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Ichthyotoxic Brominated Diphenyl Ethers from a Mixed Assemblage of a Red Alga and Cyanobacterium: Structure Clarification and Biological Properties

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Abstract

Primary fractions from the extract of a tropical red alga mixed with filamentous cyanobacteria, collected from Papua New Guinea, were active in a neurotoxicity assay. Bioassay guided isolation led to two natural products (**1**, **2**) with relatively potent calcium ion influx properties. The more prevalent of the neurotoxic compounds (**1**) was characterized by extensive NMR, mass spectrometry, and X-ray crystallography, and shown to be identical to a polybrominated diphenyl ether metabolite present in the literature, but reported with different NMR properties. To clarify this anomalous result, we synthesized a candidate isomeric polybrominated diphenyl ether (**3**), but this clearly had different NMR shifts than the reported compound. We conclude that the original isolate of 3,4,5-tribromo-2-(2,4-dibromophenoxy)phenol was contaminated with a minor compound, giving rise to the observed anomalous NMR shifts. The second and less abundant natural product (**2**) isolated in this study was a more highly brominated species. All three compounds showed a low micromolar ability to increase intracellular calcium ion concentrations in mouse neocortical neurons as well as toxicity to zebrafish. Because polybrominated diphenyl ethers have both natural as well as anthropomorphic origins, and accumulate in marine organisms at higher trophic level (mammals, fish, birds), these neurotoxic properties are of environmental significance and concern.

Keywords

Hydroxylated polybrominated diphenyl ether; red alga; cyanobacteria; environmental neurotoxin; ichthyotoxin; Ca²⁺ modulation

Introduction

Polybrominated diphenyl ethers (PBDEs) have recently attracted considerable attention due to an increasing awareness that these compounds accumulate in higher trophic level animals,

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

including sperm whales (Marsh et al., 2005; Vos et al., 2003), sea gulls (Verreault et al., 2007), seals (Vos et al., 2003), polar bears (Kelly et al., 2008), as well as in humans (Betts et al., 2002; Athanasiadou et al., 2008). Some PBDEs are industrially produced in large quantities for use as flame retardants, and therefore their presence in animals has generally been assumed to be of anthropogenic origin (Hale et al., 2003). Hydroxylated congeners of PBDEs (OH-PBDEs) have also been found in many of these same higher animals (Verreault et al., 2007; Kelly et al., 2008; McKinney et al., 2006a), and recent studies have shown that the OH-PBDEs found in large marine-associated animals may be of mixed origins with some deriving from natural sources and the others being derivatives of anthropogenic PBDEs (McKinney et al., 2006b; Kelly et al., 2008). Moreover, a variety of anthropogenic PBDEs have been shown to undergo hydroxylation in rats via oxidative metabolism, thereby producing OH-PBDE congeners (Malmberg et al., 2005; Marsh et al., 2006). Nevertheless, a number of OH-PBDEs are known to be natural products of various marine organisms, such as sponges, tunicates, and cyanobacteria (Carte and Faulkner, 1981; Carte et al., 1986; Unson et al., 1994). Environmental concerns have been raised over the occurrence of PBDEs in higher animals because PBDEs have been linked to endocrine disruption (Vos et al., 2003; Mariussen and Fonnum, 2006). Activities similar to those of chlorinated aromatic compounds have been observed with PBDEs, such as aryl hydrocarbon-receptor agonist and antagonist activities, thyroid toxicity, and effects on the immune system (Hwang et al., 2008). Neonatal exposure to PBDEs has been shown to cause neurotoxicity in adult animals (Mariussen and Fonnum, 2006). OH-PBDEs have also been shown to disrupt thyroid hormone homeostasis presumably due to their structural similarity to the thyroxine-type endogenous thyroid hormones (Kelly et al., 2008). Recently, BDE-47 and 6-OH-BDE-47 have been shown to trigger an increase in cytosolic Ca^{2+} concentration as well as exocytosis of catecholamines in neuronal cells within a few minutes (Dingemans et al., 2008 and 2007). These findings indicate that PBDEs and OH-PBDEs have the potential to acutely disrupt normal neuronal communication in animals.

Our program has been systematically screening marine algae and cyanobacteria for secondary metabolites with neuropharmacological properties, partially because of the environmental impacts these substances may impose, and partially to detect agents with potentially useful biomedical properties (Berman and Murray, 2000; Li et al., 2001; Rogers et al., 2002; Dravid and Murray, 2003). Because a variety of cellular events can trigger an increase in cytosolic Ca^{2+} levels in neuronal cells, we have employed a FLIPR-based fluorescence assay which detects cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in mouse neocortical neurons. Herein, we describe the assay-guided isolation, structural determination and neurotoxicological evaluation of two OH-PBDE congeners (**1** and **2**, Figure 1) from a mixed assemblage of marine cyanobacteria and a red alga. In the course of this work, we have also corrected the assignment of a ^{13}C NMR signal for **1** in the literature which could be a source of confusion in future efforts with OH-PBDE congeners.

Materials and Methods

2.1 Reagents and algal material

All solvents used were of HPLC grade from EMD and were used without purification. TLC grade silica gel from Sigma-Aldrich was used for vacuum liquid chromatography (VLC). Flash chromatography was performed using EMD silica gel (230–400 mesh). Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals. Minimum essential medium, deoxyribonuclease (DNase), poly-L-lysine, cytosine arabinoside, veratridine, aconitine, and deltamethrin were from Sigma-Aldrich. The fluorescent dye Fluo-3, and pluronic acid F-127 were obtained from Invitrogen Corporation.

Algal material was collected at Grabo Reef in Papua New Guinea (5° 28.664 S by 150° 12.524 E) at 12–18 m depth by SCUBA on August 24, 2000 (voucher specimen available as PNG-GR 8/24/00-3). The algae was identified in the field as *Vidalia* sp., but subsequent microscopic examination of the voucher sample identified it to likely be *Leptofaucia* sp. Additionally, a small abundance of cyanobacterial filaments similar to *Oscillatoria* were observed in the sample by light microscopy.

2.2 Bioassay-guided isolation of OH-PBDEs

A total of 2 L of the red algal and cyanobacterial assemblage (dry weight 128 g) was extracted with CH₂Cl₂ / MeOH (2:1). Upon removal of the solvents *in vacuo*, a portion (4.0 g) of the crude extract (4.6 g) was fractionated into nine fractions using silica gel vacuum liquid chromatography (hexanes to EtOAc to MeOH), one of which (308 mg, eluted with 2:3 EtOAc/hexanes) possessed Ca²⁺ modulating activity in mouse neocortical neurons. A portion (306 mg) of the active fraction was further fractionated by flash column chromatography (Still et al, 1978) on silica gel (hexanes/Et₂O 1:9 to 1:8) to obtain seven sub-fractions, of which two (2 mg and 77 mg) were associated with Ca²⁺ modulation activity. The larger sub-fraction was further purified by HPLC using a Jupiter 10 μ C18 300A column (250 $\times$ 10 mm) with a gradient solvent system (2.5 mL/min, 3:2 MeCN/H₂O to 4:1 over 30 min, then to MeCN over 10 min) to obtain 15 mg (0.38% of extract) of compound **1** (R_t = 52 min) and ~0.5 mg (0.01% of extract) of compound **2** (R_t = 55 min), both of which were confirmed to elevate neuronal [Ca²⁺]_i.

2.3 Neocortical neuron cultures

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice. Briefly, pregnant mice were euthanized by CO₂ asphyxiation and their neocortices were collected. Isolated neocortices were then removed of their meninges, minced by trituration using a Pasteur pipette, and treated with trypsin for 25 minutes at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and resuspended in Eagle's minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.4. Cells were plated onto poly-L-lysine-coated 96-well clear-bottomed black-well plate at a density of 1.5 $\times$ 10⁵ cells/well. Plates were incubated at 37°C with 5% CO₂ and 95% humidity. To prevent proliferation of nonneuronal cells, cytosine arabinoside (10 μ M) was added to the culture medium on day 2 after plating. The culture media was changed both on days 5, 8 and 11 using a serum-free growth medium containing Neurobasal Medium supplemented with B-27, 100 I.U./mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine. Neocortical neurons were used between 11–13 days *in vitro* (DIV) for Ca²⁺ influx assay. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

2.4 Neuronal [Ca²⁺]_i assay

Neocortical neurons were cultured in 96-well plates, and subsequently, the medium was removed and replaced with dye loading buffer (100 μ L/well) containing 4 μ M Fluo-3 and 0.04% Pluronic F-127 in Locke's buffer (in mM: 8.6 Hepes, 5.6 KCl, 154 NaCl, 5.6 Glucose, 1.0 MgCl₂, 2.3 CaCl₂, 0.0001 glycine, pH 7.4). After 1 h incubation in dye loading buffer, cells were washed five times with Locke's buffer using automated cell washer (Biotek instrument Inc., VT, USA) leaving a final volume of 150 μ L in each well. The plate was then transferred to Fluorescence Laser Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA) chamber. Cells were excited by the 488-nm line of argon laser and Ca²⁺ bound fluo-3 emission in the 500–600 nm range was recorded with the charge coupled device (CCD) camera. Fluorescence readings were taken once every 9 s for 3 min to establish the baseline and then 50 μ L of test compound containing solution (4x) was added to each well from the compound

plate, yielding a final volume of 200 μL /well. The cells were exposed to test compounds for another 42 min.

2.5 Assay data analysis

Concentration-response relationships were generated by non-linear regression analysis using GraphPad software (Version 4.0; San Diego, CA).

2.6 Structure elucidation

All NMR data were collected in CDCl_3 using Varian Inova 300 MHz and 500 MHz instruments. ^1H and ^{13}C NMR signals were referenced to the residual solvent peaks at 7.26 ppm and 77.0 ppm, respectively. High resolution mass spectra were recorded on a Thermo-Finnigan MAT900XL spectrometer. A crystal was grown from a CH_2Cl_2 solution of **1** by diffusional exchange with hexanes for X-ray crystallographic analysis on a Bruker APEX CCD detector.

2.7 Ichthyotoxicity assay

Various quantities of the natural product **1** in 174 μL of EtOH was added to 50 mL Erlenmeyer flasks containing 40 mL of aquarium water. For controls, 174 μL of EtOH was added to the water. Both treatments and controls were run in duplicate. Adult male zebra fish of approximately 4 cm length were randomly selected from aquaria and individually placed in the Erlenmeyer flasks. Time of death was determined by observing a lack of gill movement and no visible responses to swirling of the flask for one full minute.

Results and Discussion

Fractions from the crude lipid extract of a Papua New Guinea mixed collection of a red marine alga, tentatively identified as *Leptofauchea* sp. and a marine cyanobacterium *Oscillatoria* sp., showed an ability to increase $[\text{Ca}^{2+}]_i$ in mouse neocortical neurons. Bioassay guided isolation of the active compounds sequentially used flash column chromatography and HPLC, and yielded one more prevalent (**1**) and one minor compound (**3**).

Analysis by ESI-LCMS (negative mode) revealed the polybrominated nature of compound **1** and indicated a molecular weight of 1160 (obs. $[\text{M}^-]$ m/z 1159). Subsequent analysis by ^{13}C NMR showed 4 protonated and 8 quarternary ^{13}C signals, indicating that the compound was either a symmetric dimer having a molecular weight of 1160 or that it was a monomer with a molecular weight of 580 that easily dimerizes in the mass spectrometer. High resolution EIMS clarified that it was a monomer with an exact mass of m/z 575.6211, yielding a molecular formula of $\text{C}_{12}\text{H}_5\text{O}_2\text{Br}_5$ (calc. 575.6201). ^1H and ^{13}C NMR, COSY, HSQC, and HMBC data readily identified two benzenoid rings connected to one another by an ether linkage. By ^{13}C NMR chemical shifts and consideration of the molecular formula, one of the rings possessed 2 bromines while the other had 3 bromines and a hydroxyl group. Further analysis of the HMBC spectrum revealed the A ring to be a 2,4-dibromo-phenoxy group. The regiochemical assignment of the B ring, however, could not be made confidently based on NMR data alone. Further, the ^{13}C NMR data for **1** did not match that reported for any known OH-PBDE. Because **1** deposited well-formed crystals from CH_2Cl_2 /hexanes, an X-ray crystallographic analysis was conducted to give an excellent quality crystal structure (Figure 2). Comparison of this structure with the literature for naturally occurring OH-PBDEs showed it to be identical to one previously reported from *Dysidea herbacea* (Bowden et al, 2000; Agrawal and Bowden, 2005).

In comparing our ^{13}C NMR data to those reported in the literature for compound **1**, a significant discrepancy was noted in the chemical shift for C-3 (Table 1). Only the Bowden data are

included in this table because the only other ^{13}C NMR data available were recorded in DMSO and not in CDCl_3 (Fu and Schmitz, 1996), but they were similar to our data. One interpretation for this anomalous NMR data was that the previous workers had mis-assigned structure **1** to their material, and that it was in fact the data for another structure. Of the 59 possible regioisomers of **1**, 50 could be eliminated based on the fact that the A ring of Bowden's isolation of **1** clearly had three protons and the HMBC and COSY correlations were sufficient to confidently assign this sub-structure (e.g. ring A). Of the remaining 9 structural possibilities, two could be disregarded based on HMBC correlations observed by Bowden. Specifically, the lone proton on the B ring showed HMBC correlations to 4 carbons (Bowden et al., 2000), and thus, the only carbon for which there was not an HMBC correlation should be *para* to the carbon bearing this proton. Based on the predicted chemical shifts of the oxygen bearing carbons, six other isomers could be disregarded (see Supporting Information), leaving only a single regioisomer that required further consideration (**3**). Regioisomer **3** is also a known naturally occurring compound having also been isolated from the marine sponge *Dysidea herbacea* (Carte and Faulkner, 1981).

Unfortunately, because the ^{13}C NMR data for **3** in CDCl_3 had not been published (Carte and Faulkner, 1981), this compound was newly synthesized by Marsh's route (Figure 3; Marsh et al., 2003). Comparison of the ^{13}C NMR shifts of synthetic **3** with those reported by Bowden revealed that they were considerably different (Table 1). Subsequently, a close inspection of the ^{13}C NMR spectrum from the Bowden material revealed a small peak at 121.1 ppm (B. F. Bowden, personal communication), which corresponds well with our data for C-3 in compound **1**. Because C-3 is *para* to the only protonated carbon in ring B, this ^{13}C atom is predicted to relax slowly such that its detection may require longer delay times (Crews et al., 1998). Moreover, during our purification of **1–3**, we found that it was difficult to remove minor contaminants that had similar NMR signatures to these OH-PBDEs. In this regard, it is possible that the 113.6 ppm signal reported for C-3 by Bowden et al. was actually the quickly relaxing (e.g. protonated) resonance of a minor contaminant in the sample. Additionally, during the preparation of this manuscript, a new isolation of compound **1** was reported and the discrepancy in the ^{13}C NMR chemical shift of C-3 was also briefly noted (Calcul et al, 2009).

Having unequivocally established the structure of **1**, a minor compound was also characterized that eluted very closely to **1** by RP-HPLC and showed Ca^{2+} modulating activity. A molecular formula of $\text{C}_{12}\text{H}_4\text{O}_2\text{Br}_6$ was determined for compound **2** based on low resolution negative ion ESI-LCMS (obs. $[\text{M}-\text{H}]^- m/z$ 658.74). The isotopic pattern observed for the molecular ion was consistent with the presence of 6 bromine atoms. Although NMR characterization of this compound was challenging due to the small quantity isolated (~0.5 mg) and the presence of chromatographically similar impurities, ^1H NMR and ^1H - ^1H COSY analysis revealed a three proton spin system consisting of a vicinally coupled pair (7.29 ppm and 6.39 ppm) and a third (7.79 ppm) which was *meta* coupled to δ 7.29. These shifts and coupling pattern matched those reported for synthetic **2** and thus, this material was identified as 3,4,5,6-tetrabromo-2-(2',4'-dibromo-phenoxy)-phenol (**2**).

Compounds **1–3** were tested in the cytoplasmic $[\text{Ca}^{2+}]_i$ assay in mouse neocortical neurons at various concentrations. Exposure to compounds **1–3** evoked a rapid, robust and concentration-dependent elevation of neuronal $[\text{Ca}^{2+}]_i$, and their corresponding EC_{50} values were 16.4 μM (9.8 – 27.2, 95% confidence intervals), 27.2 μM (12.3 – 59.8, 95% confidence intervals) and 42.4 μM (23.2 – 77.5, 95% confidence intervals), respectively (Figure 4). The robust nature of the increment in Fluo-3 fluorescence is of similar magnitude to that previously reported for brevetoxin, and represents peak $[\text{Ca}^{2+}]_i$ values in the micromolar range (Berman and Murray, 2000). We have previously shown that marine natural products such as brevetoxin and antillatoxin produce Ca^{2+} entry in neurons from the extracellular compartment through three routes: the NMDA receptor ion channel, the L-type voltage-sensitive Ca^{2+} channel, and the

Na⁺/Ca²⁺ exchanger (Berman and Murray, 2000; Li et al., 2001). It was therefore of interest to establish the source of the elevation in cytoplasmic Ca²⁺ produced by **1**. To define the roles of extra- and intra-cellular Ca²⁺ pools, we exposed neocortical neurons to 30 μM **1** in the presence of both Ca²⁺ free and low Ca²⁺ extracellular media. The Ca²⁺ response to **1** was abrogated in the presence of Ca²⁺ free buffer containing 200 μM EGTA, and was significantly diminished in the low Ca²⁺ (0.2 mM) buffer (Figure 5). To confirm the importance of extracellular Ca²⁺ as the source of elevated [Ca²⁺]_i in response to **1**, we loaded the neocortical neurons with 50 μM BAPTA-AM, the acetoxymethylester of the calcium chelator BAPTA. Depletion of intracellular Ca²⁺ with BAPTA-AM did not alter the maximum Ca²⁺ response produced by 30 μM **1**. These data therefore indicate that **1** produces an influx of Ca²⁺ from the extracellular compartment. Another polybrominated diphenyl ether and its hydroxylated analog have recently been shown to elevate cytoplasmic [Ca²⁺]_i in PC12 cells (Dingemans et al, 2008). In PC12 cells the hydroxylated compound 6-OH-BDE-47 produced an initial increase in [Ca²⁺]_i produced by Ca²⁺ influx and a delayed response derived from intracellular stores, primarily mitochondria. These results therefore differ from those reported herein for compound **1** in neocortical neurons where the source for elevated [Ca²⁺]_i was exclusively extracellular. Either the use of PC12 versus neocortical neurons, or distinct actions of individual hydroxylated polybrominated diphenyl ethers, may be responsible for the differing results.

In light of the finding that **1** may act as an environmental neurotoxin (Dingemans et al., 2008), its toxicity to zebrafish was also evaluated. At 5 μg/mL (8.6 μM) media concentration, the fish immediately attempted to escape out of the container and their apparent rate of respiration visibly increased; such behaviors were not observed for the control group. Within 5 minutes, the treated fish appeared to have difficulty maintaining their proper orientation, and within 20 minutes, both died. Similar effects were seen at 1 μg/mL (1.7 μM), but it took 50 min for the fish to expire. At 0.5 μg/mL (0.86 μM), the fish appeared to be unaffected for the duration of the 3 hour assay. Regioisomer **3** had the same effect but was less potent (8.6 μM, death at 25 min; no effect at 1.7 μM), suggesting that many congeners of OH-PBDEs may display ichthyotoxicity.

Both the long term and the short term neurotoxic effects of polychlorinated biphenyls (PCBs) have been studied extensively. Notably, PCBs disrupt the intracellular Ca²⁺ homeostasis, which is critical for a variety of cell functions, including release of neurotransmitters (Mariussen and Fonnum, 2006). As for the longer term effects, PCBs can modulate muscarinic and nicotinic receptors and also disrupt neurological development (Mariussen and Fonnum, 2006). Similarly, PBDEs have been shown to have detrimental effects to learning and memory functions in mice. These behavioral effects are likely related to the observation that PBDEs reduced the densities of nicotinic receptors in the hippocampus of mice (Viberg et al., 2003). Given the ability of **1**, **2**, and **3** to alter cytoplasmic concentrations of Ca²⁺ ions, the observed ichthyotoxicity of **1** and **2** may involve calcium ion induced neurotoxicity.

These findings are significant in light of recent reports that OH-PBDEs have wide-spread occurrence in the marine environment (Verreault et al., 2007; Kelly et al., 2008; McKinney et al., 2006a). In *Disidea herbacea*, at least 1~6% of the dry weight of the organism is composed of OH-PBDEs and visible crystals of these compounds have been observed in the sponge tissue (Unson et al., 1994). In addition to sponges, tunicates, and blue-green algae, we report here that a red alga and/or cyanobacterium living in association with the red alga as a source of OH-PBDEs (Malmvärn et al., 2005). Since most of the world's seaweeds are red algae (Garrison, 2005) and many invertebrates feed on these algae, the presence of OH-PBDEs in marine invertebrates may actually reflect the natural occurrence and bioaccumulation of these compounds into higher trophic levels. Among PBDEs, pentabrominated congeners are known to be environmentally more mobile due to their greater water solubility, volatility, K_{ow} (octanol/water partition coefficient), and lipophilicity (Hale et al., 2003). OH-PBDEs are

expected to be reasonably lipophilic and yet more water soluble than PBDEs. Therefore, they may be even more prone to bioaccumulation due to their increased mobility in the environment.

The possibility raised by these findings is that many OH-PBDEs are of natural occurrence and may possess neurotoxic properties of environmental significance. In this regard, it is possible that human populations have been exposed to these materials through seafood consumption for a lengthy period and that this exposure may have had historical as well as current health implications. PCBs and brominated flame retardants other than PBDEs are suspected to be responsible for abnormal behavior phenomena such as the 'beaching of cetaceans' (Symons et al., 2003; Law et al, 2006). Because of their structurally related nature, it is possible that OH-PBDEs of natural or anthropogenic origins may contribute to such events. Thus, it is important to both characterize the origin and occurrence of OH-PBDEs as well as details of their molecular neurotoxicology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Selected microscopic photos of the voucher sample of the algae, ^1H and ^{13}C NMR, COSY, HSQC, and HMBC spectra of **1** and **3**, ^1H NMR and COSY spectra of **2**, and X-ray crystallographic analysis data on **1**. This material is available free of charge via the Internet at <http://www.sciencedirect.com/>.

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Figure 1.
OH-PBDE congeners isolated from the red alga/cyanobacteria assembly (**1** and **2**) and an alternative regioisomer of **1** (**3**).

Figure 2.
X-ray crystal structure of **1**.

Figure 3.
Synthesis of **3** by Marsh's route.

Figure 4. Assay of changes in cytoplasmic Ca^{2+} concentration in mouse neocortical neurons for compounds 1-3. Time-response relationships for compound 1 (a), compound 2 (b) and compound 3 (c). The concentration-response relationships for compounds 1-3 are depicted in panel (d).

Figure 5.

Assay of changes in cytoplasmic Ca^{2+} concentration in mouse neocortical neurons for compounds **1** in control, low and calcium-free media.

Table 1

Comparison of NMR data for two isolates of **1** and **3** (CDCl₃)

Position	1^a		1^b		3	
	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
1	148.9		148.9		144.5	
2	139.9		139.5		143.1	
3	113.6		121.1		120.7	7.00
4	119.3		119.2		114.9	
5	122.9		122.9		122.4	
6	120.8	7.42	120.7	7.44	121.5	
1'	151.8		151.7		151.3	
2'	112.8		112.7		115.6	
3'	136.4	7.79	136.3	7.79	136.5	7.81
4'	116.3		116.2		118.4	
5'	131.6	7.29	131.6	7.29	132.1	7.45
6'	115.8	6.41	115.7	6.41	121.5	6.89

^a Agrawal and Bowden, 2005

^b This study.