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Unequivocal ^{13}C NMR assignment of cyclohexadienyl rings in bromotyrosine-derived metabolites from marine sponges

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ABSTRACT

Bromotyrosine-derived compounds are commonly isolated from Verongida sponges and are a major class of marine natural products. Here we report on the unequivocal ^{13}C NMR assignment of the brominated carbons at positions C-2 and C-4 of the cyclohexadiene ring, two carbons whose resonances are often incorrectly assigned. Interpretation of HMBC data acquired for a series of known bromotyrosine analogues, which included ianthesine E (**1**), aerothionin (**2**), 11-hydroxyaerothionin (**3**), and 11,19-dideoxyfistularin-3 (**4**), allowed us to unequivocally assign the carbons in question, C-2 and C-4, through the observance of unique HMBC correlations from the C-1 hydroxyl proton. Here we present the complete 2D NMR data sets recorded in DMSO-d_6 for **2**-**4** that were used to confirm the assignment and establish the working model. Using this model, a survey of the literature revealed that many members of this structure class had been wrongly assigned. This paper serves to reassign those compounds whose ^{13}C NMR assignment at positions C-2 and C-4 of the cyclohexadiene ring should be reversed.

INTRODUCTION

Bromotyrosine-derived metabolites from sponges of the order Verongida represent a major structure class of marine natural products.[1] The first reported examples of this compound class included aerothionin [2,3] and homoaerothionin,[4] which were published in the early 1970s. To date over fifteen different structure types have been reported including the predominant aerothionin, fistularin, psammaplin, psammaplysin, bastadin, and purealidin families of molecules.[1,5] The rich chemodiversity associated with sponges of the Verongida order has ensured that they continue to be investigated vigorously, not only for new chemistry but also for their biological activities.[6-8] Common to many of these molecules is the bromotyrosine-derived spirocyclohexadiene isoxazoline moiety, which is the focus of this paper.

Known analogues of these well precedented bromotyrosine-derived sponge metabolites are often reisolated in the process of identifying new analogues from Verongida sponges. Rather than collecting what may seem superfluous 2D NMR data, the focus is directed toward interpreting signals which are clearly different to those of the known analogues. This less than rigorous approach of comparing 1D NMR data with reported chemical shifts in order to identify both new and known metabolites, has led to the common misassignment of two clearly distinguishable ^{13}C NMR resonances in the brominated cyclohexadienyl ring for this class of compounds. Furthermore a recent review by McPhail and co-authors, in which they surveyed marine natural product structure revisions reported between 2005 and 2010, identified that a large proportion (~22%) of all misassignments resulted from comparing NMR data with those in the literature.[9]

Our interest in this class of molecule was stimulated by our previous discovery of ianthesine E (**1**) from the Australian sponge *Pseudoceratina* sp., whose extract showed binding activity towards the adenosine A_1 receptor.[10] Together with **1**, which has also been reported from a Floridian *Aplysina fulva* and named araplysillin-I N^{20}-sulfamate,[11] we isolated the known aerothionin (**2**), 11-hydroxyaerothionin (**3**), and 11,19-dideoxyfistularin-3 (**4**)[10] (Figure 1).

The two ^{13}C NMR chemical shifts of the brominated carbons C-2 and C-4 in cyclohexadienyl rings rarely differ across the suite of known compounds irrespective of other substituents or solvent choice, and thus, less attention has seemingly been paid to these resonances when assigning NMR data. As a result, there exist many instances where these carbons have been wrongly assigned.

The likely reason for the initial discrepancy in the C-2/C-4 assignment was explained by Fattorusso and co-authors as far back as 1994 and relates to the structure elucidation of the related metabolites, psammaplysins A and B.[12] Nevertheless, the corresponding NMR data across the suite of related compounds continues to be incorrectly assigned, and this was highlighted as recently as 2011 by Ley and co-authors who corrected the assignment for the subereamollines.[13] Due to this inconsistency, a survey of the ^{13}C NMR data reported for molecules possessing cyclohexadienyl rings seemed warranted. Giving weight to our survey is the recently published review of bromotyrosine-derived natural products from *Aplysina* spp.[14] This comprehensive review includes a compilation of ^{13}C resonances, as published, for those compounds in question here. It is

apparent that approximately half of the relevant compounds from *Aplysina* spp. are not correctly assigned. [14]

Our unambiguous assignment of the dibromocyclohexadienyl rings was achieved through careful examination of HMBC spectra recorded in DMSO-d_6 for the natural products, ianthesine E (**1**), aerothionin (**2**), 11-hydroxyaerothionin (**3**), and 11,19-dideoxyfistularin-3 (**4**) (Tables 1 and 2). These spectra showed key, and importantly, unique correlations that have enabled us to unambiguously assign the brominated carbons in question. This paper serves to provide unequivocal, experimental evidence, for the assignment of ^{13}C NMR shifts around the bromotyrosine-derived cyclohexadienyl ring, and alert those reporting new compounds of this class, to the correct ^{13}C NMR assignment. For reference purposes, we present our findings and cite examples of compounds incorrectly assigned (Table 3). We also report complete HMBC, HMQC and COSY data recorded in DMSO-d_6 for **2**-**4** (Tables 1-2). NMR data for **1** have been published previously.[10]

RESULTS AND DISCUSSION

The assignment of NMR data to any complex natural product occasionally proves problematic due to almost co-incident or even overlapping signals. In the case of the brominated cyclohexadienyl system the difference in the ^{13}C chemical shifts for the brominated C-2 and C-4 are quite significant, usually in the order of at least 5 ppm, and thus are easily distinguishable.

We collected complete data sets including HMBC spectra for four bromotyrosine-derived molecules **1**-**4** in DMSO-d_6. All of these molecules contain at least one bromotyrosine-derived cyclohexadienyl ring, and all ring and ring substituent protons showed two and three bond HMBC correlations.

Key to the unequivocal ^{13}C NMR assignment of the cyclohexadienyl ring and thus the correct chemical shift assignment of the two brominated carbons were the observed two and three bond HMBC correlations from the position one hydroxyl (1-OH) proton. Using the data for **2** as an example (Figure 2), the ^{1}H chemical shift of the hydroxyl proton doublet, (δ 6.33, J = 7.8 Hz, 1-OH,) was assigned based on coupling constants and a COSY to the hydroxy methine doublet (δ 3.91, J = 7.8 Hz, H-1). In previous reports of bromotyrosine-derived molecules, where the ^{1}H chemical shifts of the hydroxyl protons were assigned, no HMBC correlations were reported. Fortunately, our HMBC spectra for **2** (Figure 2) revealed strong correlations from this often exchangeable hydroxyl proton to carbon atoms at 73.6 (C-1), 90.1 (C-6), and 113.1 ppm (C-2). Almost identical sets of correlations were clearly evident in the HMBC spectra acquired for all molecules (**1**-**4**) purified from the Australian *Pseudoceratina* sp. extract. To our knowledge these are the only reported instances of HMBC correlations from 1-OH. This allowed a reliable model for the assignment of carbon shifts around the ring to be established. For compounds **1**-**4**, the ^{13}C resonances for carbons C-2 and C-4, and equivalent carbons (e.g C-21 and C-19 in **2**-**3**), were in the ranges of 112.5-113.1 ppm and 120.5-120.8 ppm respectively.

The non-exchange of the cyclohexadienyl hydroxyl protons (1-OH), and the subsequent observation of HMBC correlations, lends support to the use of DMSO-d_6 as the preferred solvent for molecules of this family. This is exemplified by the report of ianthesines A-D where NMR data for ianthesines A and B, collected in the protic CD_3OD, lacked ^1H chemical shifts attributable to hydroxyl protons.[15] In contrast, the ring hydroxyl proton shifts of ianthesines C and D acquired using DMSO-d_6 as the solvent of choice, were indeed assignable.[15] The broadness of the OH signal could explain, in this case why HMBC correlations from the cyclohexadienyl ring hydroxyls were not reported.[15]

Similarly, the description of araplysillin-I N^{20}-sulfamate (=**1**) reported the 1-OH proton resonances collected in DMSO-d_6 but these lacked any HMBC data.[11] Incidentally, it appears that the incorrect assignment of C-2/C-4 was based on comparison with data that were assigned using chemical shift prediction software. Those comparative data were collected from an impure mixture of araplysillin-I N^{20}-sulfamate and a related analogue, in a CD_3OD-$CDCl_3$ (2:1) solvent system, presumably, in this case to overcome solubility issues.[11]

Reasons for the widespread misassignment of the C-2/ C-4 resonances for this particular structure class are varied. From our examination of the literature it is apparent that comparison of 1D experimental, and sometimes 2D NMR data with reported values, is the major cause. Solvent selection has seemingly played some role, however it is notable that even in cases where HMBC spectra have been collected in DMSO-d_6, correlations from 1-OH were either not observed or reported (Table 3). [11,16-33]

CONCLUSION

Unequivocal NMR assignment of carbons C-2 and C-4 positioned around the brominated cyclohexadiene moiety, a structural feature commonly encountered in sponge-derived natural products, was achieved via interpretation of HMBC spectra recorded in DMSO-d_6. Unique to our data set are strong HMBC correlations from ring hydroxyl protons (1-OH), which allowed the unambiguous ^{13}C NMR assignment of both brominated carbons. In summary, we have established a reliable model for the ^{13}C NMR assignment of these brominated carbons in which C-2 and C-4, and their equivalent carbons, should be assigned in the approximate ranges of 112-113 ppm and 120-122 ppm, respectively.

EXPERIMENTAL

General

General experimental details, as well as sponge collection details, and the isolation of **1-4**, have been published elsewhere.[10]

HMBC data acquisition

The gradient HMBC data was recorded at 30 ^{o}C on a Varian 600 MHz Unity INOVA equipped with a 5mm inverse detection probe. The ^{1}H and ^{13}C chemical shifts were referenced to the residual protonated solvent peak of DMSO-d_6 at δ 2.49 and 39.5 ppm, respectively. The gradient HMBC was acquired using 128 transients per increment, with 256 F1 increments. The ^{1}H and ^{13}C sweep widths were set at 6023 and 33195 Hz, respectively. A one-bond coupling constant of 140 Hz, and a long-range coupling

constant of 8 Hz were used to set the delays in the pulse sequence. A sine bell weighting was applied to both ^{1}H and ^{13}C dimensions and zero filled to 4K and 1K, respectively.

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Table Headings

Table 1 NMR data for **2-3** acquired in DMSO-d_6 at 30 ^{o}C

Table 2 NMR data for **4** acquired in DMSO-d_6 at 30 ^{o}C

Table 3 Bromotyrosine-derived sponge compounds reported with incorrectly assigned ^{13}C NMR resonances.

Figure Captions

Figure 1. Structures of bromotyrosine-derived sponge metabolites ianthesine E (**1**), aerothionin (**2**), 11-hydroxyaerothionin (**3**), and 11,19-dideoxyfistularin-3 (**4**).

Figure 2.

a) Partial HMBC spectrum for **2**. Highlighted are key correlations from the hydroxyl proton at position 1 (δ 6.33) to C-1 (73.6 ppm), C-2 (113.1 ppm), and C-6 (90.1 ppm) and from H-5 (δ 6.56) to C-1, C-2, C-6, C-4 (120.8 ppm) and C-3 (147.3 ppm). These correlations were used to unambiguously assign ^{13}C NMR resonances around the dibrominated cyclohexadiene rings in **1**-**4**. H-5 also shows a weaker (4 bond) HMBC correlation to C-2, and a 3 bond correlation to C-7 (39.1 ppm – not displayed).

b) Structure fragment of **2** showing observed two and three bond correlations from 1-OH and H-5 used to establish model for the assignment of ^{13}C NMR resonances for C-2 and C-4 and their equivalent carbon atoms.

Table 1

Position	aerothionin **2**				11-hydroxyaerothionin **3**			
	^{13}C [a] (δ)	^{1}H (δ, mult., *J* in Hz)	HMBC	COSY	^{13}C [a] (δ)	^{1}H (δ, mult., *J* in Hz)	HMBC	COSY
1	73.6	3.91 (d, 7.8)	C2-C4, C6, C7	1-OH	72.9	3.91 (d, 7.8)	C2, C3, C5, C6	1-OH
2	113.1	-			112.5	-		
3	147.1	-			146.4	-		
4	120.8	-			120.1	-		
5	131.3	6.56 (s)	C1-C4, C6, C7,		130.5	6.57 (s)	C1-C4, C6, C7	
6	90.1	-			89.7	-		
7	39.4	3.19 (d, 18.0) 3.61 (d, 18.0)	C1, C5, C6, C8, C9 C1, C5, C6, C8, C9	H7b H7a	38.9	3.61 (d, 18.1) 3.19 (d, 18.1)	C1, C5, C6, C8 C1, C5, C6, C8	H7b[b] H7a[b]
8	154.5	-			153.8	-		
9	158.8	-			158.3	-		
10	38.5	3.14 (br m)	C9, C11, C12	9-NH, H11	44.5	3.15 (m)	C9, C11, C12	9-NH, H11[b]
11	26.3	1.45 (br m)	C10, C12, C13	H10	66.4	3.60 (m)	-	11-OH, H10[b], H12a/b
12	26.3	1.45 (br m)	C10, C11, C13	H13	33.2	1.60 (m) 1.40 (m)	- C10[b], C11[b], C13[b]	H11 H12b, H13 H11, H12a, H13
13	38.5	3.14 (br m)	C11, C12, C14	H12, 14-NH	35.6	3.20 (m)	C11, C14	H12a, H12b, 14-NH
14	158.8	-			158.1	-		
15	154.5	-			153.8	-		
16	39.1	3.19 (d, 18.0) 3.61 (d, 18.0)	C14, C15, C17, C18, C22 C14, C15, C17, C18, C22	H16b H16a	38.9	3.61 (d, 18.1) 3.19 (d, 18.1)	C15, C17, C18, C22 C15, C17, C18, C22	H16b[b] H16a[b]
17	90.1				89.7	-		
18	131.3	6.56 (s)	C16, C17, C19-C22		130.5	6.57 (s)	C16, C17, C19-C22	
19	120.8				120.1	-		
20	147.1				146.4	-		
21	113.1				112.5	-		
22	73.6	3.91 (d, 7.8)	C16, C17, C19-C21	22-OH	72.9	3.92 (d, 7.8)	C17, C18, C20, C21	22-OH
11-OH	N/A				-	4.83 (d, 5.1)	C10, C11, C12	H11
1-OH	-	6.33 (d, 7.8)	C1, C2, C6	H1	-	6.33 (d, 7.8)	C1, C2, C6	H1
22-OH		6.33 (d, 7.8)	C17, C21, C22	H22	-	6.34 (d, 7.8)	C17, C21, C22	H22
3-OCH_3	59.6	3.64 (s)	C3		58.9	3.64 (s)	C3	
20-OCH_3	59.6	3.64 (s)	C20		58.9	3.64 (s)	C20	
9-NH		8.47 (t, 5.6)	C9, C10, C11[b]	H10	-	8.27 (t, 5.8)	C9	H10
14-NH		8.47 (t, 5.6)	C12[b], C13, C14	H13	-	8.41 (t, 5.8)	C14	H13

a ^{13}C shifts determined from 2D NMR experiments [b]Weak correlation

Table 2

Position	11,19-dideoxyfistularin-3 **4** ^{13}C (δ)[a]	^{1}H (δ, mult., *J* in Hz)	HMBC	COSY
1	73.5	3.91 (d, 7.9)	C2, C3, C5, C6	1-OH
2	113.1	-		
3	147.0	-		
4	120.5	-		
5	131.1	6.56 (d, 0.8)	C1, C2, C3, C4, C6,C7	
6	90.1	-		
7	39.5	3.18 (d, 18.2)	C1, C5, C6, C8	H7b
		3.62 (d, 18.2)	C1, C5, C6, C8	H7a
8	154.4	-		
9	158.8	-		
10	36.1	3.40 (m)	C9, C11, C12	9-NH, H11
11	29.3	1.99 (m)	C10, C12	H10, H12
12	71.1	3.95 (t, 6.1)	C10, C11, C13	H11
13	150.5	-		
14	117.2	-		
15	133.0	7.50 (s)	C13, C14, C17, C19	
16	138.5	-		
17	133.0	7.50 (s)	C13, C15, C18, C19	
18	117.2	-		
19	33.0	2.75 (t, 7.1)	C16, C17, C20	H20
20	39.1	3.39 (m)	C16, C19, C21	H19, 21-NH
21	158.8	-		
22	154.4	-		
23	39.5	3.20 (d, 18.2)	C22, C24, C25, C29	H23b
		3.60 (d, 18.2)	C22, C24, C25, C29	H23a
24	90.1	-		
25	131.1	6.57 (d, 0.8)	C23, C24, C26, C27, C28, C29	
26	120.5	-		
27	147.0	-		
28	113.1	-		
29	73.5	3.91 (d, 7.9)	C24, C25, C27, C28	29-OH
3-OCH_3	59.3	3.64 (s)	C3	
27-OCH_3	59.3	3.64 (s)	C27	
1-OH	-	6.32 (d, 7.9)	C1, C2, C6	H1
29-OH	-	6.33 (d, 7.9)	C24, C28, C29	H29
9-NH	-	8.53 (t, 5.7)	C9, C10	H10
21-NH	-	8.56 (t, 5.7)	C20, C21	H20

a ^{13}C shifts determined from 2D NMR experiments

Table 3

Incorrectly assigned compound(s)	Solvent used	Assignment method	1-OH observed ?	Ref
aerophobin-1 aerophobin-2	CD_3OD $CDCl_3/CD_3OD$	^{13}C NMR of the acetate derivatives	No (acetate derivatives)	[16]
purealin	DMSO-d_6	COSY/HETCOR/Spectra Comparison[c]	Yes[d]	[17]
11,12-dihydroxyaerothionin	DMSO-d_6 (1% TFA)	HMBC/Spectra Comparison[c]	No	[18]
araplysillin-1, -2	CD_3OD	Spectra Comparison[c]	No	[19]
11,19-dideoxyfistularin-3 11-hydroxyaerothionin	$(CD_3)_2CO$ $CDCl_3$	Spectra Comparison[c]	Yes 1D in $(CD_3)_2CO$	[20]
14-debromoaraplysillin-1	$CDCl_3$	Spectra Comparison[c]	No	[21]
purealidin B	DMSO-d_6	HMQC/Spectra Comparison[c]	Yes	[22]
11-oxoaerothionin	DMSO-d_6	Spectra Comparison[c]	Yes	[23]
11-ketofistularin-3	C_5D_5N	Spectra Comparison[c]	No	[24]
aplysinamine-I, -II aplysinamine-III	DMSO-d_6 CD_3OD	Spectra Comparison[c]	Yes No	[25]
hemifistularin-3	$(CD_3)_2CO$	Spectra Comparison[c]	Yes[e]	[26]
pseudoceratine A, C	CD_3OD	HMBC/Spectra Comparison[c]	Yes (1D in DMSO-d_6)	[27]
oxohomoaerothionin 11-hydroxyfistularin-3	CD_3OD	DEPT/COSY/HETCOSY/COLOC	No	[28]
11-deoxyfistularin-3	C_5D_5N	DEPT/COSY/Spectra Comparison[c]	No	[29]
2 × $C_{16}H_{19}Br_2N_3O_7$ isomers [a]	DMSO-d_6	DEPT/COSY/HMQC/HMBC	Yes[d]	[30]
araplysillin III hexadellin C	DMSO-d_6	COSY/HMQC/HMBC	No	[31]
araplysillin-I N^{20}-sulfamate[b] and precursor $C_{14}H_{16}Br_2N_2O_6$ ("**2**")[a]	DMSO-d_6	COSY/HMQC/HMBC	Yes[d]	[11]
subereamolline A, B	CD_3OD	COSY/HMQC/HMBC	No	[32]
19-hydroxy-araplysillin-I N^{20}-sulfamate	DMSO-d_6	COSY/HSQC/HMBC	Yes[d]	[33]

[a] No trivial name was assigned
[b] Originally named araplysillin-I N^9-sulfamate[11] and later corrected on re-isolation.[33]
[c] 1D NMR spectra comparison with previously published data
[d] HETCORs/HMBCs from 1-OH not reported
[e] Corresponding hydroxyl in hemifistularin-3 is at position 17 rather than 1.