SUPPLEMENTARY MATERIAL

Bioinspired benzoxanthene lignans as a new class of antimycotic agents: synthesis and *Candida* spp. growth inhibition

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Abstract

In this work we synthetized the bioinspired benzoxanthene lignans (BXLs) **3**, **14** - **22**, and the phenazine derivative **23** as potential antimycotic agents. MICs and MFCs against *Candida* strains were determined. In a preliminary screening, compounds **3**, **15**, **20**, **21**, **22** were substantially inactive. Compounds **14** and **17** showed antifungal activity, being able to inhibit the growth of the majority of *Candida* strains with MIC values in the range 4.6 - 19.2 μM (**14**) and 26.0 - 104.3 μM (**17**); for three strains, the MICs were lower than those obtained using the antimycotic drug fluconazole. The three BXLs **18**, **19** and **23** showed some MIC values lower than that of fluconazole; **18** was also active against two non-*albicans* *Candida* strains resistant to fluconazole. Phenazine **23**, although active only against one strain (MIC = 1.3 μM), was one order of magnitude more potent than fluconazole. All the BXLs were fungicidal.

Keywords: Benzoxanthenes; Lignans; Antimycotic agents; *Candida*

Experimental details

Antimicrobial assay

Candida samples (thirteen isolates of *C. albicans*, seven of non-*albicans Candida*) were obtained from women with a clinical diagnosis of vaginal candidiasis. An ATCC type *Candida* strain (*Candida albicans* ATCC 90028) was acquired from the American Type Culture Collection (ATCC, Gaitherburg, MD, USA) and used as the control. All *Candida* isolates were plated on Sabouraud Dextrose Agar medium and maintained at 35°C, for 48 h, to standardize the inoculum. The antifungal agents evaluated were **3**, **14 − 23**; fluconazole (**Flu**) was employed as reference standard (Table S1). The procedure was performed according to standards published in Clinical and Laboratory Standards Institute (CLSI 2008). In order to choose the appropriate range of concentrations, the MICs against *Candida* strains were determined by broth microdilution method using twofold serial dilutions. The culture medium used was bicarbonate-free RPMI 1640 (Sigma-Aldrich) with L-glutamine, buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich). The medium was sterilized by filtration through a 0.22 μm Millipore membrane. Fluconazole was dissolved in sterile distilled water and prepared in concentrations ranging from 0.4 to 208.9 μM. The compounds were solubilized in DMSO and twofold dilutions were prepared in culture medium. Solutions of compounds **14** were prepared at concentrations from 0.04 to 92.5 μM. A negative control test was performed to determine the effect of 1% DMSO on the growth of microorganisms. The yeast suspensions were prepared in 5 ml of 0.145 mol/L sterile saline solution, then vortexed for 15 s. The cell density was adjusted to an equivalent of 0.5 on the McFarland scale and standardized in a spectrophotometer at 530 nm. This procedure yielded a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells/mL. The suspensions were then diluted sequentially in RPMI 1640 liquid medium to obtain a 1:100 dilution followed by a 1:20 dilution, resulting in a concentration of $5x10^2$ to $2.5x10^3$ cells/mL. Sterile microtiter plates (Thermo Fisher Scientific) containing 96 U-wells were used to perform the test procedure. 100 μL of each concentration of compounds and **Flu** were placed on separate plates, in rows from 1 to 10. 100 μL of RPMI 1640 medium was deposited in well rows 11 and 12, in which the controls and the sterilization medium were grown, respectively. These plates were stored at -20°C until use. At test time, 100 μL of

standardized inoculum was placed in each well, and the microtiter plates were incubated at 35°C. The reference strain *Candida albicans* ATCC 90028 was included in each batch of susceptibility tests to ensure quality. The CLSI describes the broth microdilution method, specifying a defined test medium as well as a standardized inoculum, and recommends the visual determination of the MIC end points after incubation at 35°C for 48 h for *Candida* spp. By this method the end point is defined as the lowest drug concentration at which a *"prominent decrease in turbidity"* is observed compared with the growth in the control drug-free medium (CLSI 2008). However, it has become apparent that the specified decrease in turbidity in the microdilution test more closely corresponds to a 50% reduction in growth as assessed by spectrophotometric readings (Odds et al. 1995; Pfaller et al. 1995; Lozano-Chiu et al. 1999; Nguyen and Yu 1999). The MIC end points were read after 48 h of incubation with a microplate spectrophotometer set at 405 nm. The plates were agitated prior to reading. Spectrophotometric MICs were calculated based on the density of the growth control and were the lowest drug concentrations that resulted in a 50% reduction in growth compared with that of the drug-free growth control. The interpretive CLSI breakpoints for **Flu** were: Susceptible [S]: ≤ 26.1 µM; Susceptible Dose Dependent [SDD]: from 52.2 to 104.4 µM; and Resistant [R]: ≥ 208.9 µM. To obtain the MFC, samples (100 µl) were removed from all wells of the MIC microplates and subcultured onto Sabouraud dextrose agar. The colony forming units were counted after 48 h of incubation at 35°C. MFC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed. The MFC concentration are given in Table S1. The MFC/MIC ratio was calculated in order to determine if the compound had a fungistatic (MFC/MIC ratio > 4) or fungicidal (MFC/MIC ratio ≤ 4) activity. The results are showed in Table S2 (Chen et al. 2010; Pfaller et al. 2004). Three rounds of experiments were performed.

Table S1: MICs and MFCs (μM) of **14**, **16**–**19**, **23** and fluconazole (**Flu**) towards *Candida albicans* and non-*albicans Candida* strains.

Entry	Strain[b]	MICs and MFCs (μM)[a]														
		14		**16**		**17**		**18**		**19**		**23**		**Flu**		**I. C.[c]**
		MFC	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	
1	*C. albicans* 001/050	19.2	19.2	-	-	104.3	104.3	-	-	-	-	-	-	13.0	1.6	S
2	*C. glabrata* 002/050	19.2	19.2	-	-	104.3	52.1	11.7	11.7	-	-	-	-	-	-	R[d]
3	*C. glabrata* 003/050	19.2	9.5	97.5	48.7	104.3	104.3	11.7	5.8	5.6	2.8	1.3	1.3	104.4	13.0	S
4	*C. krusei* 004/050	19.2	19.2	-	-	-	-	11.7	11.7	-	-	-	-	-	-	R
5	*C. albicans* 005/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
6	*C. albicans* 006/050	-	-	-	-	-	-	-	-	-	-	-	-	13.0	0.81	S
7	*C. albicans* 007/050	19.2	19.2	-	-	104.3	104.3	-	-	-	-	-	-	52.2	6.5	S
8	*C. glabrata* 008/050	-	-	-	-	-	-	-	-	-	-	-	-	ND[e]	52.2	S
9	*C. albicans* 009/050	-	-	-	-	-	-	-	-	-	-	-	-	13.0	1.6	S
10	*C. albicans* 010/050	-	-	97.5	97.5	104.3	104.3	-	-	-	-	-	-	52.2	3.2	S
11	*C. albicans* 011/050	-	-	-	-	-	-	-	-	-	-	-	-	104.4	6.5	S
12	*C. glabrata* 012/050	-	-	-	-	104.3	52.1	-	-	-	-	-	-	ND	52.2	S-DD
13	*C. albicans* 013/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
14	*C. albicans* 014/050	-	-	-	-	-	-	-	-	-	-	-	-	104.4	6.5	S
15	*C. krusei* 015/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
16	*C. albicans* 016/050	9.5	9.5	-	-	52.1	52.1	-	-	-	-	-	-	26.1	3.2	S
17	*C. albicans* 017/050	9.5	4.7	12.9	12.9	52.1	26.1	11.7	5.8	5.6	5.6	-	-	52.2	6.5	S
18	*C. albicans* 018/050	19.2	9.5	-	-	104.3	104.3	-	-	-	-	-	-	52.2	6.5	S
19	*C. krusei* 019/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
20	*C. albicans* 020/050	19.2	19.2	-	-	104.3	104.3	-	-	-	-	-	-	26.1	3.2	S
21	*C. albicans* ATCC 90028	19.2	19.2	97.5	97.5	52.1	52.1	-	-	-	-	-	-	6.5	0.81	S

Notes: [a] For each *Candida* isolate, the experiment was performed in triplicate and identical MIC and MFC values were obtained. [b] Strain numbers refer to an internal directory for clinical isolates. [c] I.C.: MIC Interpretive Criteria for fluconazole (CLSI M27-A3): Susceptible [S]: ≤ 26.1 μM, Susceptible Dose Dependent [SDD]: from 52.2 to 104.4 μM, Resistant [R]: ≥ 208.9 μM; [d] Susceptibility testing is not recommended, as the species is a poor target for therapy with the drug; [e] ND: not done (fungal growth observed at the highest tested concentration of **Flu**).

Table S2: MFC/MIC ratios and effect of **14**, **16**–**19**, **23** and fluconazole (**Flu**) towards *Candida albicans* and non-*albicans Candida* strains.

Entry	Strain[b]	MFC/MIC ratios and effect[a]													
		14		**16**		**17**		**18**		**19**		**23**		**Flu**	
		MFC/MIC ratio[c]	**Effect**	**MFC/MIC ratio**	**Effect**	**MFC/MIC ratio**	**Effect**	**MFC/MIC ratio**	**Effect**	**MFC/MIC ratio**	**Effect**	**MFC/MIC ratio**	**Effect**	**MFC/MIC ratio**	**Effect**
1	*C. albicans* 001/050	1	FC[d]	-	-	1	FC	-	-	-	-	-	-	8	FS[e]
2	*C. glabrata* 002/050	1	FC	-	-	2	FC	1	FC	-	-	-	-	-	-
3	*C. glabrata* 003/050	2	FC	2	FC	1	FC	2	FC	2	FC	1	FC	8	FS
4	*C. krusei* 004/050	1	FC	-	-	-	-	1	FC	-	-	-	-	-	-
5	*C. albicans* 005/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	*C. albicans* 006/050	-	-	-	-	-	-	-	-	-	-	-	-	16	FS
7	*C. albicans* 007/050	1	FC	-	-	1	FC	-	-	-	-	-	-	8	FS
8	*C. glabrata* 008/050	-	-	-	-	-	-	-	-	-	-	-	-	ND[f]	ND
9	*C. albicans* 009/050	-	-	1	FC	-	-	-	-	-	-	-	-	8	FS
10	*C. albicans* 010/050	-	-	-	-	1	FC	-	-	-	-	-	-	16	FS
11	*C. albicans* 011/050	-	-	-	-	-	-	-	-	-	-	-	-	16	FS
12	*C. glabrata* 012/050	-	-	-	-	2	FC	-	-	-	-	-	-	ND	ND
13	*C. albicans* 013/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	*C. albicans* 014/050	-	-	-	-	-	-	-	-	-	-	-	-	16	FS
15	*C. krusei* 015/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	*C. albicans* 016/050	1	FC	-	-	1	FC	-	-	-	-	-	-	8	FS
17	*C. albicans* 017/050	2	FC	1	FC	2	FC	2	FC	1	FC	-	-	8	FS
18	*C. albicans* 018/050	2	FC	-	-	1	FC	-	-	-	-	-	-	8	FS
19	*C. krusei* 019/050	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	*C. albicans* 020/050	1	FC	-	-	1	FC	-	-	-	-	-	-	8	FS
21	*C. albicans* ATCC 90028	1	FC	1	FC	1	FC	-	-	-	-	-	-	8	FS

Notes: [a] For each *Candida* isolate, the experiment was performed in triplicate and identical MIC/MFC values were obtained; [b] Strain numbers refer to an internal directory for clinical isolates; [c] MFC/MIC ratio > 4: Fungistatic, MFC/MIC ratio ≤ 4: Fungicidal; [d] FC: Fungicidal; [e] FS:Fungistatic; [f] ND: not done (fungal growth observed at the highest tested concentration of **Flu**).

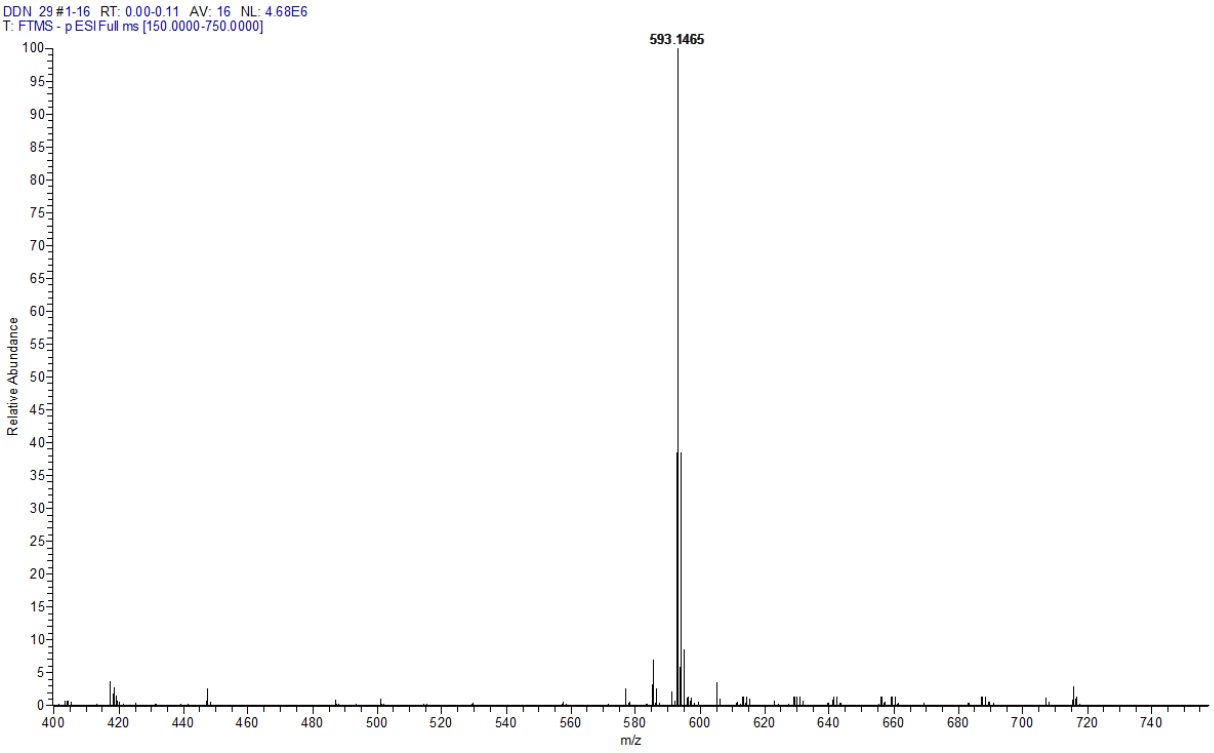

Figure S1. HRESIMS spectrum of **18**.

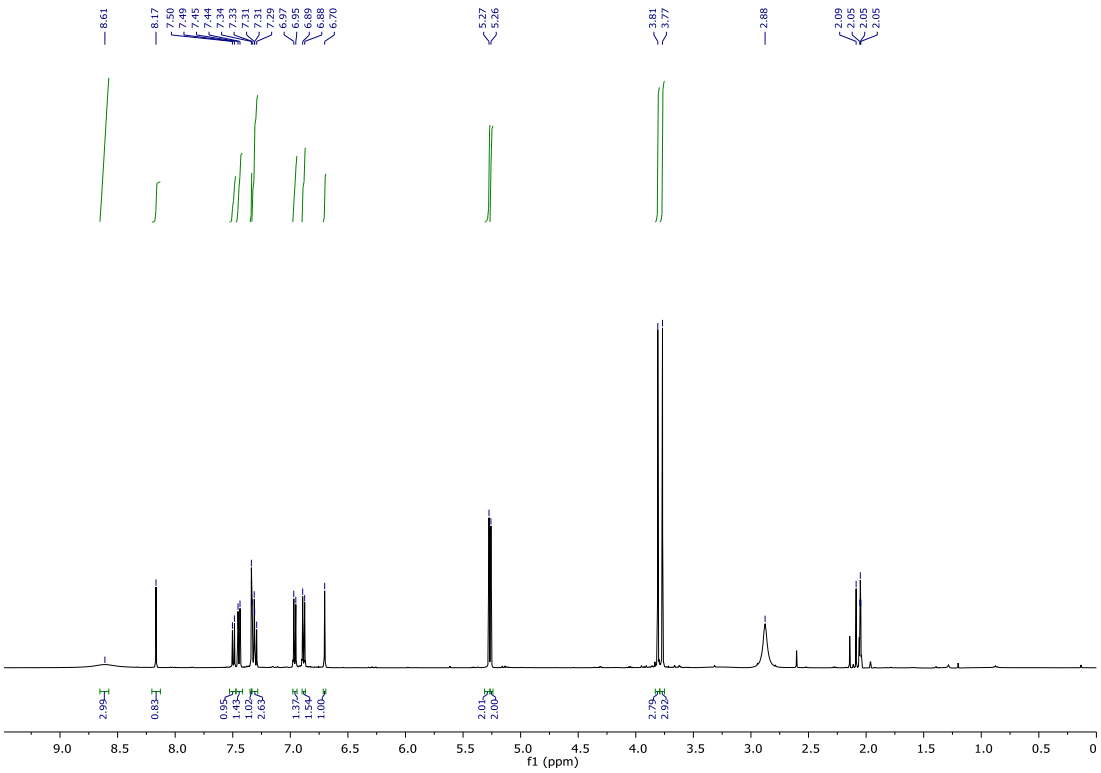

Figure S2. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of **18**.

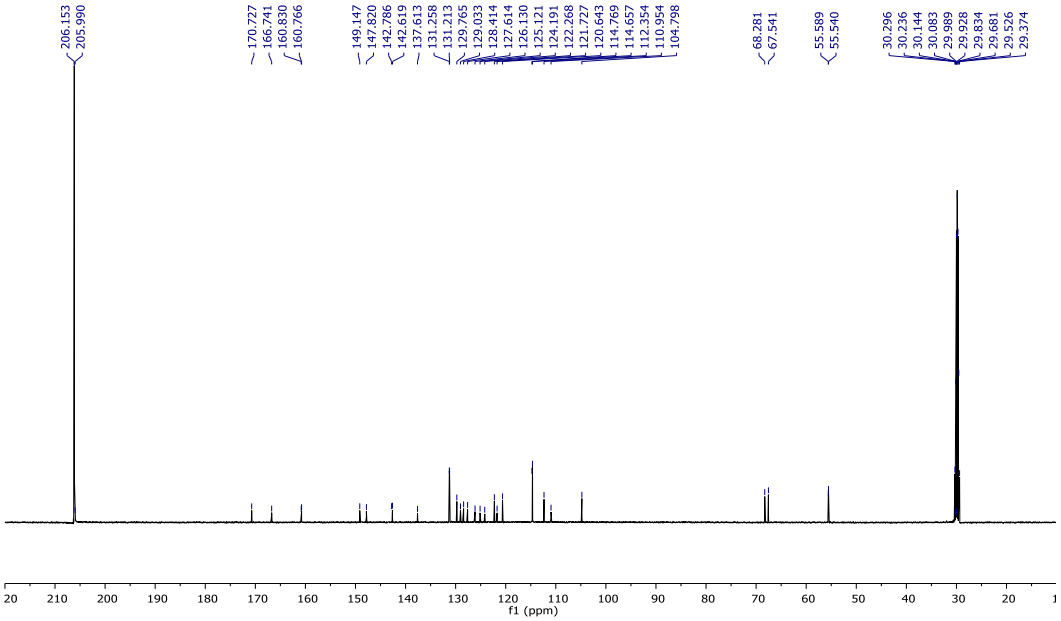

Figure S3. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of **18**.

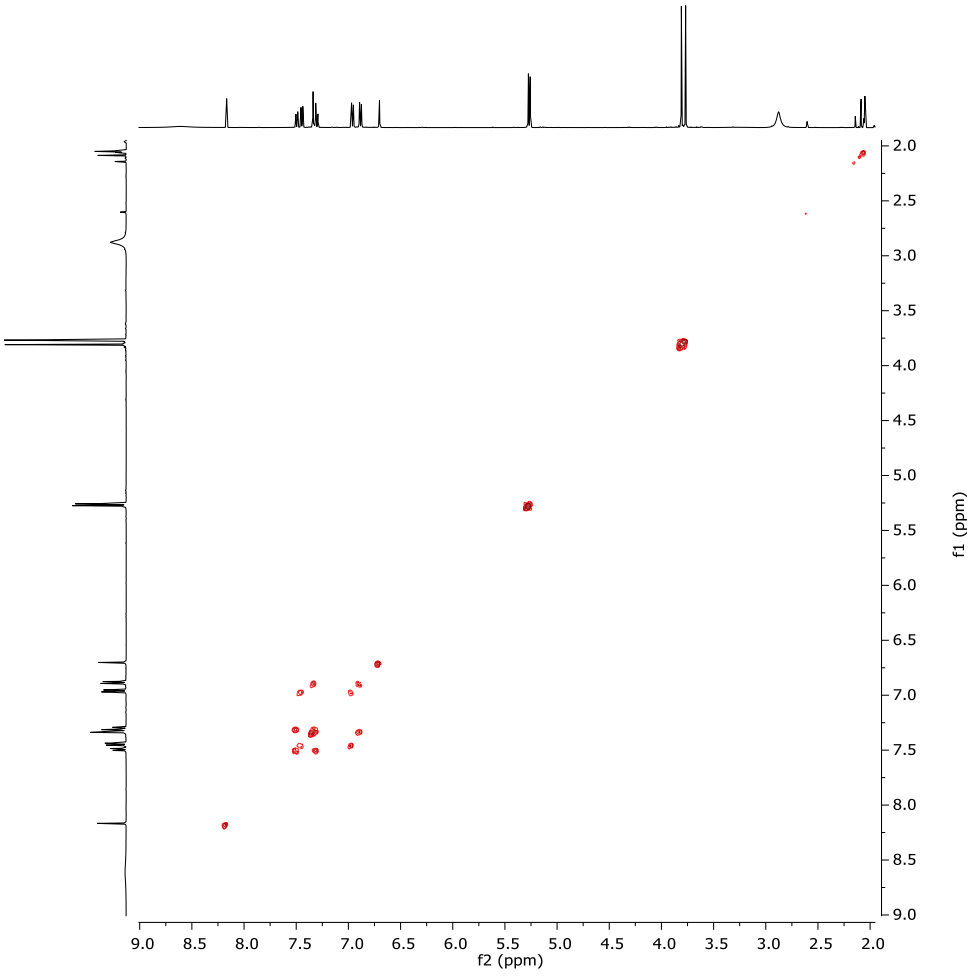

Figure S4. gCOSY spectrum of **18**.

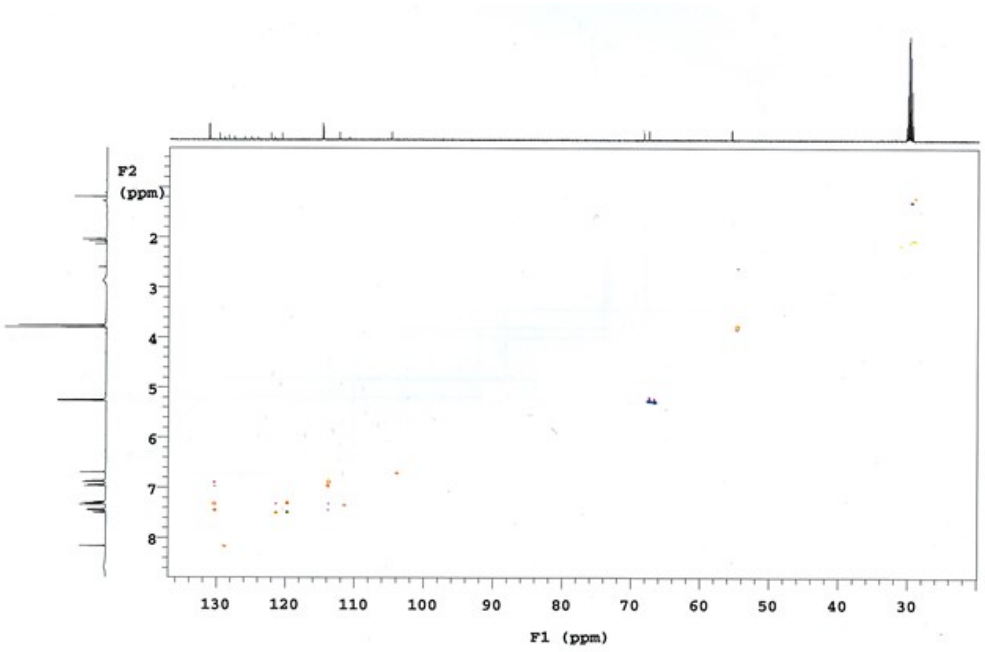

Figure S5. gHSQCAD spectrum of **18**.

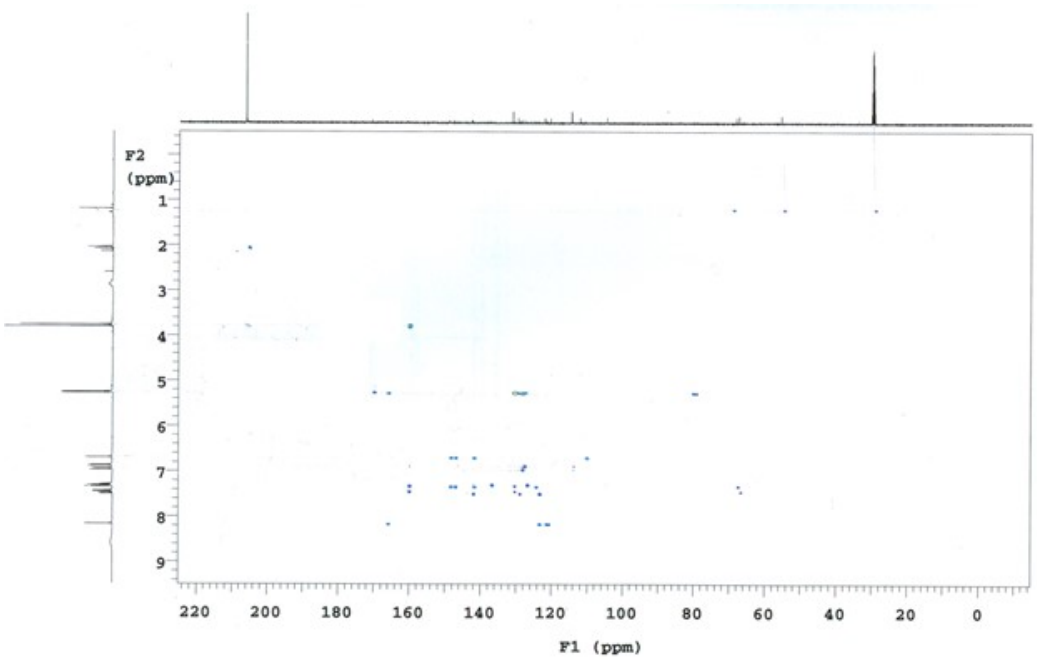

Figure S6. gHMBCAD spectrum of **18**.

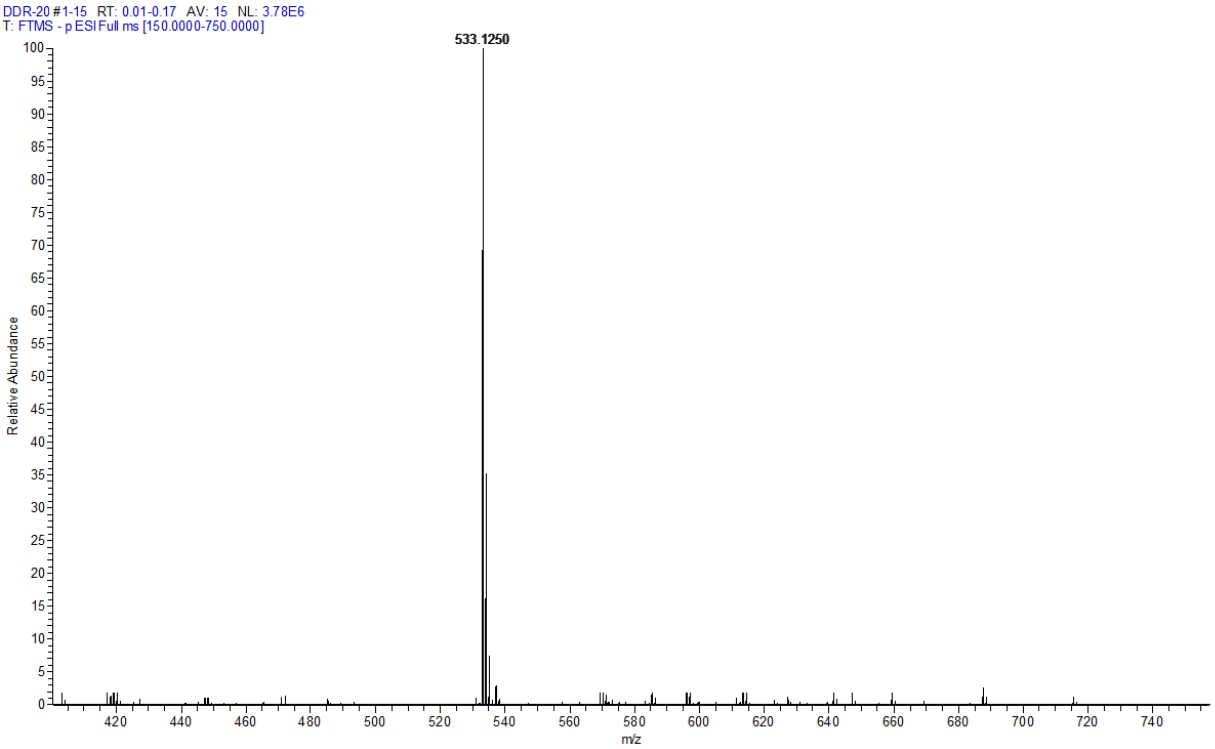

Figure S7. HRESIMS spectrum of **19**.

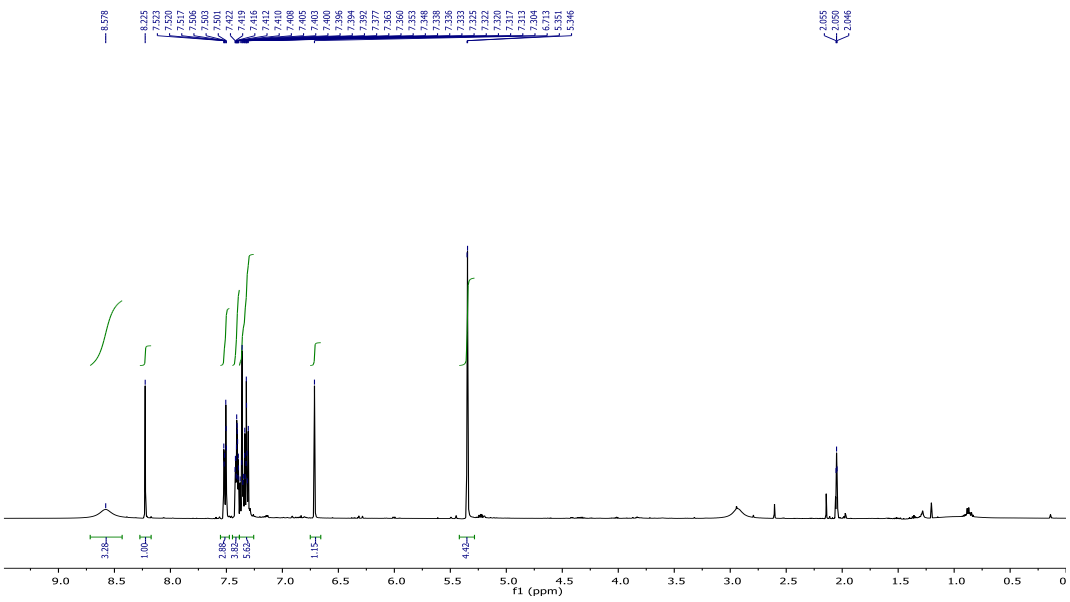

Figure S8. ^{1}H NMR spectrum (500 MHz, Acetone-d_6) of **19**.

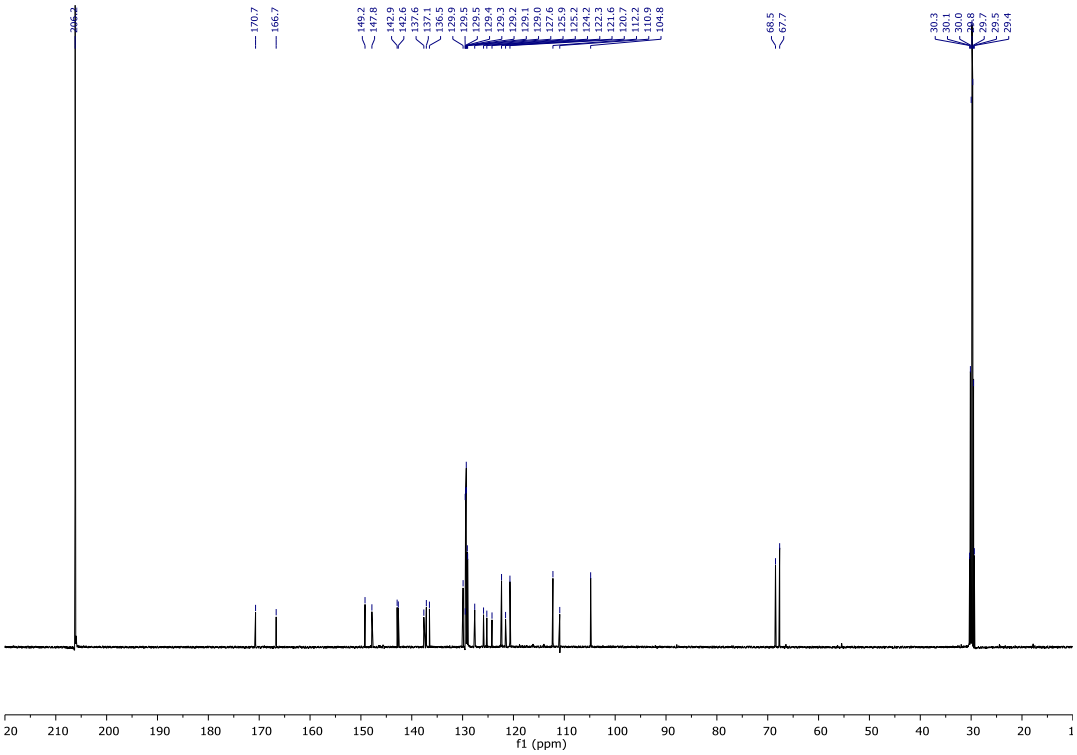

Figure S9. ^{13}C NMR spectrum (125 MHz, Acetone-d_6) of **19**.

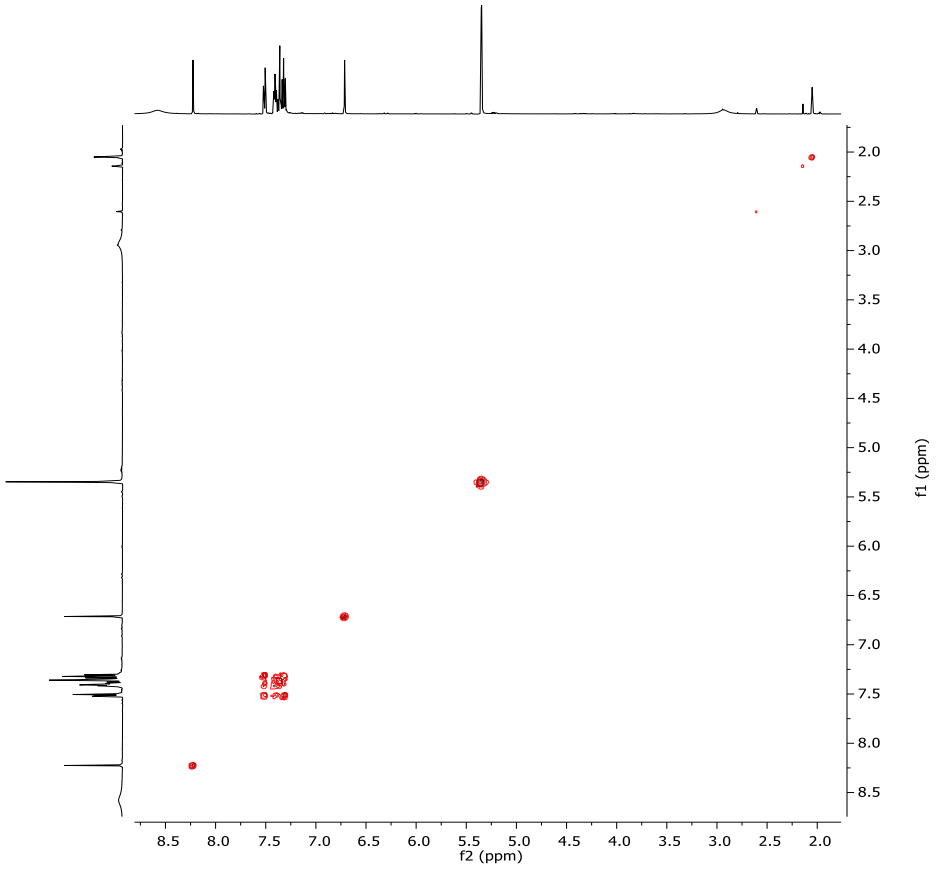

Figure S10. gCOSY spectrum of **19**.

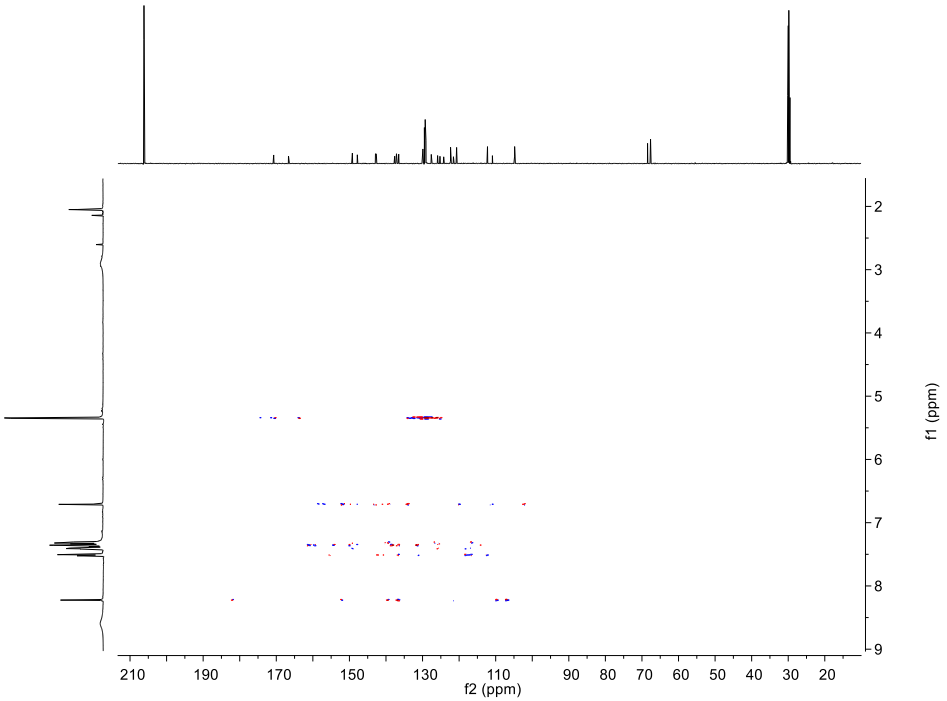

Figure S11. gHMBCAD spectrum of **19**.

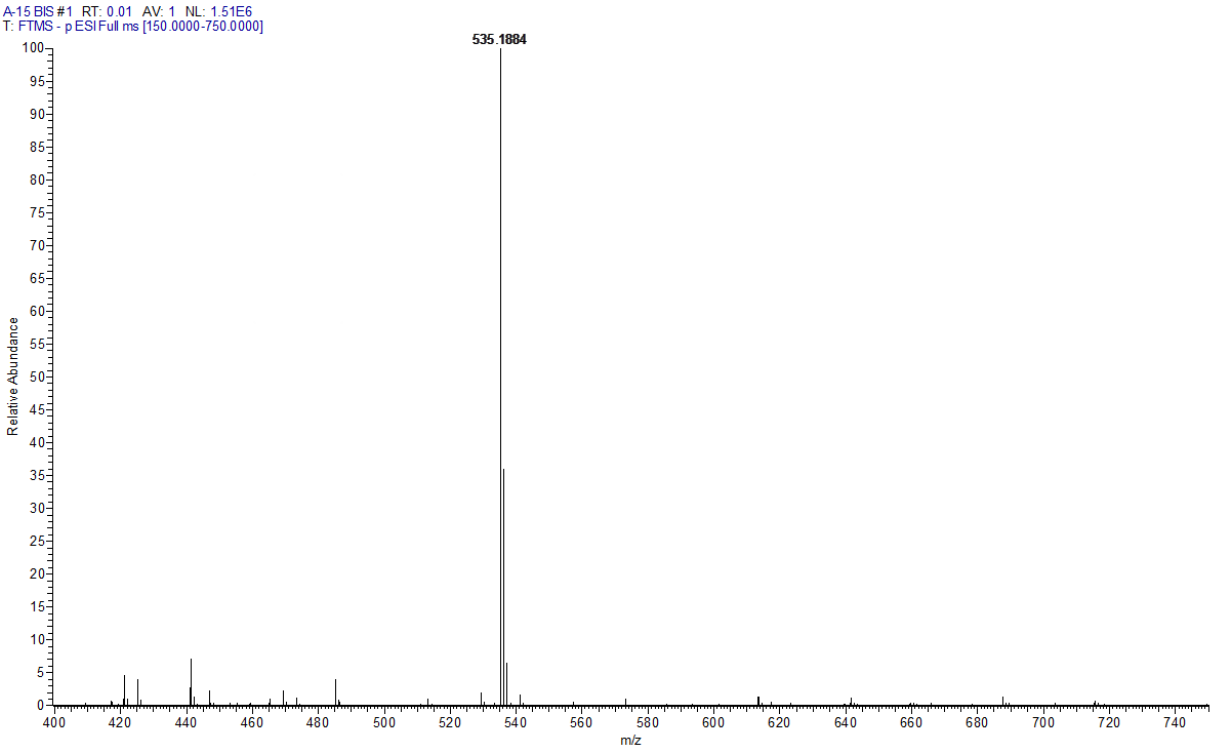

Figure S12. HRESIMS spectrum of **23**.

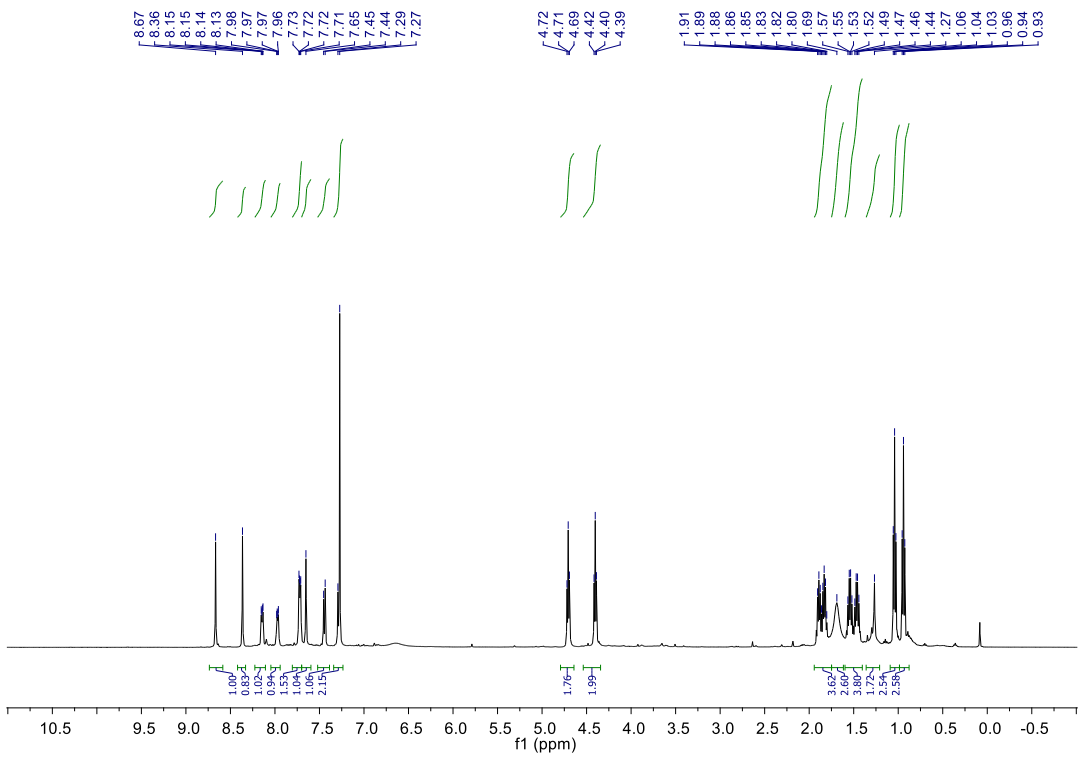

Figure S13. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **23**.

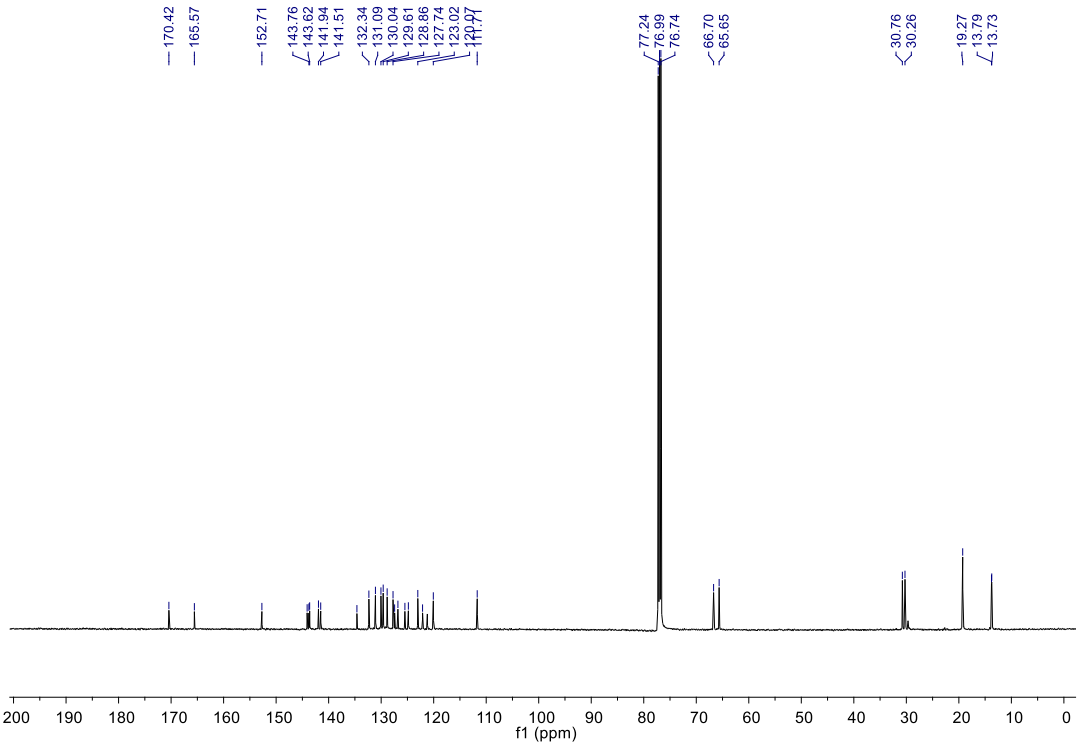

Figure S14. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **23**.

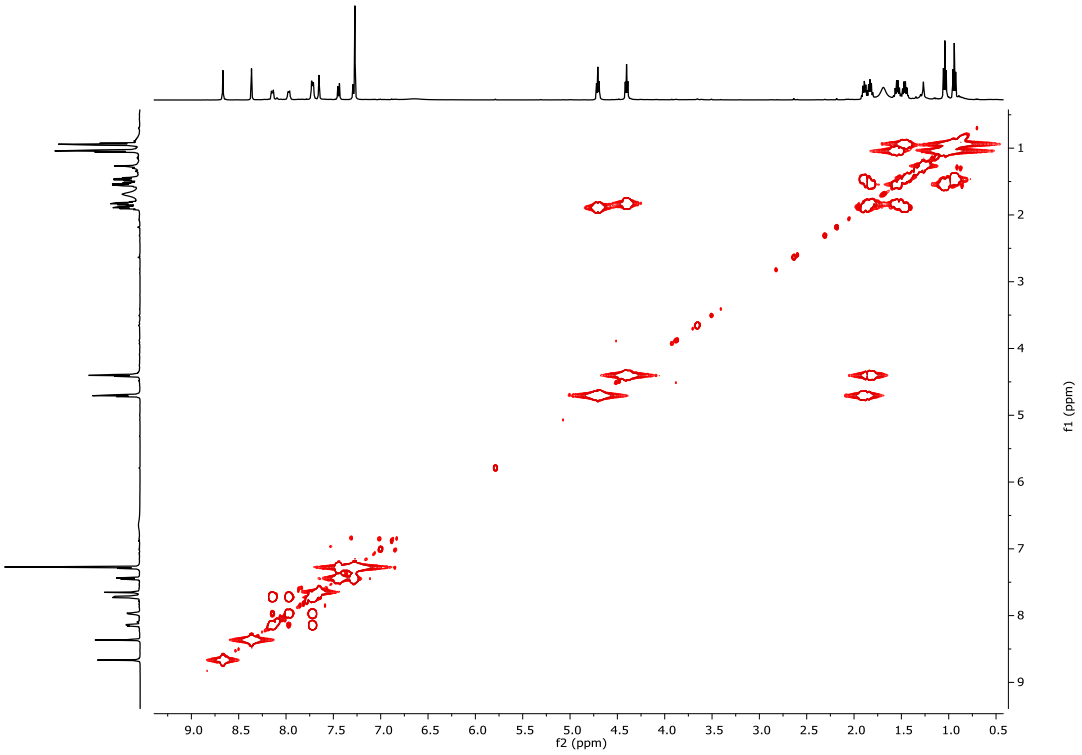

Figure S15. gCOSY spectrum of **23**.

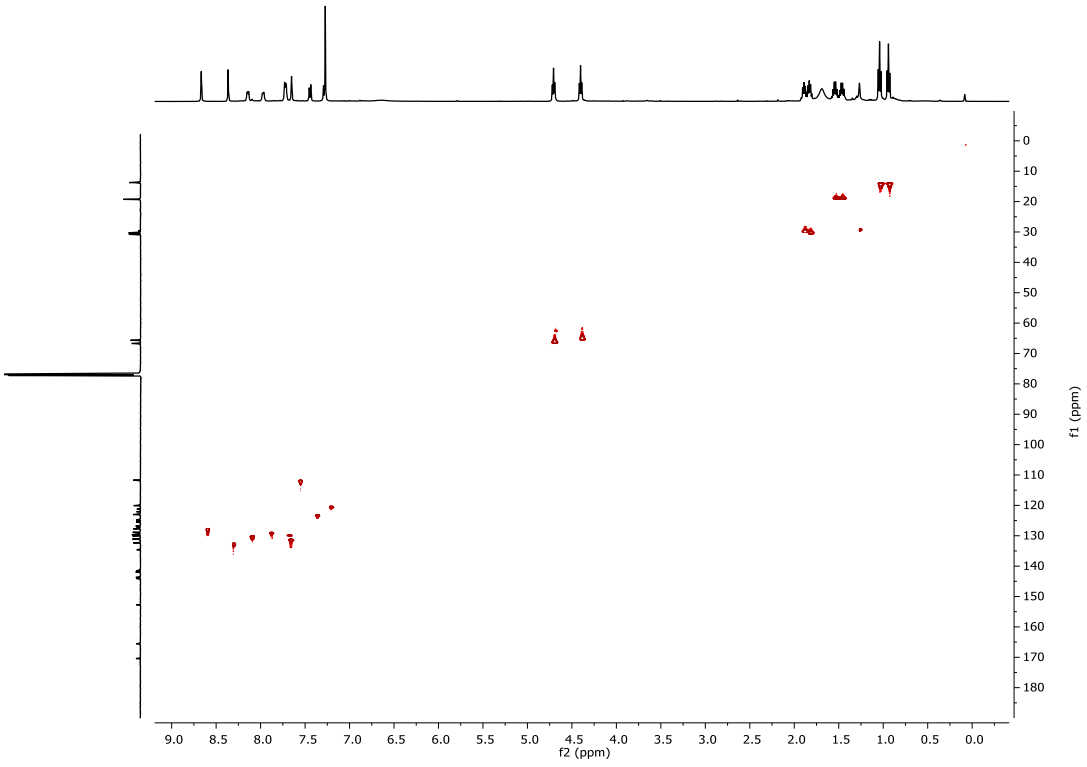

Figure S16. gHSQCAD spectrum of **23**.

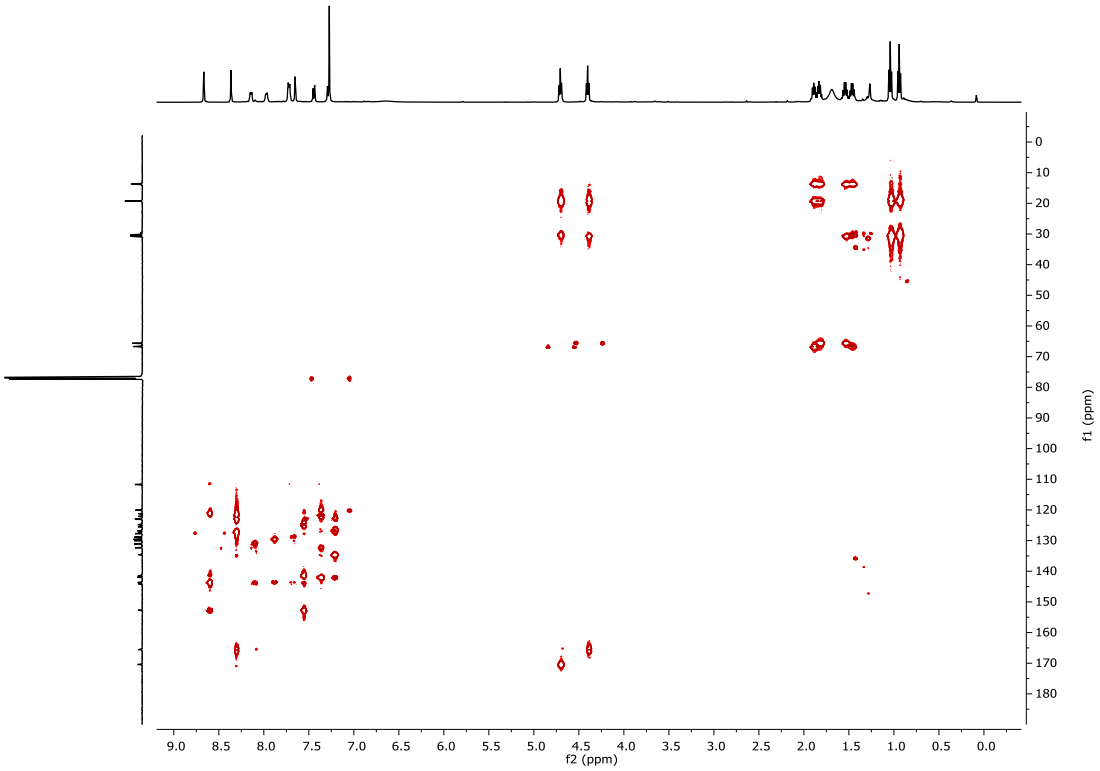

Figure S17. gHMBCAD spectrum of **23**.

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