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STUDIES ON THE STRUCTURE OF CALLIACTINE, THE ZOOCHROME OF THE SEA ANEMONE *CALLIACTIS PARASITICA*[1]

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Abstract - Chemical and spectroscopic investigations led to four possible structures (9-12) for calliactine, the pigment of the sea anemone *Calliactis parasitica*. Structure 9 is preferred, although structures 10-12 cannot be definitively ruled out.

In 1940 Lederer et al.[3] reported the isolation of a pigment from the sea anemone *Calliactis parasitica*, named calliactine, for which a $C_{21}H_{20}N_4O_5$ molecular formula was proposed by elemental analysis. Calliactine acts as a pH indicator, changing its colour from orange (acid) to blue (alkaline), and is transformed[3] sequentially into "chlorocalliactine" by boiling dilute HCl and "neocalliactine" by boiling water.

The structural elucidation of calliactine was a difficult problem , as experienced by others[4], since attempts to get derivatives or degradation products afforded complex mixtures, nor suitable crystals of calliactine, "chlorocalliactine" or "neocalliactine" were obtained for the X-ray analysis.

In addition, in previous work[4] calliactine was regarded as a paramagnetic substance since it failed to give useful signals in ^1H- and ^{13}C-NMR using a variety of solvents.

Our attempts occasionally made during several years did not give different results, until an isolation procedure different from that proposed by Lederer[3] was set up.

Absorption of the n-butanol solubles of the ethanolic extract of *Calliactis parasitica* on cellulose column chromatography, followed by elution with H_2O and then with CH_3OH, yielded in a very simple way pure samples of calliactine[5]. Freshly prepared material can be dissolved in methanol containing few drops of acetic acid; tipically ca. 10 mg of calliactine can be dissolved in 1 ml of CH_3OH-CH_3COOH. Usually calliactine precipitated from these or more dilute solutions during a 8-48 h period; to dissolve again this material a passage through a short cellulose column was necessary. Although ^1H-NMR spectra can be obtained in CD_3OD solutions (Fig. 1a), the amount required for ^{13}C spectra (Fig. 1b) or for 2D NMR spectra can be dissolved only in CD_3OD-CD_3COOD solutions. Because of the precipitation of calliactine from these solutions some problems arose for obtaining NMR data requiring longer acquisition time. Therefore the previously experienced failure in obtaining NMR spectra[4] should be ascribed to the extremely unfavourable solubility properties of calliactine when not freshly prepared as reported here.

The molecular composition of calliactine was established as $C_{18}H_{14}N_4O_2$ on the following evidence. The FAB-MS spectrum displayed a week quasi-molecular ion (M+H) at m/z 319, while into the EI-MS spectrum the molecular ion was absent. In this spectrum an ion at m/z 300 (M^+-H_2O) showed a $C_{18}H_{12}N_4O$ composition by high resolution measurements.

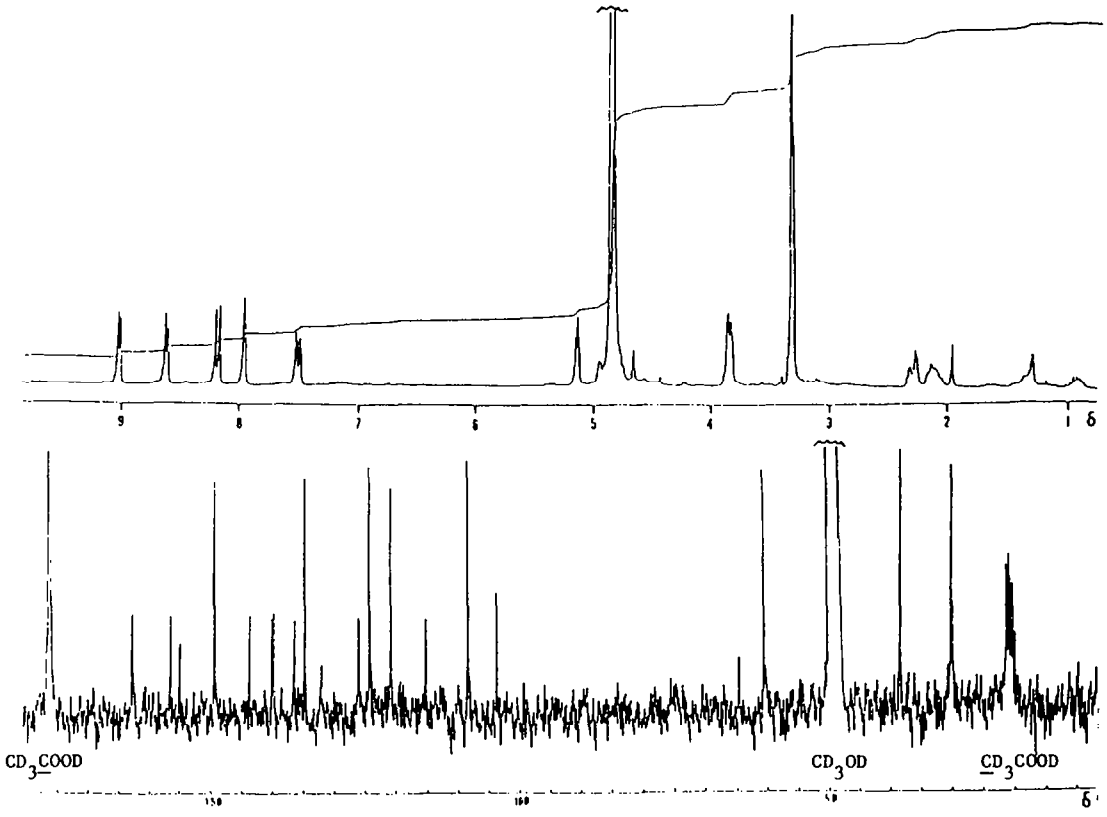
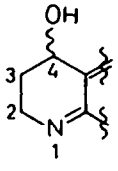


Fig. 1 - ^{1}H-NMR (a; CD_3OD; 500 MHz) and ^{13}C-NMR (b; CD_3OD-CD_3COOD; 67.9 MHz) spectra of calliactine.

The proposed molecular formula was corroborated by the ^{13}C-NMR spectrum (Table I) showing 18 distinct resonances, three sp^3 and 15 sp^2 carbons, whose multiplicity ($2xCH_2$, 6xCH, 10xqC; by DEPT sequence) accounted for 10 of the 14 hydrogen atoms, leaving four hydrogens linked to heteroatoms.

The ^{1}H-NMR spectrum (Table I) paralleled the ^{13}C spectrum in showing five protons linked to sp^3 carbons and five aromatic protons. Decoupling experiments and the ^{1}H and ^{13}C chemical shift values suggested that the higher field resonating protons ($2xCH_2$, 1xCH) could be accomodated in a hydroxy-tetrahydropyridine ring (partial structure a). Three of the five aromatic protons showed the typical pattern compatible with partial structure b, while the remaining two protons were ortho-coupled on a N-heteroaromatic ring as in partial structure c.



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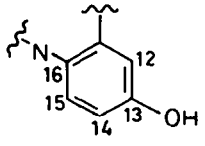

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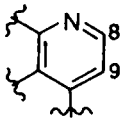

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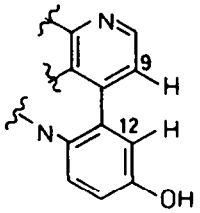

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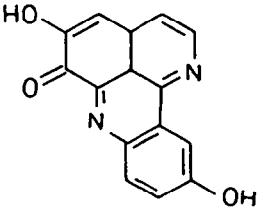

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TABLE I - Proton and Carbon-13 Data for Calliactine (9)

C	δH^a at C	multiplicity J, Hz	δC^b	multiplicity[d]
2	3.86	m	38.3	t
3	2.1-2.3	m	30.0	t
4	5.10	m	60.2	d
5			103.5	s
6			143.4	s
8	9.02	d, 5.8	149.1	d
9	8.61	d, 5.8	120.6	d
10			136.2	s
11			125.8	s
12	7.96	d, 2.3	108.2	d
13			162.6	s
14	7.49	dd, 8.9 and 2.3	124.0	d
15	8.18	d, 8.9	134.5	d
16			139.5	s
18			139.8	s
19			156.2[c]	s
20			154.7[c]	s
21			115.0	s

[a] 500 MHz, CD_3OD. [b] 67.9 MHz, CD_3OD/CD_3COOD. Assignments were aided by direct and long range ^1H-^{13}C 2D heterocorrelations and by comparison with Necatorone (1) and Amphimedine (8), where relevant. [c] May be interchanged. [d] By DEPT sequence.

TABLE II - Proton and Carbon-13 Data for Neocalliactine Acetate (2)

C	δH^a at C	multiplicity J, Hz	δC^b	multiplicity J, Hz[d]
2	9.04	dd; 3.9, 1.6	155.3	ddd; 183.4, 8.9, 3.6
3	7.62	dd; 6.9, 3.9	125.8	dd; 175.6, 9.0
4	8.69	dd; 6.9, 1.6	136.5	dd; 168.6, 5.4
5			128.8	d; 7.2
6			149.4[c]	d; 10.8
8	9.15	d; 5.1	149.5	d; 182.8
9	8.43	d; 5.1	117.0	dd; 165.9
10			137.6	dd
11			124.4	m
12	8.39	d; 2.2	115.0	dd; 163.5
13			152.2	m
14	7.69	dd; 7.8, 2.2	126.6	dd; 166.4
15	8.51	d; 7.8	134.1	d; 167.7
16			143.2	m
18			145.3	s
19			181.4	d; 3.8
20			151.9[c]	m
21			117.8	d; 8.1
$\underline{CH_3}CO$	2.35	s	20.9	q; 131.0
$CH_3\underline{C}O$			169.0	q; 7.6

[a] 270 MHz, $CDCl_3/CD_3OD$. [b] 125.8 MHz, $CDCl_3/CD_3OD$. The chemical shifts of the protonated carbons were assigned by a direct ^1H-^{13}C 2D heterocorrelation. The C-5, C-10, C-11, C-12 and C-16 chemical shifts were assigned by INAPT[15], on irradiation of H-4, H-8, H-12 and H-15. [c] May be interchanged. [d] Gated ^{13}C spectrum; poor resolution prevented an exact measurement of all long range coupling constants.

A direct ^{1}H-^{13}C 2D hetero-correlation allowed the assignment of the protonated carbons whose chemical shift values were in accord with the partial structures a-c.

A long range ^{1}H-^{13}C 2D hetero-correlation[6] showed couplings between C-16 and H-14 and between C-13 and H-15 allowing the assignment of the chemical shift values to C-16 and C-13. From these values it was argued that C-13 (δ 162.6) is linked to an oxygen atom while C-16 (δ 139.5) is connected to a nitrogen atom, as depicted in partial structure b. Moreover the chemical shift values from C-11 to C-16 are very close to those reported for the corresponding carbons of necatorone (1) whose structure has been confirmed by synthesis[8].

Furthermore, in a series of NOE difference spectra a positive enhancement of H-12 was experienced when H-9 was irradiated , and viceversa, thus suggesting that H-9 and H-12 are close in the space. On this ground structures b and c were connected as depicted in d.

The partial structures a and d account for $C_{16}H_{10}N_3O_2$, leaving a C_2N fragment and the exchangeable hydrogen atoms to be assigned.

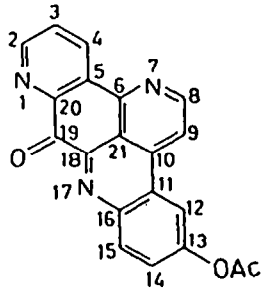
That the fourth nitrogen atom was implied in an easily hydrolizable imino function was argued by the following transformation. Calliactine was treated with 2N HCl and worked up in the conditions described by Lederer[3] to afford "neocalliactine"[9]. The resulting product, poorly soluble in the common solvents and too polar for chromatographic purifications, was acetylated with Ac_2O in pyridine affording a compound which was purified by SiO_2 column chromatography.

Inspection of the molecular formula, $C_{20}H_{11}N_3O_3$ (by HRMS), and of the spectroscopic properties of neocalliactine acetate revealed that two main modifications of calliactine occurred: aromatization of the hydroxy-tetrahydropyridine ring and hydrolysis of the imino group.

In the IR spectrum of neocalliactine acetate there are two carbonyl bands, 1765 (acetate) and 1684 cm^{-1}, this latter accounting for the carbonyl arising by hydrolysis of the imino group.

The ^{1}H-NMR spectrum (Table II) besides the aromatic protons due to partial structure d, shows three new aromatic protons at δ 9.04, 7.62 and 8.69, having a typical ABC coupling pattern, and lacks the protons due to partial structure a, suggesting that dehydration and aromatization of the hydroxy-tetrahydropyridine ring occurred. Also in the case of neocalliactine acetate a NOE was observed between H-9 and H-12 confirming the arrangement depicted in partial structure d[10].

These results,taken altogether, allow the formulation of neocalliactine acetate as one of the 2-5 alternative structures.



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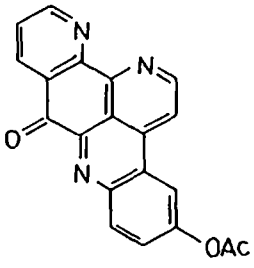

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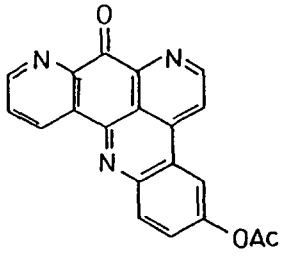

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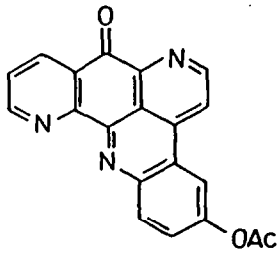

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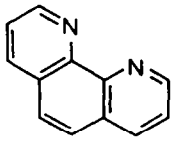
Conflicting chemical and spectroscopic results prevented us to safely select one of the above structures, although structure 2 could be favoured on the following grounds.

Inspection of structures 3 and 5 reveals the presence of nitrogen atoms in the

same relative position as in 1,10-phenanthroline (6), the well known metal chelating agent. 1,10-Phenanthroline and its derivatives give in a very sensitive test a red complex with iron salts; when neocalliactine acetate was subjected to this test the formation of the red complex was not observed. This failure does not support structures 3 and 5.

In an other attempt to gain proofs for selecting one of the 2-5 alternative structures, a NOE difference spectrum was recorded irradiating the H-4. In this spectrum, besides the obvious enhancement of H-3, was unexpectedly observed the enhancement of the H-8. This result could be compatible only with structure 2, since in the other cases H-4 and H-8 are very far in the space.

However this result could raise some doubts since also in structure 2 H-4 and H-8 are quite far in the space (~4.2 Å; Dreiding models). Although NOE enhancements have been observed on protons over longer distances, as far as 4.5 Å[11], for having further support for the observed result, the model compound 6-methoxy-1,7-phenanthroline (7) was synthesized[12].



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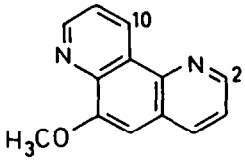

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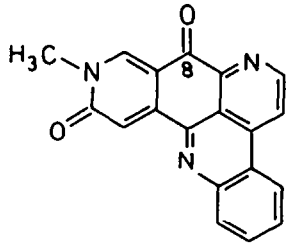

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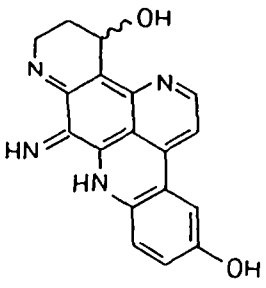
Irradiation of H-10 in a NOE difference spectrum of 7 resulted in the enhancement of H-2, thus paralleling the behaviour of the related protons of neocalliactine acetate.

This result, taken alone and/or in connection with the failure in giving a red complex with iron salts, would indicate structure 2 for neocalliactine acetate and, accordingly, structure 9 for calliactine.

However a somewhat contrasting evidence comes from the gated ^{13}C-NMR spectrum of neocalliactine acetate (Table II), in which the main feature was the finding of the C-19 carbonyl carbon as a doublet (J = 3.8 Hz). This carbon was coupled to H-4 as showed both by a ^{1}H-^{13}C long range correlation (using 5 Hz as J value) and by a H-4 irradiated ^{13}C-gated spectrum. A ^{3}J coupling between C-19 and H-4 is likely since the J value is similar to that (3.5 Hz) of the C-8 carbonyl carbon in the related alkaloid amphimedine (8) isolated from a Pacific sponge[13]. This result would suggest a choice between structures 3 and 5 since the structural arrangement of 2 and 4 requires a ^{4}J coupling between C-19 and H-4, which, although not unlikely, usually does not display large values[14]. Since this evidence cannot be overlooked, structures 3 and 5 at least, and in consequence structures 10 and 12 for calliactine, cannot be definitively ruled out.

Another derivative of calliactine was obtained in low yield by gently warming methanolic solutions of calliactine adsorbed on silica gel, followed by acetylation. Inspection of the spectral properties of this compound, formulated as 13 in the hypothesis that structure 9 is to be assigned to calliactine, revealed that the above treatment induced aromatization of the tetrahydropyridine ring without hydrolysis of the imino group.

The molecular formula $C_{22}H_{16}N_4O_2$ was deduced from MS data (Experimental), while comparison of the NMR data with those of neocalliactine acetate (Table II)



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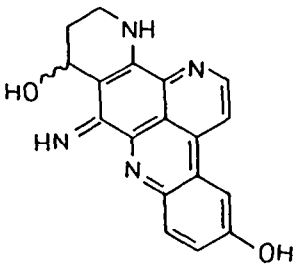

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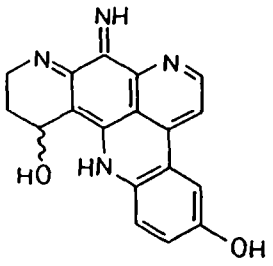

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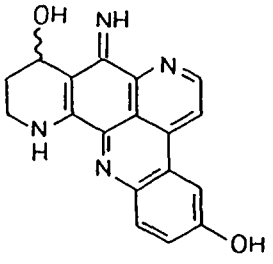

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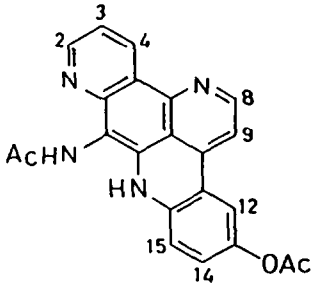

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allowed us to propose structure 13 to this calliactine derivative. Extensive spectroscopic investigation on 13 was prevented by the limited amount available. However 13 does not add further support to the choice within the 9-12 alternative structures of calliactine.

EXPERIMENTAL

NMR spectra were recorded on a Bruker WM 500 and 250 spectrometers. Mass spectra were obtained on AEI MS-30, MS-50 and MS-902 instruments. IR spectra were recorded on a Nicolet 5DXB spectrometer. UV spectra were obtained on a Shimadzu Baush & Lomb Spectronic 210 spectrometer. Cellulose chromatography was performed using Whatman cellulose powder CF11.

Isolation of calliactine (9).

In a typical isolation procedure, 200 specimens of *Calliactis parasitica*, collected in the Bay of Naples, were deprived of internal organs and extracted with EtOH (600 mL x 3) at room temperature for two days. The solvent was removed and the aqueous residue was extracted sequentially with Et_2O and n-BuOH. The butanolic extract was evaporated under reduced pressure giving a brown solid (0.470 g) which was dissolved in water and chromatographed on a cellulose column (150 g) eluting with H_2O (3 L), MeOH (1.5 L) and MeOH containing 1% AcOH (0.5 L). Fractions were monitored by silica gel TLC using n-BuOH-AcOH-H_2O (60:15:25) as the eluant and spraying the plates with 1M NaOH. In these conditions calliactine appears as blue spots.

The appropriate fractions of the MeOH and MeOH-1% AcOH eluates were combined to yield pure calliactine (80 mg).

FAB-MS, m/z 319 $(M+H)^+$; EIMS, m/z 300.1056 ($C_{18}H_{12}N_4O$ requires 300.1011),

299, 150.

^{1}H- and ^{13}C-NMR, Table I.

UV, λ_{max} (MeOH), 454 (ε 8,500), 299 (ε 9,200), 272 (ε 11,700) and 267 (ε 12,000) nm; λ_{max} (MEOH-NaOH) 580 nm.

Neocalliactine acetate (2).

Neocalliactine acetate was prepared following two different procedures.

a) Following the procedure described by Lederer[3], a 2N HCl solution of calliactine (20 mg) was boiled until a color change to green of the solution was observed. Without isolation of "chloro-calliactine", the solution was evaporated under reduced pressure and repeatedly dissolved in water and evaporated.

The crude reaction product was acetylated with Ac_2O (0.5 mL) in pyridine (1 mL) at room temperature overnight. Usual work up and column silica gel chromatography (eluant: $CHCl_3$) afforded neocalliactine acetate (8 mg).

EIMS, m/z 341 (M^+), 299 (M^+-CH_2CO), 271 (M^+-CH_2CO-CO); HREIMS, found m/z 341.0795 ($C_{20}H_{11}N_3O_3$ requires 341.0801).

^{1}H- and ^{13}C-NMR, Table II.

IR ν_{max} ($CHCl_3$) 1765 and 1684 cm^{-1}.

b) 20 mg of calliactine were dissolved in 5 mL of 0.05 N HCl and the solution was boiled for 15 min. The crude product obtained after evaporation of the solvent was acetylated and purified as above affording 5 mg of neocalliactine acetate.

Compound 13.

A methanolic solution of calliactine (15 mg) was adsorbed on silica gel (500 mg) and warmed for 10 min with hot air.

The mixture was eluted with MeOH and, after evaporation of the solvent, treated with Ac_2O (0.5 mL) in pyridine (1 mL) at reflux for 5 min. Usual work up and purification by chromatography on a Pasteur pipette (eluant: $CHCl_3$) gave ca. 1.5 mg of 13 contaminated by a small amount of neocalliactine acetate (2).

EIMS, m/z 384 (M^+), 342 (M^+-CH_2CO), 324 (M^+-AcOH), 283 (M^+-CH_2CO-$AcNH_2$); HREIMS, m/z 324.1016 ($C_{20}H_{12}N_4O$ requires 324.1011).

^{1}H-NMR δ($CDCl_3$; 500 MHz), 9.13 (H-2, dd, J 4.4 and 1.5 Hz), 7.64 (H-3, dd, J 8.1 and 4.4 Hz), 8.83 (H-4, dd, J 8.1 and 1.5 Hz), 9.29 (H-8, d, J 5.9 Hz), 8.39 (H-9, d, J 5.9 Hz), 8.42 (H-12, d, J 2.2 Hz), 7.74 (H-14, dd, J 9.6 and 2.2 Hz), 8.35 (H-15, d, J 9.6 Hz), 2.59 (3H, s), 2.46 (3H, s).

^{13}C-NMR δ ($CDCl_3$; 125.8 MHz; protonated carbons only) 154.1 (C-2), 125.5 (C-3), 135.8 (C-4), 150.2 (C-8), 116.3 (C-9), 115.0 (C-12), 126.5 (C-14), 133.0 (C-15), 24.8, 21.2.

6-Methoxy-1,7-phenantroline (7).

6-Methoxy-1,7-phenantroline was synthesized following literature procedure[12].

^{1}H-NMR δ ($CDCl_3$) 9.47 (1H, dd, J 8.3 and 1.7 Hz), 9.04 (1H, dd, J 4.3 and 1.7 Hz), 8.80 (1H, dd, J 4.4 and 1.6 Hz), 8.02 (1H, dd, J 8.1 and 1.6 Hz), 7.63 (1H, dd, J 8.3 and 4.3 Hz), 7.44 (1H, dd, J 8.1 and 4.4 Hz), 7.03 (1H, s), 4.12 (3H, s).

^{13}C-NMR δ ($CDCl_3$) 153.4, 150.2, 146.6, 142.6, 141.5, 134.0, 132.5, 127.3, 126.7, 122.2 (2C), 103.4, 55.7.

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5. Chromatography on a LH-20 column (CH_3OH) also gave quite satisfactory results, but the calliactine fractions were contaminated by some amino acid impurities.

6. A J value of 8 Hz was used in this experiment. A total of 128 experiments were taken during 62.5 h.

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9. The same product has been obtained by boiling a solution of calliactine in very dilute acidic conditions (Experimental).

10. This result was obtained from a NOESY experiment, since the chemical shift values of the two protons (δ 8.39 and 8.43) are too close for recording good NOE difference spectra.

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