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Screening a natural product-based combinatorial library using FTICR mass spectrometry

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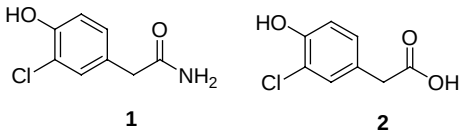
Abstract—This manuscript reports the use of Fourier transform ion cyclotron resonance mass spectrometry to screen a combinatorially generated natural product-based library for binding affinity to bovine carbonic anhydrase II (bCAII). The fungal natural product 3-chloro-4-hydroxyphenylacetamide was the library template, with 11 secondary amide analogues of this template constituting the combinatorial library. 2-(3-Chloro-4-hydroxyphenyl)-*N*-(4-sulfamoylphenethyl)acetamide (compound **11**) of this library was identified as a tight binding inhibitor of bCAII, by detection of a noncovalent complex corresponding to [bCAII + **11**] in the mass spectrum. A competitive bCAII enzyme binding assay validated the mass spectrometry screening result. The equilibrium dissociation constant (K_i) for **11** was measured as 77.4 nM. Preliminary structure–activity investigations of the bioactive natural product analogue are also reported.

1. Introduction

The relevance of natural products (NPs) to the drug discovery process is highlighted by the fact that 15 new natural product-derived drugs were launched by the pharmaceutical industry between 2000 and 2003.[1] The use of NPs as templates for construction of biologically relevant chemical libraries now represents a logical extension of the classical combinatorial library synthesis protocol. Numerous libraries incorporating a NP motif have been published[2–7] and several examples exist where the biological activity of a NP has been improved with small libraries that have integrated only simple functional group modifications.[8] Davis et al. have recently reported the isolation and structure elucidation of a new fungal natural product, 3-chloro-4-hydroxyphenylacetamide (**1**) (Fig. 1).[9] A small, high-purity secondary amide library based on this NP has subsequently been reported.[10] This NP-based combinatorial library was synthesized through EDCI mediated coupling of a variety of primary amines to the commercial reagent 3-chloro-4-hydroxyphenylacetic acid (**2**) (Fig. 1). This library (compounds **3**–**13**) was added to the Eskitis Institute's chemical repository and made accessible for random biological screening (Fig. 2).



1 2

Figure 1. Natural product-based combinatorial library template (**1**) and carboxylic acid analogue (**2**).

The carbonic anhydrase (CA) family of Zn(II) metalloenzymes (EC 4.2.1.1) catalyses the interconversion of CO_2 and HCO_3^-, a regulatory reaction that underpins many physiological processes associated with pH control, ion transport and fluid secretion.[11,12] Classically, an aromatic or heteroaromatic sulfonamide moiety (Ar-SO_2NH_2) is the primary recognition element for small molecules to bind the active site of CA.[11,12] Coordination of the ionised sulfonamide functional group with the active site Zn(II) of CA enables this protein:small molecule interaction.[11,12] The inhibition of CAs by aromatic sulfonamides has been exploited clinically for several decades for the treatment of a variety of conditions including glaucoma, epilepsy, bacterial infections and gastric ulcers. More recently, a role for this class of

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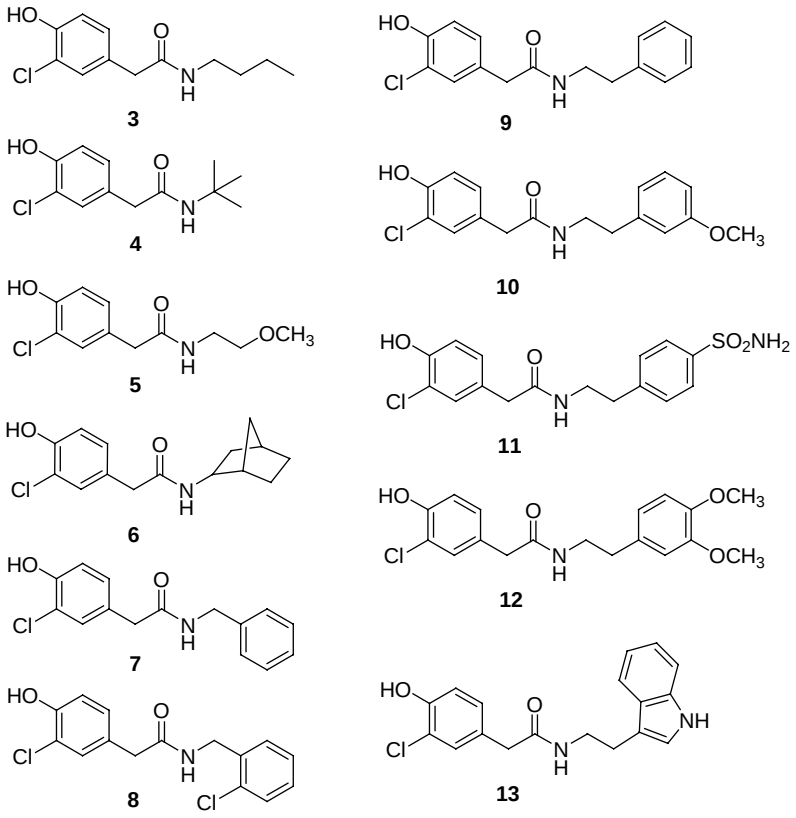

Figure 2. Natural product-based combinatorial library members **3**–**13**.[10]

compounds as anticancer agents has been identified, also as a result of CA inhibition.[13,14]

Our aim was to utilise a mixture-based screening methodology that would both reveal the presence and confirm the identity, in one step, of any members from our NP-based library (**3**–**13**) with affinity for bCAII. The methodology adopted, bioaffinity characterization mass spectrometry (BACMS), stems from the pioneering work of Smith and co-workers.[15–17] This manuscript reports the application of this mass spectrometry screening technique, with a potent bCAII binder identified from random screening of a NP-based synthetic library. A competitive bCAII enzyme binding assay has validated the mass spectrometry screening results. Preliminary structure–activity investigations of the bioactive natural product analogue are also reported.

2. Screening of the NP-based combinatorial library using ESI-FTICR-MS

Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) analysis of bCAII from 10 mM NH_4OAc solution, 1% DMSO (pH 7.0) yielded the ESI positive ion mass spectrum of Figure 3A. Peaks corresponding to the +8 to +10 charge states of bCAII were observed, with the +9 charge state predominating. This charge state envelope (low charge states and few charge states) is typical for bCAII when in a compact, tightly folded structure.[17] Deconvolution of this mass spectrum gave an average mass for bCAII of 29,090 Da, in good agreement with N-terminal acetylated bCAII with the Zn cofactor (calculated average mass equals 29089.7 Da). A mixture of bCAII (30 μM) and the synthetic library (30 μM for each of **3**–**13**) in 10 mM NH_4OAc, 1% DMSO (pH 7.0) was incubated for 1 h at room temperature and then analysed by ESI-FTICR-MS under identical conditions to those for the free protein (Fig. 3B). The same charge state envelope as for bCAII (Fig. 3A) was observed; however, each charge state now consisted of a grouping of two peaks: a lower intensity peak that corresponded to unmodified bCAII and a more intense peak at a higher m/z value that corresponded to a bCAII–ligand complex. Within a charge state grouping the mass increment between complexed bCAII and unbound bCAII multiplied by the charge state permits calculation of the mass of the small molecule complexed to bCAII. For the +9 charge state (Fig. 3B):

$$\begin{aligned}\text{mass of bound ligand} &= [m/z(\text{bCAII–ligand complex}) \\ &\quad - m/z(\text{bCAII unbound})] \times (z) \\ &= [3274.1 - 3233.2] \times 9 \\ &= 368.1 \text{ Da.}\end{aligned}$$

The library members **3**–**13** each have a different mass (Table 1) and a mass of 368.1 Da corresponded to **11** (368.06 Da). Similarly the +8 and +10 charge state mass increments confirmed the nominal mass of the bound ligand as 368 Da. This result revealed that compound **11** from the library had binding affinity for bCAII. Next an ESI mass spectrum of an equimolar mixture of bCAII and **11** (30 μM each, 10 mM NH_4OAc, pH 7.0) was

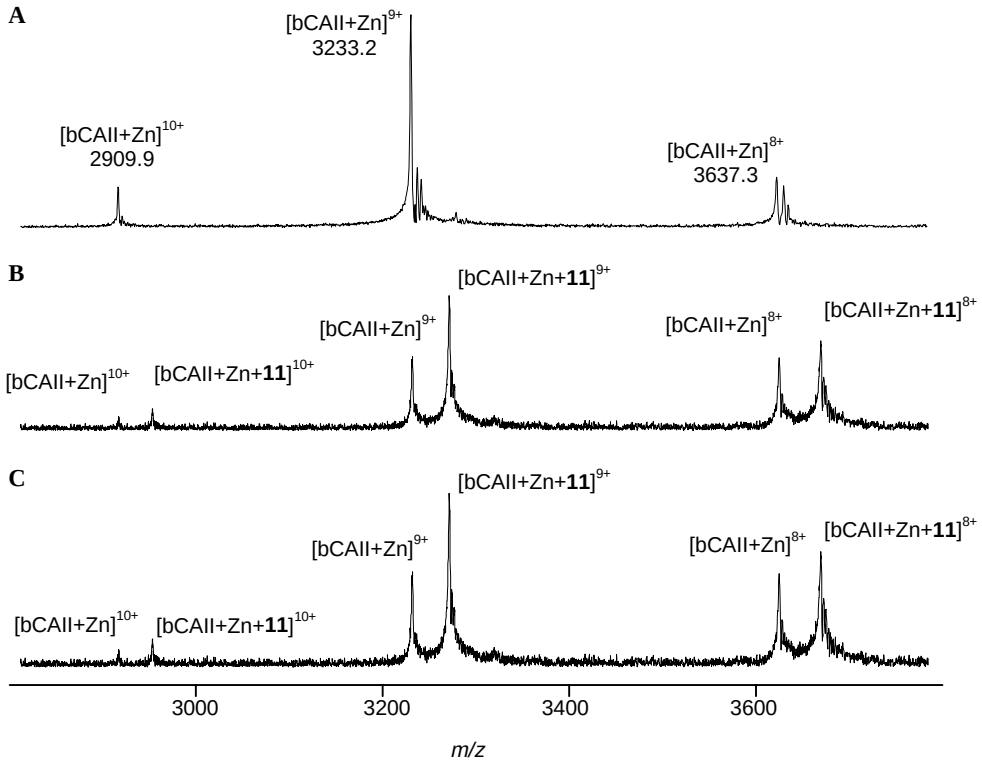

Figure 3. (A) ESI-FTICR positive ion mass spectrum of bCAII (30 μM) from 10 mM NH_4OAc solution, 1% DMSO (pH 7.0). (B) ESI-FTICR positive ion mass spectrum of a mixture of bCAII (30 μM) and NP library (30 μM for each of **3**–**13**) in 10 mM NH_4OAc, 1% DMSO (pH 7.0). (C) ESI-FTICR positive ion mass spectrum of an equimolar mixture of bCAII and **11** (30 μM each, 10 mM NH_4OAc, pH 7.0).

Table 1. Molecular weights for library members **3**–**13**

Compound	Molecular weight (Da)
3	241.09
4	241.09
5	243.07
6	279.10
7	275.07
8	309.03
9	289.09
10	319.10
11	368.06
12	349.11
13	328.10

acquired (Fig. 3C). This ESI mass spectrum contained an identical pattern of peaks resulting from ESI analysis of the full library complement **3**–**13** with bCAII (Fig. 3B). Similarly, this spectrum verified the mass of the bound ligand as 368 Da. An identical experiment with bCAII and synthetic **9**, which only lacks the sulfonamide moiety of **11**, yielded no bCAII complex in the ESI mass spectrum (data not shown). The results with individual library members were in full agreement with ESI mass analysis of the complete library mixture.

3. Screening of the NP-based library using a solution competitive binding assay

In order to validate the mass spectrometry screening results, we turned our attention to a conventional solution-phase competitive binding assay for bCAII. The fluorescence-based assay relies on the competition for the active site of bCAII between the ligand 5-(dimethylamino)-1-naphthalenesulfonamide (DNSA) and the test compounds.[18,19] Upon excitation at 290 nm (an absorption minimum for DNSA) fluorescence is detected at 460 nm (from the bCAII–DNSA complex). The equilibrium dissociation constant (K_d) of bCAII–DNSA was measured as 0.3 μM. Each of the library compounds **3**–**13**, as well as **1** and **2** were individually assessed for their ability to inhibit the binding of DNSA to bCAII. An initial screen at 1 and 10 μM was carried out and the results are presented in Chart 1. With the exception of **11**, none of the library members displaced DNSA. This result was in full agreement with and validates the mass spectrometry screening results for this library.

4. Structure–activity analysis of compound 11

Compound **11** was the only member of the NP-based library with affinity for bCAII. The entries in Chart 1 for **1** and **2** demonstrated that the NP core template had no affinity for bCAII. Also compound **9**, which lacks the sulfonamide moiety of **11**, had no affinity for bCAII. Together these results indicate that it is the sulfonamide moiety that is the key bCAII recognition descriptor for **11**, a conclusion consistent with literature precedent for aromatic sulfonamide moieties as the classical recognition motif for CAII.[11,12] The primary amine reagent used in the synthesis of **11** was 4-(2-aminoethyl)benzene-

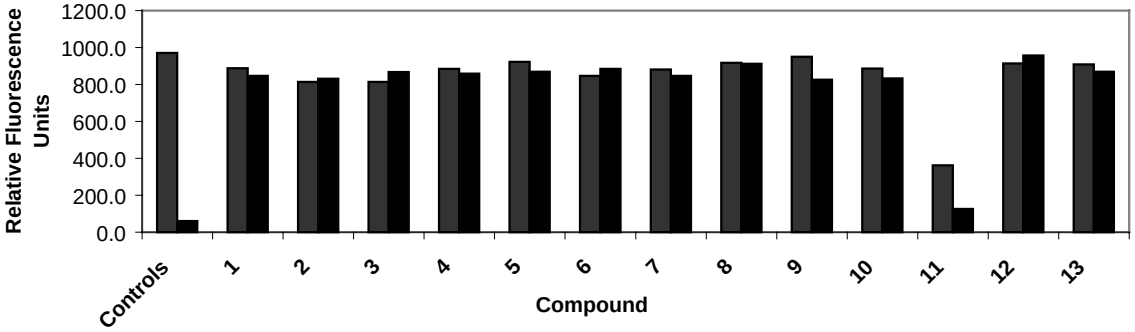

Chart 1. bCAII enzyme binding screen results at 1 μM and 10 μM for **1**, **2** and library members **3**–**13**.

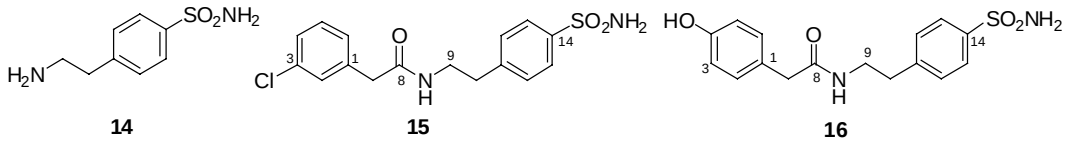

Figure 4. Compounds for structure–activity analysis: **14**, **15** and **16**.

Table 2. bCAII enzyme binding assay results for **14**, **11**, **15** and **16** expressed as K_i in nanomolar

Compound	bCAII K_i (R^2)[a]
14	1690 (0.97)
11	77.4 (0.99)
15	66.5 (0.98)
16	175 (0.98)

[a] bCAII binding data utilising competitive displacement of DNSA from bCAII, experiments performed in triplicate. K_d of DNSA was 0.3 μM.

sulfonamide (**14**) (Fig. 4). We wanted to determine if the cause of bCAII affinity for **11** was due solely to the sulfonamide component of **11** or if the NP template was acting as a secondary recognition motif alongside the sulfonamide motif of **14**. The bCAII binding constants for **11** and **14** were determined, Table 2. The bCAII affinity of the parent amine **14** is moderate (K_i = 1690 nM), while the NP derivative **11** has greater bCAII affinity (K_i = 77.4 nM). Specifically derivatization of the amine **14** with the NP scaffold to generate **11** results in a 22-fold increase in bCAII affinity. While the sulfonamide partner is necessary for binding (**9** compared to **11**), it is not responsible in isolation for the level of affinity for bCAII by **11.** Hence, we concluded that the NP template of **11** was substantially contributing to the molecular recognition for bCAII.

These results prompted us to further investigate the structure–activity relationships for the NP template of **11**, in particular the influence of the –Cl and –OH functionality. Compounds **15** and **16** were synthesized by EDCI coupling of 4-(2-aminoethyl)benzenesulfonamide with 3-chlorophenylacetic acid and 4-hydroxyphenylacetic acid, respectively (Fig. 4). Both new synthetics were purified using reversed-phase C18 HPLC and were spectroscopically characterized using 1D and 2D NMR (^{1}H, ^{13}C, DEPT, gCOSY, gHSQC, gHMBC and ROESY), IR, UV and MS data. Compound **15** lacks the –OH and retains the –Cl of **11**, while **16** lacks the –Cl and retains the –OH of **11**. The bCAII K_i's for each of **15** and **16** were determined, and the results are presented in Table 2. Compound **15** (–Cl only) displayed similar affinity to **11** (K_i = 66.5 nM), while **16** (–OH only) had slightly (2.3-fold) reduced affinity (K_i = 175 nM) compared to **11**. Notable from these structure–activity data is that the replacement of a –Cl with a –H in the NP template leads to lessened bCAII affinity, while the replacement of an –OH with a –H has minimal affect on bCAII affinity.

5. Conclusions

This work has applied bioaffinity characterization mass spectrometry (BACMS) to the analysis of a NP-based combinatorial library in the presence of the protein target bCAII. ESI-FTICR-MS was able to reveal and identify, in one step, a member from the NP-based library mixture with affinity for bCAII. This mixture-based screening strategy permitted both a rapid and informative analysis. An extension of this approach to bioactive-guided fractionation following high-throughput screening of natural product extracts is possible. Therapeutic lead compounds identified by this strategy may then enter more conventional medicinal chemistry pathways for structure–activity analysis as demonstrated here.

6. Experimental

6.1. Procedure for bCAII enzyme binding assay

Compounds **1**–**16** were assessed for their ability to inhibit the binding of DNSA to bCAII (CAII from bovine erythrocytes, Sigma–Aldrich, catalogue number C2522, lot number 044K6064). Enzyme assays were carried out in 96-well microtitre plates (Nunc F96) in an assay volume of 200 μL. Each assay contained bCAII (180 nM);

DNSA (3 μM, equals 10 times the K_d value), incubation buffer (phosphate buffer, pH 7.2) and test compound in DMSO. The final DMSO concentration in the assay was 1%, this concentration of DMSO did not decrease control binding. The assay was incubated for 4 h at 25 °C. Fluorescence measurements were carried out on a Varian Cary-Eclipse spectrophotometer in fluorescence mode using a multiwell plate reader at 25 °C (excitation wavelength of 290 nm, emission wavelength of 460 nm). Known compounds (acetazolamide and ethoxazolamide) were used to characterize this assay procedure. Test compounds were either screened at two concentrations (1 and 10 μM, triplicate determinations) for **1**–**13** or a full assay performed (test compound at 15 concentrations, triplicate determinations) to determine K_i's for **11**, **14**, **15** and **16**. Data were fitted to a sigmoidal dose–response equation using nonlinear regression analysis (GraphPad Prism V4, San Diego, California, USA). The measurement of the K_d of DNSA was determined by titrating bCAII (180 nM in pH 7.2 phosphate buffer) with DNSA (100–3500 nM) and monitoring the fluorescence as described above. Data were fitted to an equilibrium one-site binding model using nonlinear regression analysis. The K_d of DNSA was measured as 0.3 μM and is comparable with literature values.[12]

6.2. Synthesis

All synthetic reagents used were purchased from Sigma–Aldrich. NMR spectra were recorded at 30 °C on a Varian 500 MHz Unity INOVA spectrometer. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peak for DMSO-d_6 at δ_H 2.49 and δ_C 39.51, respectively. LRESIMS were recorded on a Fisons mass spectrometer. HRESIMS were recorded on a Bruker Daltonics Apex III 47e FTICR mass spectrometer. IR and UV spectra were recorded on a Bruker Tensor 27 spectrometer and a Camspec M501 spectrophotometer, respectively. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC separations. A Thermo Electron Betasil C18 5 μm, 143 Å semi-preparative column (21.2 mm × 150 mm) was used for HPLC work. All solvents used for chromatography, UV and MS were of Lab-Scan HPLC grade, and the H_2O used was Millipore Milli-Q PF filtered.

The synthesis and characterization of the secondary amide library **3**–**13** has been reported elsewhere.[10] Synthesis of 2-(3-chlorophenyl)-*N*-(4-sulfamoylphenethyl)acetamide (**15**) was carried out as follows: 3-chlorophenylacetic acid (200 mg, 1 mmol), EDCI (288 mg, 1.5 mmol) and DMAP (12 mg, 0.1 mmol) were stirred in anhydrous DMF (3 mL) at rt for 1 h, then 4-(2-aminoethyl)benzenesulfonamide (400 mg, 2 mmol) was added and the solution stirred for a further 16 h at rt. The reaction mixture was poured into 2 N HCl (50 mL), saturated with NaCl and then extracted with DCM (2 × 50 mL). The DCM-soluble material was subjected to C18 HPLC using a linear gradient from 99% H_2O/1% TFA to 99% MeOH/1% TFA in 35 min at a flow rate of 9 mL/min. This yielded **15** as a white amorphous solid (25.2 mg, 7% yield); UV (CH_3OH) λ_{max} (log ε) 221 (3.81) 262 (2.45) nm; IR ν_{max} (NaCl) 1624, 1571, 1457, 1338, 1200, 1156, 1095, 589, 538 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.77 (2H, t, J = 7.0 Hz, H-10), 3.30 (2H, dt, J = 5.5, 7.0 Hz, H-9), 3.38 (2H, s, H-7), 7.15 (1H, d, J = 7.0 Hz, H-6), 7.26 (2H, s, 14-SO_2NH_2), 7.28 (1H, m, H-4), 7.30 (1H, m, H-5), 7.30 (1H, s, H-2), 7.34 (2H, d, J = 8.5 Hz, H-12, H-16), 7.72 (2H, d, J = 8.5 Hz, H-13, H-15), 8.14 (1H, t, J = 5.5 Hz, 8-NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 34.7 (C-10), 39.8 (C-9), 41.8 (C-7), 126.3 (C-4), 127.6 (C-6), 128.8 (C-2), 129.9 (C-5), 132.7 (C-3), 138.8 (C-1), 125.6 (2C, C-13, C-15), 129.1 (2C, C-12, C-16), 142.0 (C-14), 143.7 (C-11), 169.5 (C-8); (−)-LRESIMS m/z (rel int) 351 (100), 353 (30). Synthesis of 2-(4-hydroxyphenyl)-*N*-(4-sulfamoylphenethyl)acetamide (**16**) from 4-hydroxyphenylacetic acid proceeded similarly to **15**. This yielded **16** as a white amorphous solid (9.5 mg, 3% yield); UV (CH_3OH) λ_{max} (log ε) 226 (3.42) 274 (2.60) nm; IR ν_{max} (NaCl) 1648, 1546, 1515, 1444, 1332, 1240, 1159, 1022, 582, 547 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.75 (2H, t, J = 7.0 Hz, H-10), 3.23 (2H, s, H-7), 3.27 (2H, dt, J = 5.5, 7.0 Hz, H-9), 6.66 (2H, d, J = 8.5 Hz, H-3, H-5), 6.99 (2H, d, J = 8.5 Hz, H-2, H-6), 7.26 (2H, s, 14-SO_2NH_2), 7.33 (2H, d, J = 8.0 Hz, H-12, H-16), 7.71 (2H, d, J = 8.0 Hz, H-13, H-15), 7.96 (1H, t, J = 5.5 Hz, 8-NH), 9.16 (1H, s, 4-OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 34.7 (C-10), 39.8 (C-9), 41.5 (C-7), 114.9 (2C, C-3, C-5), 125.6 (2C, C-13, C-15), 126.4 (C-1), 129.1 (2C, C-12, C-16), 129.8 (2C, C-2, C-6), 142.0 (C-14), 143.7 (C-11), 155.8 (C-4), 170.6 (C-8); (−)-LRESIMS m/z (rel int) 333 (100).

6.3. Procedure for ESI-FTICR-MS experiments

The experimental results presented in this paper were performed on an APEX® III 4.7 Tesla FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) fitted with an Apollo™ ESI source operated in positive ion mode. XMASS NT V7.0.2 mass spectrometry software on a PC platform was used for data acquisition. Broadband excitation was used to analyse a mass range from m/z 100 to 4500, with 512 K data points acquired. Samples were infused into the ESI source at 2 μL min^{-1}. Relevant parameters include the ESI source pressure (6.2×10^{-7} mbar), high-vacuum analyser region pressure (1.3×10^{-10} mbar). The drying gas temperature and capillary exit voltage were 125 °C and 180 V, respectively, for all experiments. Agilent ES tuning mix (catalogue number G2421A) was used for an external four-point calibration.

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