observed value		−240

arabinofuranosyl-(1 → 4)-β-D-glucuronopyranosyl-(1 → 3)]-3β-hydroxy-olean-12-en-28-oic acid (**3**).

Narcissiflorinine, $C_{47}H_{74}O_{17}$, gave on hydrolysis oleanolic acid, D-glucuronic acid, L-rhamnose and L-arabinose. The sugars were in equimolar proportions (1:1:1) as revealed by colorimetric estimation [15] and by the yield of the sapogenin (50%) in quantitative hydrolysis. The sequence of the sugar moieties in the saponin was determined by partial hydrolysis which resulted in the isolation of two prosapogenins, designated PS_3 and PS_4. PS_3 was identical to PS_1 (isolated by partial hydrolysis of narcissiflorine) in all respects. This led to the conclusion that C-1 of the D-glucuronic acid moiety in its pyranose form was attached to the C-3 hydroxyl of oleanolic acid.

On reduction with $LiAlH_4$ the dimethyl ester of PS_4 gave a glycosidic compound designated G_1. Hydrolysis of G_1 produced erythrodiol, D-glucose and L-rhamnose. As PS_4 contained D-glucuronic acid, which was not present in G_1, it may be concluded that the D-glucose unit present in G_1 resulted from D-glucuronic acid during reduction of the dimethyl ester of PS_4.

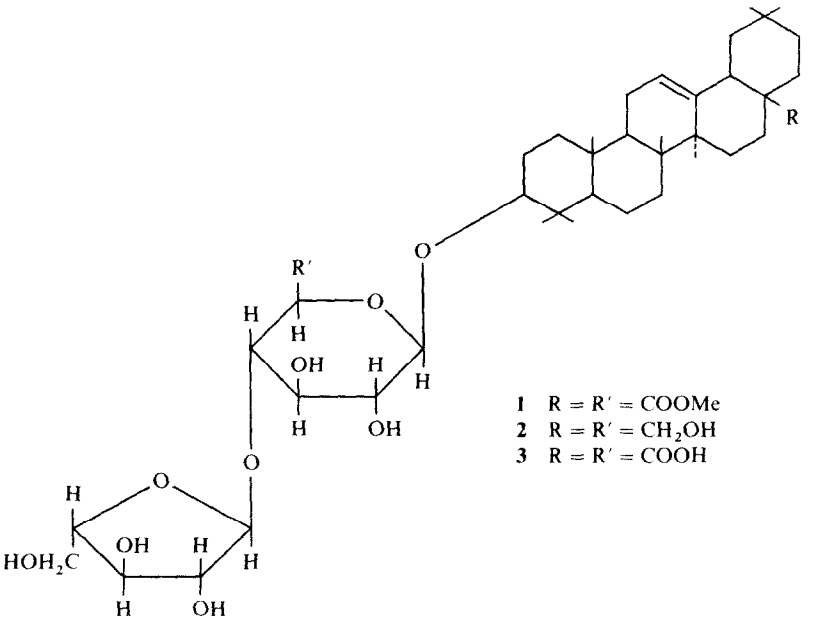
Narcissiflorinine dimethyl ester, on $LiAlH_4$ reduction, yielded another glycoside, G_2, which on hydrolysis gave erythrodiol, D-glucose, L-rhamnose and L-arabinose. The sugar linkages were established by permethylation and subsequent hydrolysis of G_1 and G_2. 2,3,4-Tri-*O*-methyl-L-rhamnose was obtained from G_1 and 3,4-di-*O*-methyl-L-rhamnose and 2,3,5-tri-*O*-methyl-L-arabinose were produced from G_2. 2,3,6-Tri-*O*-methyl-D-glucose was obtained from both G_1 and G_2. These results led to the conclusion that C-1 of L-rhamnose was linked to C-4 of D-glucose in G_1 and C-1 of L-arabinose was attached to C-2 of L-rhamnose, while C-1 of L-rhamnose was linked to C-4 of D-glucose in G_2.

The results obtained with the above permethylated derivatives of the sugars also suggested that D-glucose and L-rhamnose were present as pyranosides but L-arabinose occurred as the furanoside. Periodate oxidation of G_1 and G_2 further confirmed these facts.

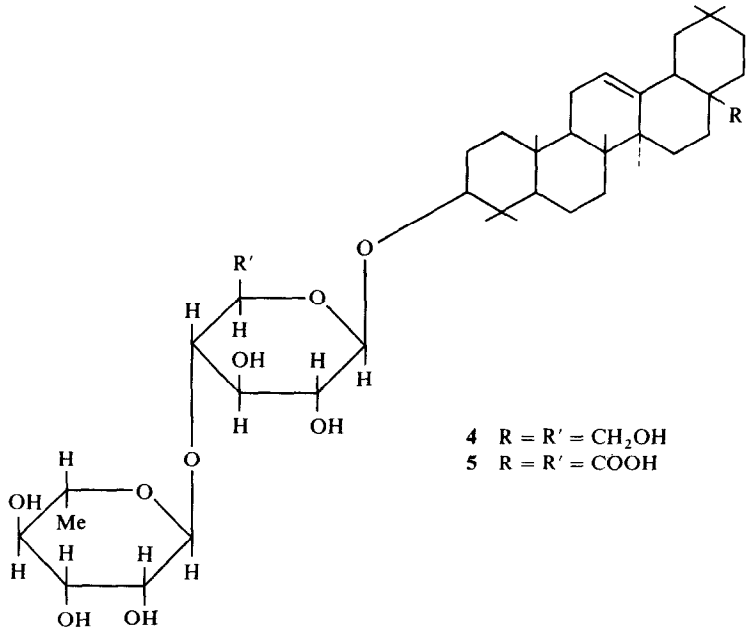
As glycosides G_1 and G_2 are the reduced methyl esters of PS_4 and narcissiflorinine, the structure of PS_4 and narcissiflorinine may be represented by structures **5** and **7**, respectively.

Hydrolysis of narcissiflorinine with the enzyme diastase yielded L-rhamnose and L-arabinose, indicating that these two sugars were involved in the formation of α-glycosidic linkages and D-glucuronic acid was in the β-linkage. The nature of the glycosidic linkages in the saponin was further supported by the calculated molecular rotation value $[M]_D$ [18–20] for the dimethyl ester of narcissiflorinine, which was in good agreement with the experimental value (Table 1). Hence, narcissiflorinine is [α-L-arabinofuranosyl-(1→2)-α-L-rhamnopyranosyl-(1→4)-β-D-glucuronopyranosyl-(1→3)]-3β-hydroxy-olean-12-en-28-oic acid.

Narcissifloridine, $C_{47}H_{76}O_{16}$, gave on hydrolysis oleanolic acid, D-glucose, L-rhamnose and L-arabinose. The sugars were equimolar in proportions (1:1:1) as revealed by colorimetric estimation [15] and the yield of the saponegin was 50.60% (quantitative hydrolysis) against 50.88% calculated for 1 unit each of oleanolic acid, D-glucose, L-rhamnose and L-arabinose.



1 R = R′ = COOMe
2 R = R′ = CH_2OH
3 R = R′ = COOH



4 R = R′ = CH_2OH
5 R = R′ = COOH

Narcissifloridine on methylation with CH_2N_2 furnished a monomethyl ester, $C_{48}H_{78}O_{16}$, which on reduction with $LiALH_4$ gave a glycosidic compound. It was identical with G_2 (**6**) in all respects. Therefore, narcissifloridine is [α-L-arabinofuranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)]-3β-hydroxy-olean-12-en-28-oic acid.

EXPERIMENTAL

Extraction. The defatted plant (10 kg) was exhaustively extracted with EtOH. The EtOH extract (8.6 l.) was concd *in vacuo*. The residue was washed successively with Et_2O, $CHCl_3$ and Me_2CO and was finally dissolved in MeOH, filtered and the filtrate poured into excess Et_2O whereby a brown mass pptd. The ppt. was dissolved in MeOH, adsorbed onto a column of Si gel and eluted with MeOH–Me_2CO (2:1) to give four fractions. All four fractions were rechromatographed over a column of Si gel separately using MeOH as eluting solvent to yield olean-3-*O*-α-L-rhamnopyranosyl-Δ^{12}-en-28-oic acid, mp 198–200° (directly identified by mmp and co-TLC with an authentic sample) [9]; narcissiflorine, mp 232–234°; narcissiflorinine, mp 266–267° and narcissifloridine, mp 280–82°. (Found for narcissiflorine: C, 64.46; H, 8.34, $C_{41}H_{64}O_{13}$ requires: C, 64.39; H, 8.37%. Found for narcissiflorinine: C, 62.00; H, 8.10. $C_{47}H_{74}O_{17}$ requires: C, 61.97; H, 8.13%. Found for narcissifloridine: C, 62.99; H, 8.44. $C_{47}H_{76}O_{16}$ requires: C, 62.94; H, 8.48%).

Identification of the sugars in the hydrolysate and isolation and study of sapogenin from narcissiflorine, narcissiflorinine and narcissifloridine. The saponins (0.9 g each) were hydrolysed separately by refluxing with 7% H_2SO_4 in EtOH (100 ml) for 4 hr on a steam bath. The products were poured in H_2O (500 ml), and EtOH was removed by distillation *in vacuo*. The sapogenins were separated from the aq. hydrolysates and purified by the K salt method [21]. They were crystallized from $CHCl_3$ into colourless crystals (0.450, 0.396 and 0.403 g, respectively). All three sapogenins were found to be the same compound as revealed by mmp and TLC ($CHCl_3$–C_6H_6–EtOAc, 1:2:3: spray—30%, $SbCl_3$ in $CHCl_3$; R_f 0.35). (Found: C, 78.89; H, 10.61. $C_{30}H_{48}O_3$ requires: C, 78.94; H, 10.52%). IR ν_{max}^{KBr} cm^{-1}: 3420, 2900, 2840, 1701, 1464, 1390, 1366, 1347, 1325, 1305, 1264, 828, 818, 804. MS *m/z*: 456 (M^+), 441, 411, 410, 395, 300, 248, 207, 203 (base peak), 189, 175, 133. Methyl ester: $C_{31}H_{50}O_3$, mp 198–199°. MS *m/z* 470 (M^+), 455, 411, 410, 262, 249, 207, 203 (base peak), 189, 133; ^1H NMR ($CDCl_3$): δ 0.75 (3 H), 0.80 (3 H), 0.97 (6 H), 1.00 (6 H), 1.16 (3 H), 3.60 (3 H), 5.28 (1 H). All three hydrolysates were separately neutralized with $BaCO_3$. The neutral aq. hydrolysate from narcissiflorine revealed the presence of D-glucuronic acid (R_f 0.11) and L-arabinose (R_f 0.20). The hydrolysate from narcissiflorinine showed the presence of D-glucuronic acid, L-rhamnose and L-arabinose, and the hydrolysate from narcissifloridine revealed the presence of D-glucose, L-rhamnose and L-arabinose by co-PPC with authentic samples (BuOH–HOAc–H_2O, 4:1:5; spray—aniline hydrogen phthalate).

Quantitative estimation of the sugars in the saponin hydrolysate. The ratio of sugars in the saponin was determined colorimetrically [15] in a Klett–Summerson photoelectric colorimeter using a blue filter (420 nm), with the help of standard curves of authentic sugars. Ten solns (5, 10, 15, 20 ... 50 μg in 0.03 ml H_2O) of each of D-glucuronic acid, D-glucose, L-rhamnose and L-arabinose were applied to Whatman No. 1 filter papers (50 × 55 cm, spot distance 5 cm). The chromatograms were developed by the descending technique with BuOH–HOAc–H_2O (4:1:5) for 24 hr, dried in air, sprayed with aniline hydrogen phthalate and dried at 110° for 15 min. The coloured spots were cut out in equal rectangles eluted by immersing in 50% HOAc (5 ml each) and the colour intensity of each eluate measured. The sugars in the hydrolysates of narcissiflorine, narcissiflorinine and narcissifloridine were assayed as described above.

Partial hydrolysis of narcissiflorine (**3**), *erythrodiol glycoside* (**2**) *narcissiflorinine* (**7**); *isolation of prosapogenins* PS_1, PS_2, PS_3 *and* PS_4 (**5**). Narcissiflorine, erythrodiol glycoside (**2**) and narcissiflorinine (1 g each) and 1% H_2SO_4 in MeOH (75 ml) were kept for 6 days separately at 35°. MeOH was removed and H_2O (20 ml) was added. The aq. solns were extracted with BuOH. The BuOH extract of the acid hydrolysate of narcissiflorine, on concn to a syrup and CC over Si gel, solvent $CHCl_3$–MeOH (3:2), yielded prosapogenin PS_1. The BuOH extract of the acid hydrolysate of erythrodiol glycoside on concn and CC over Si gel,

solvent $CHCl_3$–MeOH (3:2), gave PS_2. The BuOH extract of the acid hydrolysate of narcissiflorinine on concn and CC over Si gel, solvent $CHCl_3$–MeOH (3:2), yielded two prosapogenins PS_3 and PS_4 (**5**). PS_3 was found to be identical to PS_1.

Preparation of the dimethyl ester of narcissiflorine, narcissiflorinine and PS_4. Samples (150 mg) of **3**, **7** and **5** were treated separately with an ethereal soln of CH_2N_2. The products obtained were separately crystallized from $CHCl_3$–MeOH (1:2) to yield narcissiflorine dimethyl ester (**1**), mp 240–241°; narcissiflorinine dimethyl ester, mp 271–272° and the dimethyl ester of prosapogenin PS_4, mp 262–263°. (Found for narcissiflorine dimethyl ester): C, 65.38; H, 8.55. $C_{43}H_{68}O_{13}$ requires: C, 65.15; H, 8.58%. Found for narcissiflorinine dimethyl ester; C, 62.59; H, 8.34. $C_{49}H_{78}O_{17}$ requires: C, 62.68; H, 8.31%. Found for dimethyl ester of PS_4: C, 65.55; H, 8.62. $C_{44}H_{66}O_{13}$ requires: C, 65.51; H, 8.68%.

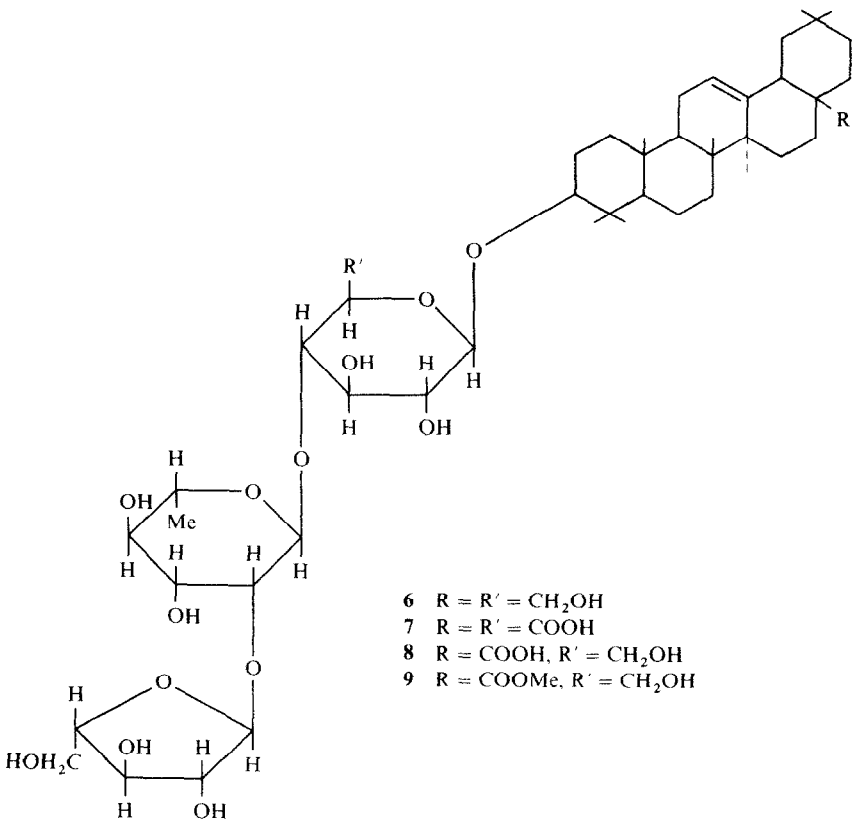
Reduction of narcissiflorine dimethyl ester (**1**), *narcissiflorinine dimethyl ester and dimethyl ester of PS_4; isolation of erythrodiol glycoside* (**2**), *G_1* (**4**) *and G_2* (**6**). The dimethyl esters (70 mg each) were reduced separately under reflux with $LiAlH_4$ in Et_2O–THF for 10 hr. The excess of $LiAlH_4$ was decomposed with moist EtOAc. The products were acidified with HCl and extracted with BuOH. The solvent was removed and the residues were crystallized from Et_2O–MeOH to yield erythrodiol glycoside (**2**), mp 266–8°; G_1 (**4**), mp 284–285° and G_2 (**6**), mp 274–275° respectively.

Permethylation of PS_2, erythrodiol glycoside (**2**), *G_1* (**4**) *and G_2* (**6**) *and the hydrolysis of the permethylated derivatives.* The glycosides (60 mg each) were treated with MeI (2 ml) and Ag_2O (1 g) in DMF (4 ml) separately for 48 hr at room temp. The contents were filtered and the residue washed with a little DMF. The filtrate was evapd to dryness and the residue taken up in EtOH (25 ml). The syrups obtained after removal of EtOH were hydrolysed with Killiani's mixture (HOAc–HCl–H_2O, 7:3:10) and the product worked up in the usual way. The products were analysed by PPC (BuOH; EtOH–H_2O, 5:1:4) [22]. The hydrolysate from PS_2 contained 2,3,4,6-tetra-*O*-methyl-D-glucose (R_g, 1.0); **2** hydrolysate contained 2,3,6-tri-*O*-methyl D-glucose (R_g, 0.83) and 2,3,5-tri-*O*-methyl-L-arabinose (R_g, 0.95); G_1 hydrolysate contained 2,3,4 tri-*O*-methyl-L-rhamnose (R_g, 1.01) and 2,3,6-tri-*O*-methyl-D-glucose (R_g, 0.83), G_2 hydrolysate contained 3,4-di-*O*-methyl-L-rhamnose (R_g, 0.84), 2,3,5-tri-*O*-methyl-L-arabinose (R_g, 0.95) and 2,3,6-tri-*O*-methyl-D-glucose (R_g, 0.83).

Periodate oxidation of erythrodiol glycoside (**2**), *G_1* (**4**) and *G_2* (**6**). Periodate oxidations were carried out separately by the method of ref. [23]. Samples (40 mg) of each were dissolved in 20 ml EtOH and 20 ml 0.25 M sodium metaperiodate soln added. The oxidation was allowed to take place at room temp. for 60 hr. Aliquots (5 ml) were withdrawn in duplicate from the reaction mixture at different intervals of time and analysed for periodate and formic acid.

Methyl ester of narcissifloridine. Methyl ester (**9**) was prepared from **8** (400 mg) by the method described above for the preparation of the dimethyl ester of narcissiflorine. The product was crystallized from MeOH to give an amorphous powder (410 mg), mp 288–90°. (Found: C, 63.18; H, 8.55. $C_{48}H_{78}O_{16}$ requires: C, 63.29; H, 8.57%).

Reduction of narcissifloridine methyl ester (**9**). Compound **9** (350 mg) was reduced with $LiAlH_4$ in Et_2O–THF. The product was purified by CC with $CHCl_3$–MeOH (2:3) and was crystallized from MeOH as a colourless solid, mp 296–297°. (Found: C, 63.88; H, 8.81. $C_{47}H_{78}O_{15}$ requires: C, 63.93; H, 8.84%).



6 R = R′ = CH_2OH
7 R = R′ = COOH
8 R = COOH, R′ = CH_2OH
9 R = COOMe, R′ = CH_2OH

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