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Renieramycins H and I, two novel alkaloids from the sponge *Haliclona cribricutis* Dendy

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The known alkaloid, mimosamycin **1**, along with its mono- and di hydroxy derivatives, 4-hydroxy mimosamycin **2** and 1,4-dihydroxymimosamycin **3** and two new dimeric alkaloids: namely renieramycins H and I **4** and **5** have been isolated from the MeOH extract of the bright blue sponge *Haliclona cribricutis*. These compounds have been purified on reverse phase HPLC and their structures finalized from their respective spectral data.

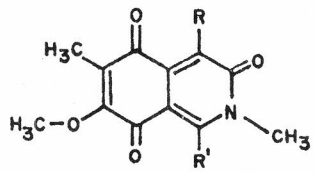
The methanol extract of the sponge *Haliclona cribricutis* exhibited promising antiviral and antibacterial properties[1]. The crude extract was subsequently partitioned into hexane, EtOAc, *n*-BuOH and aqueous fractions. Antibacterial studies revealed the hexane and EtOAc fractions to be more active than the latter two polar fractions. Several fatty acids, sterols and a sphingosine analog were isolated from the hexane fraction[2]. The hexane and EtOAc fractions were rich in alkaloids. We had earlier isolated two major metabolites, *O*-demethyl renierone **6** and its dimer **7** from this fraction[1]. Further studies of this fraction led to the isolation of five more compounds, *viz*, mimosamycin **1**, 4-hydroxy-mimosamycin **2** and 1,4-dihydroxymimosamycin **3**, renieramycin H **4** and renieramycin I **5.**

Compound **1**, a yellow solid, appeared as a bright yellow fluorescent spot on TLC plates. The IR spectrum of this compound had multiple peaks in the region of 1600-1700 cm^{-1}, indicating the presence of quinone, amide and C=C groups. The EIMS revealed the molecular weight to be 233. Presence of hydroxyl groups are ruled out as the compound had no IR absorption around 3500 cm^{-1}. The ^1H NMR spectrum had two vinyl proton signals at δ 7.15 and 8.30 (1H each, s) besides three methyl singlets (δ 2.1, 3.7 and 4.2). These values were identical to that of mimosamycin, previously reported from the sponge *Reniera* sp[3]. and the bacteria *S. lavendulae*[5].

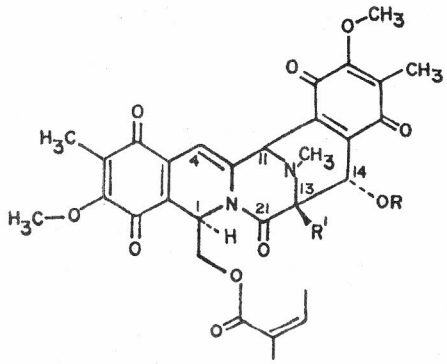
Compound **2**, isolated as an orange-red crystalline solid, mp 225°C, had IR absorptions at 3420, 1660 and 1640 cm^{-1}, suggesting the presence of hydroxyl, quinone and amide groups. The compound had strong UV-Vis absorptions at 227.5 and 485 nm. The EIMS revealed its molecular weight to be 249 amu., which is 16 mass units more than mimosamycin **1**.

Its ^1H NMR spectrum was similar to that of mimosamycin, but for the absence of one vinyl proton and a shift of the other signal to δ 7.60. The additional broad proton signal at δ 8.85 (br, exchanged with D_2O), indicated the replacement of one of the vinyl protons by hydroxyl group. In view of the negligible influence of this hydroxyl group on the chemical shift of N-Me signal, its position was fixed at C-4. This is also supported by the fact that this compound could be directly obtained from 14-hydroxy- renieramycins as proposed by He *et al* [4]. From the above results the structure of this compound was finalized as 4-hydroxymimosamycin **2**.

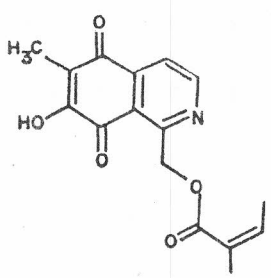
Compound **3**. The UV-Vis spectrum of compound **3** was similar to that of compound **2** while its ^1H NMR spectrum had all three methyl signals as found in compounds **1** and **2**. However, signals due to the ring protons 1-H and 4-H found in compound **1** were absent in it. Instead, two D_2O-exchangeable signals appeared at δ 8.85 and 13.2, indicating replacement of both the ring protons by OH groups. Both EIMS and CIMS



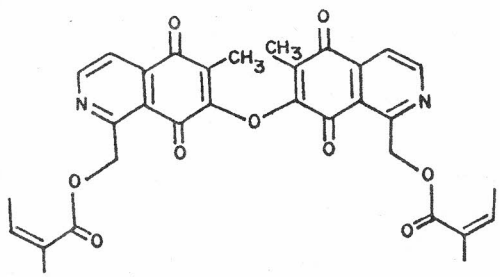
1 R = R' = H, Mimosamycin
2 R = OH, R' = H, 4-Hydroxy Mimosamycin
3 R = R' = OH, 1,4-Dihydroxy Mimosamycin



4 : R = H, R' = OH, Renieramycin H
5 : R = CH_3, R' = H, Renieramycin I



6 O-Demethyl Renierone



7 Dimeric ether of compound 6

revealed its molecular weight to be 265 amu, which is 32 mass units more than mimosamycin **1**. From this, its structure was finalized as 1,4-dihydroxymimosamycin **3**. The position of second hydroxyl group at C-1 was also supported by the upfield shift by 0.2 ppm of the N-Me signal in its ^{1}H NMR spectrum as compared to compounds **1** and **2**. Our observation that compounds **1** and **2** undergoes air-oxidation upon storage, yielding compound **3** further confirms its structure.

Compound **4**, (10 mg, R_t=41.7 min) was isolated as a red amorphous powder. CIMS and EIMS revealed its molecular weight to be 594 amu. The elemental composition of the prominent peak at *m/z* 580 $[M-OH+3H]^+$ was determined to be $C_{30}H_{32}N_2O_{10}$ by HRMS (calculated : 580.2057, experimental: 580.2066). From this, the molecular formula was deduced to be $C_{30}H_{30}N_2O_{11}$.

Its IR spectral bands at 3425, 1726, 1690 and 1648cm^{-1} were indicative of the presence of hydroxyl, ester, quinone and amide groups. The quinonoid nature was also indicated by its strong UV-Vis absorptions at 225, 275, 365 and 520 nm and the ^{13}C NMR peaks at δ 179, 184 and 192[3,4]. Its ^{1}H NMR spectrum had signals due to two vinyl methyls (δ 2.15 and 1.95), two OMe's (δ 4.05 and 3.85), one NMe (δ 2.56), one angelate moiety [δ 1.46(3H,d,1.5Hz), 1.74(3H,dq,7.25 & 1.5 Hz), and 5.91(1H,qq,7.25 & 1.25 Hz) and two D_2O-

exchangeable protons (δ 5.7 and 11.34]. The "dimeric" nature of the molecule was indicated by the presence of characteristic pairs of carbon signals between δ 119 and 192[3].

Comparison of the ^{13}C and ^{1}H NMR spectral values with those of renieramycins[3-5] and saframycins[6] indicated its close structural similarity to renieramycin C. A notable difference was the absence of aliphatic 3-H and 4-H_2 signals which appear at δ 4.19, 3.02 and 3.86 respectively in renieramycin C. Instead, a vinyl proton signal appeared at δ 6.26 (1H,s). The corresponding carbon was located at δ 100.0(d) by ^{1}H-^{13}C COSY spectrum. Comparison of these values with the chemical shifts of C-4 and H-4 in *N*-formyl-1,2-dihydrorenierones[3] indicated the presence of unsaturation at C-3. The new carbon signal at δ 134.7(s) could be assigned to C-3. This is also supported by the downfield chemical shifts of 1-H and 11-H compared to renieramycins C and D (δ 6.20 and 4.80 in **4** as compared to δ5.48 and 4.19 in renieramycin C and δ 5.49 and 4.22 in renieramycin D[3]). Another notable difference was the absence of 13-H signal in this compound. In renieramycins C and D this proton appears at δ 3.73 and 3.67 respectively. On the other hand, compound **4** has two D_2O-exchangeable protons [δ 5.7 and 11.34 (1H each)] as compared to only one such proton in renieramycin C (C_{14}-OH). From this it may be presumed that the second hydroxyl group is present at C-13. The ^{13}C NMR spectrum of compound **4** had a new signal at δ 108(s), which could be assigned to C-13. The downfield shift of this carbon is expected as it is linked to carbonyl, nitrogen and hydroxyl groups. The ^{1}H NMR spectrum of this compound also revealed a long range coupling between 11-H and 14-H (1.3 Hz). The allylic coupling between 11-H and 4-H is ruled out as the latter signal appears as a sharp singlet.

Spectral properties of compound **5** (R_t=54.4 min, 2 mg) were similar to those of renieramycin H (**4**). Thus, it had strong IR bands at 1716, 1652 and 1600-1500 cm^{-1}, probably due to ester, amide and vinyl groups respectively. Its UV-Vis absorptions at 220, 260, 340 & 520 nm were similar to other renieramycins[3-4]. As its IR spectrum had no absorption around 3500 cm^{-1} presence of hydroxyl groups in the molecule could be ruled out. EIMS and FABMS revealed its molecular weight to be 592 amu., the elemental composition of which was determined to be $C_{31}H_{32}N_2O_{10}$ by HRMS (Experimental: 592.2086, Calculated: 592.2057).

Further support for the renieramycin type structure was provided by the characteristic signals due to OMe's (δ 4.05 and 3.85), one N-Me (δ2.55) and two vinyl methyl groups δ 1.96 and 1.94(3H each). The signal at δ 3.62(3H,s) was assigned to 14-OMe group by analogy with renieramycin F[4]. Comparison of the ^{1}H NMR spectrum with those of renieramycins H, C and D[3] led to the assignment of all proton signals. Thus, the signal at δ 4.54(1H,d,0.93Hz) and 4.34(1H,d,1.83 Hz) were assigned to 11-H and 14-H. The signals at δ 1.55 (3H,d,1.6 Hz), 1.73 (3H,dq,7.25 and 1.6 Hz) and 5.91 (1H,qq,7.25 and 1.6 Hz) could be easily assigned to the angelate ester moiety. The signal at δ 3.73(1H, brs) must be due to 13-H[3]. The very small vicinal coupling between 13-H and 14-H indicated them to be orthogonal to each other[4].

Further support for these structures were provided by their similar MS fragmentations (Table IV).

Renieramycins are very labile compounds, readily decomposing to 'monomeric' renierone, mimosamycin and their analogs. He *et al.*[4] have shown that the crude extract is richer in dimeric renieramycins, which decompose upon storage/silica gel column chromatography to yield renierone / mimosamycin and their analogs. Frincke *et al.*[3] had indicated 1-H and 3-H in renieramycins A-D to be *trans* , whereas, they are *cis* in saframycin C as determined by X-ray crystallographic studies[7]. Subsequent low power NOEDS studies have shown that 1-H and 3-H are indeed *cis* in renieramycins A-D[4]. The same results were arrived at by Fukuyama *et. al.*, from synthetic studies of saframycins[8] and renieramycins[9]. since the ^{1}H and ^{13}C NMR spectra of compounds **4** and **5** are very similar to those of other renieramycins and saframycins, we believe that their stereochemistry are also identical.

The alkaloids **1-7** have close structural similarities to those reported from similar sponges, the bacteria *S. lavendulae*[6] and their predator nudibranch[10]. The notable difference in the structures of renieramycins H and I from the known compounds C and D are in the presence of a new double bond at C-3 in the former pair. In

addition, 13-H has been oxidized to hydroxyl group in renieramycin H **4**. The major monomeric alkaloids O-demethyl renierone and mimosamycin are found both in this sponge and in *Reniera* sp. Biologists are of the opinion that the names *Reniera* and *Haliclona* denote one and the same genus. This means that similar bacteria are endemic to these sponges all over the world or that these compounds are genuine sponge metabolites.

Experimental Section

HPLC separations were carried out on a Spectra Physics Model, 8800, fitted with an ODS column (5μ, 250X8 mm, MeOH-Water=3:1, 254 nm).The IR and UV-Vis spectra were recorded on Perkin Elmer Model 1640 and Beckman Model DU-6 spectrophotometers. ^{1}H and ^{13}C NMR spectra were recorded on Bruker WM-200 and 400 MHz instruments using $CDCl_3$ as solvent and TMS as internal standard. CIMS was obtained on a BIOSPET Mass Spectrometer using CH_4 as reagent gas at a source temperature of 180° C. EIMS and LSMIS (Liquid Secondary Ion Mass Spectra) were obtained on a Finnigan MAT-90 Spectrometer, having ultra high resolution double focussing facilities and operating at an accelerating voltage of 5 Kev. EIMS was recorded at 70 ev at a probe temp. of 150° C while the source temperature was maintained at 250° C. For LSIMS experiments, samples dissolved in DMSO (2-5 μg/mL) was deposited on a stainless steel probe tip and then mixed with a thin layer of glycerol-thioglycerol (1:1) mixture. These samples were ionised by bombardment with cesium ions of an energy of 20 Kev. The temp. of the source was maintained at 25° C.

Extraction and isolation of compounds

The animals (10 Kg), collected from the intertidal region in Okha, Gujarat, were immediately soaked in MeOH and transported to the laboratory. After two weeks, the solvent was drained off, concentrated under vacuum and fractionated into pet. ether, EtOAc, *n*-BuOH, and water-soluble fractions in the usual way. The fractions were tested for antimicrobial properties.

Table I — Antibacterial activity of various fractions*

Fraction	*S.typhi-murum*	*E. coli*	*B subt-ilis*	*P.mira-bilis*	*V..parahaemolyticus*	*S. aureus*	*Arthrobacter sp.*	*B. polymirc*	*M. aureus*
Pet. Ether	20	26	20	11	19	30	28	12	18
EtOAc	20	10	18	8	20	12	10	11	16
n-BuOH	--	--	8	--	--	--	--	--	--
Aqueous	8	16	12	--	12	16	16	15	12

[a] Expressed as inhibition diameter against 6mm dia. disks containing 50 μg of the compounds per disk

Table II —^{1}H NMR spectral data of renieramycins H and I

Proton	$^{1}HNMR$ (δ, ppm) Renieramycin H	Renieramycin I
1-H	6.20(dd,6.3 & 2.7 Hz)	6.07(dd,5.28 & 2.67 Hz)
4-H	6.26(s)	6.25(s)
11-H	4.80(d,1.3 Hz)	4.54(d,0.93 Hz)
13-H	----	3.73(brs)
14-H	4.10(d,1.3 Hz)	4.34(d,1.83 Hz)
25-Me	1.46(d,1.5Hz)	1.55(d,1.6 Hz)
26-H	5.91(qq,7.25 & 1.5 Hz)	5.91(qq,7.25 & 1.5 Hz)
26-Me	1.74(dq,7.25 & 1.5 Hz)	1.73(dq,7.25 & 1.55 Hz)
Vinyl Me	2.15(s)	1.96(s)
Vinyl Me	1.95(s)	1.94(s)
22-H	4.05(dd,11.8 & 6.8 Hz)	4,16(dd,12.1 & 2.67 Hz)
22-H'	3.82(dd,11.8 & 3.1 Hz)	4.01(dd,12.1 & 2.67 Hz)
Vinyl OMe	4.05(s)	4.05(s)
Vinyl OMe	3.85(s)	3.98(s)
14-OMe	----	3.62(s)
NMe	2.56(s)	2.55(s)
OH	11.34(s)	----
OH	5.70(s)	----

Tests showed that the pet.ether and EtOAc fractions are very active. Repeated chromatography of these fractions over silica gel helped in separating lipids from alkaloids[1,2]. The alkaloid fraction was purified by silica gel chromatography and HPLC to yield compounds **1-7.**

Antimicrobial screening

The antibiotic assays were done using the agar diffusion method[1]. The test cultures (The bacteria: *Salmonella typhimurium* MTCC-98, *Micrococcus luteus* MTCC-106, *Klebsiella pneumoniae* MTCC-109, *Escherichia coli* MTCC-118, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC-96, *Proteus mirabilis* MTCC-425 and *Vibrio parahaemolyticus* MTCC-451 the fungi : *Candida tropicalis* MTCC-230, *Rhodotorula rubra* MTCC-248 and *Saccharomyces cerevisiae* MTCC-249) were grown overnight in nutrient broth which was then spread on nutrient agar plates to form a uniform lawn. Paper discs (HIMEDIA, Bombay; 6 mm diameter) bearing 50 μg of the compounds were placed on the media and incubated at 37° C in a bactereological incubator. After 24 hrs., the diameter of the zone of inhibition (in mm) was recorded. All test cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The results are given in Table I.

Mimosamycin **1**: R_t=12.2 min., was isolated as an yellow solid. IR (KBr): 3050, 2957, 1686, 1646, 1636, 1618, 1584, 1545, 1368 and 1320 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.3 (1H,s,1-H), 7.15(1H,s,4-H), 4.2 (3H,s,OMe), 3.7(3H,s,NMe), 2.1(3H,s, vinyl Me); EIMS *m/z*(%): 235($[M+2H]^+$,19), 234-($[M+H]^+$,100), 219($[M+H-CH_3]^+$,16.1), 218($[M-CH_3]^+$,36.8), 205($[M-CO]^+$,48.2), 190(44.6), 177(27.3).

4-Hydroxy mimosamycin **2**: R_t 14.5 min.,MP 225°C, IR(KBr): 3420, 3290, 3030, 2920, 2840, 1660, 1640, 1580, 1500, 1440, 1280, 1220, 900, 800 and 745 cm^{-1}; UV-Vis [MeOH, (log ε)]: 227.5(4.05), 485(3.58) nm; ^1H NMR ($CDCl_3$): δ 8.85 (1H,brs, OH, exchangeable with D_2O), 7.62(1H,s, 1-H), 4.06(3H,s,OMe), 3.64(3H,s,NMe) 2.07(3H,s,vinyl Me); PCIMS(CH_4): 250$[M+H]^+$.

1,4-Dihydroxymimosamycin **3**: UV-Vis [MeOH, (log ε)]: 220(4.11), 255(3.75), 500 (3.58) nm; EIMS *m/z*(%): 265(M^+, 59), 247(8.3), 222(9.1), 219(7.8), 181(18.4); ^1H NMR ($CDCl_3$): 13.28 and 8.85 (1H each, s,OH exchanged with D_2O), 4.0(3H, s, OMe), 3.46(3H, s, NMe), 2.16(3H, s, vinyl Me).

Table III—^{13}C NMR spectral data of renieramycin H

Carbon No.	Chem shifts (δ ppm)	Carbon No.	Chem shifts (δ ppm)
C-1	46.96(d)	C-17	156.42(s)[d]
C-3	134.70(s)	C-18	Not obsvd.
C-4	100.00(d)	C-18a	124.28(s)[e]
C-4a	139.95(s)[b]	C-21	161.17(s)
C-5	184.00(s)	C-22	62.12(t)
C-6	119.93(s)[c]	C-23	Not obsvd.
C-7	153.36(s)[d]	C-24	127.10(s)
C-8	179.00(s)	C-25	15.39(q)
C-8a	126.74(s)[e]	C-26	139.09(d)
C-11	56.32(d)	C-27	19.85(q)
C-13	108.00(s)	Vinyl Me	8.54(q)
C-14	72.60(d)	Vinyl Me	8.93(q)
C-14a	138.18(s)[b]	Vinyl OMe	61.10(q)
C-15	192.00	Vinyl OMe	61.20(q)
C-16	119.16(s)[c]	N-Me	41.22(q)

[a] NMR spectrum was obtained on Bruker WM-400 instrument in $CDCl_3$ solution with TMS as internal standard and the multiplicities confirmed by 2D HETCORR experiments.

[bcde] Values with identical superscripts may be interchanged.

Table IV —EIMS Data of Renieramycins H (4) and I (5))

Renieramycin H 4 *m/z* (%)	Renieramycin I 5 *m/z* (%)
594(M^+, 1.5)	594($[M+2H]^+$,5)
580($[M-OH+3H]^+$,12)	593($[M+H]^+$,4)
550($[M-28-18+2H]^+$,1	562($[M-28-2H]^+$,12.5)
522(550–28, 1.5)	534(562–28, 3.5)
481($[M-4\times28-H]^+$, 2.5)	482($[M-4\times28]^+$, 3)
467($[M-4\times28-15]^+$, 11)	479($[M-4\times28-H]^+$, 15)
453($[M-5\times28-H]^+$, 10.5)	451($[M-5\times28-H]^+$, 75)
439($[M-5\times28-15]^+$, 75)	421($[M-6\times28-3H]^+$, 100)
396($[M-7\times28-2H]^+$, 4.5)	-----
279(31)	250(57)
234(5)	218(43)
167(40)	-----
149(100)	149(11)

Renieramycin H (**4**): R_t=41.8min; IR(KBr): 3425, 2964, 2921, 2850, 1726, 1705, 1648, 1563, 1457, 1379, 1279, 1123, 1027, 801 and 744 cm^{-1}; UV-Vis [MeOH (log ε)]: 225(4.5), 275(4.0), 365(3.5), 520(3.0) nm; For ^1H and ^{13}C NMR spectra, see Tables II and III; PCIMS *m/z*: 594$[M]^+$, 580$[M-OH+3H]^+$; LSIMS *m/z*: 580(3.53), 579(3.85), 466 (2.6), 453(2.85), 438(4.94), 414(20.31) and 413(29.8); HREIMS of the peak at *m/z* 580 determined its elemental composition as $C_{30}H_{32}N_2O_{10}$ (Calculated: 580.2057, experimental: 580.2066). The EI mass fragmentation values are given in Table IV.

Renieramycin I, **5**: R_t 51.4 min., IR(KBr): 2936, 2850, 1716, 1652, 1567, 1453, 1375, 1233, 1205, 1148 & 1091 cm^{-1}; UV-Vis [MeOH, (log ε)]: 220(4.4), 260(4.1), 340(3.5) & 520(3.1) nm; For ^1H NMR spectrum see Table 2; PCIMS *m/z*: 592$[M]^+$, 564$[M-CO]^+$; LSIMS *m/z* (%): 595($[M+3H]^+$,4.53), 594($[M+2H]^+$,4.2), 593($[M+H]^+$, 7.34), 563($[M+H-CO]^+$,3.77), 561(4.22), 453(3.7) and 451(4.74); The EI mass fragmentation is given in Table 4. HRMS of the peak at *m/z* 592 determined its elemental composition as $C_{31}H_{32}N_2O_{10}$ (Calculated: 592.2057, experimental: 592.2086).

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