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Complete Assignment of the ^{1}H and ^{13}C NMR Spectra and Solution Conformation of the Antitumour Antibiotic, Aclacinomycin A.

John A. Parkinson, Ian H. Sadler*

University of Edinburgh, Department of Chemistry, West Mains Road, Edinburgh. EH9 3JJ. U.K.

Michael B. Pickup, Alethea B. Tabor*

Department of Chemistry, Christopher Ingold Laboratories, University College London, 20 Gordon Street, London WC1H 0AJ. U.K.

Abstract: As a prelude to the study of the molecular recognition of DNA by anthracycline antibiotics, and as a basis for comparison with synthetic analogues the proton and carbon-13 NMR spectra of Aclacinomycin A in chloroform have been fully assigned and the likely solution conformation determined using COSY, HMQC and 1-D TOCSY and 1-D ROESY techniques. The D-ring of the fused tetracyclic ring system adopts a half-chair conformation and the sugar residues are shown to lie essentially in chair conformations with the glycosidic links diaxial. This imposes considerable restriction in the rotation about the glycosidic links.

Many anthracycline antibiotics, such as daunomycin and adriamycin, are widely used in the clinical treatment of cancer.[1] Such compounds bind to DNA via intercalation of the anthracycline ring system, with further binding strength and specificity being conferred by the interaction of the sugar moieties with the DNA.[2] It is believed that the anti-cancer activities of this class of drugs derives from their ability to inhibit DNA replication and transcription.[3] Aclacinomycin A (Figure 1A),[4] another anthracycline antibiotic, shows antitumour activity superior to adriamycin in some models[5] and has milder cardiac toxicity.[6] It differs from daunomycin and adriamycin both in the structure of the aglycone, aklavinone, and also in possessing a trisaccharide portion attached to the C-7 position of the aglycone. There is increasing evidence that the polysaccharide portions of DNA-binding drugs play a major part in the binding and sequence-recognition of DNA.[7,8] In order to elucidate the role that is played by these polysaccharides, it is important to investigate the structure of such anthracyclines, both bound to DNA and in solution.

The original determination[4] of the structure of aclacinomycin A was carried out by degradative methods and 1-D NMR studies at low magnetic field strengths, and a full assignment of the ^{1}H and ^{13}C signals was not attempted. In a recent paper[9] a 2-D proton NMR study of the structures of the 2:1 complexes of aclacinomycins A and B with d(CGTACG) was reported. However the paper is largely concerned with Aclacinomycin B and ^{1}H and ^{13}C spectra of Aclacinomycin A are not given. As part of our research programme in this area of molecular recognition, we therefore undertook the full assignment of the ^{1}H and ^{13}C spectra of aclacinomycin A using appropriate 1-D and 2-D techniques. The assignments and other pertinent data are set out in Table 1.

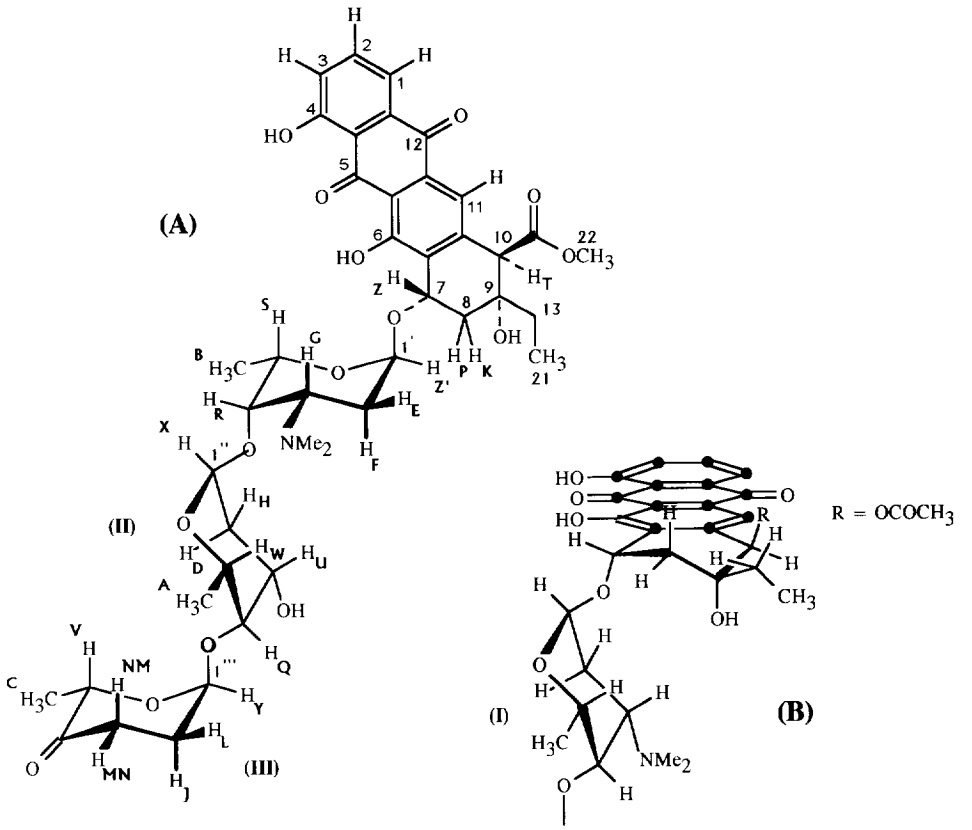

Figure 1 (A) Structure of Aclacinomycin A showing spatial relationships of hexose rings
(B) Spatial relationship of aglycone and adjacent hexose.

INTERPRETATION AND ASSIGNMENT OF SPECTRA

The ^{1}H NMR spectrum of Aclacinomycin-A in deuteriochloroform obtained at 14.1T (600 MHz), is complex with considerable overlap of resonances in the 1.8 - 2.5δ region and coincidence of two resonances at *ca.* 4.1δ (Figure 2A). Only the resonances arising from **H-2, H-11, H-13**(methylene), **H-21**(methyl), **H-22**(methoxy), and the dimethylamino protons can be umambiguously assigned by inspection. To simplify the ensuing discussion all other proton resonances, except those from the hydroxyl and aromatic protons, are labelled **A** to **Z,** and **Z'** (omitting letters **I** and **O**) from low to high frequency. Thus the three sugar methyl proton resonances are **A**, **B** and **C**,

The 2-D HMQC C-H one-bond correlation spectrum show resonances **X**, **Y** and **Z'** to arise from the three sugar anomeric protons. It also allows the identification of the resonances of geminal pairs of methylene protons as follows: **D** with **H**, **E** with **F**, **J** with **L**, **K** with one of the resonances **M**, **N** and **P** and the remaining two of this group with each other. The corresponding characteristic cross-peak coupling patterns in the 2-D DQFCOSY spectrum (Figure 2B) confirm these pairings and show clearly that **K** pairs with **P**.

TABLE 1 ^{1}H and ^{13}C NMR Spectra of Aclacinomycin A.

Site		δ_C	δ_H*	J_{HH} (Hz)	Proton-Carbon long-range correlations
1		120.0	7.805 (dd)	7.7, 1.1	C-3, C-4a, C-12
2		137.2	7.671 (dd)	8.4, 7.5	C-12a
3		124.7	7.288 (dd)	8.4, 1.1	C-1, C-4a
4		162.4			
4-OH			12.014[x] (bs)		
4a		115.7			
5		192.6			
5a		114.5			
6		162.0			
6-OH			12.657[x] (bs)		
6a		131.3			
7	Z	70.5	5.253 (dd)	4.4, 2.1	C-8, C-9, C-6, C-10a, C-11, C-1'
8 (β)	P	33.6	2.494 (dd)	14.8, 4.0	C-9
(α)	K		2.290 (dt)	14.8, 2 x 1.6,	C-7, C-9, C-10, C-13, C-6a
9		71.6			
9-OH			4.57 (bs)		
10	T	57.0	4.094 (s)		C-6a, C-8, C-9, C-10a, C-11, C-15
10a		142.5			
11		120.8	7.665 (s)		C-10, C-12, C-5a, C-6, C-6a
11a		132.7[y]			
12		181.2			
12a		133.3[y]			
13	(h)	32.0	1.731 (dq)	14.0, 3 x 7.3	C-8, C-9, C-10, C-14
	(l)		1.488 (dq)	14.0, 3 x 7.3	C-8, C-9, C-10, C-14
15		171.2			
21	(Me)	6.5	1.073 (t)	7.3	C-9, C-13
22	(MeO)	52.4	3.680 (s)		C-15

x, y: in each of these pairs the assignments may be reversed

Site		δ_C	δ_H*	J_{HH} (Hz)	Proton-Carbon long-range correlations
1'	Z'	101.5	5.495 (bd)	4.2	C-7, C-3', C-5'
2'	E (eq)	29.1	1.808 (ddt)	12.9, 4.4, 2 x 1.2	C-3', C-4'
	F (ax)		1.873 (td)	2 x 12.9, 4.1	C-3'
3'	G	61.4	2.046 (dd)	12.9, 4.4	
4'	R	73.8	3.716 (bs)		C-2', C-3', C-1''
5'	S	68.2	3.984 (q)	6.6	C-4', C-6'
6'	B	17.7	1.260 (d)	6.6	C-4', C-5'
3'-NMe_2		43.2	2.146 (s)		C-3'
1''	X	99.3	5.018	3.4	C-3'', C-5'', C-4'
2''	D (ax)	34.2	1.808 (td)	2 x 12.2, 3.7	C-3''
	H (eq)		2.090 (ddt)	12.3, 4.7, 2 x 1.2	C-3'', C-4''
3''	U	65.2	4.087 (bm)		
4''	Q	82.9	3.653 (bs)		C-2'', C-3'', C-1'''
5''	W	66.6	4.533 (bq)	6.5, 0.9	C-4'', C-6''
6''	A	16.8	1.146 (d)	6.5	C-4'', C-5''
3''-OH			3.66 (bd)	9.3	
1'''	Y	100.0	5.060 (t)	2 x 6.3	C-5''', C-4''
2'''	J (ax)	27.5	2.14 (cm)		C-1''', C-3''', C-4'''
	L (eq)		2.42 (cm)		
3'''	M,N	33.4	2.4-2.5 (cm)		C-2'''
4'''		209.7			
5'''	V	71.6	4.476 (q)	6.5	C-1'', C-4''', C-6'''
6'''	C	14.7	1.311(d)	6.5	C-4''', C-5'''

* b = broad, c = complex, d = doublet, m = multiplet, q = quartet, s = singlet, t = triplet

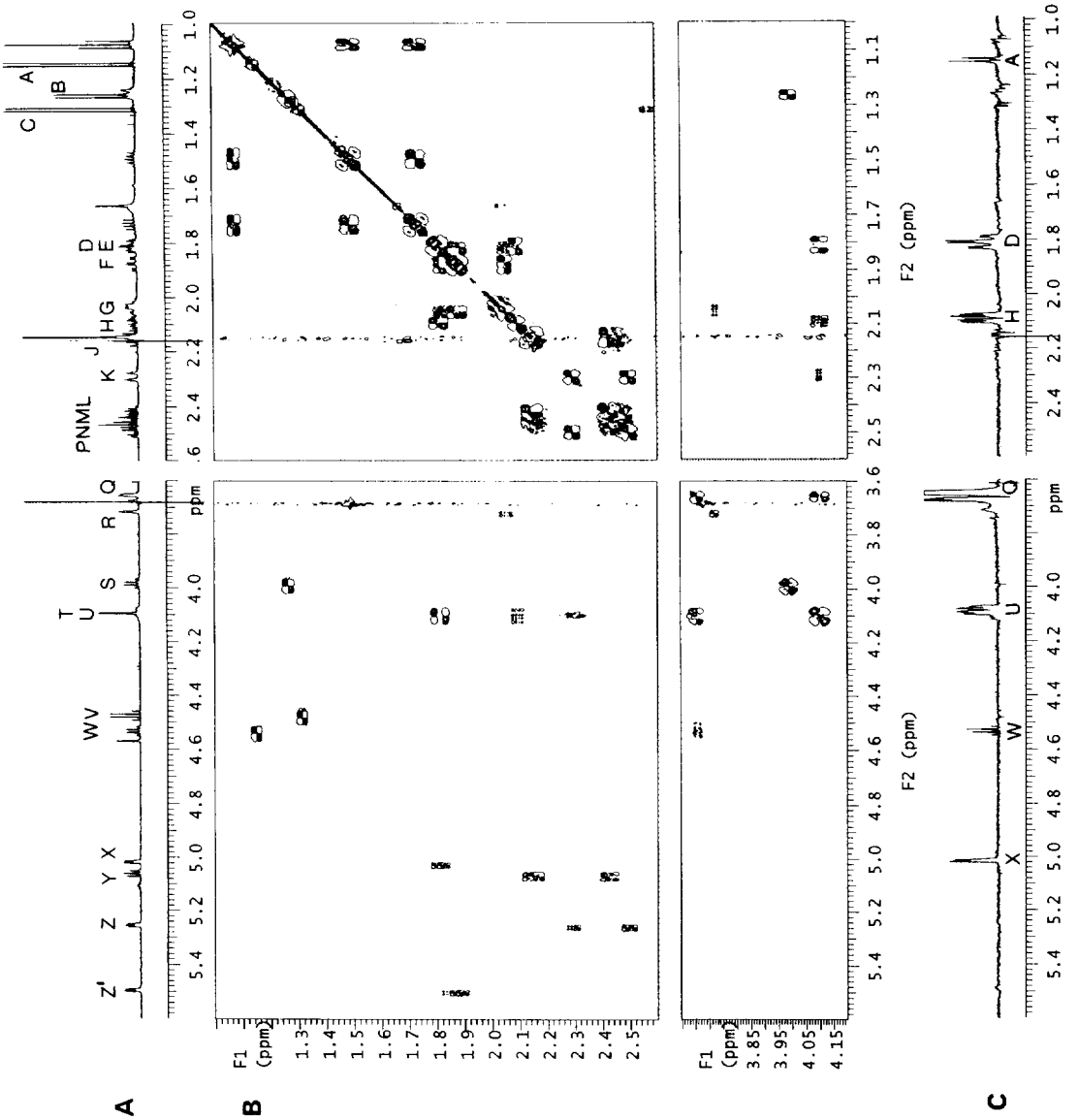

Figure 2: (A) Aliphatic Region of Proton NMR Spectrum of Aclacinomycin A.
(B) Part of the two-dimensional DQFCOSY Spectrum of Aclacinomycin A corresponding to (A).
(C) One-dimensional TOCSY spectrum of Aclacinomycin A showing resonances of the 2'-deoxyfucose residue (**II**) only by selective excitation of the **H-4''** resonance.

The assignments of the sugar and tetracycline D-ring proton resonances were made by analysis of the 2-D DQFCOSY spectrum. In this discussion off-diagonal peaks in the 2-D DQFCOSY spectrum showing coupling between two nuclei i and j are referred to as ij crosspeaks for simplicity. Thus, starting with the anomeric proton **X**, it is possible to sequentially trace and completely assign the proton resonances (Table 1) of one sugar residue via the cross-peaks **XD**, **DH**, **DU**, **HU**, **UQ**, **QW** and **WA.** This was identifed as the 2'-deoxyfucose residue (**II**) from the chemical shift of **H-3"** (**U**) at *ca.* 4.1δ (the corresponding proton in the rhodosamine residue (**I**), the alternative 7-spin system, resonates *ca.* 2.0δ). This spin system was confirmed by a 1-D TOCSY spectrum in which resonance **Q** (**H-4"**) was selectively excited and only the resonances of this spin system were obtained (Figure 2C). Similarly, starting at anomeric proton **Z'**, the cross-peaks **Z'F**, **FE**, **FG**, **EG**, **GR**, **RS**, and **SB** allow the complete assignment of the protons (Table 1) of the rhodosamine residue (**I**) spin system, again confirmed by 1-D TOCSY spectra. The **YJ**, **YL, JL**, **JM** and **VC** cross peaks allow the assignment of the protons (Table 1) to the cinerulose residue (**III**); cross-peaks corresponding to coupling between protons **L**, **M** and **N**, lie too close to the diagonal to be distinguished. The separate spin systems of this residue were confirmed, as before, by 1-D TOCSY spectra. The remaining aliphatic proton resonances arise from the aglycone D-ring protons, crosspeaks confirming the coupling of **H-7** (**Z**) to both **H-8** protons (**P** and **K**); a 1-D TOCSY spectrum indicates a long-range coupling to **H-10** (**T**). The resonances of aromatic protons **H-1** and **H-3** were distinguished by the reference to a 2-D HMBC long-range C-H correlation spectrum, optimised for a coupling of *ca.* 8. Hz. The **H-11** resonance and the proton resonance at 7.8δ (but not at 7.3δ) each show a long-range correlation to a carbonyl resonance. Thus the resonance at 7.8δ corresponds to **H-1** and that at 7.3δ to **H-3**. This agrees with assignments of the analogous resonances in the proton spectrum of 1,8-dihydroxy-9,10-anthraquinone[10]. The phenolic proton resonances at ca. 12.0δ and 12.7δ could not be uniquely assigned, however irradiation of either in a saturation difference spectroscopy experiment located the C-9 and C-3" hydroxyl resonances (see Table 1) as a broad singlet (4.57δ) and broad doublet (3.66δ, coincident with **H-4"**) respectively.

The assignment of the non-quaternary carbon resonances follows directly using the 2-D HMQC C-H one-bond correlation spectrum with no ambiguities (see Table 1). Long-range correlations obtained from the 2-D HMBC spectrum between **H-1** and **C-4**, and **H-4** and **C-1** of adjacent sugar rings, between **H-7** and **C-1'** and between **H-1'** and **C-7** confirmed the assignments and the connectivity of the sugar residues. The assignment to **C-9** of the unique aliphatic quaternary carbon resonance at 71.6δ was confirmed by long-range correlation to **H-7**. The other quaternary carbon atom resonances were assigned using the 2-D HMBC long-range C-H correlation spectrum and by comparison with the carbon-13 spectrum of 1,8-dihydroxy-9,10-anthraquinone[10]. Correlation of **H-2'"** and **H-6'"** of the cinerulose residue to the carbonyl resonance at 209.7d confirms the assignment of this resonance to **C-4'"**. Correlation of **H-10** and the methoxy protons with the carbonyl resonance at 171.2δ confirms the assignment of this resonance to **C-15**. In substitued anthraquinones 2-bond proton carbon couplings are very small (<2 Hz) whereas 3-bond proton carbon couplings lie in the range 7 - 8 Hz and will give responses in the HMBC spectrum. The correlation identifying **H-1**, described previously, also identifies the carbonyl resonance at 181.2δ as arising from **C-12** and hence the carbonyl resonance at 192.6δ correponds to **C-5**. Correlations of **H-1** and **H-11** with carbon resonances at 114.5δ and 115.7δ allow the assignment of these shifts to **C-4a** and **C-5a** respectively. Correlations with **H-8** and **H-11** indicate that the carbon resonance at 131.3δ correponds to **C-6a,** and correlations with **H-7** and **H-**

10 indicate that the carbon resonance at 142.5δ correponds to **C-10a**. Correlation with **H-7** shows that the carbon resonance at 162.0δ correponds to **C-6** and the correlation with **H-2** shows that the carbon resonance at 162.4δ correponds to **C-4**. The two remaining carbon resonances at 132.7δ and 133.3δ therefore correspond to **C-11a** and **C-12a**, not necessarily respectively. The shifts of carbon atoms of the anthraquinone sub-unit of the aglycone agree well with those of the analogous resonances in the carbon-13 spectrum of 1,8-dihydroxy-9,10-anthraquinone[10].

STEREOCHEMICAL RELATIONSHIPS

The large coupling (12 - 13 Hz) between **H-3** and one **H-2** for the rhodosamine (**I**) and 2'-deoxyfucose (**II**) residues confirms that the dimethylamino and hydroxyl substituents at **C-3** lie in equatorial postitions on rings that are essentially in the chair conformation. However near-zero values for the vicinal **H-4/H-5** and **H-3/H-4** couplings and one of the **H-1/H-2** couplings suggests slight deviations from perfect chair conformations for these two rings and also confirms that the glycosidic links are axial at **C-4**. The absence of large couplings for **H-1** in all three residues confirms that the glycosidic links are also axial at **C-1**. Attempts to obtain conventional NOE difference spectra were unsuccessful, probably because the tumbling rate of this size of molecule places it in the zero NOE region. However satisfactory results were obtained using a 1-D ROESY sequence with irradiation at fully resolved resonances. For all three sugar residues the **H-3** resonances are enhanced on irradiation of the corresponding **H-5** resonances supporting a chair conformation for all three rings. For the cinerulose residue (**III**) both **H-2/H-1** couplings are similar suggesting that this ring is less distorted from a perfect chair than the other two. As expected for all three residues, the **H-4** resonances are enhanced on irradiating the corresponding **H-5** resonances, and the **H-4** and **H-5** resonances are enhanced on irradiation of the corresponding methyl resonances.

The 1-D ROESY spectra also suggest that the D ring of the aglycone adopts a half-chair conformation (Figure 1B) with **H-7**, **H-10**, and the ethyl group occupying equatorial (or psuedo-equatorial) positions. For example, irradiation of **H-7** enhances both **H-8α** and **H-8β**; irradiation of **H-21** (Me) enhances **H-10**, **9-OH**, **H-13l**, **H-13h**, and **H-8α** but not **H-8β**. Irradiation of either **H-13** resonance enhances the other and also enhances **H-21**. In addition **H-10** is enhanced by irradiation of **H-13h** and the **H-8α** and **H-8β** resonances by irradiation of **H-13l**; irradiation of **9-OH** enhances **H-10** and only **H-8α**. Thus the glycosidic link occupies an axial (or pseudoaxial) position.

Rotation of sugar residues about glycosidic links are known to be very restricted and for two pyranose rings linked 1'-4 via axial bonds on both rings, only a very small region of conformational space is available[11]. The minimum energy conformation corresponds to a position where **H-1'** on one residue lies near both **H-4** and **H-6** of the other. The 1D ROESY spectra support a similar picture for Aclacinomycin A (Figure 1A); mutual enhancements are observed between **H-1'''**, **H-4''** and **H-6''** and between **H-1''**, **H-4'** and **H-6'**, also **H-2''** is enhanced on irradiation of **H-5'''**. A similar situation is observed for the relative position of the D-ring of the aglycone and the rhodosamine residue (Figure 1B). For example, mutual enhancements are observed between **H-1'** and **H-7**, and between **H-5'**, **H-8a** and **9-OH**.

EXPERIMENTAL

Aclacinomycin A (aclacin) was a gift from Lundbeck Ltd. (U.K.), and supplied in vials each containing 20 mg aclacinomycin. The sample was prepared for NMR spectroscopy by dissolving the contents of each vial in water (20 ml); this solution was washed with chloroform (30 ml) which had been previously neutralised by extraction with saturated aqueous sodium bicarbonate. This extraction was repeated until no yellow colour persisted in the aqueous layer. The combined organic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo* to yield aclacinomycin A as a yellow solid.

The spectra were measured in deuteriochloroform using a Varian VXR600 spectrometer operating at 599.9 MHz for protons and 150.9 MHz for ^{13}C nuclei.

1-D TOCSY proton spectra were obtained using the sequence[12]: D1 -- 180^o_{sel} -- 90^o -- D2 -- 90^o -- 90^o -- AQ with D1 = 2 s, 180^o_{sel} = 300 ms (= 20Hz), D2 = 0.12 s (pulsed spin lock[13]), AQ = 2.56 s. An 8 step phase cycle was used. Other parameters were SW = 7000 Hz; 32K data points. A shaped (IBURP)[14] selective spin inversion pulse was applied on- and off-resonance on alternate scans and the FID's alternatively added and subtracted to give a "difference" FID which, on transformation, gave spectra showing only TOCSY responses.

1-D ROESY proton spectra were obtained using the sequence[13]: D1 -- 90^o_{sel} -- 90^o -- 90^o -- D2 -- 90^o -- AQ with D1 = 2 s, 90^o_{sel} = 150 ms, D2 = 0.3 s (spin lock), AQ = 2.56 s. An 8 step phase cycle was used. A shaped (TOPHAT)[14] selective 90^o pulse was used. Spectra show ROESY responses as positive signals; small negative signals are TOCSY responses.

The 2-D DQFCOSY phase sensitive proton spectrum was obtained using the sequence[15]: D1 -- 90^o -- t_1 -- 90^o -- 90^o -- AQ with D1 = 3 s, AQ = 0.2936 s. An 8 step phase cycle (hypercomplex acquisition) was used. Other parameters were SW =3493 Hz; 2K data points; 512 increments each with 16 transients per FID were used. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1-data to 2K data transformation resulting in digital resolution of 3 Hz/pt.

The 2-D proton detected one-bond ^1H-^{13}C correlation (HMQC) spectrum was obtained using the sequence[16]: D1 -- $90^o(^1H)$ -- D2 -- $180^o(^1H)$;$180^o(^{13}C)$ -- D2 -- $90^o(^1H)$ -- D3 -- $90^o(^1H)$ -- D2 --$90^o(^{13}C)$ -- $t_1/2$ -- $180^o(^1H)$ -- $t_1/2$ -- $90^o(^{13}C)$ -- D2 -- AQ. The delays used were D1 = 1.8 s, D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 500 ms (to minimise signals from protons bonded to ^{12}C nuclei). The experiment was preceeded by 200 dummy scans to establish thermal equilibrium. A 16 step phase cycle (hypercomplex acquisition) was used with ^{13}C broad band decoupling during acquisition of the proton signals. 256 Increments with 48 scans per FID were obtained. Other parameters were SW(^1H) = 5000 Hz; 2K data points; SW(^{13}C) = 35000 Hz, AQ = 0.205 s. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 256W to 512W before transformation. The 2-D proton detected multiple-bond ^1H-^{13}C correlation (HMBC) spectrum was obtained using the sequence[16]: D1 -- $90^o(^1H)$ -- D2 -- $90^o(^{13}C)$ -- D3 -- $90^o(^{13}C)$ -- $t_1/2$ -- $180^o(^1H)$ -- $t_1/2$ -- $90^o(^{13}C)$ -- AQ. The delays used were D1 = 1.8 s, D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 60 ms (optimised for signals from protons with couplings to carbon of ca. 8 Hz). A 16 step phase cycle (hypercomplex acquisition) was used with no ^{13}C broad band decoupling during acquisition of the proton signals. 192 Increments with 48 scans per FID were obtained. Other parameters were as for the HMQC experiment. The HMQC and HMBC data were both processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data to 512W before transformation.

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