Electronic Supporting Information

Bisucaberin biosynthesis: An adenylating domain of the BibC multienzyme catalyzes cyclodimerisation of *N*-hydroxy-*N*-succinylcadaverine

N. Kadi, L. Song and G.L. Challis*

1. Materials, methods and procedures

1.1 Cloning and overexpression in *E. coli* of the region of *bibC* encoding the $BibC^C$ domain

The region of *bibC* encoding the $BibC^C$ domain (nucleotides 600-2460) was amplified by PCR from genomic DNA of *Vibrio salmonicida* strain LFI1238 (kindly provided by Prof. Nils-Peder Willassen, University of Tromso). A CACC sequence was introduced at the 5'-end of the amplimer, to allow the directional TOPO® cloning of blunt-end PCR products into pET151/D-Topo (invitrogen). The PCR mixture (50 μL) contained the genomic DNA as template (50 ng), 2 μM of each primer (forward: 5'-CACCATGAAAAATAGCAGTAAGAATCC-3' and reverse: 5'-CCCCTAAATAATGCGATGAGTCTTAGC-3'), 0.1 mM of each dNTP (Fermentas), 5% dimethyl sulfoxide and 3.5 U Expand high fidelity DNA polymerase (Roche) in 1X Expand reaction buffer with $MgCl_2$. Reaction conditions consisted of an initial denaturation step of 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 55°C for 60 s, and 72°C for 1 min. The reaction mixture was separated by electrophoresis on a 1% agarose gel (SeaKem® LE agarose, Rockland, USA), visualised by staining with 0.2 μg/mL ethidium bromide. The major product having the expected size of ~ 1.9 kb was cut from the gel and extracted using a QIAquick Gel Extraction Kit (Qiagen).

The purified PCR product (30 ng) was ligated with the pET151/D Topo vector (15-20 ng) using the Champion™ pET Directional Topo Expression Kit (Invitrogen). The resulting mixture was used to transform One Shot® Top10 chemically-competent *E. coli* cells. The transformation mix was plated on LB agar supplemented with ampicillin (50 μg/mL).

DNA sequencing (Molecular Biology Service, Department of Biological Sciences, University of Warwick) was used to confirm that the cloned coding sequence in the pET151/D-Topo expression vector was correct. One clone (pNK003) was used to transform *E. coli* BL21Star(DE3) (Invitrogen) for expression of the $BibC^C$ encoding sequence as an N-terminal His_6 in-frame fusion.

For overexpression of the gene encoding His_6-$BibC^C$, 200 mL of LB medium supplemented with ampicillin (50μg/mL) was inoculated with a 1 mL overnight culture of *E. coli* BL21Star(DE3) / pNK003 and incubated with shaking at 180 rpm and 37°C. Incubation continued until the optical density at 600 nm of the culture reached 0.6-0.8, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression. The culture was then incubated overnight at 180 rpm and 15° C.

1.2 Purification of His_6- $BibC^C$

Cells were harvested by centrifugation, the pellet was resuspended in 10 mL of 20 mM Tris buffer-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole and 10 % glycerol, and lysed in the presence of 1mM Phenylmethylsulphonyl fluoride (PMSF) using a French Press (17,000 psi internal cell pressure) followed by sonication for 2 min (Ultasonic processor, Jencons). After removal of cellular debris by centrifugation (18,000 x g for 20 min, at 4°C), the supernatant was applied to a 1mL HiTrap™ HP affinity column (Nickel Sepharose High Performance, GE Healthcare) equilibrated with a solution containing 20 mM Tris-HCl, pH8.0, 100 mM NaCl, 20 mM imidazole, and 10% glycerol. Unbound proteins were removed by washing with 8 mL of equilibration buffer and the His_6-$BibC^C$ fusion protein was eluted with 4 mL of elution buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 300 mM imidazole, 10% glycerol).

Fractions were analysed by electrophoresis (8% SDS-PAGE, Figure 1), and those containing His_6-$BibC^C$ were pooled, washed and concentrated to 1 mL using Amicon® Ultrafiltration with a 30,000 MWCO membrane (Millipore) in buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 % glycerol. His_6-$BibC^C$ was then aliquoted and frozen at -80° C. The protein was used for all subsequent experiments without further purification, unless otherwise noted.

1.3 Confirmation of $BibC^C$ identity and analysis of native oligomerization state

Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. The molecular weight of native His_6-$BibC^C$ was determined by gel filtration on a 110 mL superose 12 prep grade gel filtration resin poured in a XK 16/50 column (Amersham Biosciences), equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10% glycerol, at a flow rate of 0.75 mL/min (Figure 1). The column was calibrated with the Kit for Molecular Weights 12,000-200,000 (Sigma), consisting of Cytochrome c (12,400 Da), Carbonic Anhydrase (29,000 Da), Bovine Serum Albumin (66,000 Da), Alcohol Dehydrogenase (150,000 Da), β-Amylase (200,000 Da), and Blue dextran (2,000,000 Da).

(a) (b)

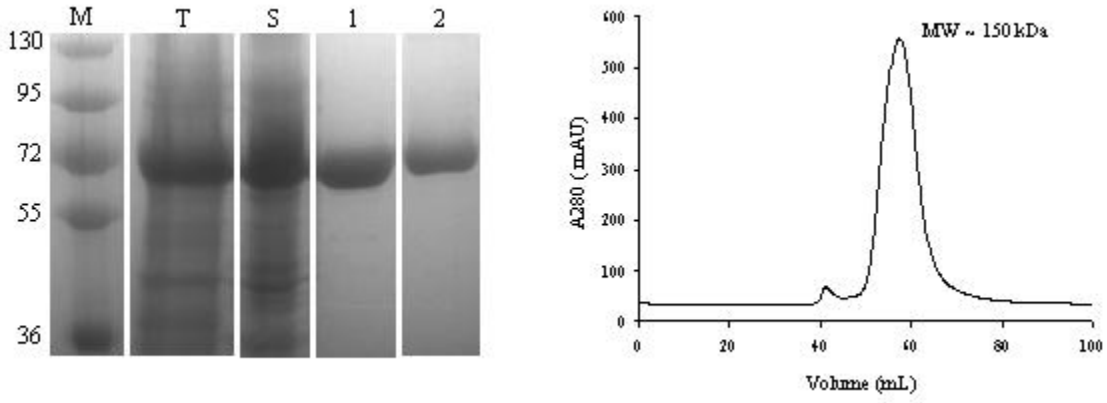

Figure 1. Overexpression of the region of *bibC* encoding $BibC^C$ in *E. coli*, and purification and analysis of recombinant His_6-$BibC^C$. (**a**) 8% SDS-PAGE analysis of His_6-$BibC^C$ overproduction and purification. Lane M = molecular weight standards (kDa), Lane T = total protein after lysis, lane S = soluble protein fraction after lysis,

lane 1 = protein eluted from Ni-NTA column with 300mM imidazole, lane 2 = His_6-$BibC^C$ after purification by gel filtration chromatography. (**b**) Chromatogram from gel filtration analyis of His_6-$BibC^C$. The observed retention volume indicates that it is a dimer.

To confirm the identity of purified His_6-$BibC^C$, electrospray ionization mass spectrometry (ESI-MS) was performed on tryptic digests of the protein (The Biological Mass Spectrometry and Proteomics Facility in the Department of Biological Sciences, University of Warwick). 17 predicted tryptic fragments of $BibC^C$ were identified by this analysis.

1.4 Incubation of His_6-$BibC^C$ with HSC

1 mM of HSC was incubated with 1.5 mM ATP, 15 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0) and 3 μM His_6-$BibC^C$ (after Ni-NTA purification and desalting) in a final volume of 140 μL for 20 min at 37°C. The reaction was initiated by addition of the enzyme and was stopped by addition of 3 μL of 10% aqueous trichloroacetic acid solution to precipitate the enzyme. No products could be detected in control incubations with enzyme inactivated by boiling (100°C for 10 minutes prior to addition to the incubation mixture).

LC-MS analysis of the reaction mixture was carried out using an Eclipse XDB-C18 column (150 x 4.6mm, 5μm, Agilent) connected to an Agilent 1100 HPLC instrument. The outflow was connected via a splitter (10% flow to MS, 90 % flow to waste) to a Bruker HCT+ mass spectrometer fitted with an electrospray source operating in positive ion mode. Absorbance was monitored at 471 nm. The compounds were eluted using the profile in table 1, with a flow rate of 1 mL/min. The retention times for bisucaberin **1** and prebisucaberin **5** were 13.1 min and 11.7 min, respectively.

Table 1

Time (min)	Water (0.1% Formic Acid)	Acetonitrile (0.1% Formic Acid)
0	95	5
5	95	5
25	0	100
33	0	100

1.5 Purification of bisucaberin 1 from scaled-up incubation of His_6-$BibC^C$ with HSC.

2 mM ATP, 15 mM $MgCl_2$, 1.5 mM HSC, 25 mM Tris-HCl, 5 μM His_6-$BibC^C$ (after Ni-NTA purification and concentration) in a final volume of 10 mL were incubated at 37°C for 15 hours. The reaction was stopped with 0.2 mL of 10% aqueous trichloroacetic acid solution to precipitate the enzyme, the mixture was centrifugated for 10 min at 4,000 rpm and the supernatant was recovered.
Bisucaberin **1** was purified from the mixture by semi-preparative HPLC on an Agilent Zorbax C18 column (21.2 X 100 mm, 5μ) using the elution profile in table 2, monitoring absorbance at 210 nm.

Table 2

Time (min)	Water (0.1% Formic Acid)	Acetonitrile (0.1% Formic Acid)	Flow (mL / min)
0	97	3	5
5	97	3	5
25	0	100	5
33	0	100	5

The collected fractions with a retention time around 14.4 minutes were analysed by ESI-MS and those containing the compound with *m/z* 401 $[M+H]^+$ were freeze dried. ESI-MS/MS, ESI-TOF-MS and NMR spectroscopy (^{1}H, COSY, HMQC and HMBC; Bruker AM500 and AV700 equipped with a TCI cryoprobe) unambiguously confirmed the structure of the isolated compound as biscauberin (Figure 2).

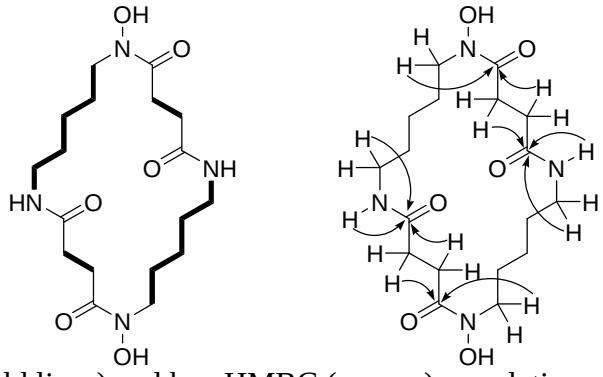

Figure 2. COSY (bold lines) and key HMBC (arrows) correlations observed for bisucaberin **1** isolated from incubation of HSC **3** with ATP, Mg^{2+} and His_6-$BibC^C$.

1.6 Purification of pre-bisucaberin 5 from scaled-up incubation

The incubation procedure described in section 1.5 was used, except that the incubation was carried out at 37 °C for 5 minutes to maximize the quantity of prebisucaberin accumulated. The reaction was stopped with 0.2 mL of an aqueous solution of 10% trichloroacetic acid. The precipitated enzyme was pelleted by centrifugation (10 min at 4000rpm) and the decanted supernatant was separated on an Agilent Zorbax C18 column (21.2 X 100 mm, 5μ) using the elution profile in table 3, monitoring absorbance at 210nm.

Table 3

Time (min)	Water (0.1% Formic Acid)	Acetonitrile (0.1% Formic Acid)	Flow (mL / min)
0	100	0	4.5
15	100	0	4.5
35	0	100	4.5
40	0	100	4.5

The collected fractions with retention time around 22.5 min, were analysed by ESI-MS and those containing the compound with *m/z* 419 $[M+H]^+$ were freeze dried. Analysis of the isolated compund by ESI-MS/MS (Bruker HCT+ spectrometer equipped with an electrospray source in positive ion mode) and ESI-TOF-MS (Bruker MicroTof) suggested that it had structure **5**.

1.7 Incubation of His_6-$BibC^C$ with pre-bisucaberin 5. An ~2:1 mixture of pre-bisucaberin **5** and bisucaberin **1** dissolved initially in 100 μL dionised water was incubated (15 μL/ reaction volume) with 2 mM ATP, 15 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0) and 2.7 μM His_6-$BibC^C$ in a final volume of 140 μL. The reaction was initiated by addition of the enzyme and the incubation was carried out for 2 h 40 min at 37°C. The reaction was stopped by addition of 3 μL of 0.5 M aqueous $FeCl_3$, which precipitated the enzyme and converted **1** and **5** to their ferric complexes. LC-MS analysis of the reaction mixture was carried out using the procedure described in section 1.4 (Figure 3). These analyses showed that all of the **5** in the mixture had been converted to **1**. No conversion of **5** to **1** could be detected in control incubations with enzyme inactivated by boiling at 100 °C for 10 minutes prior to addition to the incubation mixture.

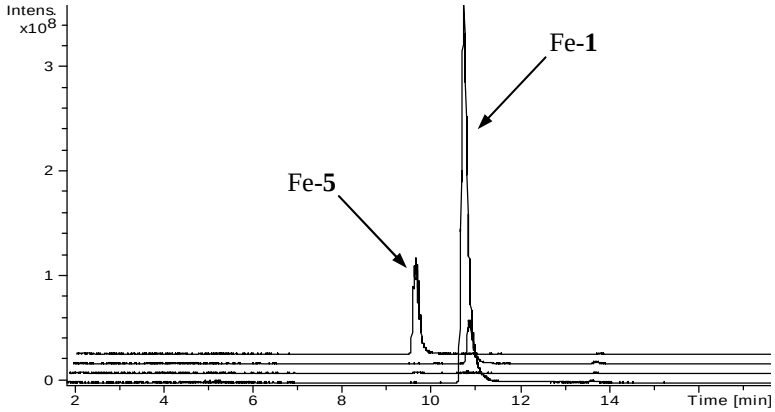

Figure 3. LC-MS analyses of the conversion of bisucaberin intermediate (**5**) to bisucaberin by recombinant His_6-$BibC^C$. Extracted ion chromatograms at m/z = 472.3 (ferri-pre-bisucaberin) and 454.3 (ferri-bisucaberin) from LC-MS analyses of incubations of pre-bisucaberin **5**, ATP, Mg^{2+} with His_6- $BibC^C$ (bottom two traces) and with boiled His_6- $BibC^C$ (top two traces), respectively.

1.8 Time course of the production of bisucaberin 1 and pre-bisucaberin 5 from HSC by His_6-$BibC^C$. The incubation procedure described in section 1.5 was used**,** except that 2.5 μM His_6-$BibC^C$ was used in a total volume of 140 μL and the reactions were stopped after 2.5, 5, 7.5, 10, 20, 30, 45 and 60 minutes by addition of 3 μL of 0.5 M aqueous $FeCl_3$. The relative quantity of bisucaberin **1** and pre-bisucaberin **5** in each incubation mixture was determined using HPLC monitoring absorbance at 471nm by integrating the peaks with retention times of 10.7 min and 9.5 min, corresponding to the ferric complexes of **1** and **5**. A plot of relative concentrations against time is shown in Figure 4.

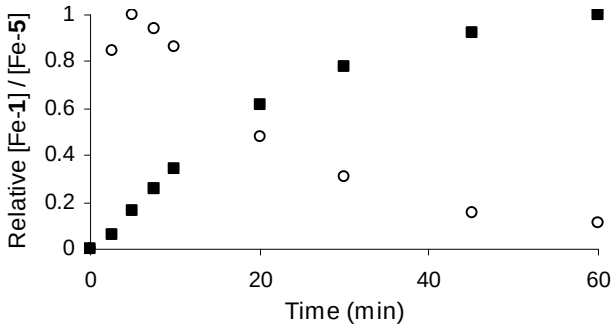

Figure 4. Change in relative quantities of ferri-bisucaberin (filled squares) and ferri-pre-bisucaberin (open circles) with time in incubations containing HSC, His_6-$BibC^C$, Mg^{2+} and ATP.

1.9 Analysis of AMP and ADP formation in incubation mixtures. For analysis of AMP formation, reactions containing 25 mM Tris-HCl buffer (pH 8.0), 1 mM ATP, 15 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, 1.0 mM NADH, 2 μM His_6-$BibC^C$, 20.5 units of lactate dehydrogenase, 26.6 units of pyruvate kinase, 4 units of myokinase and 1.5 mM HSC in a total volume of 140 μL were incubated at 37°C for 6 min in a quartz cuvette in a Varian Cary 1 UV-Vis spectrometer. The decrease in absorbance at 340 nm with time due to oxidation of NADH was monitored. A control reaction was carried out using heat-inactivated His_6-$BibC^C$. For the ADP assay, the same procedure was used except that myokinase was omitted from the reaction. The results of these experiments are shown in Figure 5.

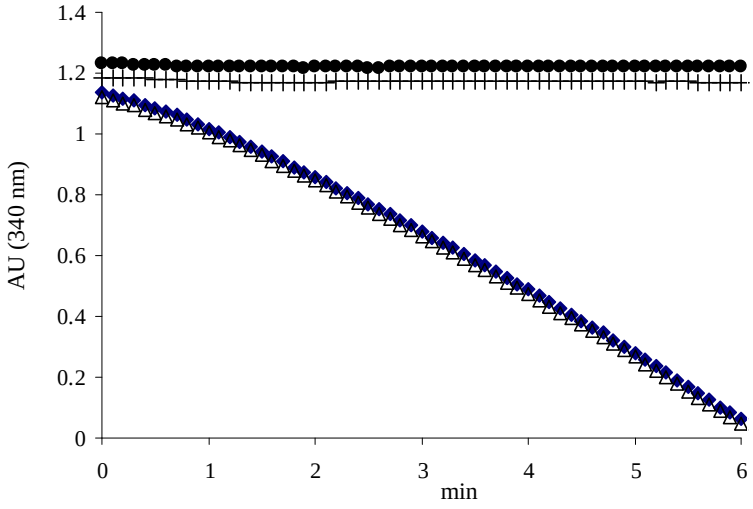

Figure 5. Results of coupled assay for AMP (♦, Δ) and ADP (+) production in the reaction of HSC with ATP and Mg^{2+} catalysed by $BibC^C$. The decrease in absorbance at 340 nm resulting from conversion of NADH to NAD^+ was monitored against time. No activity was observed in the coupled assay for AMP production (●) using $BibC^C$ enzyme inactivated by boiling for 10 minutes prior to addition.

2.0 Analysis of phosphate and pyrophosphate formation in incubation mixtures. Pyrophosphate (PP_i) production was measured by a coupled continuous spectrophotometric assay using the EnzChek Pyrophosphate Assay Kit (Molecular

Probes). The reactions contained 20 mM Tris-HCl (pH 8), 7.5 mM $MgCl_2$, 0.2 mM MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside), 1 unit of purine nucleoside phosphorylase, 0.1 unit of inorganic pyrophosphatase, 1.5 mM ATP, 1 mM HSC in a total volume of 200 μL. This mixture was pre-incubated at 37ºC for 10 min, then His_6-$BibC^C$ (3.4 μM final concentration) was added and absorbance at 360 nm was monitored for 20 min (Figure 6). The increase in absorbance at 360 nm results from hydrolysis of released PP_i by inorganic pyrophosphatase and reaction of the resulting phosphate (P_i) with MESG (2-amino-6-mercapto-7-methylpurine ribonucleoside) to yield 2-amino-6-mercapto-7-methylpurine and ribose-1-phosphate, catalyzed by purine nucleoside phosphorylase. P_i production was monitiored ommiting inorganic pyrophosphatase from the reaction.

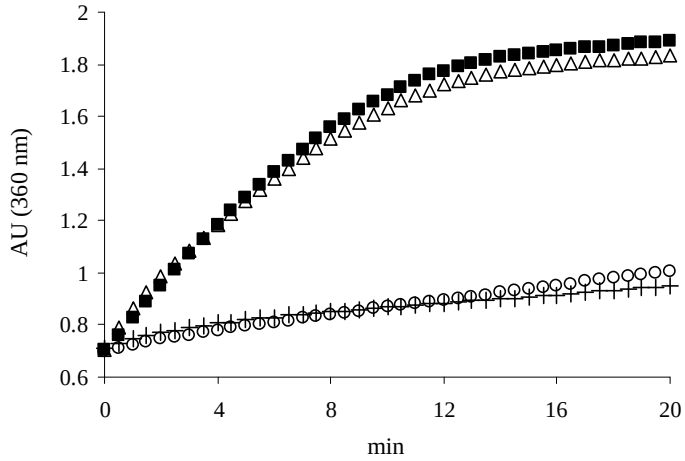

Figure 6. Results of coupled assay for pyrophosphate (■, Δ) and phosphate (+) release in the reaction of HSC with ATP and Mg^{2+} catalysed by His_6-$BibC^C$. No activity was observed in the coupled assay for pyrophosphate production using enzyme inactivated by boiling for 10 minutes prior to initiation of the reaction (○).

2.1 Identification of BibC homologues in other bacteria

The BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify other proteins in the database with significant sequence similarity to BibC across its entire length, indicating that these proteins have the same bi-domain organization as this multienzyme. The hits retrieved were as follows [locus tag (species, % similarity)]: Csal_1053 (*Chromohalobacter salexigens* DSM 3043, 62%); ETA_30300 (*Erwinia tasmaniensis* Et1/99, 62%); PTD2_05460 (*Pseudoalteromonas tunicata* D2, 62%); plu4630 (*Photorhabdus luminescens subsp. laumondii* TTO1, 61%); RED65_16581 (*Oceanobacter sp.* RED65, 60%); M23134_04758 (*Microscilla marina* ATCC 23134, 60%); M23134_04759 (*Microscilla marina* ATCC 23134, 59%); PPSIR1_41959 (*Plesiocystis pacifica* SIR-1, 60%); ATW7_00745 (*Alteromonadales bacterium* TW-7, 57%); CMS_1135 (*Clavibacter michiganensis subsp. sepedonicus*, 55%); CMM_2093 (*Clavibacter michiganensis subsp. michiganensis* NCPPB 382, 55%); BlinB01001962 (*Brevibacterium linens* BL2, 54%).

2.2 Spectroscopic data

• ESI-TOF-MS spectra of bisucaberin **1** and pre-bisucaberin **5** isolated from incubation of HSC, ATP and Mg^{2+} with His_6-$BibC^C$ (figures 7 and 8).

• ESI-MS/MS spectra of bisucaberin **1** and pre-bisucaberin **5** isolated from incubation of HSC, ATP and Mg^{2+} with His_6-$BibC^C$ (figures 9 and 10)

• ^1H, COSY, HMBC and HMQC spectra of bisucaberin **1** isolated from incubation of HSC, ATP and Mg^{2+} with His_6-$BibC^C$ (figures 11-14)

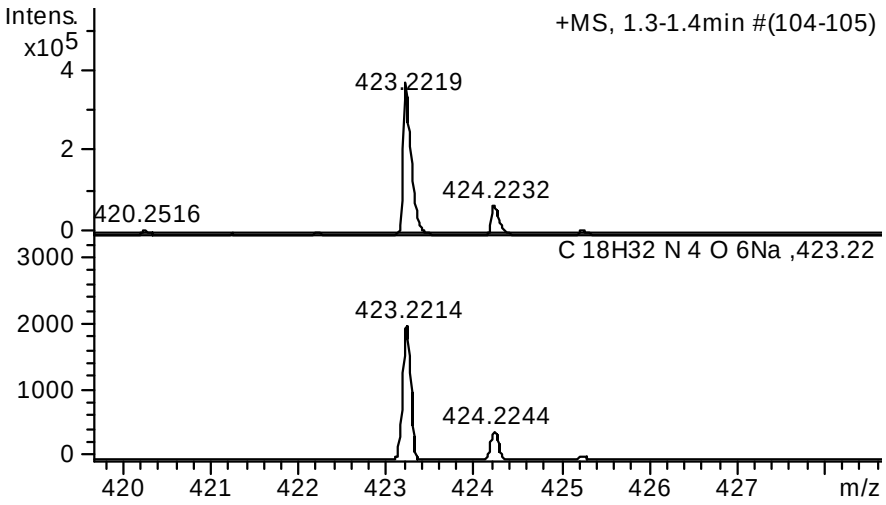

Figure 7. ESI-TOF-MS analysis of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, Mg^{2+} and ATP. Top panel: measured spectrum. Bottom panel: simulated spectrum.

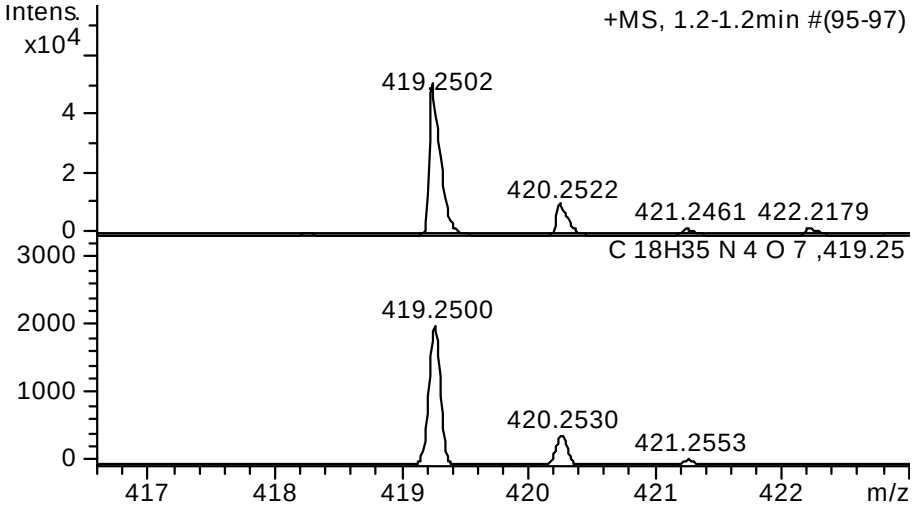

Figure 8. ESI-TOF-MS analysis of pre-bisucaberin **5** isolated from the incubation of HSC with recombinant BibC^{C}, Mg^{2+} and ATP. Top panel: measured spectrum. Bottom panel: simulated spectrum.

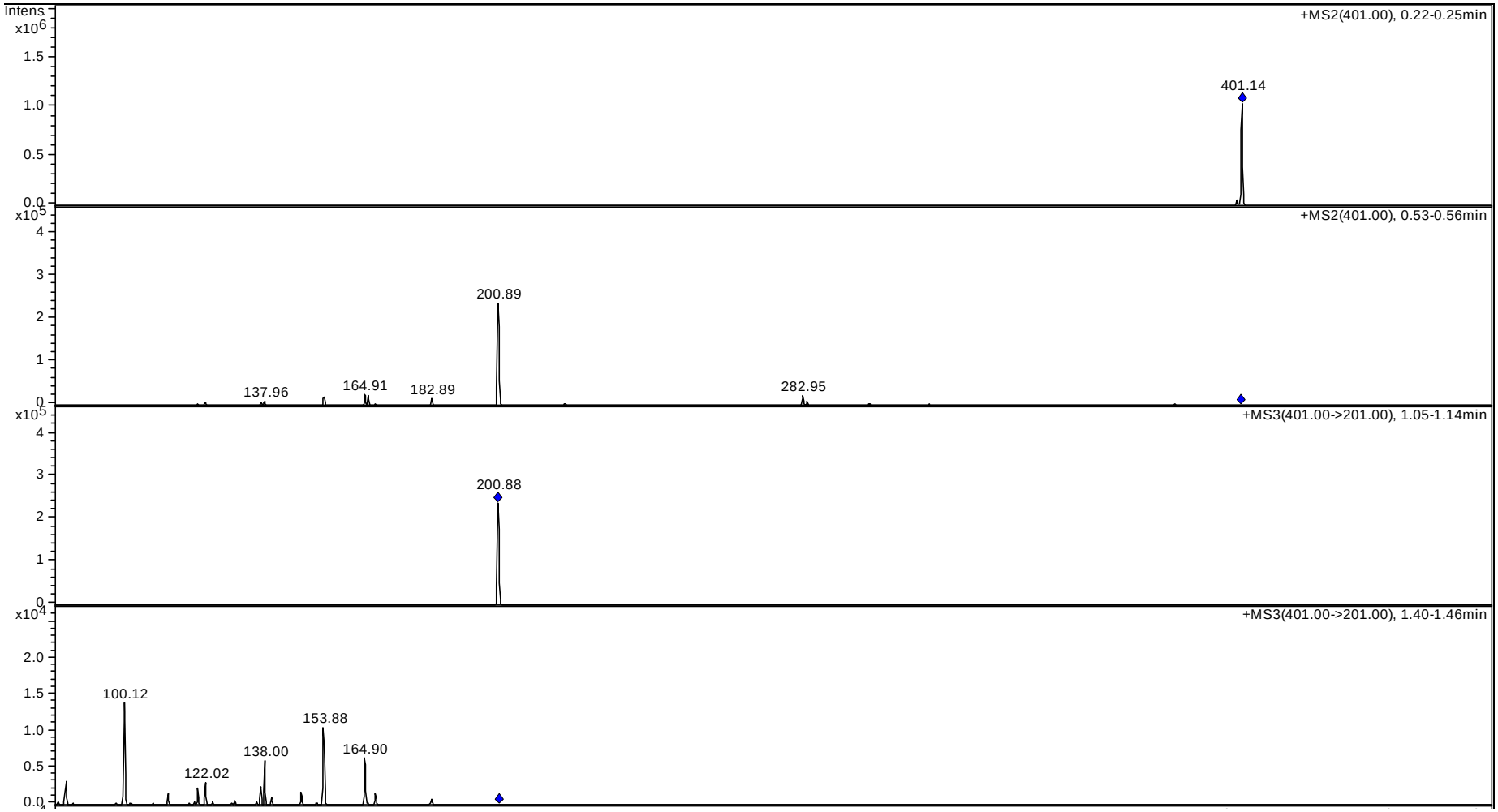

Figure 9. ESI-MS-MS analysis of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, Mg^{2+} and ATP. Panels from top to bottom are: spectrum of *m/z* 401.14 parent ion $[M+H]^{+}$; daughter ions produced by CID of *m/z* 401.14 ion; spectrum of selected *m/z* 200.89 ion; daughter ions produced by CID of *m/z* 200.89 ion.

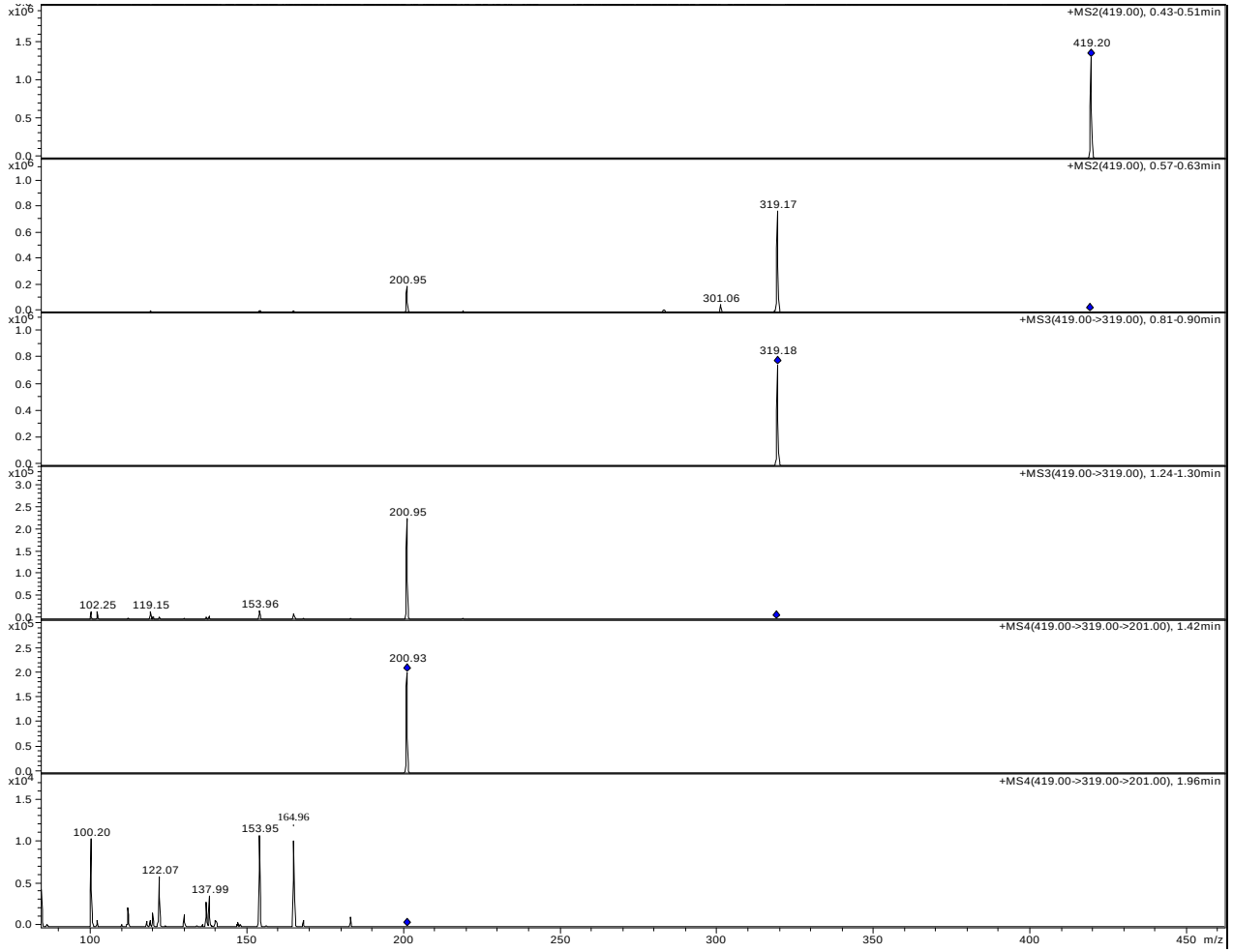

Figure 10. ESI-MS-MS analysis of pre-bisucaberin **5** isolated from the incubation of HSC with recombinant BibC^{C}, Mg^{2+} and ATP. Panels from top to bottom are: spectrum of *m/z* 419.20 parent ion $[M+H]^{+}$; daughter ions produced by CID of *m/z* 419.20 ion; spectrum of selected *m/z* 319.18 ion; daughter ions produced by CID of *m/z* 319.18 ion; spectrum of selected *m/z* 200.93 ion; daughter ions produced by CID of *m/z* 200.93 ion.

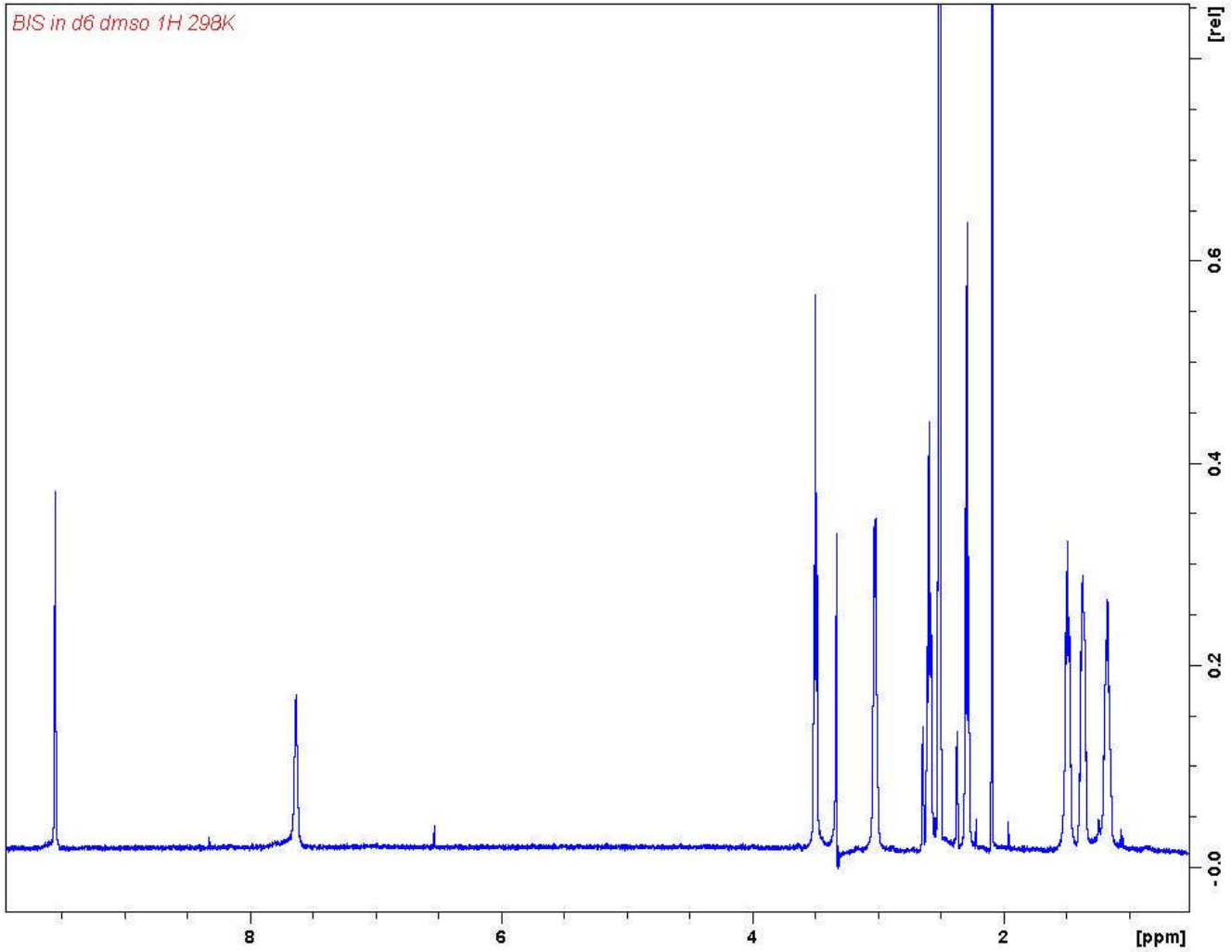

Figure 11. ^{1}H NMR spectrum (DMSO, 500 MHz) of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, ATP, and Mg^{2+}.

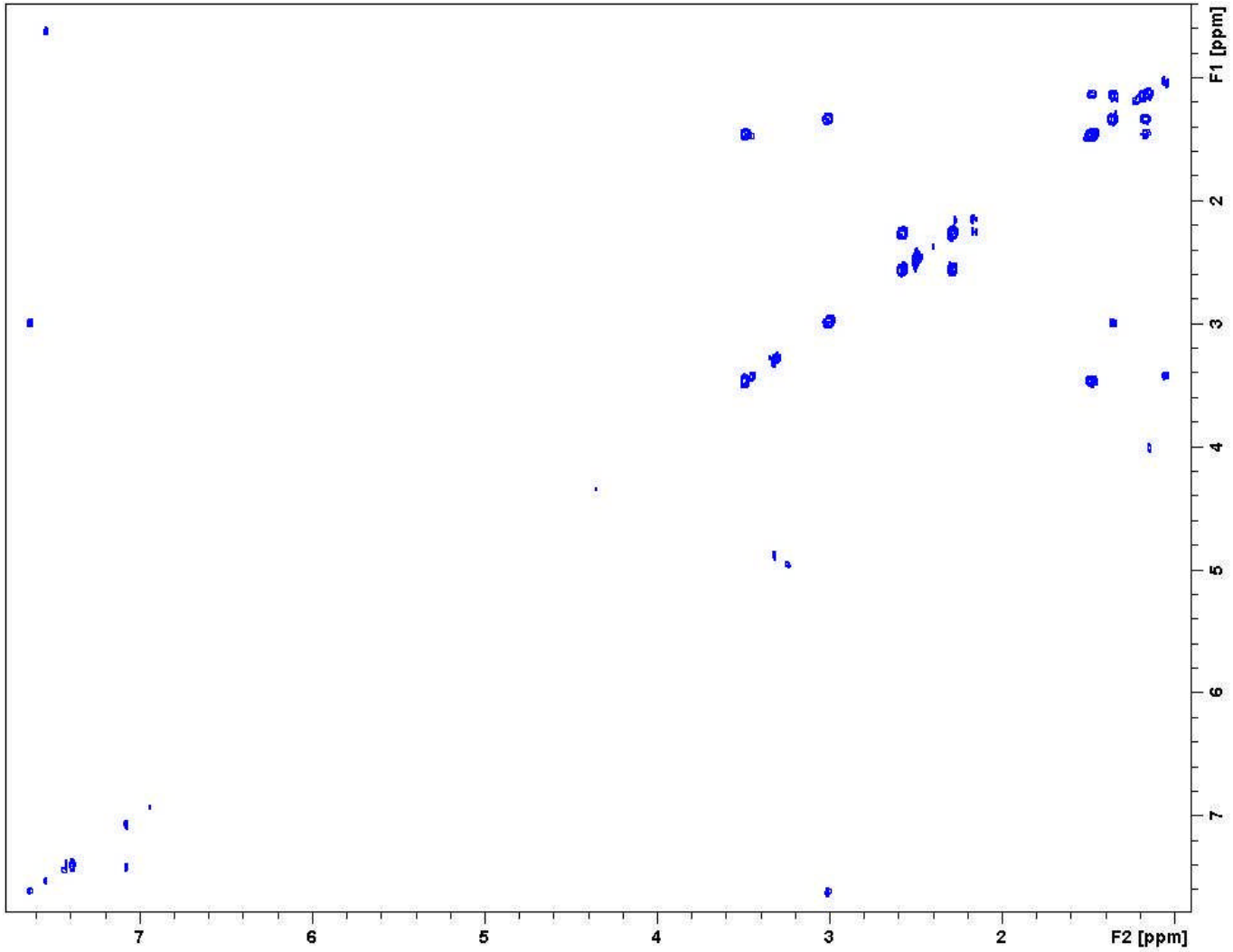

Figure 12. COSY spectrum (DMSO, 700 MHz) of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, ATP, and Mg^{2+}.

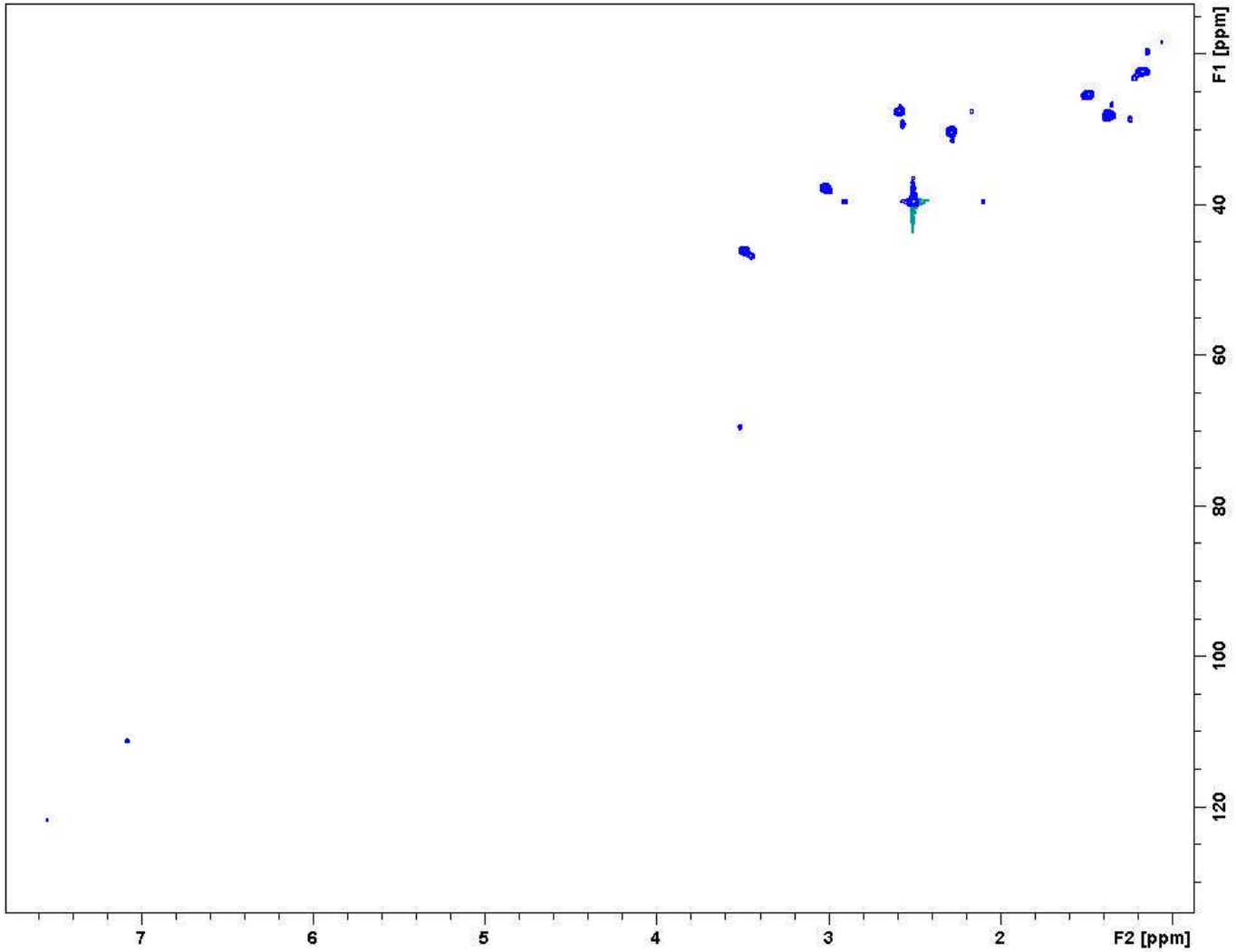

Figure 13. HSQC spectrum (DMSO, 700 MHz) of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, ATP, and Mg^{2+}.

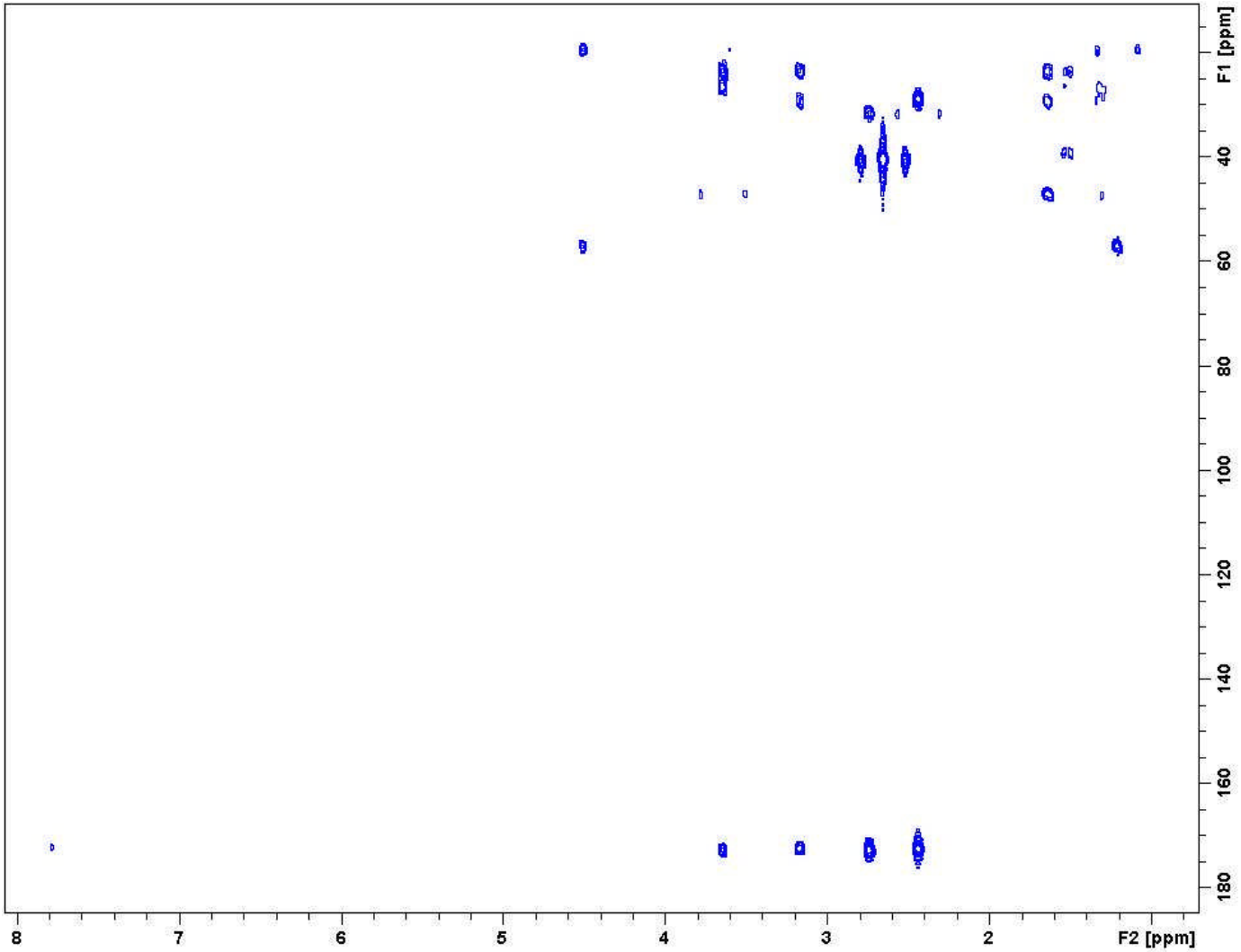

Figure 14. HMBC spectrum (DMSO, 700 MHz) of bisucaberin **1** isolated from the incubation of HSC with recombinant BibC^{C}, ATP, and Mg^{2+}.