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Misenine, a Novel Macrocyclic Alkaloid with an Unusual Skeleton from the Mediterranean Sponge *Reniera* sp.

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Abstract: Misenine (**1**/**1a**), a new marine alkaloid possessing an unprecedented tetracyclic cage-like core with two macrocyclic rings, has been isolated from the Mediterranean sponge *Reniera* sp. and its structure was determined by extensive spectroscopic studies. A suggestion is made as to its biogenetic origin.

Polycyclic marine alkaloids containing 3-alkylpyridine or partially reduced 3-alkylpyridine as basic building blocks represent an emerging and intriguing group of bioactive natural products from marine sponges. Since halitoxin,[2] the first example of this kind of alkaloid, was reported in 1978, many related alkaloids have been discovered from sponges of the order Haplosclerida. All of these alkaloids, in spite of formally exhibiting quite different frameworks, could biogenetically derive from *bis*-3-alkylpyridine or reduced *bis*-3-alkylpyridine units. In the last decade, the knowledge of this fascinating group of compounds has increased remarkably. A recent review has exhaustively studied occurrence, distribution, plausible biogenetic origin and relatedness, as well as biological activities, of 3-alkylpyridine derived marine alkaloids.[3]

The Mediterranean sponge *Reniera sarai* is a rich source of 3-alkylpyridine derived alkaloids. Nine macrocyclic alkaloids, named saraines, which occupy two positions of the eleven novel skeleta hitherto reported in the literature,[3] were found by our group.[4-10] During our continuing effort aimed at finding new bioactive compounds from marine organisms, another *Reniera* sponge was collected in the Bay of Naples. Chemical investigations of this sponge led to the isolation of the novel macrocyclic alkaloid, misenine (**1**). The structure of **1**, the first example of a new class of macrocyclic diamine alkaloids, is reported here.

The sponge *Reniera* sp. was collected off Capo Miseno (the locality suggested the name assigned to the new alkaloid), Naples, Italy in 1994 and kept frozen until used. The acetone extract of the sponge was first partitioned between Et_2O and water and subsequently between *n*-butanol and water. The Et_2O soluble material was fractionated by silica gel column chromatography (gradient from $CHCl_3$ to MeOH). The fractions eluted with $CHCl_3$/MeOH (9:1 and 8:2) were further purified by silica gel column ($CHCl_3$ as eluant) affording pure misenine (**1**). Compound **1** was also obtained from the *n*-butanol soluble fraction by using the same work-up.

Misenine (**1**) was isolated as an optically active colourless oil {$[\alpha]_D$ + 6.4° ($CHCl_3$)} that gave an intense FABMS peak at *m/z* 495 $(M+H)^+$. The molecular formula, $C_{33}H_{54}N_2O$, of misenine was established by HREIMS (*m/z* 494.4230, M^+, Δ = + 0.6 mmu of that calculated). The structural determination of misenine was aided by the previous experience acquired during the structural elucidation of saraines. In fact, **1** showed

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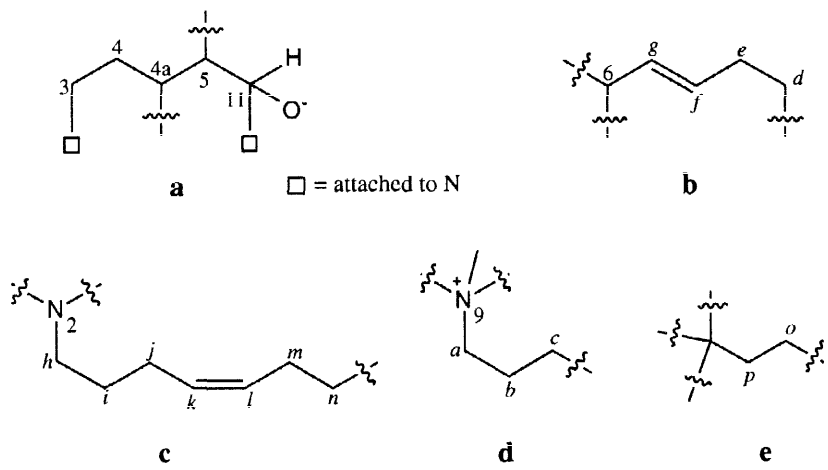
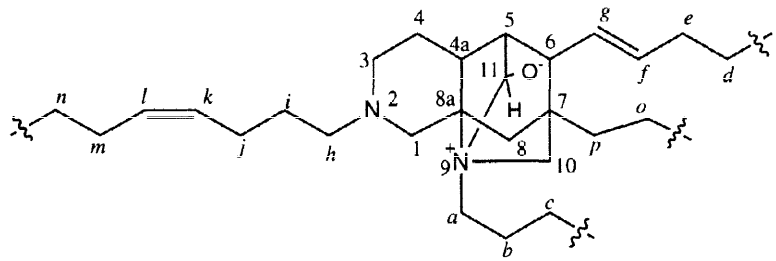
spectral and chemical properties very similar to those of saraines. Its NMR spectra were also strongly influenced by the concentration of the sample, by the pH value of the solution and by traces of inorganic impurities. Due to these reasons the NMR spectra of the same sample of misenine were seldom reproducible. Unfortunately, it was impossible to obtain crystalline derivatives of misenine suitable for a resolved diffractometric analysis. By analogy with saraines, a $CHCl_3$ solution of **1** was washed with a saturated $NaHCO_3$ solution and then immediately submitted to NMR analysis obtaining well-resolved NMR spectra. All 1D and 2D NMR experiments (^1H-^1H COSY, TOCSY, HMQC, HMBC, NOESY) including DEPT and spin-decoupling experiments were performed on the same sample very quickly.

The ^{13}C-NMR data of **1** disclosed four sp^3 methines, four sp^2 methines, two sp^3 quaternary carbons and twenty-three sp^3 methylenes. All the protons were connected to carbons (**Table 1**) by HMQC experiments. The presence of two disubstituted double bonds was easily recognized by ^1H- and ^{13}C-NMR resonances (δ_H 5.85, 5.42, 5.42, 5.35; δ_C 131.3, 129.0, 129.6, 130.0). These data account for two of the required eight sites of unsaturation. Consequently, misenine must possess six rings containing two nitrogens. In the ^1H-NMR spectrum, the signal at δ 4.98, correlated by HETCOR experiment to a ^{13}C-NMR resonance at δ 94.9, was assigned to a methine between a nitrogen and an oxygen (N-CH-O-) which was strongly reminiscent of the aminal proton of saraine-A.[6] In order to confirm this relation, CD_3COOD was added to a $CDCl_3$ solution of **1**. ^1H- and ^{13}C-NMR spectra of this sample (**1a**) (**Table 1**) displayed a new signal at δ 8.71, correlated by HETCOR experiment, to a carbon resonating at δ 174.5, assignable to an aldehydic proton meanwhile the signal at δ 4.98 disappeared. This experiment provided an important anchor point to begin the substructure analysis on the basis of ^1H-^1H COSY, TOCSY, HMQC, HMBC, and NOESY spectral data.



Analysis of the ^1H-NMR spectra of **1**, aided by ^1H-^1H COSY, HMQC, TOCSY and HMBC, revealed the proton connectivities for five partial structures **a**-**e** and also allowed recognition of three pairs of diastereotopic methylenes which should be located either between a nitrogen and a quaternary carbon (δ 63.0, 58.8) or between two quaternary carbons (δ 43.0) based on their downfield ^{13}C-NMR chemical shifts. For the partial structure **a**, the nitrogen-bearing methylene (δ 2.89, H-3_{eq}; 2.07, H-3_{ax}; δ 54.8, C-3) exhibited clear correlations with the adjacent methylene protons (δ 1.72, H-4_{ax}, 1.53, H-4_{eq}) which, in turn, were further correlated with the methine at δ 1.43 (H-4a); on the other hand, the sharp doublet signal at δ 4.98 (H-11) was

linked to the methine proton at δ 2.37 (H-5) which, in turn, was also coupled with H-4a. For the partial structure **b**, the doublet signal at δ 2.18 was assigned to an allylic proton (H-6) linked to a double bond with resonances at δ 5.85 (H-*g*) and δ 5.42 (H-*f*). Further correlations between H-*f* and H_2-*e* (δ 2.07, 1.97), between H_2-*e* and H_2-*d* (δ 1.43) were also observed. The *trans* nature of the double bond was assigned on the basis of the large coupling constant between H-*f* and H-*g* (*J*=15.8 Hz). Strangely, no coupling was observed between H-5 and H-6. For the partial structure **c**, starting from the nitrogen-bearing methylene (δ 2.34 and 2.30), assigned to H_2-*h* (δ_C 57.4), the ^{1}H-^{1}H COSY, TOCSY and HMBC indicated the presence of three consecutive methylene units, the last one (δ 2.04, 1.94, H_2-*j*) being linked to the second isolated double with the resonances at δ 5.42 (H-*k*) and δ 5.35 (H-*l*) which was in turn connected to H_2-*m* (δ 2.04, 1.98) and H_2-*n* (δ 1.37, 1.33). The *cis* geometry of this double bond was suggested on the basis of the ^{13}C-NMR resonances of the vinyl carbons C-*j* (δ 27.3), C-*m* (δ 27.1). Analogously, starting from H_2-*a* (δ_H 2.74, 2.66; δ_C 53.4), a series of distinct correlations between H_2-*a* and H_2-*b* (δ 1.48, 1.33), between H_2-*b* and H_2-*c* (δ 1.61, 1.22) were observed according to the partial structural **d**. Finally, the HMQC spectra of **1** showed another two well resolved methylenes (δ_H 1.34, 1.26; δ_C 20.7 and δ_H 1.28, 1.20; δ_C 38.5) which were assigned to C-*o* and C-*p* (partial structural **e**), respectively, being linked to a quaternary carbon. NMR data were completed by assigning the residual overlapping resonances to the methylenes in the aliphatic chains.



f

All the subunits, bearing in mind three unassigned AB type methylenes (C-1, δ 63.0; C-8, δ 43.0; C-10, δ 58.8) and two quaternary carbons (C-7, δ 37.7; C-8a, δ 76.0), were connected by extensive interpretation of well-resolved HMBC spectra. Significant ^{1}H-^{13}C long-range correlations, as shown in **Table 1**, connected C-1 (δ 63.0) to H-3_{eq} (δ 2.89), H_2-*h* (δ 2.34, 2.30), and H-8_{eq} (δ 1.22); C-4a (δ 47.1) to H_2-1 (δ 2.81, 1.88), H_2-3 (δ 2.89, 2.07), H-5 (δ 2.37), H-6 (δ 2.18) and H-8_{eq}; C-8a (δ 76.0) to H-4a (δ 1.43), H-1_{eq} (δ 2.81), and H-8_{ax} (δ 2.41); C-7 (δ 37.7) to H-5 (δ 2.37), H_2-10 (δ 2.72, 2.42), H-8_{eq}, and H-*g* (δ 5.85); C-11 (δ 94.9) to H_2-*a* (δ 2.74, 2.66) and H-6; C-*p* (δ 38.5) to H-6. Thus, the partial structure **f** was unambiguously assigned.

The relative stereochemistry around the tetracyclic core (**Figure 1**) was established by detailed analysis of NOE difference, NOESY, as well as decoupling spectra. The NOE's between H-6 and H_2-4 (δ 1.72, 1.53), H-8_{ax} (δ 2.41), H_2-*p* (δ 1.28, 1.20); between H-4_{ax} (δ 1.72) and H-8_{ax} indicated, for the A and B rings of the isoquinoline moiety, a *cis*-fused junction adopting chair conformations. Analogously with A and B, C ring, resulting from the interaction between C-11 and N-9, also adopted a chair conformation. In addition, analysis of the Drieding model constructed on the basis of NOE experiments revealed that the dihedral angle between H-5 and H-6 was almost 90° according to a coupling constant very close to zero and according to the absence of cross-peaks between H-5 and H-6 in the ^{1}H-^{1}H COSY experiments.

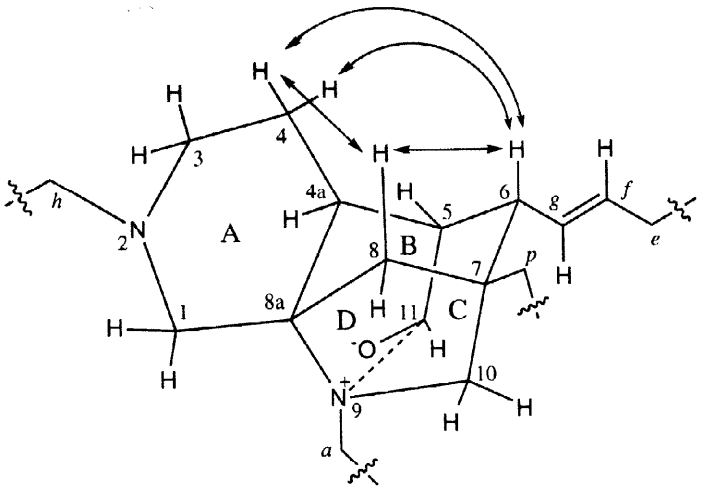
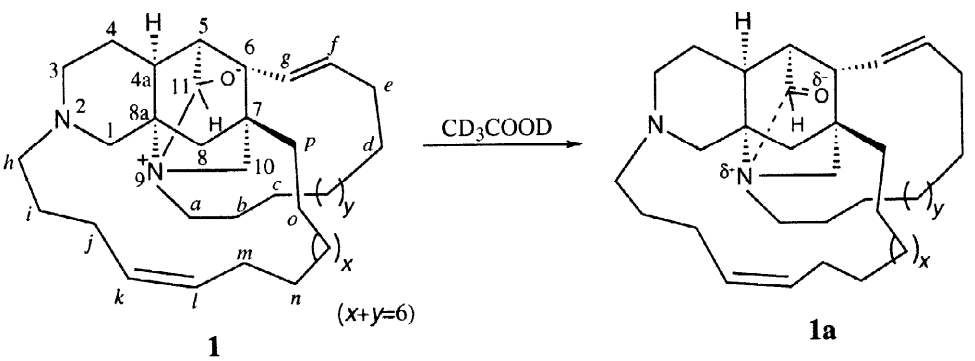
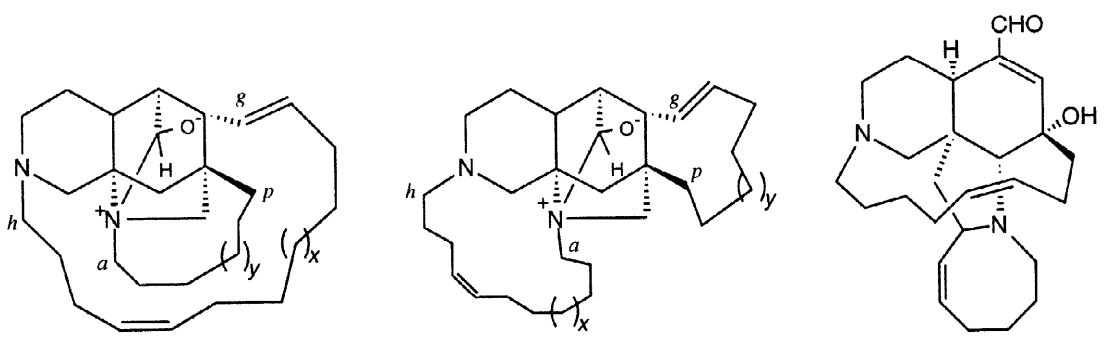


Figure 1. NOE analysis of misenine (**1**)

Subtraction of the atoms present in the above elaborated tetracyclic core and aliphatic side-chains from the molecular formula of misenine (**1**) indicated that the other 6 methylenes have to complete the alkyl chains to form two linear bridges between the four appendages at N-2, N-9, C-6, and C-7 according to the unsaturation number indicated by the molecular formula. Unfortunately, it was not possible to determine the individual lengths of the two alkyl chains, because the aliphatic ends of **b**-**e** showed couplings with high field protons (δ 1.3-1.5), which, in turn, were further coupled with protons resonating in the same range. As a consequence, three spectroscopically indistinguishable alternatives (**1**-**3**) can be depicted. Biogenetic considerations, which are discussed below, suggest **1** as the most reasonable structure for misenine.



1 **1a**



2 **3** **4**

Table 1. ^{1}H- and ^{13}C-NMR Data[a] of Misenine (**1/1a**) and HMBC[b] Correlations Observed for **1**

No.[e]	**1**			**1a**	
	^{13}C, *m*[c]	^{1}H[d], *m* (*J*=Hz)	HMBC (C-H)	^{13}C, *m*[c]	^{1}H[d], *m* (*J*=Hz)
1	63.0 *t*	2.81eq, *d* (9.8) 1.88ax, *d* (9.8)	H-3eq, H_2-*h*, H-8eq	64.4 *t*	2.70cq, *d* (11.0) 1.88ax, *m*
3	54.8 *t*	2.89eq, *br. d* (11.0) 2.07ax, *m*	H_2-1, H_2-*h*, H-4a	54.8 *t*	2.80eq, *br. d* (9.8) 2.18ax, *m*
4	24.8 *t*	1.72ax, *dddd* (12.5,12.5,12.5,4.4) 1.53eq, *m*	H_2-3, H_2-*h*	28.3 *t*	1.78, *m* 1.64, *m*
4a	47.1 *d*	1.43ax, *m*	H_2-1, H-3eq, H-4ax, H-5, H-6, H-8eq	43.8 *d*	2.33ax, *m*
5	45.4 *d*	2.37eq, *m*	H-4a, H-6, H-*g*	42.8 *d*	3.01eq, *br. s*
6	38.8 *d*	2.18ax, *d* (7.9)	H-5, H-6, H_2-8, H-*f*, H-*g*	34.9 *d*	2.52ax, *br. s*
7	37.7 *s*	-	H-1eq, H-5, H-6 H-8eq, H_2-10, H-*g*	37.7 *s*	-
8	43.0 *t*	2.41ax, *d* (14.1) 1.22eq, *m*	H_2-1, H-10ax	42.5 *t*	2.40eq, *d* (14.6) 1.41ax, *m*
8a	76.0 *s*	-	H_2-1, H-4a, H-5 H_2-8, H-10eq, H-2	71.0 *s*	-
10	58.8 *t*	2.72ax, *d* (9.8) 2.42eq, *d* (9.8)	H-6, H_2-8, H-*a*, H-11	59.3 *t*	3.38eq, *d* (15.6) 3.25ax, *d* (15.6)
11	94.9 *d*	4.98, *d* (5.9)	H-6, H-10ax, H-*a*	174.5 *d*	8.71, *s*
a	53.4 *t*	2.74, *m* 2.66, *m*	H-10eq, H-11	61.4 *t*	3.93, *br. t* (11.6) 3.38, *m*
b	25.1 *t*	1.48, *m* 1.33, *m*	H_2-*c*	24.3 *t*	1.64, *m* 1.57, *m*
c	25.3 *t*	1.61, *m* 1.12, *m*	-	23.3 *t*	-[f]
d	29.3 *t*	1.43, *m*	-	-[f]	-[f]
e	30.1 *t*	2.07, *m* 1.97, *m*	H_2-*d*, H-*f*, H-*g*	31.7 *t*	2.08, *m* 1.98, *m*
f	129.0 *d*	5.42, *m*	H-6, H-*e*	127.2 *d*	5.46, *m*
g	131.3 *d*	5.85, *dd* (15.8, 7.9)	H-5, H-6, H_2-*e*	131.9 *d*	5.53, *m*
h	57.4 *t*	2.34, *m* 2.30, *m*	H_2-1	57.1 *t*	2.32, *m*
i	28.3 *t*	1.40, *m*	-	29.1 *t*	1.65
j	27.1 *t*	2.04, *m* 1.98, *m*	H-*k*	27.7 *t*	2.20, *m* 1.92, *m*
k	129.6 *d*	5.42, *m*	H_2-*j*	129.6 *d*	5.42, *m*
l	130.0 *d*	5.35, *m*	H_2-*m*	130.0 *d*	5.35, *m*
m	27.3 *t*	2.04, *m* 1.98, *m*	H-*l*	27.8 *t*	2.10, *m* 1.92, *m*
n	27.6 *t*	1.37, *m* 1.33, *m*	-	-[f]	-[f]
o	20.7 *t*	1.34, *m* 1.26, *m*	-	20.6 *t*	1.41, *m*
p	38.5 *t*	1.28, *m* 1.20, *m*	H-6	37.7 *t*	1.46, *m* 1.30, *m*

[a] Bruker AMX 500 MHz; δ values are reported in ppm referenced to $CHCl_3$ (δ_H 7.26 and δ_C 77.0).

[b] J = 10Hz.

[c] Deduced by DEPT sequence.

[d] The assignments were aided by ^{1}H-^{1}H COSY, TOCSY, HMQC, and decoupling experiments.

[e] Other ^{13}C-NMR resonances for **1** at δ 29.1, 28.5, 26.6, 26.3, 24.3, 24.2 all assigned to carbons of methylenes with protons resonanting between δ 1.1-1.5; while other ^{13}C-NMR resonances for **1a** at δ 29.0, 28.7, 27.1, 26.9, 24.2, 23.8, 23.0, 22.0 all assigned to carbons of methylenes with protons resonanting between δ 1.1-1.5

[f] The NMR resonances at these positions were indistinguishable.

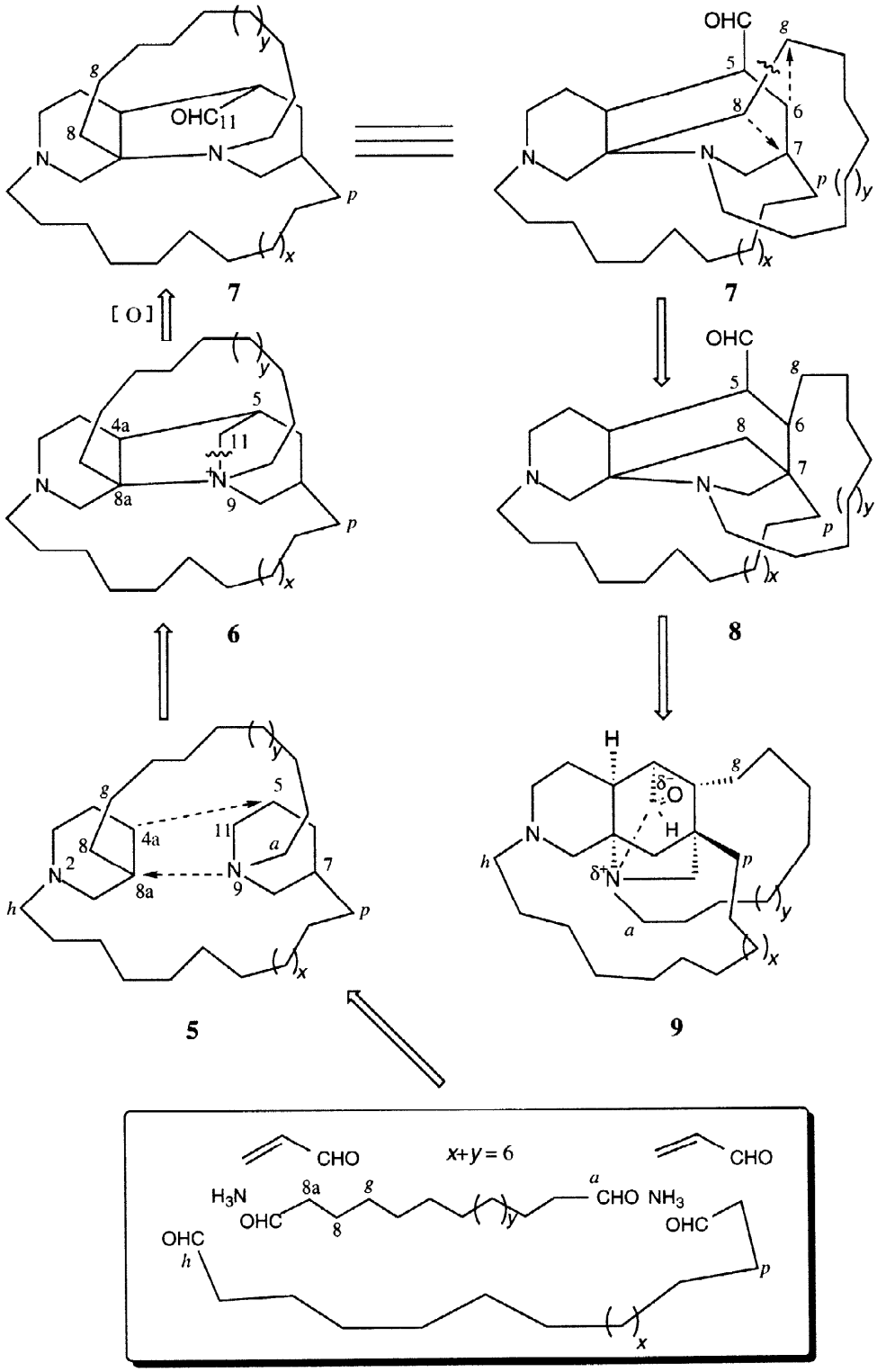
In order to ascertain the proposed structure of misenine and to understand its NMR pH-dependent peculiarity, a sample of **1** was treated, as mentioned above, with increasing amounts of CD_3COOD. As expected, the NMR spectra of misenine changed with the addition of CD_3COOD. The best-resolved and reproducible NMR spectrum was obtained when the sample contained an equimolar amount of **1** and CD_3COOD. Based on this observation, a new sample containing this ratio was used for an extensive NMR analysis. Detailed interpretation of ^1H-^1H COSY, TOCSY, HMQC, and HMBC spectra of this sample (**1a**) allowed assignments of four segments (H_2-3 to H-6, H_2-*a* to H-*g*, H_2-*h* to H_2-*p*, H-5 to H-11) and three AB type methylenes (H_2-1, H_2-8 and H_2-10). The ^{13}C chemical shifts at C-1 (δ 64.4), C-3 (δ 54.8), C-8a (δ 71.0), C-10 (δ 59.3), C-*a* (61.4), and C-*h* (δ 57.1) indicated that each carbon was attached to a nitrogen atom. Moreover, the newly appeared aldehydic proton (δ 8.71, H-11) displayed not only coupling with the adjacent methine at δ 3.01 (H-5) but also couplings (faint but clearly visible) with H_2-10 (δ 3.38, 3.25) indicating the continued presence of an interaction between C-11 and N-9. HMBC data allowed us to connect the four segments corresponding to above mentioned partial structures **a-e** via two nitrogens, two quaternary carbons and three pairs of diastereotopic methylenes. Important long-range correlations included those from C-11 (δ 174.5) to H_2-*a* (δ 3.93, 3.55), to H-10_{eq} (δ 3.38); from C-8a to H-1_{ax} (δ 2.70), to H-4a (δ 1.64), to H-5 (δ 3.01), to H-8_{ax} (δ 1.41); from C-7 (δ 37.7) to H-5, to H_2-10 (δ 3.38, 3.25); from C-6 (δ 34.9) to H-10_{ax} (δ 3.25), to H-*g* (δ 5.53); from C-5 (δ 42.8) to H-4a, and to H-*g*. Finally, the relative stereochemistry around the tetracyclic system was confirmed to be the same as that depicted in **1** (**Figure 1**) by NOE experiments. Thus the structure of **1** in the presence of CD_3COOD was unequivocally established to be **1a**. Of course, the alternative structures derived for **2** and **3** could also be possible.

Now, we can conclude that the structure of **1** is characterized by a central tricyclic nucleus (*cis*-fused decahydro-8a,7-azamethano-isoquinoline-5-aldehyde) surrounded by two alkyl chains. The spatial proximity between the carbonyl (C-11) and the tertiary amine moiety (N-9) induces an interaction, namely "proximity effect", which was well described many years ago by Leonard *et al*[11] and that had also been observed in saraines -A[6] and -B.[9] This transannular N/C=O-interaction is very sensitive to the pH environment. Because of this, the spectral and chemical properties of both the carbonyl and amine moieties can be strongly modified by solvent concentration, temperature and acidity. As a consequence, misenine (**1**) could be present in two forms (**1** or **1a**) in solution. The formation of the former is favoured in neutral or weakly basic condition while the latter is preferred when increasing the acidity. It may be worth pointing out that increasing acidity weakened the N/C=O-interaction of **1**. On the contrary, in analogous conditions, the proton attached the oxygen atom of the carbonyl of saraine-A[6] enhancing the C-N linkage.

Misenine (**1**) has an unprecedented skeleton related to ircinals/ircinols,[12,13] such as **4**.[12] However, there is no easy way to explain the biogenetic origin of **1** by analogy with that of **4**.[14] A hypothetical pathway (**Scheme 1**), incorporating principles proposed for manzamines,[14] saraines[4,9,10] and other macrocyclic diamine alkaloids,[15-20] involves the formal coupling of two long chain dialdehydes with two acroleins and two ammonias to give the tricyclic carbon skeleton **5**. Subsequent cyclizations (9-8a; 4a-5) lead to **6** which, after cleavage between C-11 and N-9 bond, gives the intermediate structure **7**. Finally, couplings between C-8 and C-7, C-g and C-6 with subsequent break of the linkage between C-8 and C-g should give the skeleton (**9**) of misenine.

The discovery of misenine has added to an extremely diverse and complex array of marine macrocyclic alkaloids which is rapidly expanding. Now, there is a strong interest in performing further studies aimed at

experimentally proving the true biogenetic origin and the effective biological role that misenine, and related alkaloids, play in the life cycle of the sponges and finally at confirming their structural peculiarities by synthesis.



Scheme 1. Formal biosynthetic pathway of misenine

EXPERIMENTAL SECTION

General Procedures: ^{1}H- and ^{13}C-NMR spectra were measured on a 500 MHz Bruker AMX 500 spectrometer. 2D experiments were performed using standard micro programs of Bruker software. AEI MS-30 (EIMS), Kratos MS-50 (HREIMS) instruments were used for obtaining mass spectra. FABMS spectrum was recorded on ZAB VG tandem mass spectrometer using *m*-nitrobenzyl alcohol (positive-ion mode) as matrix. IR spectra were recorded in liquid film with Bio-Rad FTS 7 spectrometer. Optical rotation was measured with a Jasco DIP-370 digital polarimeter.

Merck precoated Silica gel plates were used for TLC; Spots were detected by exposure to iodine vapour. Commercial Merck Silica gel 60 (70-230 mesh ASTM) was used for column chromatography.

Collection of Animal Material: Specimens of *Reniera* sp. were collected by hand using SCUBA on reefs at a depth of -2 m, off Capo Miseno, in the Bay of Naples in 1994. A voucher sample (S-036-94) is available for inspection at the ICMIB.

Extraction and Isolation of Misenine: Specimens of *Reniera* sp. (240g, dry wt.) were thawed and exhaustively extracted with acetone (500 ml x 3). The acetone extract was filtered and concentrated *in vacuo* to give a dark brown aqueous suspension which was then diluted with H_2O to 300 ml and partitioned sequentially against Et_2O (400 ml x 3) and *n*-butanol (150 ml x 3). The Et_2O soluble material (4.2 g) was subjected to silica gel column chromatography using gradient elution [$CHCl_3$/MeOH (10:0 to 0:10)]. The fractions eluted with $CHCl_3$/MeOH (9:1 and 8:2) were further purified via a second silica gel column chromatography separation using $CHCl_3$ as eluant to afford pure misenine (**1**) (22.3 mg). A portion (1 g) of *n*-butanol soluble material (4 g) was also chromatographed on the silica gel column using the same procedure as described above to give pure misenine (**1**) (7.7 mg).

Misenine (1): Colorless liquid, $[\alpha]_D$ + 6.4° (*c* 2.23, $CHCl_3$); IR ν_{max} (liquid film): 3441, 2928, 1462, 1026, 744 cm^{-1}; EIMS, *m/z* (%): 494 (M^+, 100), 437 (91); HREIMS: *m/z* 494.4230 ($C_{33}H_{54}N_2O$ requires 494.4236); FABMS: *m/z* 495 $(M+H)^+$.

A sample of 7.7 mg of **1** (after washing with saturated $NaHCO_3$) in 0.5 ml $CDCl_3$ was used for NMR experiments; ^{13}C- and ^{1}H-NMR data are listed in **Table 1**.

Influence of Acidity on NMR Features of Misenine: The evaluation of the influence of acidity on misenine was performed following the procedure previously reported for saraine-A.[6] A sample of **1** (1.0 mg) was used for this study. Following the addition of the CD_3COOD, the ^{1}H- and ^{13}C-NMR spectra varied. In particular, the aldehydic proton (H-11) resonance appeared range between δ 8.5 to 9.2 whereas carbonyl resonances were detected in the δ 200-175 ppm region of the ^{13}C-NMR spectra. The best-resolved NMR spectra were obtained when the sample contained equimolar amounts of **1** and CD_3COOD. Due to this observation, a new sample (**1a**) (10 mg) containing this ratio was used for recording 2D NMR spectra. ^{13}C-

and ^{1}H-NMR data of **1a** are listed in **Table 1**. IR[ν_{max} (liquid film)] data of **1a:** 2928, 1714, 1724 (in presence of DCl), 1544, 1462, 1251, 755 cm^{-1}.

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