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ACYLATED C-21 STEROIDAL BISDESMOSIDIC GLYCOSIDES FROM *CARALUMA UMBELLATA*

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Key Word Index—*Caraluma unbellata*; Asclepiadaceae; C-21 steroidal glycosides; Bisdemosidic glycosides, Caralumagenin; Carumbelloside III; Carumbelloside IV; Carumbelloside V; Structure elucidation; NMR assignments; Two-dimensional NMR techniques.

Abstract—From the whole plant of *Caraluma umbellata*, three new C-21 steroidal glycosides, named as carumbellosides III–V, were isolated and their structures elucidated by extensive spectroscopic experiments, devoid of any derivatisation, as caralumagenin 3-*O*-*β*-D-glucopyranosyl(1→4)-*β*-D-digitalopyranoside-20-*O*-*β*-D-glucopyranoside, caralumagenin 3-*O*-*β*-D-glucopyranosy(1→4)-*β*-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-*β*-D-glucopyranoside and caralumagenin 3-*O*-[6-*O*-benzoyl-*β*-D-glucopyranosyl(1→4)]-*β*-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-*β*-D-glucopyranoside. The determination of the absolute configuration of the aglycone as (20*R*), the conformations of the sugars and the unambiguous assignments of their NMR spectroscopic signals were achieved by a combination of 2D-NMR techniques. The isolates were devoid of significant cytotoxity in the UIC human cancer cell panel. © 1997 Elsevier Science Ltd

INTRODUCTION

Caralluma umbellata Haw. (syn. *Boucerosia umbellata* W. and A.) (Asclepiadaceae) is a succulent, perennial herb growing wild in Tirupathi and surrounding places of Andhra Pradesh, India. Previously, we reported on the isolation and identification of two new pregnane glycosides, carumbellosides I and II, from this plant [1]. In this report, we present the isolation, characterization and complete NMR assignments of three further new C-21 steroidal glycosides carumbellosides III (**1**), IV (**2**) and V (**3**) from this plant.

RESULT AND DISCUSSION

Carumbelloside III (**1**) gave a positive Liebermann–Burchard reaction, as a steroidal glycoside, and exhibited a molecular formula $C_{40}H_{66}O_{17}$ (MW = 818) based on its ^{13}C NMR (DEPT) spectrum and FAB-MS. In the latter, a quasi-molecular ion was observed at *m/z* 817 $[M-H]^-$ in the negative ion FAB-mass spectrum and a sodiated molecular ion at *m/z* 841 $[M+Na]^+$ in the positive ion FAB-mass spectrum.

Compound **1** displayed no absorption in the UV spectrum indicating the absence of any conjugated double bonds. The IR spectrum revealed no other significant absorptions except for a strong hydroxyl group band at 3500 cm^{-1} and glycosidic bonds (C—O) at 1110 cm^{-1}. The ^1H NMR spectrum of **1** displayed three doublet anomeric proton signals δ 5.09, 4.82 and 4.73 with coupling constants of 8.0 Hz, diagnostic of an axial orientation for all three sugar moieties. This is in good agreement with the negative FAB-mass spectral fragmentation pattern of **1**, which showed *m/z* 817 $[M-H]^-$, 655 $[M-H-162]^-$ and 493 $[M-H-162-162]^-$, suggesting the presence of two hexose units. The third sugar could be inferred as 6-deoxy-3-*O*-methyl-hexose based on the observed predominant fragmentation at *m/z* 479, which was probably generated from the quasi-molecular ion at *m/z* 819, corresponding to $[M+H]^-$ from the positive mode FAB-mass spectrum, by the loss of a hexose unit and a

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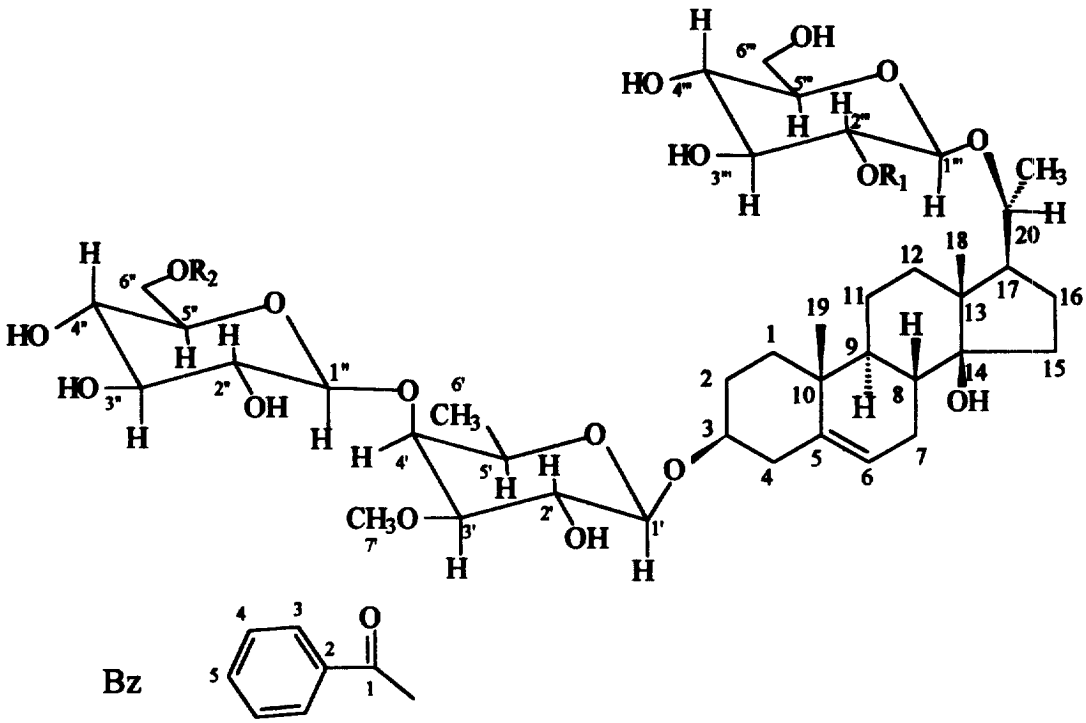

Carumbelloside III (**1**) $R_1 = R_2 = H$
Carumbelloside IV (**2**) $R_1 = Bz$, $R_2 = H$
Carumbelloside V (**3**) $R_1 = R_2 = Bz$

molecule of water. Furthermore, the combination fragmentation patterns of positive and negative FAB-mass spectra suggest that **1** is a bisdesmosidic glycoside, i.e. with one hexose attached directly to the aglycone, and another hexose linked to the aglycone-bound deoxyhexose. The NMR spectral outline of the aglycone derived by subtraction of the sugar chain from that of the glycoside, taking into account the 'glycoside shift' effect, is analogous to those of calogenin, isolated by Khare *et al.* from *Periploca calophylla* [2] and *Hemidesmus indicus* [3], with the exclusion of the configuration at the stereogenic centers C-17 and C-20, since these centres in calogenin remained to be assigned stereochemically.

In classical approaches, the structure of glycosides are traced by analysis of the fragments obtained by chemical and/or enzymic degradation, and can be associated with disadvantages such as artifact formation and tedious workup of various derivatization reactions [4]. Moreover, these degradative methods consume precious sample which is frequently difficult to isolate and could be preserved for biological evaluation. In the current study, an effort was made to characterise the intact glycoside structure primarily based on NMR spectroscopic techniques. The structure characterization and stereochemical assignment of the glycosides were achieved through the unambiguous rationalization of the ^1H and ^{13}C NMR resonances using a combination of DQF-COSY, HOHAHA, HETCOR, HMBC and ROESY techniques. Proton connectivities were determined by DQF-COSY and HOHAHA experiments and the signals of all carbons with directly attached protons were assigned using the HETCOR spectrum. The ROESY spectrum was employed to assign the stereochemistry of the aglycone along with the configurations and conformations of the sugars and the sugar-chain sequences and its attachment to the aglycone. Finally, the HMBC spectrum was used to assign the quaternary carbons and to verify the correctness of the connectivities established by the interpretation of the other spectra with consequent confirmation of the oligosaccharide chain linkage and sequences.

The ^{13}C DEPT experiment of **1** revealed a total of 40 carbon resonances, of which five corresponded to methyl carbons, ten to methylene carbons, twenty-one to methine carbons, and four to non-proton bearing carbons, corresponding to a C-21 steroid triglycoside. Two olefinic carbons at δ 122.67 and 139.63 and three anomeric carbons resonating at δ 102.57, 105.29 and 104.86 could be recognized readily. The ^1H NMR spectrum displayed two distinct chemical shift regions as a set of oxygenated methine and methylene protons (olefinic proton included) in the range δ 3.50–5.50, and the aliphatic methylene and methine protons in the range δ 0.80–2.70 arising mainly from the aglycone portion. Except for the well-resolved signals of the methyl groups and some of the non-equivalent methylene protons, most of the signals at high field were severely overlapped and could only be distinguished in the HETCOR spectrum in a 'fuzzy logic' fashion. However, it was still possible to assign

the corresponding ^{13}C resonances unambiguously through the investigation of the DQF-COSY, HOHAHA and HMBC spectra.

The proton singlet at δ 0.83, assignable to the methyl CH_3-19, showed a cross-peak with the quaternary carbon signals at δ 37.54 and δ 139.63, a methylene carbon at δ 37.35 and a methine carbon at δ 46.39 in the HMBC spectrum, resulting in their assignment to C-10, the olefinic carbon C-5, and C-1 and C-9, respectively. Accordingly, the proton singlet at δ 1.33 should be assigned to the CH_3-18, since it correlated with the quaternary carbons C-13 at δ 47.40 and C-14 at δ 83.90, the methylene carbon at δ 40.62 and a methine carbon at δ 57.20 ppm, leaving the latter to be assigned to C-12 and C-17, respectively. One of the two doublet methyl signals at δ 1.37 (*d*, $J = 6.0$ Hz) could be assigned to CH_3-21 based on a correlation with the C-17 carbon signal in the HMBC spectrum.

Following the interpretation of the coupling pattern using DQF-COSY of the HOHAHA coupling fragments C_1-C_2-C_3-C_4, C_6-C_7-C_8-C_9-C_{11}-C_{12} and C_{15}-C_{16}-C_{17}, H_2-2, H-3, H_2-4, H_2-7, H-8, H-9, H_2-11, H_2-12, H_2-15, H_2-16, H-17 were identified, resulting in the ^{13}C assignments of C-2, C-3, C-4, C-7, C-8, C-9, C-11, C-15 and C-16 by means of the HETCOR experiment. The stereochemical assignments of the methine and non-equivalent methylene protons, except for the almost coincident H_2-15, were achieved with the aid of the ROESY spectrum, in a similar way as with gymnemarsgenin [5] and marstenacigenins A and B [6]. The H_3-19 showed a strong correlation contour with the C-1 and C-4 methylene proton signals at δ 1.72 and δ 2.33, which should be assigned to H-1β and H-4β, leaving their partners at δ 1.05 and δ 2.64 to be assigned to H-1α and H-4α, respectively. The β-configuration of H-8 was also evident from its strong ROE correlation contour with H_3-19.

It is a well-established feature of pregnane steroids with a $\Delta^{5,6}$-double bond that H-6 and H-7β are spatially close and afford an easily observed ROE cross-peak [5], which enables δ 2.59 and 2.02 to be assigned to H-7β and H-7α, respectively. The α-orientation of H-9 was confirmed by the ROE correlation contour between H-7α and H-9, and in addition, H_2-11, H_2-12 and H_2-16 could also be differentiated by relative configuration analogies accordingly.

The H-17 signal appeared as a doublet of doublets (δ 1.55, *dd*, $J = 11.5$, 6.5 Hz), therefore, the configuration of the side-chain was deduced to be β-orientated (i.e. H-17α) by coupling pattern analysis [7]. The absolute configuration of C-20, which is left unassigned in most published papers on pregnane steroids bearing a hydroxyl group at the C-20 positions [8, 9], was finally determined to be *R*- by the ROESY spectrum and molecular modelling (see below). Thus, the aglycone of **1** was deduced to be 3β,14β,20(*R*)-trihydroxy-pregnane and was designated caralumagenin to avoid confusion with the C-17 and C-20 stereochemically undefined pregnane calogenin [2, 3].

Proton-3 of the aglycone showed a strong ROE correlation contour with a doublet anomeric proton H-1′ at δ 4.73 (*d*, $J = 8.0$ Hz), which in turn was coupled with a double doublet signal H-2′ at δ 4.37 (*dd*, $J = 9.5$, 8.0 Hz) and was relay coupled with a double doublet signal H-3′ at δ 3.52 (*dd*, $J = 9.5$, 0.5 Hz) in the HOHAHA (45 ms) spectrum and an overlapping signal H-4′ at δ 4.07 in the HOHAHA (55 ms) spectrum. The quadruplet signal at δ 3.69, coupling with a methyl signal at δ 1.52 (*d*, $J = 6.5$ Hz), with no observed cross-peak with H-4′ due to a small coupling constant, could be attributed to H-5′ based on its ROE effects on both H-1′ and H-3′ in the ROESY spectrum. From the ongoing evidence, the inner sugar of the oligosaccharide chain could be established as β-D-digitalopyranose in a $^4C_1$ conformation.

The doublet proton signal at δ 5.09 (*d*, $J = 8.0$ Hz) was readily assigned to that of the terminal sugar H-1″ from its strong ROE cross contour with H-4′, implying attachment to the C-4′ position of the inner sugar digitalose. Protons-2″ (δ 3.92, *t*, $J = 8.5$ Hz) and H-3″ (δ 4.18, *t*, $J = 8.5$ Hz) are well-resolved and were assigned by the DQF-COSY spectrum; consequently, the ^{13}C assignments of C-1″, C-2″ and C-3″ are straightforward. Protons-4″ and H-5″ could not be well-defined due to the limits of resolution. However, the ^{13}C assignments of C-4″, C-5″ and C-6″ (Table 2) could be made after careful interpretation of DQF-COSY, HOHAHA, HETCOR and HMBC spectra and were found to be almost superimposable with those of methyl β-D-glucopyranoside [10]. Thus, the disaccharide unit should be β-D-glucopyranosyl-β-D-digitalopyranose.

The third sugar was deduced to be β-D-glucose and its ^{13}C assignments (Table 2) were accomplished in a similar way as mentioned above. The notable ROE cross-peaks (Fig. 1) between H-1‴ at δ 4.82 (*d*, $J = 8.0$ Hz) with the doublet signal of H-21 at δ 1.37 (*d*, $J = 6.0$ Hz) and the singlet signal of H_3-18 at δ 1.33 (*s*) allowed the conclusion that it was attached to the aglycone directly at the C-20 hydroxyl group. This could be further confirmed by key observations of correlation contours between H-1′ and C-3, H-1″ and C-4′, as well as H-1‴ and C-20, in the HMBC spectrum.

The ROE results were also applicable for the determination of the absolute configuration of C-20. The individual structures of the 20(*R*)- and 20(*S*)- isomers were generated by the molecular modelling program PCMODEL V 5.0 for Windows, using the MMX force field calculations for energy minimization. The calculated distances between H-1‴ and H_3-18, H_3-21 and H-20 were 2.32, 2.19 and 2.45 Å, respectively, for the C-20(*R*) isomer, consistent with the strong ROE correlations observed between each of these pairs; while for the C-20(*S*)- isomer, the calculated distance between H_3-21 and H-1‴ was 4.53 Å, which was considered to be too far away to account for the observed ROE correlation.

Table 1. ^{13}C NMR data of Carumbellosides III, IV and V (**1**–**3**)

	1	**2**	**3**
Aglycone			
1	37.35 (*t*)	37.23 (*t*)	37.22 (*t*)
2	30.13 (*t*)	30.15 (*t*)	30.17 (*t*)
3	77.90 (*d*)	77.86 (*d*)	77.89 (*d*)
4	39.04 (*t*)	39.01 (*t*)	39.03 (*t*)
5	139.63 (*s*)	139.20 (*s*)	139.19 (*s*)
6	122.67 (*d*)	122.76 (*d*)	122.75 (*d*)
7	27.81 (*t*)	27.65 (*t*)	27.66 (*t*)
8	37.30 (*d*)	35.98 (*d*)	35.97 (*d*)
9	46.39 (*d*)	45.99 (*d*)	45.97 (*d*)
10	37.54 (*s*)	37.50 (*s*)	37.51 (*s*)
11	19.72 (*t*)	20.11 (*t*)	20.25 (*t*)
12	40.62 (*t*)	40.15 (*t*)	40.14 (*t*)
13	47.40 (*s*)	47.00 (*s*)	46.99 (*s*)
14	83.90 (*s*)	83.22 (*s*)	83.25 (*s*)
15	21.40 (*t*)	21.09 (*t*)	21.09 (*t*)
16	33.30 (*t*)	32.72 (*t*)	32.67 (*t*)
17	57.20 (*d*)	56.65 (*d*)	56.64 (*d*)
18	15.19 (*q*)	15.15 (*q*)	15.17 (*q*)
19	19.48 (*q*)	19.28 (*q*)	19.28 (*q*)
20	77.92 (*d*)	79.24 (*d*)	79.59 (*d*)
21	21.61 (*q*)	21.75 (*q*)	21.76 (*q*)
Digitalose			
1′	102.57 (*d*)	102.53 (*d*)	102.55 (*d*)
2′	71.35 (*d*)	71.33 (*d*)	71.33 (*d*)
3′	85.37 (*d*)	85.43 (*d*)	85.44 (*d*)
4′	76.16 (*d*)	76.04 (*d*)	76.06 (*d*)
5′	70.39 (*d*)	70.41 (*d*)	70.42 (*d*)
6′	17.67 (*q*)	17.69 (*q*)	17.70 (*q*)
-OCH_3	58.85 (*q*)	58.86 (*q*)	58.90 (*q*)
Glucose			
1″	105.29 (*d*)	105.36 (*d*)	105.37 (*d*)
2″	76.01 (*d*)	76.20 (*d*)	76.26 (*d*)
3″	78.22 (*d*)	78.27 (*d*)	78.29 (*d*)
4″	71.76 (*d*)	71.78 (*d*)	71.79 (*d*)
5″	78.96 (*d*)	78.56 (*d*)	75.38 (*d*)
6″	62.99 (*t*)	62.44 (*t*)	65.08 (*t*)
Glucose			
1‴	104.86 (*d*)	102.63 (*d*)	102.67 (*d*)
2‴	75.02 (*d*)	75.42 (*d*)	75.19 (*d*)
3‴	78.55 (*d*)	77.14 (*d*)	76.88 (*d*)
4‴	71.31 (*d*)	71.51 (*d*)	71.70 (*d*)
5‴	78.46 (*d*)	78.89 (*d*)	78.61 (*d*)
6‴	62.72 (*t*)	63.03 (*t*)	63.04 (*t*)
2‴-Bz			
1		166.01 (*s*)	166.00 (*s*)
2		132.19 (*s*)	133.22 (*s*)
3		128.05 (*d*)	128.79 (*d*)
4		130.22 (*d*)	130.24 (*d*)
5		132.48 (*d*)	132.09 (*d*)
6″-Bz			
1			166.45 (*s*)
2			130.74 (*s*)
3			128.07 (*d*)
4			129.89 (*d*)
5			132.55 (*d*)

ppm for internal standard TMS in pyridine-d_5

Multiplicity by DEPT experiments in parentheses, *s*, quaternary; *d*, methine; *t*, methylene and *q*, methyl carbons.

Therefore, the absolute configuration of aglycone caralumagenin was assigned to be 20(*R*), and the structure of **1** was deduced as caralumagenin 3-*O*-*β*-D-glucopyranosyl-*β*-D-digitalopyranoside-20-*O*-*β*-D-glucopyranoside.

The results described above would imply an alternative method for the determination of absolute configuration of pregnane steroids with a free hydroxyl group at the C-20 position, which are based on the fundamental principle that the free rotation of C_{17}-C_{20} bond of C_{20}-OH pregnanes was inhibited after glycosylation at C_{20}-OH. Thus, the favourable conformation could be 'frozen out' and could be suitable for spatial relationship analysis by NOED or ROESY (NOESY) experiments.

Carumbelloside IV (**2**) gave a positive Liebermann–Burchard test for steroids, and its FAB-MS revealed a molecular formula of $C_{47}H_{70}O_{18}$ (MW = 922), in which quasi-molecular ions were detected at *m/z* 923 $[M+H]^+$ in a positive-ion mode and *m/z* 921 $[M-H]^-$ in a negative-ion mode respectively, in combination with a ^{13}C NMR (DEPT) experiment. In the IR spectrum, bands for hydroxyl groups at 3440 cm^{-1}, a carbonyl group at 1700 cm^{-1}, and characteristic absorptions of a benzene ring at 1600 and 1450 cm^{-1} were apparent. Its mass spectrum presented a base peak fragment at *m/z* 105 (benzoyl cation) and a strong ion at *m/z* 122 (benzoic acid ion), indicative of the presence of a benzoyl group. The appearance of the ^1H and ^{13}C NMR of **2** are very similar to those of **1**, and therefore, it was deduced that **2** is an analogue of **1** with an additional benzoyl group.

Following the same methodology as described for **1**, the aglycone of **2** was deduced as caralumagenin and its NMR spectral data assigned. The inner sugar was shown to be *β*-D-digitalopyranose with a $^4C_1$ conformation by analysis of its ^1H coupling pattern and spatial relationship with reference to H-3, since all of the signals were well resolved.

Anomeric protons of the two glucose protons H-1″ at *δ* 5.14 (*d*, *J* = 7.6 Hz) and H-1‴ at *δ* 5.16 (*d*, *J* = 7.9 Hz) could be readily differentiated by the observed ROE cross contour between H-1″ and H-4′, and also the correlation of H-1″ with the ^{13}C signal of C-4′ in the ROESY and HMBC spectra, respectively. In the DQF-COSY spectrum, H-1‴ exhibited a cross-peak with a triplet signal at *δ* 5.75 (*t*, *J* = 7.9 Hz), assignable to a benzoylated geminal proton by its correlation with the ^{13}C signal of the carbonyl group at *δ* 166.11, indicating that the benzoyl group was attached to the C-2‴ position of the isolated glucose. This, in turn, was attached directly to the aglycone at the C-20 hydroxyl group from the strong ROE correlations between the proton pairs of H-1‴/H_3-18, H-1‴/H_3-21 and H-1‴/H-20, and also the cross-peak between H-1‴ and C-20 by the ROESY and HMBC experiments. Thus, the structure of **2** was elucidated as caralumagenin 3-*O*-*β*-D-glucopyranosyl (1→4)-*β*-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-*β*-D-glucopyranoside.

Table 2. ^{1}H NMR data of carumbellosides III, IV and V (**1**–**3**)*

	1	**2**	**3**
1α	1.05 (*dt*, 13.5, 3.0)	0.83 (*m*)	0.85 (*m*)
1β	1.72 (*m*)	1.69 (*m*)	1.69 (*m*)
2α	2.09 (*m*)	2.10 (*m*)	2.09 (*m*)
2β	1.64 (*m*)	1.65 (*m*)	1.64 (*m*)
3α	3.86 (*m*)	3.86 (*dt*, 10.5, 7.5)	3.85 (*dt*, 11.9, 4.9)
4α	2.64 (*m*)	2.62 (*m*)	2.61 (*m*)
4β	2.33 (*br t*, 12.0)	2.30 (*m*)	2.28 (*m*)
6	5.43 (*br s*)	5.31 (*s*)	5.30 (*s*)
7α	2.02 (*m*)	2.03 (*m*)	2.03 (*m*)
7β	2.59 (*m*)	2.48 (*m*)	2.46 (*m*)
8β	1.94 (*m*)	1.87 (*m*)	1.87 (*m*)
9α	1.19 (*m*)	1.22 (*m*)	1.23 (*m*)
11α	2.12 (*m*)	2.08 (*m*)	2.07 (*m*)
11β	1.74 (*m*)	1.80 (*m*)	1.80 (*m*)
12α	1.26 (*m*)	1.26 (*m*)	1.24 (*m*)
12β	1.15 (*m*)	1.18 (*m*)	1.18 (*m*)
15-H_2	1.13 (*m*)	1.28 (*m*)	1.29 (*m*)
16α	1.69 (*m*)	1.72 (*m*)	1.72 (*m*)
16β	1.87 (*m*)	1.90 (*m*)	1.87 (*m*)
17α	1.55 (*dd*, 11.5, 6.5)	1.52 (*m*)	1.49 (*m*)
18-H_3	1.33 (*s*)	0.92 (*s*)	0.93 (*s*)
19-H_3	0.83 (*s*)	0.75 (*s*)	0.75 (*s*)
20	4.06 (*m*)	4.08 (*br q*, 6.0)	4.06 (*br q*, 6.1)
21	1.37 (*d*, 6.0)	1.43 (*d*, 6.0)	1.42 (*d* 6.1)
digitalose			
1′	4.73 (*d*, 8.0)	4.75 (*d*, 7.6)	4.75 (*d*, 8.4)
2′	4.37 (*dd*, 9.5, 8.0)	4.42 (*dd*, 9.6, 7.6)	4.41 (*dd*, 9.5, 7.8)
3′	3.52 (*dd*, 9.5, 2.5)	3.55 (*dd*, 3.2, 9.6)	3.55 (*dd*, 2.6, 9.0)
4′	4.07 (*t*, 2.5)	4.37 (*m*)	4.37 (*m*)
5′	3.69 (*q*, 6.5)	3.72 (*q*, 6.4)	3.72 (*q*, 6.3)
6′	1.52 (*d*, 6.5)	1.55 (*d*, 6.4)	1.55 (*d*, 6.3)
-OCH_3	3.63 (*s*)	3.66 (*s*)	3.66 (*s*)
glucose			
1″	5.09 (*d*, 8.0)	5.14 (*d*, 7.6)	5.14 (*d*, 7.9)
2″	3.92 (*t*, 8.0)	3.98 (*m*)	3.98 (*t*, 8.7)
3″	4.18 (*t*, 8.0)	4.21 ((*m*)	4.23 (*t*, 8.7)
4″	4.12 (*m*)	4.20 (*t*, 8.0)	4.19 (*t*, 8.7)
5″	4.08 (*m*)	3.95 (*m*)	4.21 (*dd*, 8.7, 6.3)
6″	4.50 (*br d*, 10)	4.54 (*m*)	5.23 (*br d*, 11.4)
	4.29 (*br d* 10)	4.31 (*m*)	4.90 (*dd*, 11.4, 6.3)
glucose			
1‴	4.82 (*d*, 8.0)	5.16 (*d*, 7.9)	5.17 (*d*, 8.5)
2‴	3.84 (*m*)	5.75 (*t*, 7.9)	5.76 (*t*, 8.5)
3‴	3.89 (*m*)	4.35 (*m*)	4.32 (*m*)
4‴	4.05 (*m*)	4.26 (*m*)	4.19 (*t*, 8.7)
5‴	3.83 (*m*)	3.97 (*m*)	3.95 (*dd*, 8.4, 6.5)
6‴	4.45 (*d*, 11.5)	4.52 (*m*)	4.56 (*br d*, 11.2)
	4.25 (*d*, 11.5)	4.30 (*m*)	4.35 (*m*)
2‴-Bz			
3		8.20 (*d*, 7.6)	8.23 (*d*, 7.8)
4		7.33 (*t*, 7.6)	7.39 (*t*, 7.8)
5		7.44 (*d*, 7.6)	7.45 (*d*, 7.8)
6″-Bz			
3			8.18 (*d*, 7.5)
4			7.33 (*t*, 7.5)
5			7.51 (*d*, 7.5)

* ppm from internal standard TMS in pyridine-d_5, coupling constants in Hz given in parentheses.

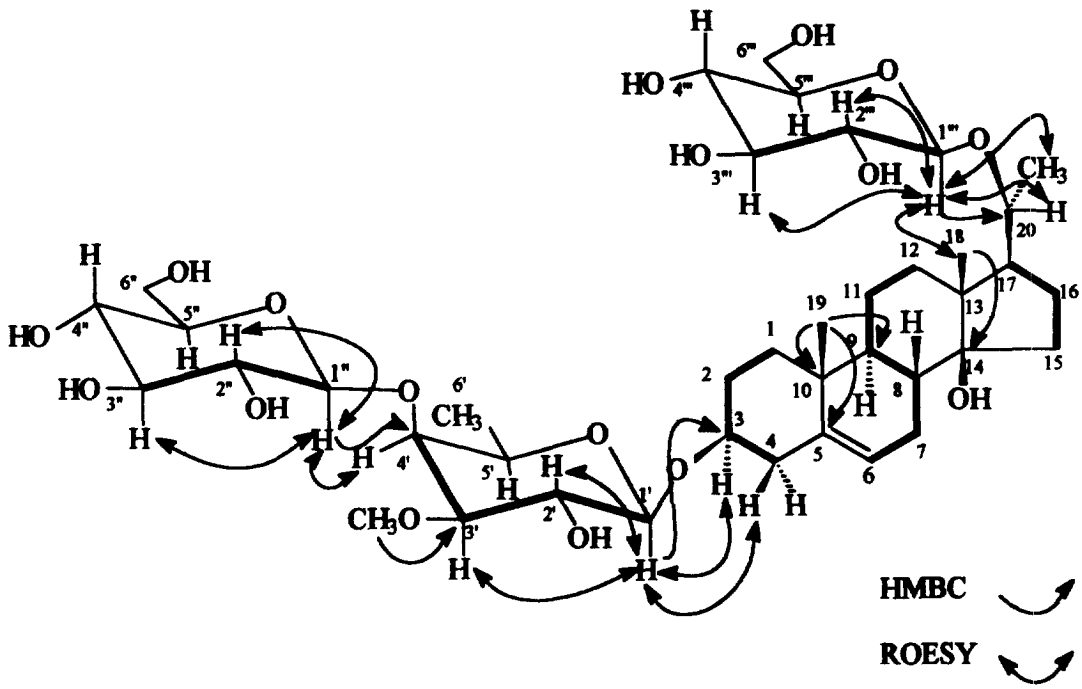

Fig. 1. The significant ROE and ^{1}H-^{13}C long range correlations of carumbelloside III (**1**) observed in the ROESY and HMBC spectra, with HOHAHA coupling fragments shown with bold C—C bonds.

Carumbelloside V (**3**) gave a positive Liebermann–Burchard test for steroids, and its negative-ion FAB-mass spectrum revealed a molecular formula $C_{54}H_{74}O_{19}$ (MW = 1026) based on the quasi-molecular ion $[M-H]^-$ observed at *m/z* 1025 in combination with the ^{13}C NMR (DEPT) experiment. Compound **3** exhibited two distinct sets of ^{1}H and ^{13}C NMR signals of a benzoyl group, besides the very close IR and UV absorption characteristics of compounds **1** and **2**, implying the presence of an additional benzoyl group in **3** by comparison with **2**. The aglycone of **3** was characterized as caralumagenin, the same as that in **1** and **2**, with the NMR spectroscopic data completely assigned by the methods as mentioned above.

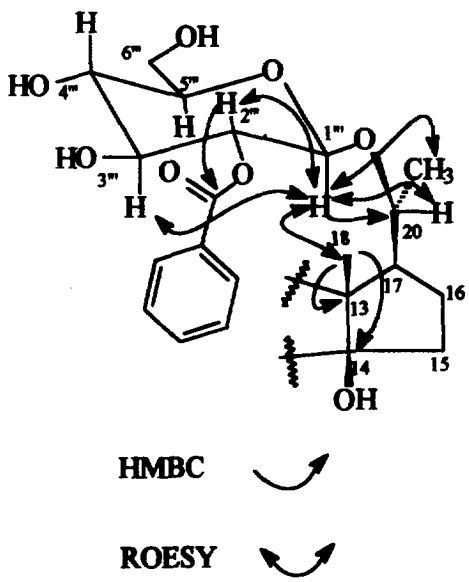

Fig. 2. Partial structure of carumbelloside IV (**2**) with the diagnostic ROE and ^{1}H-^{13}C long range correlations observed in the ROESY and HMBC spectra.

With the introduction of another benzoyl group in its molecule, compound **3** presented a more resolved ^{1}H NMR spectrum compared with those of **1** and **2** in the hydroxylated methine proton region, which enabled them to be assigned more readily. The ^{1}H NMR assignments of the inner β-D-digitalose were achieved from ROESY, DQF-COSY and HOHAHA spectral analysis using the well-defined signal of H-3 at δ 3.85 as an emanating point. Proton-3 showed a ROE cross peak with H-1′ at δ 4.75 (*d*, J = 8.4 Hz), which in turn was coupled with H-2′ and long-range coupled with H-3′ and H-4′ in the HOHAHA spectrum. Proton-5′ could only be recognized by a ROE correlation with H-1′ due to the weak coupling between H-5′ and H-4′.

The two benzoyl groups were disclosed as being associated with different spin systems from the DQF-COSY and HOHAHA spectral analysis. The doublet anomeric proton at δ 5.14 (*d*, J = 7.9 Hz), assignable to H-1″ based on the ROE cross-peak with H-4′ of the inner digitalose, showed vicinal coupling with H-2″ at δ 3.98 (*t*, J = 8.7 Hz) and relayed coupling with H-3″ at δ 4.23 (*d*, *t*, J = 8.7 Hz), which, in turn, showed vicinal coupling with H-4″, resonating coincidently with H-4‴ at δ 4.19 (*t*, J = 8.7 Hz), and was relay coupled with H-5″ at δ 4.21 (*dd*, J = 8.5 and 6.3 Hz). Thus, one benzoyl group was attached to the C-6″ hydroxyl group since H-5″ coupled with one of the H_2-6″ protons appearing as a double doublet at δ 4.90 (its partner resonating at δ 5.23 as a doublet in the

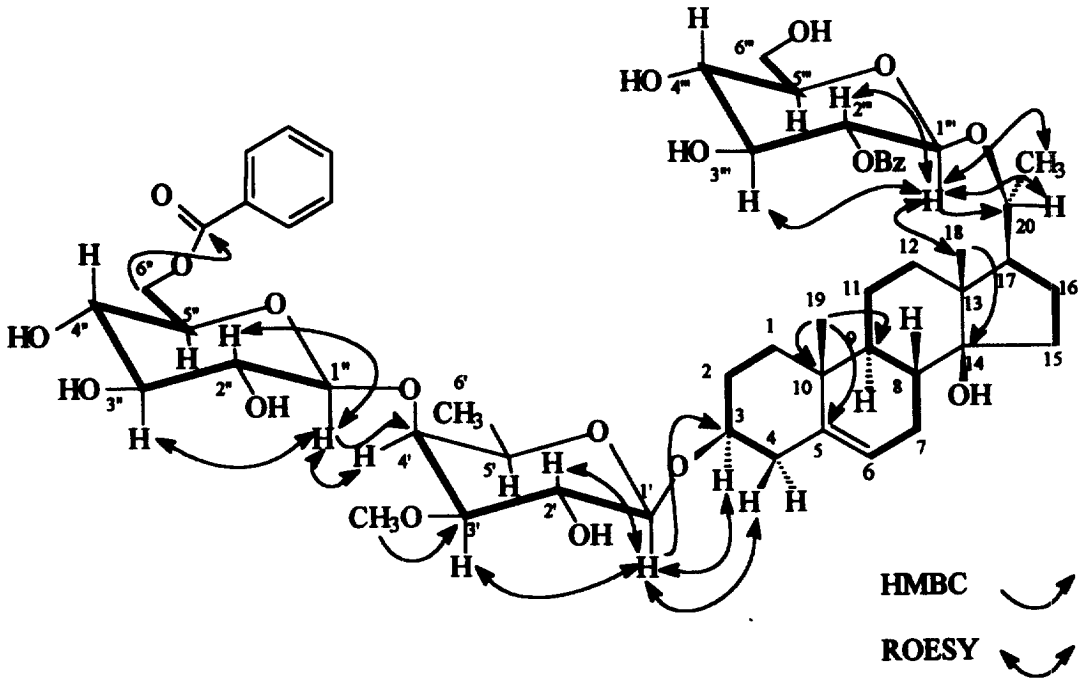

Fig. 3. The significant ROE and ^{1}H-^{13}C long range correlations of carumbelloside V (**3**) observed in the ROESY and HMBC spectra, with HOHAHA coupling fragments shown with bold C—C bonds.

HETCOR spectrum), and which further showed a correlation contour with the carbonyl of the benzoyl group at δ 166.45 from the HMBC experiment.

The remaining benzoyl group was rationalized to be bound to the C-2‴ hydroxyl group by means of vicinal and relayed coupling pattern deciphering using DQF-COSY and HOHAHA spectral analysis, as well as the HMBC experiment. The vicinal coupling of the benzoylated methine proton revealed by the HMBC spectrum at δ 5.76 (*t*, J = 8.5 Hz) with both H-3‴ at δ 4.32 and the anomeric proton H-1‴ at δ 5.17 (*d*, J = 8.5 Hz) which showed ROE cross contours with H_3-21 and H_3-18 as anticipated, were evident in the DQF-COSY spectrum. In addition, H-5‴ at δ 3.95 (*dd*, J = 8.7 and 6.3 Hz) and the methylene proton pair H_2-6‴ could be delineated by the HOHAHA spectrum despite the overlapping signal of H-4‴. Therefore, with the accomplishment of the unambiguous assignments of the NMR signals, the structure of **3** was established as caralumagenin 3-*O*-[6-*O*-benzoyl-β-D-glucopyranosyl(1→4)]-β-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-β-D-glucopyranoside.

It is rarely reported that C-21 steroidal glycosides contain sugar (s) at both C-3 and C-20 positions. To our knowledge, this is the first report of a C-21 steroidal bisdemosidic glycoside with acyl groups bound to the sugars rather than steroidal parent skeleta, usually to the C-11, C-12 and C-20 hydroxyl groups. This may be of some significance for the biosynthesis of pregnane steroidal glycosides and also the chemotaxonomy of the title plant.

Except for showing the potency of advanced 2D-NMR techniques in the structure elucidation of steroidal glycosides, the assignments of these compounds provide a basis for the confirmation of the structures and assignments published, and also facilitate the further structure characterization of related compounds. For example, the apparently uninterpretable ^{13}C assignments on C-3 of the pregnane aglycone and C-2′ of digitalose of caratubersides A and B, isolated from *C. tuberculata* [8], should be revised following the current results.

Carumbellosides III (**1**), IV (**2**) and V (**3**) were devoid of significant cytotoxity in the UIC human cancer cell panel according to established protocols [11].

EXPERIMENTAL

General. Mps: uncorr. UV spectra were taken in MeOH soln on a Beckman DU-7 spectrometer. IR spectra were recorded in a KBr pellet on a MIDAC FT-IR interferometer. The optical rotations were measured with a Perkin–Elmer 241 polarimeter. The ^{1}H, ^{13}C NMR, DEPT, DQF-COSY, HOHAHA, HETCOR and ROESY spectra were recorded at 500.12 MHz for ^{1}H, and 125.76 MHz for ^{13}C with a GE OMEGA 500 instrument, using GE standard programs in C_6D_6N soln. HMBC experiments were carried out on a Varian Plus-400 spectrometer. FAB-MS were recorded by the direct-inlet on a VG ZAB-HS mass spectrometer using glycerol as matrix. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck) and the detection was achieved by spraying with 10% H_2SO_4 followed by heating.

Plant material. The plant material of *C. umbellata*

was collected in Tirupathi, Andhra Pradesh, India, and identified by Dr V. S. Raju, Department of Botany, Kakatiya University, Warangal, India. A voucher sample was deposited in the herbarium of the University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India.

Extraction and isolation. The fresh whole plant (5 kg) of *C. umbellata* was chopped and crushed and extracted with EtOH at room temp. for 7 days. The extract was filtered and the solvent was removed *in vacuo*. To the concentrate, 1 l of H_2O was added, and extracted successively with toluene, ether, EtOAc and *n*-BuOH. After evapn of the solvent, the EtOAc ext and the *n*-BuOH ext were subjected to flash Silica gel CC to yield compounds **1**, **2** and **3**, respectively.

Isolation of carumbellosides III and IV. The *n*-BuOH extract (35 g) was dissolved in MeOH and then $CHCl_3$ was added until carumbelloside **1** pptd out. It was removed by filtration and the filtrate was subjected to flash silica gel chromatography (10–40 μm, 500 g) using EtOAc–MeOH–H_2O (81:11:8) as the eluent and frs of 50 ml each were collected. Frs 85–120 contained carumbelloside III and were purified further by a repetition of the process. Frs 15–21 contained carumbelloside IV which was purified by re-chromatography using the same system.

Carumbelloside III (**1**). (600 mg, yield 0.012%). amorphous powder, mp 183–185°; $[\alpha]_D^{19} - 18.4°$ (MeOH, *c* 0.50). IR ν_{max} (cm^{-1}): 3600–3200 (OH), 980, 920, 902, 856. FAB-MS *m/z* (negative): 817 $[M-H]^-$, 655 $[M-H-162]^-$,493 $[M-H-162-162]^-$; *m/z* (positive): 841 $[M+Na]^+$, 819 $[M+H]^+$, 801 $[M+H-H_2O]^+$, 639 $[M+H-H_2O-162]^+$, 495 $[M+H-162-162]^+$, 479 $[M+H-H_2O-162-160]^+$. ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 2.

Carumbelloside IV (**2**). (75 mg, yield 0.0015%). amorphous powder, mp 188–190°; $[\alpha]_D^{19} - 36.8°$ (MeOH, *c* 0.50). UV λ_{max}^{EtOH} nm (log ε): 218 (4.24), 224 (4.30), 281 (4.15). IR ν_{max} (cm^{-1}): 3450, 1700, 1600, 1450, 1280. FAB-MS *m/z* (positive): 923 $[M+H]^+$, 905 $[M+H-H_2O]^+$, 743 $[M+H-H_2O-162]^+$; *m/z* (negative): 921 $[M-H]^-$, 817 $[M-H-Bz]^-$, 655 $[M-H-Bz-162]^-$. ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 2.

Isolation of carumbelloside V (**3**). The EtOAc extract (around 10 g) was subjected to flash chromatography eluting with $CHCl_3$–MeOH (95:5). Frs were pooled and re-chromatographed using EtOAc–MeOH–H_2O (81:11:8) to yield carumbelloside V.

Carumbelloside V (**3**). (25 mg, yield 0.0005%), mp 160–162°; $[\alpha]_D^{19} - 32.4°$ (MeOH, *c* 0.50). UV λ_{max}^{EtOH} nm (log ε): 229 (4.05), 257 (3.30), 278 (3.21). IR ν_{max} (cm^{-1}): 3420, 1700, 1600, 1450, 1280. FAB-MS *m/z* (negative): 1025 $[M-H]^-$, 921 $[M-H-Bz]^-$, 655 $[M-H-Bz-162]^-$. ^1H NMR and ^{13}C NMR spectroscopic data are shown in Tables 1 and 2.

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