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Note

Complete assignment of ^{1}H and ^{13}C NMR spectra of vinflunine

Jean-Paul Ribet,[1]* Pascal Zalavari,[1] Jacques Fahy,[2] Alain Duflos[2] and Thierry Beltran[1]

[1] Département de Chimie Analytique, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, 81106 Castres, France
[2] Division de Chimie Médicinale V, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, 81106 Castres, France

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A new family of fluorinated *Vinca* alkaloids was synthesized in superacidic media. Superacidic media were obtained by mixing a strong Brønsted acid, such as anhydrous hydrogen fluoride, with a strong Lewis acid, such as antimony pentafluoride. Thus vinflunine was obtained when vinorelbine was treated in $HF–SbF_5$ at −40 °C in the presence of a chlorinated solvent such as CH_2Cl_2, $CHCl_3$ or CCl_4. Vinflunine was characterized by superior *in vivo* activity to vinorelbine in preclinical tumor models. The chemical structure of vinflunine was been examined using NMR spectrometry. Taking into account the complexity of the NMR spectra, the total assignment of the ^{1}H and the ^{13}C spectra required experiments using homonuclear ($^{1}H–^{1}H$) and heteronuclear ($^{1}H–^{13}C$) correlations such as gradient-selected COSY (gs-COSY), double-quantum filtered COSY (DQFCOSY), heteronuclear multiple quantum-correlation spectroscopy (HMQC) and heteronuclear multiple bond correlation spectroscopy (HMBC). We also undertook a comparative structural analysis between vinflunine base and vinflunine ditartrate in acetone solution. The conformation of the vinflunine base molecule in acetone solution was studied with a NOESY experiment. The structure of vinflunine was thus determined without ambiguity, and its conformation was verified by a single-crystal x-ray diffraction study. The results indicated that the conformation in the solution state was analogous to that observed in the solid state.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; ^{19}F NMR; vinflunine; structural analysis; 20′,20′-difluoro-3′,4′-dihydrovinorelbine

INTRODUCTION

The indole alkaloids vinblastine and vincristine, initially extracted from the common Madagascar periwinkle (*Catharanthus roseus* G. Don, Apocynaceae), have been used for 30 years in anticancer chemotherapy.[1] They are binary alkaloids composed of one indole sub-unit (cleavamine, resulting from the rearrangement of catharanthine) and one indoline sub-unit (vindoline). The first product of this type was vinblastine (Velbé), marketed in 1963, followed by vincristine (Oncovin) in 1964. Until 1972, these two alkaloids were extracted from *Catharanthus roseus* leaves. Only very small quantities could be obtained in this manner, since this plant has an alkaloid content of only ~0.005%. In 1983, a new synthetic antitumoral binary alkaloid appeared on the market, vindesine (Eldésine), which was obtained by hemisynthesis using vinblastine. Vinorelbine (Navelbine), which appeared on the French market in 1989, was the second non-natural medicinal product in this group. Vinorelbine was then the only medicinal product obtained by coupling the two monomeric alkaloids catharanthine and vindoline.

**Correspondence to*: J.-P. Ribet, Département de Chimie Analytique, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, 81106 Castres, France.

Since 1974, an original chemical reaction, the Polonovski–Potier[2] reaction, has been used to couple catharanthine *N*-oxide with vindoline, thus producing anhydrovinblastine, which has the natural configuration of C-18′. Vinorelbine was then obtained directly from anhydrovinblastine by ring C′ contraction using *N*-bromosuccinimide, then silver tetrafluoroborate.[3–5] More recently, Fahy *et al*.[6] explored an original chemical approach based on the special reactivity provided by superacidic chemistry. Under these unusual conditions, starting from vinorelbine, they obtain vinflunine or 20′, 20′-difluoro-3′, 4′-dihydrovinorelbine as shown in Fig. 1. This compound, like the other *Vinca* alkaloids, inhibited microtubule assembly at micromolar concentrations,[7,8] but exerted markedly superior *in vivo* anti-tumour activities against a panel of 13 murine and human tumour models compared with the parent compound, vinorelbine.[9,10] ^{1}H and ^{13}C NMR studies of indole alkaloids have been reported previously.[11–17] Vinflunine has been investigated in our laboratory by NMR spectroscopy to study the ^{1}H and ^{13}C NMR spectra and to understand its molecular conformation. The results of this structural study are presented on both base and ditartrate in $(CD_3)_2CO$ solution. A combination of one- and two-dimensional NMR techniques (DEPT, COSY, HMQC, HMBC and NOESY) was

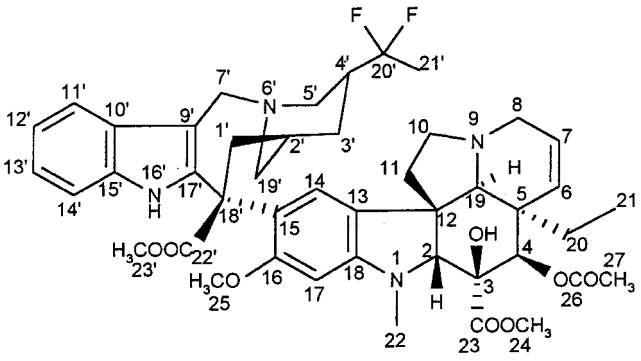

Figure 1. Structural formula of vinflunine.

used to achieve the structural elucidation of vinflunine. The ^{1}H NMR spectra were analyzed from the correlations in the COSY spectra and the NOESY experiments. Two-dimensional exchange spectroscopy was established as a powerful technique for assigning internuclear proximities through cross-relaxation. The ^{13}C NMR spectra were analyzed with the help of HMQC and HMBC experiments.

EXPERIMENTAL

Synthesis

Vinflunine ditartrate (batch LP 1) was supplied by Plantes et Industrie Pierre Fabre Santé in the form of an amorphous off-white powder. The empirical formula is $C_{53}H_{66}F_2N_4O_{20}$ and the molecular weight is 1117.117. The chemical name is *C*′-norvincaleukoblastine-4′-deoxy-20′, 20′-difluoroditartrate salt (1 : 2) and the CAS number is 194468-36-5. The chemical purity of this product, evaluated by high-performance liquid chromatography (HPLC), was >99% (w/w). Vinflunine base was obtained using the following procedure: 200 mg of vinflunine ditartrate were dissolved in 2 ml of distilled water. A 10% (w/v) solution of sodium carbonate (Na_2CO_3) was added drop by drop until pH 8. Vinflunine base was extracted with methylene chloride (2 × 2 ml), then dried on anhydrous magnesium sulfate and was filtered through Celite. After complete evaporation of the solvent, the vinflunine base (CAS number 162652-95-1) was isolated as an off-white vitreous solid. The empirical formula is $C_{45}H_{54}F_2N_4O_8$ and the molecular weight is 816.947.

Spectral measurements

All spectra were recorded non-spinning on a Bruker Avance 400 spectrometer operating at the proton nominal frequency of 400 MHz equipped with a 5 mm inverse multinuclear gradient probe-head. The spectrometer frequency was 400.13 MHz for ^{1}H and 100.58 MHz for ^{13}C. The sample was maintained at 25 °C throughout.

Vinflunine ditartrate and vinflunine base were dissolved in acetone-d_6 (isotopic enrichment 99.95%, Eurisotop D038-B). The concentrations of the solutions were ca 14 mM. To optimize the experimental conditions of the NOESY experiment, the solution was carefully filtered through an external filter tip (45 μm, Polylabo 91943) in a dry 5 mm sample tube (New Era Enterprises NE-HP5-9″). The solution was subjected to five freeze–pump–thaw cycles before being sealed under nitrogen. Chemical shifts (δ) are given in parts per million relative to tetramethylsilane ($SiMe_4$) and the coupling constants in hertz.

The gs-COSY experiment was acquired using the cosygp Bruker program with the following parameters: 90° pulse width 7.0 μs, spectral width 3434.07 Hz, acquisition time 0.3 s, relaxation delay 2 s, one transient collected for each time increment, 1024 time increments were recorded. An extension of the COSY experiment was the double-quantum filtered technique (DQFCOSY). This experiment filters out signals which result from single quantum transitions only but with a penalty in the form of reduced sensitivity. DQFCOSY was also used to reduce t_1 noise. The DQFCOSY spectrum was acquired with a spectral width of 4664 Hz in both dimensions, 512 × 2K data points before and 1K × 2K after zero-filling, resulting in a digital resolution of 9 and 4.5 Hz per point in the ω_1 and ω_2 dimensions, respectively. Processing was carried out using sine-bell squared functions shifted by $\pi/2$ and a TPPI method.

NOESY studies were acquired in the phase-sensitive mode, using the noesytp program on a Bruker Avance 400 spectrometer, over a range of mixing times between 0.05 and 1 s. One-dimensional ^{13}C NMR and DEPT spectra were recorded with Waltz16 ^{1}H decoupling; 32K complex points were acquired with a spectral width of 24 154 Hz. The data were apodized with a 1 Hz exponential function prior to Fourier transformation.

HMQC experiments were performed with a minimum of 512 time increments in the magnitude mode. Spectra were acquired using the inv4gp Bruker sequence with a $^{13}C(\omega_1)$ spectral width of 24 000 Hz. The experiment was optimized for a one-bond coupling constant of 140 Hz. Broadband ^{13}C decoupling using GARP1 modulation was used during the acquisition time of 0.12 s. Sixteen transients were collected for each time increment. A zero filling in F_1 to 1K in order to have a matrix of 1K × 1K real data points was applied. Before Fourier transformation, a phase-shifted sine function in F_2 and F_1 was used with $\pi/4$ and $\pi/3$, respectively.

The HMBC experiment was optimized for a long-range $^{1}H-^{13}C$ coupling constant of 16 Hz. Spectra were obtained with 512 time increments, 24 scans per t_1 increment and a 3.5 ms delay period for suppression of one-bond correlation signals. The spectra were acquired using the inv4gplplrnd Bruker sequence where no decoupling was applied during acquisition. The suppression of correlations via $^{1}J(C,H)$ was not perfect with the low-pass filter, and cross peaks caused by $^{1}J(C,H)$ coupling could be distinguished in the spectra.

^{19}F NMR spectra were recorded on vinflunine base at 188.2 MHz on a Bruker AC200 spectrometer, at ambient temperature, in deuterochloroform solution. Proton-decoupled and proton-coupled spectra were recorded.

RESULTS AND DISCUSSION

Assignment of ^{1}H NMR spectra

To assign the protons of vinflunine ditartrate with no ambiguity, we undertook a comparative structural analysis between vinflunine base (**1**) and vinflunine ditartrate

(**2**) (Table 1). The vinflunine molecule may be divided into a difluorocleavamine subunit (upper part) and a vindoline subunit (lower part). The molecular structure of the upper part without substitution at the 18′-position and, similarly, the molecular structure of the lower part without substitution at the 15,16-positions and partially substituted at the 3,5-positions are shown, respectively, in Figs 2 and 3. The numbering system adopted to designate the atoms of the molecule was recommended by the Organic Chemistry Nomenclature Commission of IUPAC.

In the ^{1}H NMR spectrum of **2** we observed considerable overlap in the aliphatic region, and the protonation of nitrogen atoms N-6′ (pK_a = 7.55) and N-9 (pK_a = 5.37) produced deshielding of the neighboring protons. In contrast, proton resonances were sharp and well resolved with **1**, making it fairly easy to follow connectivity networks in the COSY spectrum and to measure coupling constants in the DQFCOSY spectrum.

The aliphatic region showed signals due to methyl groups bound either to a quaternary carbon atom (H-27, δ 1.98),

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra of **1** and **2** (CD_3COCD_3): chemical shifts (δ, ppm) and coupling constants (*J*, Hz, in parentheses)

	1		**2**	
Carbon	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)
2	83.82	3.58 (s)	82.16	3.61 (s)
3	80.64	—	79.34	—
4	77.20	5.26 (s)	75.73	5.23 (s)
5	43.54	—	42.12	—
6	131.51	5.30 (d, 10.5)	130.06	5.30 (d, 10.5)
7	124.91	5.77 (dd, 10.5, 4.0)	123.45	5.76 (dd, 10.5, 4.0)
8	51.04	endo 3.26 (dd, 16.0, 4.0) exo 2.65 (d, 16.0)	49.44	endo 3.26 (dd, 16.0, 4.0) exo 2.71 (d, 16.0)
10	50.44	endo 3.19 (ddd, 9.5, 9.5, 5.0) exo 2.35 (ddd, 9.5, 9.5, 5.0)	48.72	endo 3.20 (m) exo 2.41 (ddd, 10.0 10.0. 5.0)
11	45.65	endo 2.09 (ddd, 13.5, 9.5, 5.0) exo 1.87 (ddd, 13.5, 9.5, 5.0)	44.17	endo 2.08 (m) exo 1.90 (m)
12	54.09	—	52.80	—
13	123.92	—	122.74	—
14	124.39	6.56 (s)	122.74	6.65 (s)
15	121.86	—	119.35	—
16	158.98	—	157.59	—
17	94.57	6.33 (s)	93.16	6.35 (s)
18	153.80	—	152.80	—
19	65.33	2.76 (s)	63.60	2.80 (s)
20	31.44	a 1.34 (dq, 15.0, 7.5) b 1.54 (dq, 15.0, 7.5)	30.11	a 1.33 (dq, 15.0, 7.5) b 1.51 (dq, 15.0, 7.5)
21	8.46	0.66 (dd, 7.5, 7.5)	7.00	0.63 (dd, 7.5, 7.5)
22	38.53	2.71 (s)	36.94	2.73 (s)
23	172.26	—	170.82	—
24	51.83	3.68 (s)	50.53	3.68 (s)
25	56.29	3.82 (s)	55.01	3.86 (s)
26	170.94	—	169.55	—
27	21.10	1.98 (s)	19.71	1.97 (s)

(*continued overleaf*)

Table 1. (*continued*)

	1		**2**	
Carbon	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)
1′	35.72	*endo* 3.09 (dd, 15.0, 15.0) *exo* 2.48 (dd, 15.0, 6.0)	33.12	*endo* 3.17 (dd, 15.0, 15.0) *exo* 2.65 (dd, 15.0, 6.0)
2′	30.77	1.07 (m)	26.47	1.56 (m)
3′	31.60	ax 1.65 (m) eq 1.80 (d, 12.5)	27.83	ax 1.87 (m) eq 1.94 (m)
4′	31.86 (t, 25.0)	2.88 (m)	32.69 (t, 25.0)	3.38 (m)
5′	52.01	ax 2.98 (dd, 12.0, 12.0) eq 3.26 (d, 12.0)	48.29	ax 3.35 (m) eq 3.74 (d, 10.0)
7′	47.65	*endo* 4.51 (d, 12.5) *exo* 4.32 (d, 12.5)	45.47	*endo* 5.06 (d, 14.5) exo 4.95 (d, 14.5)
9′	111.92	—	104.38	—
10′	129.59	—	127.82	—
11′	118.67	7.60 (d, 7.5)	117.70	7.76 (d, 7.5)
12′	119.86	7.04 (ddd, 7.5, 7.5, 1.5)	119.42	7.08 (ddd, 7.5, 7.5, 1.5)
13′	122.50	7.09 (ddd, 7.5, 7.5, 1.5)	121.97	7.13 (ddd, 7.5, 7.5, 1.5)
14′	112.30	7.44 (d, 7.5)	111.25	7.50 (d, 7.5)
15′	136.30	—	135.05	—
17′	134.49	—	134.74	—
18′	56.39	—	54.67	—
19′	48.72	ax 2.17 (dd, 14.0, 1.5) eq 3.38 (d, 14.0)	45.63	ax 2.89 (dd, 14.0, 2.5) eq 3.80 (d, 14.0)
20′	126.84 (t, 240)	—	124.14 (t, 240)	—
21′	21.62 (t, 28)	1.64 (dd, 19.0, 19.0)	20.37 (t, 28)	1.75 (dd, 19.0, 19.0)
22′	175.11	—	173.02	—
23′	52.36	3.59 (s)	51.28	3.62 (s)
3OH	—	8.43 (s)	—	broad 5.0
—COOH (tartaric acid)			173.14	—
—CHOH (tartaric acid)			71.36	4.33 (s)

a nitrogen atom (H-22, δ 2.7) or even an oxygen atom (H-24, δ 3.7; H-25, δ 3.8; H-23′, δ 3.6). The other singlets were attributed to protons H-2 at δ 3.58, H-19 at δ 2.8 and H-4 at δ 5.2. The intense signal at δ 4.3, appearing only in the salt spectrum, represented the two protons (CHO) of the tartaric acid. In the aliphatic region we also clearly identified nine pairs of anisochronous geminal methylene protons which are close to a chiral or prochiral center. The chemical environment of the two protons is non-equivalent and the protons are therefore diastereotopic. The high-field region showed a characteristic pseudo-triplet easily recognized as being due to methyl-21′ by its simple coupling pattern with two vicinal fluorines (dd, $^{3}J = 19.0$ Hz). The multiplet centered at δ 1.07 in **1** (δ 1.56 in **2**) may be assigned to H-2′ owing to its chemical shift and its relative integration. Following its coupling connectivities deduced by the homonuclear 2D COSY spectrum, the assignment of the H-3′, H-19′ and H-1′ protons now was possible. ≠3′ eq showed

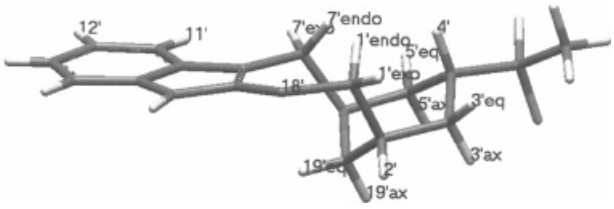

Figure 2. Molecular structure of the difluorocleavamine subunit without substitution at the C-18′ position to clarify the figure.

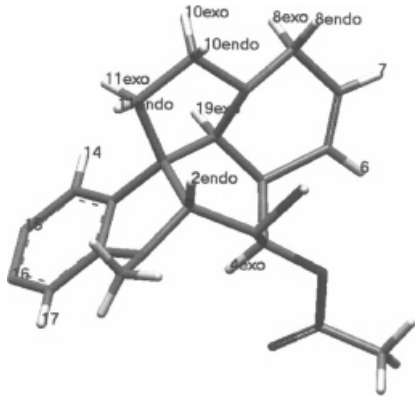

Figure 3. Molecular structure of the vindoline subunit without substitution at the C-15,16 positions and partially substituted at the C-3,5 positions to clarify the figure.

a correlation peak with H-4′ at δ 2.9 and also a long-range coupling pattern over four bonds with one of the H-5′ protons (H-5′ eq, W configuration). We also observed a long-range coupling between H-19′ eq and H-3′ eq. The conformation of the piperidine ring of the difluorocleavamine subunit may be studied from the coupling constants. A very small vicinal coupling constant should correspond to a dihedral angle that approaches 90°, while a large coupling constant should be the result of an eclipsed or an antiperiplanar configuration.

The proton H-5′$_{ax}$ at δ 2.98, being axial, exhibited a large coupling constant with the axial H-4′ (^3J = 12.0 Hz). This observation was very important to define the configuration of the C-4′ hydrogen atom. The equatorial orientation for H-2′ was corroborated by the coupling constants of H-2′ with H-1′$_{endo}$ and H-1′$_{exo}$, which are 15.0 and 6.0 Hz, respectively. These values reflect an antiperiplanar disposition between H-2′ and H-1′$_{endo}$ and a torsion angle of about 55° between H-2′ and H-1′$_{exo}$. In addition to the coupling constant study, through-space dipolar interactions between protons were used to deduce structural and particularly sterochemical information about the piperidine ring of the difluorocleavamine subunit.

The phase-sensitive NOESY spectrum of **1** and **2** showed positive NOEs because of the small correlation time of the molecule in acetone-d_6 at 25 °C ($\omega\tau_c < 1.12$).[18] The aromatic proton H-11′ at δ 7.6 showed a correlation peak with H-7′, centered at δ 4.3, confirming the *exo* orientation of this hydrogen atom. Another interaction included a cross peak of strong intensity for H-11′ and H-12′. Correlations were also observed between H-7′$_{endo}$ centered at δ 4.5 and H-1′$_{endo}$ centered at δ 3.1. The slice at δ 2.17 showed cross peaks between H-19′$_{ax}$ and the protons H-19′$_{eq}$ at δ 3.38, H-5′$_{ax}$ at δ 3.0 and H-2′ at δ 1.07. Finally, from the cross peaks observed in the NOESY spectra and from the vicinal coupling constants, it was concluded with no ambiguity that the conformation of the piperidine ring is a chair with the *R* configuration at C-4′. Chemically exchanging protons such as 3-OH at δ 8.43 in **1** and hydroxy protons of residual water at δ 2.9 exhibited an intense negative cross peak with each other.

For the vindoline subunit, the relative configuration of C-19, C-5 and C-4 was confirmed by the significant cross peaks observed between H-19, H-20/H-21 and H-4. The spatial proximity between H-2 and H-11$_{endo}$ confirmed the relative configuration of C-2 and C-12. The relatively significant NOESY cross peak observed for the H-14′,16′-NH resonances at δ 7.44 and 9.72, respectively, with the 21-methyl pseudo-triplet of the vindoline subunit (δ 0.66) suggested a preferred conformation of the difluorocleavamine moiety relative to the vindoline subunit. This indicated that the indole ring of the upper part and the 21-methyl group protons of the lower part to be in close contact (<3 Å). These results and the spatial interaction of H-14 with H-19′$_{eq}$ and with 16′-NH confirm the *S* configuration of C-18′. The conformation of vinflunine in the solution state is very similar to the conformation obtained later from a single-crystal x-ray diffraction study by J.M. Léger (in preparation).

Assignment of ^{19}F NMR spectrum

The fluorine-19 resonances were characterized by large chemical shifts and by strong ^{19}F–^{19}F and ^{1}H–^{19}F spin–spin interactions. The two fluorines cannot be brought into a chemically identical position because they are diastereotopic. The geminal ^{19}F–^{19}F coupling constant was easily obtained under proton noise decoupling conditions. The analysis of the AB spin system gave ^2J = 242 Hz. The vicinal ^{1}H–^{19}F coupling constant between H-4′ and CF_2 may be obtained by selective decoupling the 21′-methyl protons. The CF_2 signal appeared as an ABX system from which the vicinal coupling constant may be readily deduced ($^3J_{FA}$–H′$_4$ = 6.8 Hz and $^3J_{FB}$–H′$_4$ = 6.55 Hz). These typical values correspond to an averaged coupling, since at room temperature there was rapid exchange between the different rotamers. Similarly, the coupling constant between CF_2 and the 21′-methyl protons was found to 19 Hz by decoupling H-4′.

Assignment of ^{13}C NMR spectra

The ^{13}C NMR spectrum of vinflunine ditartrate exhibited 47 signals that, according to the DEPT spectrum, correspond to seven methyls, nine methylenes, 14 methines and 17 quaternary carbons. The ^{13}C NMR spectra were analyzed with the help of HMQC and HMBC experiments. Signals for carbons directly bonded to non-exchangeable protons were assigned from a ^{1}H–^{13}C HMQC spectrum. This experiment was particularly useful since signals for geminal protons are readily identified. For example, the well separated H-3′$_{ax}$ and H-3′$_{eq}$ proton signals correlated with a single carbon frequency (C-3′) at δ 31.60 for **1** and δ 27.83 for **2**. The carbon-13 resonances were almost identical for the two compounds. The most important chemical shift differences were found for the difluorocleavamine moiety around N-6′. For example,

C-5′ had a chemical shift of δ 52 in vinflunine base and δ 48.3 in the salt (Table 1).

The ^{13}C NMR spectra were characterized by large coupling constants between fluorine-19 and neighboring carbon-13 nuclei. A value of 240 Hz was found for coupling through one bond. This value is typical for fluoroaliphatic compounds. Geminal couplings were found to be ^{2}J(C-21′, F) = 28.0 Hz and ^{2}J(C-4′, F) = 25.0 Hz. Long-range couplings through more than two bonds could not be determined accurately under our experimental conditions because they were <5 Hz. Most of the remaining ^{13}C signals were assigned from $^{1}H-^{13}C$ HMBC spectra. For example, HMBC correlation signals were observed from the H-7′$_{endo}$ proton to four carbon atoms with ^{13}C chemical shifts of δ 111.9 (C-9′), 129.6 (C-10′), 134.5 (C-17′) and 52.01 (C-5′). The HMBC spectrum allowed us to define unambiguously the chemical shifts of carbons C-9′, C-10′, C-17′ and C-15′ based on their correlations with the 16′-NH. We also used proton-detected multiple-bond $^{1}H,^{13}C$ correlations (HMBC) to obtain an independent assignment of the carbonyl C-26 and C-22′ signals via their respective couplings with H-4 and H-1′$_{exo}$ protons. Correspondingly, the C-23′ and C-24 methoxy protons were assigned via their respective couplings with the carbonyl C-22′ and C-23 carbons. Similarly, C-18′ exhibited a cross peak with H-14 in accordance with the anchor position of the two parts of the molecule. The remaining quaternary signals at δ 43.5 and 54.1 were assigned to C-5 and C-12, respectively, based on HMBC connectivities involving H-7 and H-2.

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